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NON-EQUILIBRIUM MODEL OF LIQUID COLUMN CHROMATOGRAPHY

I. EXACT EXPRESSIONS FOR ELUTION PROFILE MOMENTS AND RELATION TO PLATE HEIGHT THEORY

HERBERT W. HETHCOTE* and CHARLES DeLISI*
Laboratory of Mathematical Biology, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)
(First received July 6th, 1981; revised manuscript received December 24th, 1981)

SUMMARY

A model for column chromatography that includes non-equilibrated mass transfer, diffusion, and generalized boundary conditions at the top of the column is solved by the method of moments. The theory predicts that as the macroscopic velocity u becomes very small, the plate height approaches a constant (rather than diverging as u^{-1}). As u increases, the plate height drops to a minimum and then increases. We show that the past assumptions that mass transfer and various types of diffusion contribute additively to the plate height, hold only at or beyond the minimum. We show further that our expression fits observed data with only a *single* adjustable lumped parameter. The parameter contains the mass transfer rate k_{-1} for moving out of a bead. The data fit, as well as an analytical approximation that we derive for the position of the minimum, provides a relation between k_{-1} and bead particle size (d_p) and consequently between d_p and profile dispersion. Conditions under which the peak of the elution profile is a good approximation to the mean are described. For a suitably chosen flow-rate, the mass transfer rates can be estimated from the observed dispersion in the elution profile.

INTRODUCTION

The use of column chromatography as a quantitative tool for molecular weight determination[1] and chemical reaction characterization[2,3] has increased continuously and rapidly during the past decade. However, the basis of its validity for quantitative thermodynamic (and perhaps kinetic[4]) studies is uncertain, resting largely on assumptions that local equilibrium is established instantaneously[3], and that the contribution of chemical kinetics to elution profile broadening can be made to dominate the effects of diffusion and other none-equilibrium processes[4]. A general assessment of the range of validity of these assumptions has been difficult because of the formidable problems in obtaining analytic solutions for the elution profile, even when the mathematical

* Present address: Department of Mathematics, University of Iowa, Iowa City, IA, 52242, U.S.A.

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system is linear. In this paper we extend a basic non-equilibrium model of chromatography and develop the experimental implications. This and the companion paper[5], which includes heterogeneity and non-linear mass transfer, will provide a useful foundation for generalization to more complex systems involving chemical reactions[6,7].

Of the many important mathematical contributions to the theory of liquid column chromatography (*e.g.*, refs. 2 and 8, and references cited therein), those most similar to the model presented here are the non-equilibrium random walk model of Giddings and Eyring[8,9] and the partial differential equations formulation of Thomas[10]. Our method of solution, involving explicit equations for the moments of the profile, is similar to that used by other authors (refs. 8 and 11–13, and references cited therein). Here we extend these methods by generalizing the boundary conditions at the top of the column and by pursuing the numerical consequences of the model.

After developing and solving the model, we study plate height (proportional to the dispersion in the profile divided by the square of the mean) as a function of the convection velocity u. At very low u, the plate height approaches a constant (rather than diverging as u^{-1}) which is explicitly given by the theory. As u increases, the plate height passes through a minimum in the usual way, and then increases. A good non-linear least-squares fit of our predicted equation to data is obtained with only one adjustable parameter. An analytic approximation is obtained for this minimum which relates the desorption mass transfer coefficient to bead diameter.

Criteria on the flow-rate are given which guarantee that the effects of diffusion on the means and variances can be neglected. A chromatography model which assumes local equilibration of mass transfer kinetics is described. When diffusion is negligible, this model yields a widely used formula for the peak of the elution profile. Identification of the assumptions required for its derivation provides a basis for assessing its range of validity.

Implications of our analysis are discussed. A complete procedure is outlined for estimating the equilibrium constant and the rate constants for the sorption–desorption kinetics from the mean and variance of the elution profile. Both the equilibrium constant and the rate constants can be estimated from the mean and variance, but *only* the equilibrium constant can be estimated from the mean.

A CHROMATOGRAPHY MODEL WITH DIFFUSION

We formulate an initial-boundary value problem which models a small-zone chromatography experiment with a single type of molecule in the sample. We model only non-gradient, elution chromatography with a constant temperature in the column and a constant pressure on the solvent. Consider a column where x measures the distance up from the bottom of the bed; x is h at the top of the bed and x is f at the top of the solvent above the bed. Let V_0 be the void volume, *i.e.*, the volume exterior to the beads, and let V_p be the volume interior to the beads that can be penetrated by the macromolecules. Define the void cross-sectional area as $A_0 = V_0/h$ and the penetrable cross-sectional area as $A_p = V_p/h$. The concentration (over A_0) of solute molecules in the mobile phase is $C(x,t)$ and the concentration (over A_p) of molecules in the stationary phase is $B(x,t)$.

Using a conservation of mass approach on $0 < x < h$, we have derived a system of reaction–diffusion–convection partial differential equations for the density

A_0C (in units of molecules per unit column length) of solute molecules in the mobile phase and the density A_pB of solute molecules in the stationary phase. By dividing these equations by the initial number of molecules I, the following partial differential equations for the probability density $p(x,t)$ for the mobile phase and $q(x,t)$ for the stationary phase are obtained:

$$\frac{\partial p}{\partial t} = u\frac{\partial p}{\partial x} + D\frac{\partial^2 p}{\partial x^2} - k_1 p + k_{-1} q \tag{1}$$

$$\frac{\partial q}{\partial t} = k_1 p - k_{-1} q \tag{2}$$

In eqn. 1 the first term on the right corresponds to mobile-phase flow (convection) with velocity u ($u = F/A_0$ where F is the flow-rate), and the second term corresponds to diffusion with diffusion constant D. The movement of solute molecules back and forth between the flowing solvent (mobile phase) and the inside of the beads (stationary phase) is described by the sorption and desorption rate constants, k_1 and k_{-1}. At sorption–desorption equilibrium the concentrations C and B are equal so that eqn. 2 implies that the equilibrium constant $K = k_1/k_{-1}$ satisfies $K = A_p/A_0$ or $K = V_p/V_0$. The equilibrium constant K is not equal to the partition or distribution coefficient defined as $K_d = V_p/V_i$, where V_i is the volume inside the beads that is not gel matrix; however, $K_d = KV_0/V_i$. The retention ratio R used by Giddings[8] satisfies $R = 1/(1 + K)$.

The initial layer at the top of the bed containing sample molecules is assumed to be sufficiently small so that it can be considered an instantaneous source (small zone). Thus, the initial conditions on $0 \leqslant x \leqslant f$ are

$$\begin{aligned} p(x,0) &= \delta(x - h) \\ q(x,0) &= 0 \qquad x \neq h \end{aligned} \tag{3}$$

where $\delta(x - h)$ is a Dirac delta function. We remark that alternatively the instantaneous source could have been included as a term $\delta(x - h)\,\delta(t)$ in eqn. 1.

Diffusion of molecules into the solvent above the top of the bed is possible; however, if the flow-rate is positive, then these molecules would soon be moved into the bed by the solvent flow. The partial differential equation for the mobile phase molecule density A_tC above the top of the bed ($h < x < f$) involves flow and diffusion (A_t is the cross-sectional area of the column). By dividing this equation by the initial number of molecules I, we obtain the following differential equation for the probability density $p(x,t)$:

$$\frac{\partial p}{\partial t} = u_1\frac{\partial p}{\partial x} + D_1\frac{\partial^2 p}{\partial x^2} \tag{4}$$

Above the bed the velocity u_1 of the solvent is F/A_t and the diffusion constant of the molecules is D_1. Since no molecules can move above the top of the solvent, the molecular current is zero there; that is,

$$u_1 p(f,t) + D_1 \frac{\partial p}{\partial x}(f,t) = 0 \tag{5}$$

for $t \geqslant 0$.

The number of molecules and the current must be continuous at the top of the bed so that

$$p(h^-,t) = p(h^+,t)$$

$$u_1 p(h^+,t) + D_1 \frac{\partial p}{\partial x}(h^+,t) = up(h^-,t) + D\frac{\partial p}{\partial x}(h^-,t) \tag{6}$$

for $t > 0$. Precise incorporation of diffusion below the bottom of the bed would require a differential equation like eqn. 4 for the solute molecules below the bed and matching conditions similar to eqn. 6 at the bottom of the bed. It is more convenient to assume that the elution profile is measured at the bottom of the bed ($x = 0$) and that there is an absorber (sink) there. This corresponds to $A_0 C(0,t) = 0$ or

$$p(0,t) = 0 \tag{7}$$

for $t \geqslant 0$. This condition is reasonable since the rate of movement by flow of solute molecules in the solution below the bed is large compared with the rate of movement of these molecules in the bed. This model is also applicable to affinity chromatography in which ligands, covalently bound to the surfaces of impenetrable beads, interact monovalently with solute molecules. In that case the equilibrium and rate constants describe the binding reaction.

THE MOMENTS OF THE ELUTION PROFILE

Rather than attempting to solve the initial-boundary value problem (eqns. 1–7), we show how the model can be used to obtain ordinary differential equations with boundary conditions for the mean and variance of the passage time. The mean passage time $T_1(x)$ is defined to be the mean time for molecules starting in the initial layer to move past a position x[6,14,15]. The mean elution time $M_e = T_1(0)$ (also called the mean residence time or the mean retention time) is the mean time for molecules to move out of the bottom of the bed. Definitions of higher moments are similar. The fraction of molecules per unit time moving by position x in the bed at time t is the current $up(x,t) + D\frac{\partial p}{\partial x}(x,t)$. Thus, the jth moment of the passage time at position x is defined to be

$$T_j(x) = \int_0^\infty t^j \left[up(x,t) + D\frac{\partial p}{\partial x}(x,t) \right] dt \tag{8}$$

Mean passage time

Direct integration with respect to t on the interval $(0,\infty)$ of 1 times and t times the partial differential equations, and use of the boundary conditions, leads to the following boundary value problem for $T_1(x)$:

$$D_1\frac{d^2T_1}{dx^2} + u_1\frac{dT_1}{dx} = 0 \qquad h < x < f$$

$$D\frac{d^2T_1}{dx^2} + u\frac{dT_1}{dx} = -(1 + K) \qquad 0 < x < h \tag{9}$$

The boundary conditions in eqns. 5–7 convert to

$$T_1(f) = 0, \qquad \frac{dT_1}{dx}(0) = 0$$

$$T_1(h^+) = T_1(h^-), \qquad (1 + K)\frac{dT_1}{dx}(h^+) = \frac{dT_1}{dx}(h^-) \tag{10}$$

The solution for $0 \leqslant x \leqslant h$ of the boundary value problem (eqns. 9 and 10)

$$T_1(x) = (1 + K)\left[\frac{h - x}{u} - \frac{D}{u^2}(e^{-ux/D} - e^{-uh/D})\right] + \frac{D_1}{u_1 u}(1 - e^{-uh/D}) \times [1 - e^{-u_1(f - h)/D_1}] \tag{11}$$

so that the mean elution time is

$$M_e = T_1(0) = \frac{h}{u}\left\{(1+K)\left[1 - \frac{(1 - e^{-r})}{r}\right] + \frac{(1 - e^{-r})}{r_1}(1 - e^{r_1 - r_2})\right\} \tag{12}$$

where $r = uh/D$, $r_1 = u_1 h/D_1$ and $r_2 = u_1 f/D_1$.

Variance of the passage time

If the differential equations 4, 1 and 2 are multiplied by t^2 and integrated with respect to t on $(0,\infty)$, then the boundary value problem for the variance $S(x) = T_2(x) - T_1(x)^2$ is

$$D_1\frac{d^2S}{dx^2} + u_1\frac{dS}{dx} = -2D_1\left(\frac{dT_1}{dx}\right)^2 \qquad h < x < f$$

$$D\frac{d^2S}{dx^2} + u\frac{dS}{dx} = -2D\left(\frac{dT_1}{dx}\right)^2 - \frac{2K}{k_{-1}} \qquad 0 < x < h \tag{13}$$

$$S(f) = 0,\ S(h^+) = S(h^-), \qquad \frac{dS}{dx}(0) = 0$$

$$\frac{dS}{dx}(h^-) = (1 + K)\frac{dS}{dx}(h^+) + \frac{2K}{k_{-1}}\frac{dM}{dx}(h^+) \tag{14}$$

From the solution $S(x)$ the variance of the elution time is

$$S_e = S(0) = \frac{2Kh}{k_{-1}u}\left[1 - (1 - e^{-r})/r\right]$$

$$+ \frac{2(1 + K)^2h^2}{r\,u^2}\left[1 + 2e^{-r} - 2\frac{(1 - e^{-r})}{r} - \frac{(1 - e^{-2r})}{2r}\right]$$

$$+ \frac{2(1 + K)\,h^2}{r_1u^2}\left[1 - e^{r_1 - r_2}\right]\left[\frac{(1 - e^{-r})}{r} - 2e^{-r}\right] \tag{15}$$

$$+ \frac{h^2}{r_1^2u^2}(1 - e^{-r})^2(1 - e^{r_1 - r_2})^2$$

Although the mean elution time (eqn. 12) depends only on the sorption–desorption equilibrium constant K, the variance S_e in eqn. 15 depends on both K and the desorption rate constant k_{-1}. Special cases of some of the equations in this section were obtained by Weiss[16]. When diffusion is ignored ($D = 0$, $D_1 = 0$), then the mean and variance of the elution time are

$$M_e = (1 + K)\,h/u,\ S_e = 2Kh/(k_{-1}u) \tag{16}$$

Moments of the elution volume

An elution profile is a graph of the number of molecules eluted from the bottom of the bed as a function of time or of eluted solvent volume. For positive flow F, the mean elution volume V_e is defined to be the total volume of solvent eluted up to the mean elution time M_e. Since $K = V_p/V_0$, and the volume eluted up to time t is $V = Ft = V_0ut/h$, the following mean elution volume expression can be found from eqn. 16 when diffusion is neglected:

$$V_e = V_0 + V_p \tag{17}$$

Similarly the variance found from eqn. 16 when diffusion is neglected is

$$W_e = F^2S_e = 2FV_0\,K/k_{-1} \tag{18}$$

Note that the mean elution volume V_e does not depend on the flow-rate F, but the variance increases as the flow-rate increases. Eqn. 17 is formally identical to a stan-

dard chromatography equation; however, the V_e in the standard equation is the peak of the elution profile instead of the mean. (See later for further comparison with the local equilibration and elution profile peak approach).

Thick sample layer

If the effects of diffusion can be neglected and T is the thickness of the initial layer of sample, then the means and variances given by eqns. 16–18 become

$$M_e = (1 + K)h/u + T/(2u) \tag{19}$$

$$S_e = 2Kh/(k_{-1}u) + T^2/(12u^2)$$

$$V_e = V_0 + V_p + V_0T/(2h) \tag{20}$$

$$W_e = 2FV_0K/k_{-1} + V_0^2T^2/(12h^2)$$

Third central moment

The methods above can be used to calculate higher moments, but the results are complicated. *Neglecting diffusion* we find that the third central moment (*i.e.*, around the mean) of the elution profile as a function of time is

$$N_e = 6\ Kh/(k_{-1}^2\ u) \tag{21}$$

and as a function of volume is

$$U_e = 6F^2V_0K/k_{-1}^2 \tag{22}$$

Since the third central moment of the time for a layer of thickness T to enter the bead is zero, eqns. 21 and 22 hold for both thin and thick sample layers. When the flow-rate is such that diffusion is negligible, the moment coefficient of skewness for a thin sample layer is

$$G_1 = U_e/W_e^{3/2} = 3(F/2k_1V_0)^{1/2} \tag{23}$$

Thus, the skewness increases as the flow-rate F increases, as noted in numerical solutions[17].

RELATION TO PLATE HEIGHT THEORY

Since plate height terminology is still widely used, we now present and discuss our results in that notation. The spreading of solute molecules from a thin initial layer to a bell-shaped distribution along the bed at later times is called dispersion or zone spreading or band broadening. The causes of dispersion are the sorption–desorption kinetics (also called non-equilibrium effects) and diffusion in the mobile phase. Here we use diffusion (in the mobile phase) as a general term which includes longitudinal diffusion (molecular diffusion) and diffusion-related flow phenomena such as eddy diffusion and velocity profile heterogeneity.

The relative dispersion about the mean of the elution profile is the variance S_e divided by the square of the mean M_e. If diffusion above the bed is negligibly small, then from eqns. 12 and 15 we obtain

$$\frac{S_e}{M_e^2} = \frac{\alpha r}{1 - (1 - e^{-r})/r} + \frac{2[1 + 2e^{-r} - 2(1 - e^{-r})/r - (1 - e^{-2r})/2r]}{r[1 - (1 - e^{-r})/r]^2} \quad (24)$$

where $r = uh/D$ and

$$\alpha = 2KD/[h^2 k_{-1}(1 + K)^2] \quad (25)$$

The two terms in eqn. 24 correspond to the two causes of dispersion described above.

The height equivalent to a theoretical plate has been shown[1,8,18] to satisfy $H = hS_e/M_e^2$. Division by the bead diameter d_p leads to the reduced plate height

$$\hbar = hS_e/(d_p M_e^2) \quad (26)$$

which is dimensionless. Using eqn. 24, it can be shown that $\hbar$ equals $2(\alpha + 1/3)\, h/dp$ when $r = 0$, that it decreases to a minimum when $r \approx \sqrt{2/\alpha}$ and that if then increases. Near and beyond the minimum, a good approximation is

$$\hbar = 2/v + \alpha h/d_p + \alpha(h/d_p)^2 v \quad (27a)$$

where $v = rd_p/h = ud_p/D$ is a dimensionless quantity called the reduced velocity. For typical parameter values and typical reduced velocities ($v \gtrsim 0.01$), the second term in eqn. 27a is entirely negligible so that

$$\hbar \approx 2/v + \alpha(h/d_p)^2\, v \quad (27b)$$

Eqn. 27a is similar to the van Deemter equation[1,19] given by

$$\hbar = B/v + A + Cv \quad (28)$$

where the constants B, A and C are associated with axial molecular diffusion, eddy diffusion, and non-equilibrated mass transfer. However, eqn. 28 involves three undetermined parameters, B, A and C, whereas eqn. 27a has only one undetermined parameter, α. Many equations similar to eqn. 28 have been derived for the reduced plate height[20–23]. For example, the Knox equation[23] is

$$\hbar = B/v + Av^{1/3} + Cv \quad (29)$$

Although eqn. 24 is valid for $v \geqslant 0$, eqns. 27–29 are only reasonable near and beyond the minimum, since the plate height should be a constant when $v = 0$ and should not be infinite.

Data fitting

Plots of log h against log v have been obtained experimentally[1,21,23]. Using a

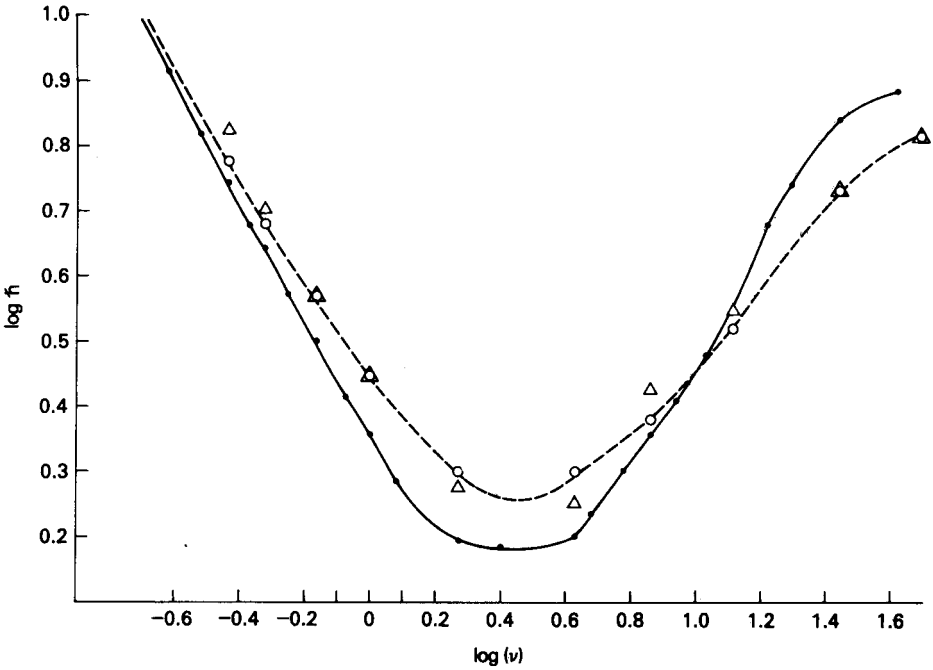

Fig. 1. Log–Log plot of reduced plate height h as a function of reduced velocity v. (-△-) are the experimental data points (ref. 21). The RMS error of the fits using the van Deemter equation (eqn. 28) and the Knox equation (eqn. 29) are virtually identical (dashed line). The best least-square parameter sets (A, B, C) are (0.83, 1.86, 0.17) for eqn. 28 and (0.62, 2.03, 0.13) for eqn. 29. The best least-square fit of eqn. 27b is shown by the solid line. The curve was generated using the experimental value $h/d_p = 3837$ and determining $\alpha(h/d_p)^2 = 0.26$ by a least-squares fit.

data set from Fig. 7 in ref. 21, we have determined the best fits of eqns. 27b, 28 and 29. The best least-square fits of the two empirical equations (28 and 29) are virtually identical in terms of root mean square error, although the parameter sets (A, B, C) that produce the fits are of course different for the two equations. Our eqn. 27b, derived from a basic chromatography model, gives a somewhat poorer fit, but it has only one adjustable parameter.

Interestingly, the best least-square parameter B in the Knox equation turns out to be 2, as predicted by our result. The strikingly close agreement is probably to some extent fortuitous. Nevertheless, the agreement suggests that the main reason for the difference between the data and the prediction based on eqn. 27b is the absence of velocity profile effects. As a check on this, we added a term $Av^{1/3}$ onto eqn. 27b, thus producing a two-parameter equation, and obtained a fit that was slightly better (in terms of RMS error) than that based on the Knox equation. One way of looking at these results is to regard eqn. 27b as providing a partial theoretical basis for the Knox equation, pinning down two of the three adjustable parameters in that equation to values determined by a molecular model.

Desorption rate constant related to bead diameter

Let v_m be the reduced velocity at which the minimum of h (or log h) occurs.

Since the minimum of eqn. 24 occurs when $r \approx \sqrt{2/\alpha}$, where α is given by eqn. 25, we obtain

$$k_{-1} \approx \frac{KDv_m^2}{(1 + K)^2 d_p^2} \tag{30}$$

From various plots of $\log h$ against $\log v$, it has been observed[23] that v_m is consistently *ca.* 3. Thus, eqn. 30 predicts that the desorption rate constant k_{-1} is inversely proportional to the square of the bead diameter d_p.

THE SIGNIFICANCE OF DIFFUSION

From eqns. 24 and 27 we see that diffusion contributes more to dispersion for low flow-rates and that non-equilibration of mass transfer contributes more for high flow-rates. For many chromatography systems an intermediate flow-rate is chosen so that the axial dispersion is minimized[21–23]. Here we choose a flow-rate (higher than the dispersion minimizing flow-rate) so that the effects of diffusion are negligible. This procedure allows us to use the information contained in the observed dispersion to estimate the sorption and desorption rate constants.

The expressions for the mean and variance of the elution time are particularly simple when the contribution of terms corresponding to diffusion are sufficiently small so that they can be neglected. From eqn. 12 we find the following bound for large flow-rate on the relative difference between the mean elution times with and without diffusion

$$\left| \frac{M_e - (1 + K)h/u}{(1 + K)\, h/u} \right| \leqslant \frac{1}{r} + \frac{1}{r_1 (1 + K)} \tag{31}$$

For large flow-rate F the relative magnitude of the terms corresponding to diffusion in eqn. 15 for the variance are given in the following inequality:

$$\left| \frac{S_e - 2Kh/(k_{-1}u)}{2Kh/(k_{-1}u)} \right| \leqslant \frac{1}{r} + \frac{k_{-1}h}{Ku} \left[\left| \frac{(1 + K)^2}{r} \right| + \frac{(1 + K)}{rr_1} + \frac{1}{2r_1^2} \right] \tag{32}$$

For positive K it is clear that the contributions to the mean elution time and to the variance of the diffusion-related terms can be neglected if the flow-rate (and hence u, u_1, r and r_1) is chosen large enough so that the right sides of eqns. 31 and 32 are small. Note that the above inequalities are also measures of the relative contributions due to diffusion for the mean and variance of the elution volume. Calculations using typical parameter values reveal that the contribution of diffusion above the bed is much less than the contribution of diffusion in the bed and that diffusion terms have a larger influence on the variance than on the mean.

Local equilibration and the elution profile peak

The widely used equation for the elution profile peak can be obtained by assuming local equilibration and negligible diffusion. If it is assumed that the mobile

phase and stationary phase molecules equilibrate locally for each fixed x, then $B(x,t) = C(x,t)$ so that $q(x,t) = Kp(x,t)$. In this case the sum of eqns. 1 and 2 reduces to

$$\frac{\partial p}{\partial t} = \frac{u}{1 + K}\frac{\partial p}{\partial x} + \frac{D}{1 + K}\frac{\partial^2 p}{\partial x^2} \tag{33}$$

The solution of the initial value problem for $-\infty < x < \infty$ consisting of eqn. 33 and $p(x,0) = \delta(x - h)$ is

$$p(x,t) = [4\pi Dt\,(1 + K)]^{-1/2} \exp\left[\frac{-[h - x - ut/(1 + K)]^2}{4\,Dt/(1 + K)}\right] \tag{34}$$

This solution has the form of a Gaussian distribution function which moves and spreads simultaneously. The elution profile $up(0,t) + D\dfrac{\partial p}{\partial t}(0,t)$ is not a Gaussian distribution as a function of t, and it is not symmetric around the peak which occurs at

$$T\,(\text{peak}) = \frac{(1 + K)h}{u}\left(1 - \frac{7}{3r} + \frac{91}{54r^2} + \ldots\right) \tag{35}$$

When diffusion is negligible ($1/r \ll 1$), the elution profile is approximately $\delta[h - x - ut/(1 + K)]$ and the peak is approximately equal to the mean (eqn. 16). Hence the corresponding formula $V(\text{peak}) = V_0 + V_p$ assumes both local equilibration and negligible diffusion while the formula $V_e = V_0 + V_p$ for the mean elution volume assumes only that diffusion is negligible. The local equilibration assumption is usually unreasonable since it implies that the variance is approximately zero. Differences between the peak and the mean are discussed further in ref. 5.

DISCUSSION

We outline a procedure for determining the sorption–desorption equilibrium constant K, the sorption rate constant k_1 and the desorption rate constant k_{-1} by liquid column chromatography. This procedure works for both small-zone and large-zone chromatography.

(1) Use eqns. 31 and 32 with estimates for the parameter values to choose the flow-rate F large enough so that the contributions of diffusion are negligible.

(2) Find the void volume V_0 experimentally by using the mean elution volume as V_0 when a weaker solvent or a non-retained larger molecule is run through the column[1].

(3) Find values of the mean volume V_e and the variance W_e of the elution profile by running the sample through the column.

(4) Calculate the equilibrium constant K from

$$K = V_p/V_0 = [V_e - V_0 - V_0T/(2h)]/V_0 \tag{36}$$

by using the values determined in steps 2 and 3. The equations in this step are

obtained from eqn. 20. Eqns. 19 would be used if the elution profile were given as a function of time. The term in eqn. 36 involving the sample thickness T can be omitted if the volume measurement is started when half of the sample has entered the column. Now calculate the desorption rate constant from

$$k_{-1} = 2F\,V_0K/[W_e - V_0^2T^2/(12h^2)] \tag{37}$$

The term involving the sample thickness T is always included but may be negligibly small if T is small. Of course, the sorption rate constant can now be found from $k_1 = k_{-1}\,K$.

(5) Use the values calculated above in eqns. 31 and 32 to verify that the contributions of diffusion were indeed negligible.

We emphasize that the sorption and desorption rates can be found by using the first and second moments, but only the equilibrium constant can be found from the first moment. The third central moment does not yield any new information; however, the observed third central moment can be used in eqn. 22 to check the estimates of K and k_{-1} obtained from the first two moments.

If the only constant of interest is the equilibrium constant (and not the rate constants), then the most accurate method of determining this value might be to choose a chromatography system so that the dispersion (band width) is minimized[21,23]. The dispersion can be made quite small by using optimum bed particle sizes, column diameters and lengths, flow-rates, pressures, sample dilutions and sample sizes in high-performance liquid chromatography[23]. In this case the mean and peak of the elution profile would essentially coincide so that the equilibrium constant could be easily estimated. We emphasize that if the goal is to also estimate the rate constants for movement in and out of the beads, then the procedure outlined at the beginning of this section should be followed. Estimates of the sorption and desorption rate constants are necessary for the quantitative analysis of more complicated systems that include chemical reaction[6,7].

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NON-EQUILIBRIUM MODEL OF LIQUID COLUMN CHROMATOGRAPHY

II. EXPLICIT SOLUTIONS AND NON-IDEAL CONDITIONS

CHARLES DeLISI*, HERBERT W. HETHCOTE* and JEFFREY W. BRETTLER
Laboratory of Mathematical Biology, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)
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SUMMARY

The analysis begun in the previous paper of a general model for liquid chromatography in a column is continued here. Explicit solutions are obtained for small-zone and large-zone non-equilibrium chromatography models both without and with diffusion. The effects on the moments of the elution profile of a distribution function which characterizes molecular heterogeneity (such as molecular size) or bead non-uniformities are analyzed. A first-order correction to the mean value of the elution profile when sorption–desorption kinetics are concentration dependent is derived.

Numerical simulations of the elution profile indicate the following. (1) The peak and mean may differ by as much as a factor of two for slow mass transfer ($k_1 \lesssim 0.01$). Since the mean is uniquely determined by the equilibrium constant but the peak is not, the use of the peak to characterize the equilibrium constant for broad asymmetric profiles may lead to serious errors. (2) When the rate of mass transfer from the void to penetrable volumes becomes comparable to u/h, a second peak will develop in the elution profile. This happens even for a completely homogeneous population under ideal conditions, and is caused by molecules that traverse the column without penetrating beads. The dispersion of this peak is therefore determined entirely by effects *other* than mass transfer. (3) In the non-linear regime (*i.e.*, when mass transfer rates are concentration dependent), the equilibrium constant is, in general, no longer uniquely determined by the mean. Uniqueness is, however, obtained in the limit as both mass transfer coefficients become very small, with their ratio remaining moderate.

INTRODUCTION

In the previous paper[1] we developed a non-equilibrium theory of chromatography which included diffusion, and we showed how the sorption–desorption kinetic parameters can be estimated from the moments of the elution profile. Expressions

* Present address: Department of Mathematics, University of Iowa, Iowa City, IA 52242, U.S.A.

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were derived for the mean, variance and skewness of the elution profile under ideal conditions of homogeneous molecules, uniform bead packing, and size- and concentration-independent rate constants. Here we extend that theory by considering other aspects of liquid column chromatography including nonideal conditions. The fundamental results derived here and in ref. 1 provide a basis for further quantitative development of chromatography as a method for thermodynamic and kinetic characterization of chemical reactions[2,3].

Solutions involving modified Bessel functions are given for both small-zone and large-zone chromatography models without diffusion. These solutions, together with a Gaussian kernel, are used to obtain solutions to a chromatography model with diffusion. Numerical simulations of the elution profile indicate that the peak and mean may differ by as much as a factor of two for slow mass transfer into the beads. Since the mean is uniquely determined by the sorption–desorption equilibrium constant, but the peak is not, the use of the peak to characterize the equilibrium constant for broad asymmetric profiles can lead to serious errors. For some low transfer rates the profile has two peaks. This happens even for homogeneous molecules under ideal conditions and is caused by molecules that traverse the bed without penetrating the beads. The dispersion in the first peak is determined by effects other than mass transfer.

We then consider heterogeneity or non-uniformity in the bed and in the molecules. When equilibrium constants are distributed, the expressions for the mean elution time involve the average equilibrium constant (or the average penetrable volume). If the sorption rate is constant, then the variance of the elution profile is proportional to the sum of the square of the average equilibrium constant and the variance of the distribution. A chromatography model with non-linear sorption–desorption kinetics is considered. When the mass transfer rates are concentration dependent, the sorption–desorption equilibrium constant is no longer uniquely determined by the profile mean. However, uniqueness is obtained in the limit as both mass transfer coefficients become small, with their ratio remaining moderate. Graphs reveal the remarkable dependence of the mean of the elution profile for the non-linear model on the mass transfer rates.

A CHROMATOGRAPHY MODEL WITHOUT DIFFUSION

Solutions for a very thin solute zone

The model discussed in ref. 1 consisted of the following diffusion–reaction–convection system

$$\frac{\partial p}{\partial t} = D\frac{\partial^2 p}{\partial x^2} + u\frac{\partial p}{\partial x} - k_1 p + k_{-1} q \tag{1}$$

$$\frac{\partial q}{\partial t} = k_1 p - k_{-1} q \tag{2}$$

together with appropriate boundary and initial conditions, where p and q are the probabilities per unit column length of finding solvent molecules at position x at time t, D is the diffusion constant, u is the convection velocity, and k_1 and k_{-1} are the rate

constants for penetrance of, and exit from, the beads. The bottom of the bed is at $x = 0$ and the top is at $x = h$ (see ref. 1 for notation). These equations were solved for quantities related to the moments of the density functions p and q. In particular, we obtained expressions for the mean, variance and skewness of the elution profile.

To obtain an expression for the entire profile, first consider eqns. 1 and 2 without diffusion ($D = 0$) subject to initial conditions

$$p(x,0) = \delta(x - h)$$
$$q(x,0) = 0 \qquad x \neq h \tag{3}$$

The number of molecules and the current must be continuous at the top of the bed so that

$$p(h,t) = 0 \qquad t > 0 \tag{4}$$

Instead of including the instantaneous source in the initial conditions (eqn. 3), it could have been included in eqn. 1 as a term $\delta(x - h)\,\delta(t)$ or in the boundary condition (eqn. 4) as $p(h, t) = \delta(u, t)$ for $t \geqslant 0$.

For the purpose of finding a solution, we assume that there is no solute buildup at the bottom of the bed; *i.e.*, we assume that flow continues as though the bed were extended below $x = 0$. We thus take the eluted current (molecules per unit time) at the bottom of a bed of height h as $uA_0C(0,t) = uIp(0,t)$. In this equation, I is the total number of solute molecules and $p(x,t)$ is the solution of the initial boundary value problem with a uniform bed on the semi-infinite interval $-\infty < x \leqslant h$, with p approaching zero as x approaches $-\infty$.

Now define $\Delta = ut + x - h$ and $\varrho = (k_1\, k_{-1}/u^2)(h - x)$; then the solution (Appendix A) of eqns. 1–4 is

$$p(x,t) = \exp[-k_1(h - x)/u][\delta(\Delta) + H(\Delta) \exp(-k_{-1}\Delta/u) \sqrt{\varrho/\Delta}\, I_1(2\sqrt{\varrho\Delta})] \tag{5}$$

$$q(x,t) = (k_1/u) \exp[-k_1(h - x)/u]\, H(\Delta) \exp(-k_{-1}\Delta/u)\, I_0(2\sqrt{\varrho\Delta}) \tag{6}$$

where the Heaviside function $H(\Delta)$ is 0 for $\Delta < 0$ and 1 for $\Delta \geqslant 0$, and the symbols I_0 and I_1 are modified Bessel functions[4]. Thomas[5] found similar solutions and also found asymptotic approximations. Giddings and Eyring[6] and Giddings[7] obtained similar solutions using a stochastic (random walk) approach.

Solutions for a large solute zone

A large zone corresponds to an initial layer of macromolecules which is thick enough so that it cannot be considered an instantaneous source. To obtain a solution for this case we again begin with the initial boundary value problem without diffusion. The partial differential equations in the bed are eqns. 1 and 2 with $D = 0$, and the initial conditions are

$$p(x,0) = 0 \qquad q(x,0) = 0 \qquad 0 \leqslant x < h \tag{7}$$

If the thickness of the initial layer of molecules is T, then the boundary condition at the top of the bed is

$$p(h,t) = [1 - H(ut - T)]/T \tag{8}$$

for $t \geqslant 0$.

The following solutions of the initial boundary value problem described above are found by Laplace transforms (Appendix A).

$$p(x,t) = \begin{cases} 0 & \Delta < 0 \\ \dfrac{1}{T}\exp\left[\dfrac{-k_1(h-x)}{u}\right]\left[1 + \displaystyle\int_0^{\Delta} \exp\left(\frac{-k_{-1}w}{u}\right)\sqrt{\frac{\varrho}{w}}\, I_1(2\sqrt{\varrho w})\, dw\right] & 0 \leqslant \Delta \leqslant T \\ \dfrac{1}{T}\exp\left[\dfrac{-k_1(h-x)}{u}\right]\left[\displaystyle\int_{\Delta - T}^{\Delta} \exp\left(\frac{-k_{-1}w}{u}\right)\sqrt{\frac{\varrho}{w}}\, I_1(2\sqrt{\varrho w})\, dw\right] & \Delta \geqslant T \end{cases} \tag{9}$$

$$q(x,t) = \begin{cases} 0 & \Delta \leqslant 0 \\ \dfrac{k_1}{Tu}\exp\left[\dfrac{-k_1(h-x)}{u}\right]\displaystyle\int_0^{\Delta} \exp\left(\frac{-k_{-1}w}{u}\right) I_0(2\sqrt{\varrho w})\, dw & 0 \leqslant \Delta \leqslant T \\ \dfrac{k_1}{Tu}\exp\left[\dfrac{-k_1(h-x)}{u}\right]\displaystyle\int_{\Delta - T}^{\Delta} \exp\left(\frac{-k_{-1}w}{u}\right) I_0(2\sqrt{\varrho w})\, dw & \Delta \geqslant T \end{cases} \tag{10}$$

As T approaches 0, eqns. 9 and 10 approach eqns. 5 and 6. Using eqn. 9 in the definitions of the moments in ref. 1, we find that the mean and variance of the elution profile agree with eqns. 19 and 20 in ref. 1.

A MODEL WITH DIFFUSION

The procedure for finding a solution including diffusion can be understood best by considering a molecular interpretation of eqns. 5 and 6. In particular, we note that $p(x,t)$ could have been obtained by finding the probability that a molecule at (x,t) had moved freely for a total time $\tau \leqslant t$, multiplying that probability by the conditional probability that a molecule moving for time τ will be at x, and then integrating over τ[6,7]. In the absence of diffusion, the kernel in the integrand, *i.e.*, the probability that a molecule moving freely for total time τ is at x is $\delta(\tau - (h - x)/u)$ since motion by convection is completely deterministic. This procedure uncouples reaction from movement down the bed; something that can always be achieved for a linear system.

If now, rather than allowing movement only by convection, we include diffusion, then the kernel will be Gaussian. In particular, we take

$$G(x,\tau) = (4\pi D\tau)^{-1/2} \exp[-(u\tau + x - h)^2/4D\tau] \tag{11}$$

where $G(x,\tau)$ is the probability that a molecule having moved freely for $\tau \leqslant t$, will be

at x. The diffusion constant D should be interpreted broadly so that it not only includes the simple Brownian diffusion one would find in a homogeneous medium, but also includes eddy diffusion and velocity profile effects[1].

To find the probability density $P(x,t)$ for free molecules at (x,t), eqn. 11 must be multiplied by the probability that a molecule, having moved freely for $\tau \leqslant t$, will be free at (x,t) and the product must then be integrated over τ from 0 to t. The probability density $Q(x,t)$ for bound molecules at (x,t) is found similarly. But we already know the solutions for the reactive probabilities since eqns. 5 and 6 are simply those solutions integrated over a δ-function kernel. Hence we find that the solutions to the model with diffusion are

$$P(x,t) = \int_0^t G(x,\tau)\, p(h - u\tau,t)u d\tau \tag{12}$$

$$Q(x,t) = \int_0^t G(x,\tau)\, q(h - u\tau,t)u d\tau \tag{13}$$

More explicitly,

$$P(x,t) = G(x,t)e^{-k_1 t} + \int_0^t (4\pi D\tau)^{-1/2} \exp\left[- \frac{(u\tau + x - h)^2}{4D\tau} - k_1\tau - k_{-1}(t - \tau)\right] \times \sqrt{\frac{k_1 k_{-1}}{u^2}\frac{\tau}{t - \tau}}\, I_1\left(2\sqrt{k_1 k_{-1}\tau(t - \tau)}\right) u d\tau \tag{14}$$

$$Q(x,t) = k_1 \int_0^t (4\pi D\tau)^{-1/2} \exp\left[- \frac{(u\tau + x - h)^2}{4D\tau} - k_1\tau - k_{-1}(t - \tau)\right] \times I_0\left(2\sqrt{k_1 k_{-1}\tau(t - \tau)}\right) d\tau \tag{15}$$

The functions P and Q above satisfy the differential equations 1 and 2, the initial conditions (eqn. 3) and boundary conditions $P(\pm\infty,t) = 0$, $Q(\pm\infty,t) = 0$.

The main approximation in eqns. 14 and 15, for which we expect the error to be negligible, is that they hold on the interval $-\infty < x < \infty$, whereas the chromatographic bed only occupies $0 \leqslant x \leqslant h$. The contribution from molecules that move above h is expected to be only a second-order effect, since only a small fraction of solute will ever be above h when movement down the bed is dominated by convection. Similarly we expect the concentration of molecules just above $x = 0$, with no bed beneath $x = 0$, to be essentially the same as it would if the bed continued below $x = 0$. Approximate solutions to the large-zone problem with diffusion can be found as above by using eqns. 9 and 10 and the Gaussian kernel (eqn. 11).

PEAKS AND MEANS OF THE ELUTION PROFILE

The number of molecules per second leaving the bottom of the bed is the elution profile $uP(0,t) + D\dfrac{\partial P}{\partial x}(0,t)$ with $P(x,t)$ given by eqn. 14. From this elution

profile we can assess the approximation involved in using the peak of the profile rather than the mean to obtain the equilibrium constant.

A number of investigators have shown[1,8–10] that the mean depends only on the equilibrium constant $K = k_1/k_{-1}$. On the other hand, the peak is not uniquely determined by K, but depends on k_1 and k_{-1} separately. This is shown clearly in Fig. 1, where the time at which the peak occurs is plotted as a function of k_1. The parameters in this example are chosen so that the mean always occurs at 2000 sec. The peak and mean differ by less than 5% for $k_1 \geqslant 0.015$ sec^{-1}, but as k_1 drops the peak decreases rapidly so that it is within a few percent of its limiting value of 1000 sec at $k_1 = 0.002$ sec^{-1}. In the low k_1 limit, the mass transfer rate k_1 is sufficiently low relative to the time for a mobile molecule to traverse the bed that essentially no adsorption occurs.

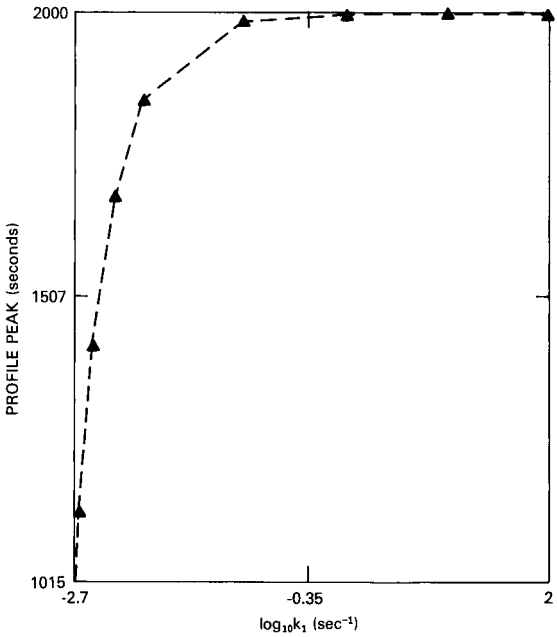

Fig. 1. The profile peak position is a function of the mass transfer rate (k_1 or k_{-1}) with K held constant. The profile mean, on the other hand, is determined only by K. The mean is at 2000 sec, $K = 1$, $h/u = 1000$ sec, $D = 0$.

As the low k_1 limit is approached an interesting effect arises: the profile begins to develop a second peak (Fig. 2). Mathematically, this peak arises from the first term in eqn. 14; physically it represents those particles that traverse the bed with no adsorption. The theory predicts that such a peak can be produced or eliminated by changing h/u so that it either approximates or exceeds $1/k_1$. The rate constant k_1 can be determined by methods discussed in ref. 1. Under usual circumstances one would want to perform separations so that an "artifactual peak" does not arise. However, if the second peak can be produced as readily as the theory suggests, it might be useful. Its breadth is free from mass transfer contributions and it therefore allows an assess-

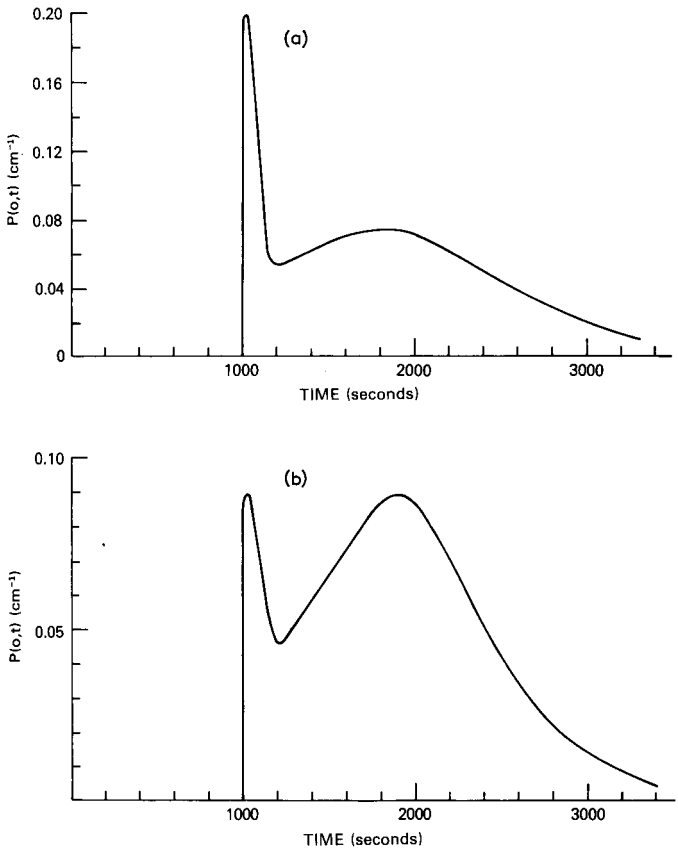

Fig. 2. The elution profile of a completely homogeneous molecular population under ideal conditions becomes bimodal when the rate of mass transfer from the void volume to interior bead volume is comparable to u/h. $D = 10^{-6}$ cm^2/sec, $u = 0.01$ cm/sec, $h = 10$ cm, $K = 1$. (a) $k_1 = 4 \times 10^{-3}$ sec^{-1}; (b) $k_1 = 5 \times 10^{-3}$ sec^{-1}.

ment of the relative contribution of diffusion and mass transfer to the overall profile dispersion.

HETEROGENEITY

The models formulated and analyzed here and in ref. 1 assume that the molecules are uniform in size, structure and weight and that the beads (gel particles are uniform in their packing, size and structure. Here we examine the effect of nonuniformities on the moments of the passage time. Assume that the equilibrium constant K is distributed with probability density function $n(K)$. Note that the distribution of K could be due to a distribution of k_1 or of k_{-1} or of both. The average over the distribution of K of the jth moment of the passage time at position x is defined as

$$T_j = \int_0^\infty n(K) \int_0^\infty t^j [up(x,t;K) + D\frac{\partial p}{\partial x}(x,t;K)] \, dt \, dK \tag{16}$$

For a small-zone experiment for which diffusion is negligible, the analogs of the expressions in ref. 1 for the moments are

$$\bar{T}_e = (1 + \bar{K})h/u, \qquad \bar{S}_e = 2\overline{(K/k_{-1})}h/u \tag{17}$$

$$\bar{V}_e = V_0 + \bar{V}_p, \qquad \bar{W}_e = 2FV_0\,\overline{(K/k_{-1})} \tag{18}$$

where $\bar{K} = \bar{V}_p/V_0$ and $\overline{(K/k_{-1})}$ is the expected value of K/k_{-1}.

If the main sources of equilibrium constant heterogeneity are non-uniformities in the beads or in their packing, then the average equilibrium constant $\bar{K}$ and the average penetrable volume $\bar{V}_p$ depend only on the column conditions. As long as the same column is used under the same conditions (for example, during molecular weight calibration and determination), this non-uniformity does not significantly affect the results since it is the same for all molecules passing through the column.

If the main sources of equilibrium constant heterogeneity are non-uniformities in the size, structure or weight of the molecules, then $\bar{K}$ and $\bar{V}_p$ are averages over these molecules. If the sorption rate k_1 is always the same for the molecules, but the desorption rate k_{-1} varies because of differences in the molecules, then $\overline{(K/k_{-1})} = \overline{K^2}/k_1$. In this case, if k_1 is known (*e.g.*, from another experiment), then the second moment $\overline{K^2}$ can be estimated from the observed variance of the elution profile. Hence the variance W_e of the elution profile satisfies

$$W_e = \frac{2FV_0\,\overline{K^2}}{k_1} = \frac{2FV_0}{k_{-1}}\{(\bar{K})^2 + [\overline{K^2} - (\bar{K})^2]\} \tag{19}$$

where the first term in the brackets is the square of the mean of the equilibrium constant distribution and the second term is the variance. Thus if k_1 is constant, both the means and the variances of the distribution can be estimated.

NON-LINEAR KINETICS

Consider the chromatography model without diffusion for a thin solute zone described earlier. Assume that the sorption–desorption kinetic rates decrease near saturation, *i.e.*, the kinetics are no longer linear. Then the eqns. 1 and 2 become

$$\frac{\partial p}{\partial t} = u\frac{\partial p}{\partial x} - k_1 p(1 - C/C^m) + k_{-1}q(1 - B/B^m) \tag{20}$$

$$\frac{\partial q}{\partial t} = k_1 p(1 - C/C^m) - k_{-1}q(1 - B/B^m) \tag{21}$$

where the parenthesized factors reflect saturation effects. The mobile and stationary phase concentrations C and B are less than the corresponding maximum concentrations C^m and B^m. Since $p = CA_0/I$ and $q = BA_p/I$, we find

$$C/C^{m} = ph(I/C^{m}A_0h) \tag{22}$$

$$B/B^{m} = qh(I/B^{m}A_ph) \tag{23}$$

where A_0h and A_ph are the void and penetrable volumes, respectively. If the total number of molecules I is much less than the molecular capacities $C^{m}A_0h$ and $B^{m}A_ph$, then a suitable small parameter is

$$\varepsilon = I/C^{m}A_0h \tag{24}$$

Since $K = V_p/V_0$, then

$$I/B^{m}A_ph = \varepsilon C^{m}V_0/B^{m}V_p = \varepsilon a/K \tag{25}$$

where $a = C^{m}/B^{m}$ is a constant near 1 since the saturation concentration C^{m} and B^{m} are nearly equal.

Let the expansion of p and q in powers of ε be

$$\begin{aligned} p &= p_0 + p_1\varepsilon + p_2\varepsilon^2 + \ldots \\ q &= q_0 + q_1\varepsilon + q_2\varepsilon^2 \ldots \end{aligned} \tag{26}$$

Substituting eqn. 26 into eqns. 20 and 21 modified to include ε by using eqns. 22–25, and equating the powers of ε, we find that p_0 and q_0 are given by eqns. 5 and 6 and p_1 and q_1 satisfy

$$\begin{aligned} \frac{\partial p_1}{\partial t} &= u\frac{\partial p_1}{\partial x} - k_1(p_1 - hp_0^2) + k_{-1}\left(q_1 - \frac{ah}{K}q_0^2\right) \\ \frac{\partial q_1}{\partial t} &= k_1(p_1 - hp_0^2) - k_{-1}\left(q_1 - \frac{ah}{K}q_0^2\right) \\ p_1(x,0) &= 0,\ q_1(x,0) = 0,\ p_1(h,t) = 0 \end{aligned} \tag{27}$$

The first-order approximation to the mean elution time is

$$M_e = \int_0^\infty tu[p_0(0,t) + \varepsilon p_1(0,t)]\mathrm{d}t = (1 + K)(h/u) + \varepsilon\int_0^\infty tup_1(0,t)\mathrm{d}t \tag{28}$$

Following the procedure in the section "The moments of the elution profile" in ref. 1, we find

$$\int_0^\infty tup_1(x,t)\mathrm{d}t = -\int_x^h [K\int_0^\infty hp_0^2(w,t) - \frac{a}{K}\int_0^\infty hq_0^2(w,t)\mathrm{d}t]\mathrm{d}w \tag{29}$$

Substituting eqns. 5 and 6 for p_0 and q_0 in eqn. 29 and using identities involving integrals and series[11], we obtain

$$\int_0^\infty tup_1(0,t)\mathrm{d}t = -(h/u)\left[\frac{2+\beta}{4\beta}(1-\mathrm{e}^{-2\alpha}) - \alpha\mathrm{e}^{-2\alpha} + \frac{1}{2}\int_0^\alpha \mathrm{e}^{-w}I_0(w)\mathrm{d}w - \left(\frac{\alpha}{2}\right)\mathrm{e}^{-2\alpha}I_1^2(\sqrt{2\alpha}) - \frac{a}{2}\int_0^\alpha \mathrm{e}^{-w}I_0(w)\mathrm{d}w\right] \tag{30}$$

where $\alpha = k_1 h/u$ and $\beta = k_{-1}h/u$.

Numerical evaluation of the integral in eqn. 30 indicates that it is well approximated, over a wide range of α, by

$$\int_0^\alpha \mathrm{e}^{-w}I_0(w)\mathrm{d}w \cong -0.1895 + 0.855\sqrt{\alpha} \tag{31}$$

In particular, this approximation holds to within 6% at $\alpha = 0.2$; 1% at $\alpha = 1$, and it becomes increasingly better as α increases. Therefore eqn. 28 can be written as

$$M_e = (1+K)(h/u) - \varepsilon(h/u)\left\{\frac{2+\beta}{4\beta}(1-\mathrm{e}^{-2\alpha}) - \alpha\mathrm{e}^{-2\alpha} - \frac{\alpha}{2}\mathrm{e}^{-2\alpha}I_1^2(\sqrt{2\alpha}) - \frac{1}{2}(1-a)(0.1895 - 0.855\sqrt{\alpha})\right\} \tag{32}$$

If α and β are small, with K remaining moderate, the result simplifies to

$$M_e \cong (1+K)(h/u) - \frac{\varepsilon hK}{u} = [1 + K(1-\varepsilon)](h/u) \tag{33}$$

Combining eqns. 28 and 30 we find that the first-order approximation to the relative error in neglecting the non-linear kinetics is given by

$$\left|\frac{M_e - (1+K)(h/u)}{(1+K)(h/u)}\right| \leqslant \frac{\varepsilon}{1+K}\left[\frac{2+\beta}{4\beta} + (1+\mathrm{a})\sqrt{\frac{\alpha}{2\pi}}\right] \tag{34}$$

Eqn. 32 predicts that in the non-linear regime, the elution profile mean depends separately on the mass transfer rates k_1 and k_{-1}, not just on their ratio K. Thus with K held fixed, changes in $\alpha = k_1 h/u$ can cause changes in the mean (see Fig. 3). The percentage difference between the non-linear and linear means is usually small, generally not more than 100ε. The maximum percentage difference between the non-linear and linear means is shown in Fig. 4. Note that the linear mean is usually larger than the non-linear mean. More interesting are the maxima and minima that occur in some parts of Fig. 3. Fig. 3 clearly illustrates that when the sorption–desorption kinetics are non-linear, the mean is not uniquely determined by the equilibrium constant K, but depends on the sorption and desorption rate constants k_1 and k_{-1}.

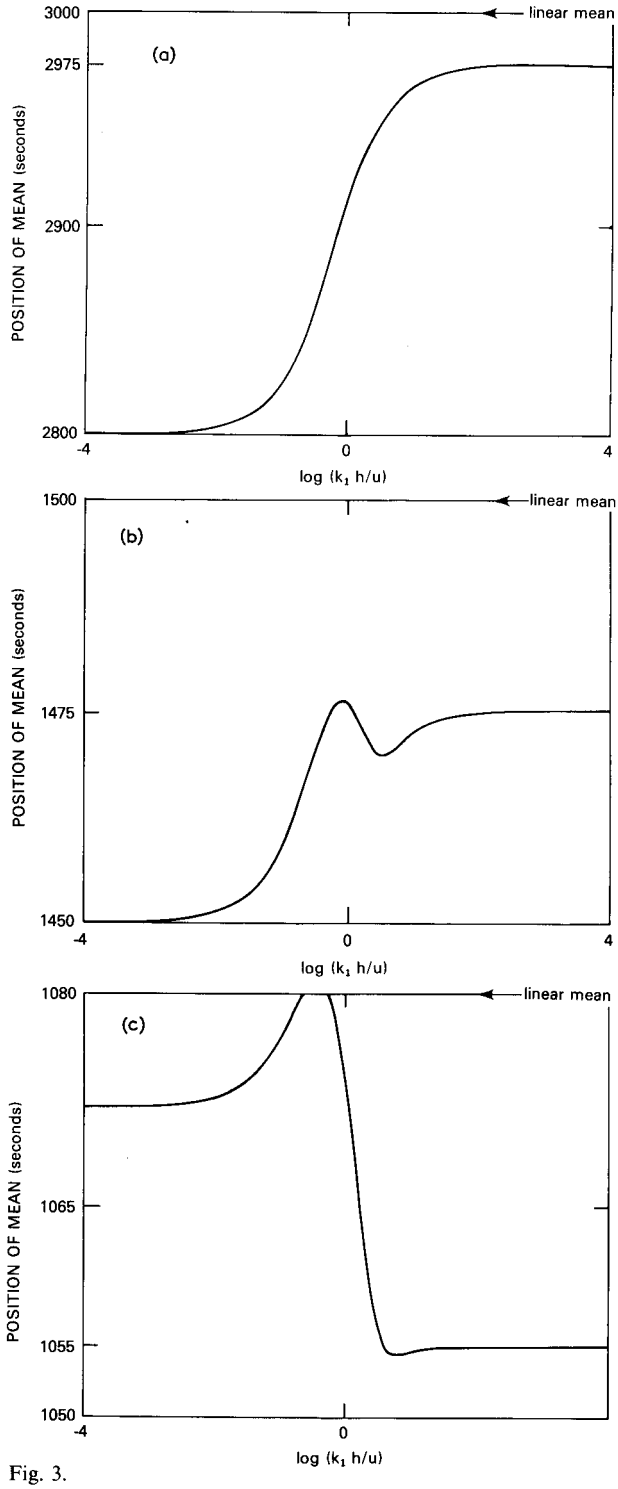

Fig. 3.

(Continued on p. 294)

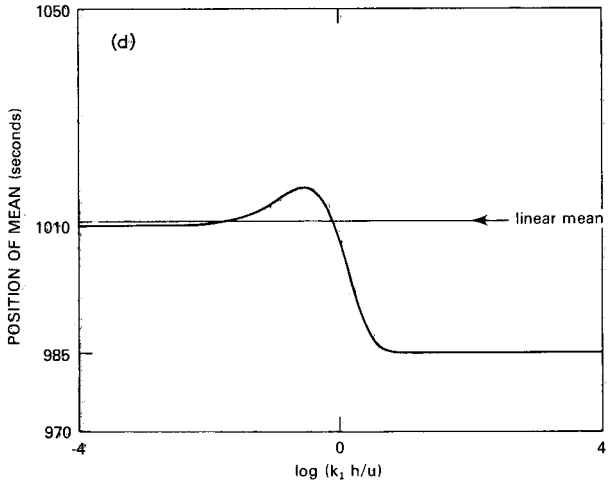

Fig. 3. The position of the mean in the non-linear problem is not uniquely determined by K. a = 1 and $\varepsilon = 0.1$ in all figures. (a) $K = 2$, (b) $K = 0.5$, (c) $K = 0.08$, (d) $K = 0.01$.

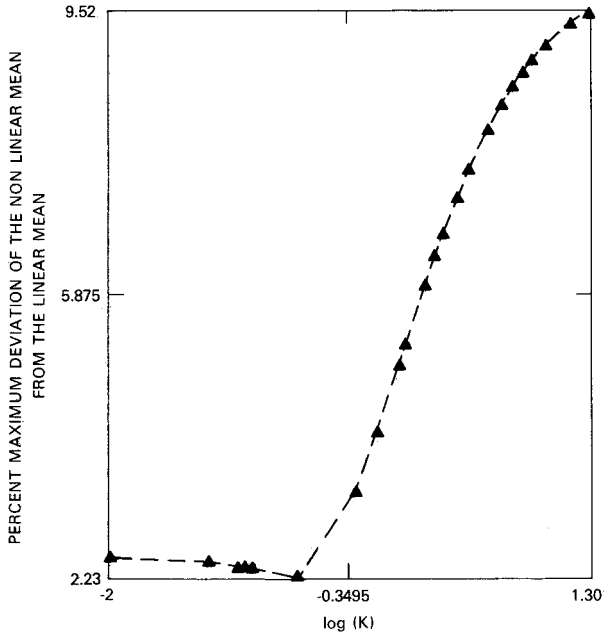

Fig. 4. Maximum deviation between the linear and non-linear means as a function of K. At high K, the maximum deviation is 10% (100ε); at low K it is 2.5%.

APPENDIX A: DERIVATION OF EXPLICIT SOLUTIONS

Let $Lp(x,s)$ and $Lq(x,s)$ be the Laplace transforms of $p(x,t)$ and $q(x,t)$. From eqns. 1 and 2 we find that

$$u\frac{\partial Lp}{\partial x} - \left(s + k_1 - \frac{k_1 k_{-1}}{s + k_{-1}}\right) Lp = -\delta(x - h),\ Lp(h,s) = 0 \tag{35}$$

$$Lq(x,s) = k_1 Lp/(s + k_{-1}) \tag{36}$$

The solution of the initial value problem (eqn. 35) is

$$Lp(x,s) = \frac{1}{u} \exp\left[\frac{-k_1(h - x)}{u} - \frac{s(h - x)}{u} + \frac{k_1 k_{-1}(h - x)}{u(s + k_{-1})}\right] \tag{37}$$

We will need the following Laplace transform formulas[12]:

$$e^{a/s} = L\{(a/t)^{1/2} I_1 [2(at)^{1/2}] + \delta(t)\} \tag{38}$$

$$e^{a/s}/s = L[I_0 (2at)^{1/2}] \tag{39}$$

Using eqns. 36–39 with the usual rules for Laplace transforms, we find the solution given by eqns. 5 and 6.

For the large-zone model

$$Lp(x,s) = \frac{(1 - e^{-Ts/u})}{Ts} \exp\left[\frac{-k_1(h - x)}{u} - \frac{s(h - x)}{u} + \frac{k_1 k_{-1}(h - x)}{u(s + k_{-1})}\right] \tag{40}$$

Using eqns. 36 and 38–40 and standard Laplace transform formulas, eqns. 9 and 10 are obtained.

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CERENKOV PHOTON ABSORPTION DETECTOR FOR LIQUID CHROMATOGRAPHY

I. PRELIMINARY EXPERIMENTS

DAVID J. MALCOLME-LAWES*
Chemistry Department, King's College London, Strand, London WC2 (Great Britain)
and
PETER WARWICK
Chemistry Laboratory, Loughborough University of Technology, Leicestershire (Great Britain)
(Received January 18th, 1982)

SUMMARY

An high-performance liquid chromatographic detector system based on the absorption of UV photons produced by the Cerenkov effect is discussed. A simple flow cell constructed around a standard commercial source of strontium-90 is described and a number of example chromatograms, recorded by monitoring the Cerenkov light intensity at 254 or 200 nm, are presented.

INTRODUCTION

During the last decade there has been a dramatic increase in the use of high-performance liquid chromatography (HPLC) as an analytical technique. The technology associated with pumping systems, column packing materials and detectors has improved considerably over this period, and the last few years in particular have seen the birth of a number of ideas which have led to new principles being applied to each of these components of the chromatographic system[1]. In spite of this the most popular form of detection system has remained the UV absorption monitor, generally based on a mercury or deuterium lamp source. While not the most sensitive detection system for some compounds, the UV absorption detector combines the advantages of relative simplicity and good sensitivity for a large number of compounds. However the conventional UV absorption detector does have some disadvantages. For example, lamp sources are prone to both short-term and long-term intensity fluctuations, requiring dual-beam operation and expensive power supplies for stability at moderate sensitivities. Mercury lamps are generally only useful for detectors operating above 254 nm, and, although deuterium lamps are used in detectors which operate down to 190 nm, these lamps do tend to have unpredictable lifetimes.

In this paper we describe some preliminary experiments with a new type of absorption detector which overcomes the difficulties mentioned above, which can be

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used over a wide range of wavelengths and which has no lamp to fail. The principle of operation of the detector is discussed in the section below, followed by a description of the experimental detection system constructed at Loughborough. Finally some examples of results obtained using the detection system are given for reversed-phase chromatography.

THE PHOTON SOURCE

In the present detector system photons are produced by the Cerenkov effect[2] from energetic electrons resulting from the decay of a radioisotope. The Cerenkov effect is based on the fact that when electrons travel through a medium with a velocity greater than the velocity of light in that medium, the electrons lose energy by generating photons (Cerenkov photons) until the electron velocity is lower than the velocity of light in that medium. The theory of the Cerenkov effect was developed on a classical basis by Frank and Tamm[3], and has been reviewed by Robin[4].

A radioactive material may be used to generate Cerenkov photons in a variety of ways. A β decay nuclide which emits electrons with an energy above the Cerenkov threshold (*ca.* 0.26 MeV in water, refractive index 1.33) may be used to generate Cerenkov photons by allowing the β particles to travel into a suitable transparent medium. Each β particle may give rise to a number of Cerenkov photons emitted in rapid succession and covering a range of wave-lengths (for example a 1 MeV β^- particle travelling through water will yield approximately 100 photons). An alternative technique is to use an energetic α decay nuclide which gives rise to energetic electrons when the α particles interact with matter. However, for the preliminary experiments reported below we have confined our attention to beta decay sources.

Any high-energy β decay radionuclide may be used for the production of Cerenkov photons, and the higher the β decay energy, the more photons are available. However, to be suitable for use in a liquid chromatography detector other criteria may be applied to the selection of the radioisotope. Firstly it is desirable that the nuclide chosen has a half-life of at least a year —so that frequent replacement of the source is not required— and yet be available at sufficiently high specific activity that a small volume source with a high β particle output may be produced. Secondly it is desirable that the β decay process of the source does not lead to any energetic γ-emitting daughter nuclides —as high energy γ radiation could give rise to problems of γ-induced fluroescence within the optical system and to radiation hazards for personnel operating the detector.

For our preliminary experiments we have chosen strontium-90 as the source of energetic electrons. ^{90}Sr is a β^- emitting nuclide with a half-life of 28 years and a short-lived β^- emitting daughter (^{90}Y) which decays to a stable nuclide (^{90}Z). ^{90}Sr is readily available at low cost and in a variety of forms from the Radiochemical Centre, Amersham, Great Britain. The source chosen in the present work was a 1 mCi "point source", mounted on a 10 × 2 mm diameter cylindrical stainless-steel holder and covered by a 50-μm stainless-steel window, which effectively protected the radioisotope from the solvents which passed over the window during some of the experiments. [The source was obtained from the Radiochemical Centre, Code Number SIF. 32].

The radioactive source was used to generate Cerenkov photons in a variety of

flow cells as described below. Photons which passed through the flow cell window were detected using a quartz window photomultiplier tube.

By placing interference filters between the flow-cell and the photomultiplier tube the Cerenkov photon flux at wavelengths of 210, 254 or 280 nm could be monitored, (bandwidth *ca.* 15 nm in each case).

THE EXPERIMENTAL SYSTEM

A schematic diagram of the experimental Cerenkov photon absorption (CPA) detector is shown in Fig. 1, and schematic detail of one of the flow cells is given in Fig. 2. The flow cell was machined from brass, having a 10 × 1.5 mm diameter flow hole closed at one end by the face of the strontium source. The other end of the flow cell was closed by a 20 mm diameter × 2 mm Spectrosil A window. Inlet and outlet tubes of 0.01-in. bore stainless-steel were soldered into the brass body of the cell.

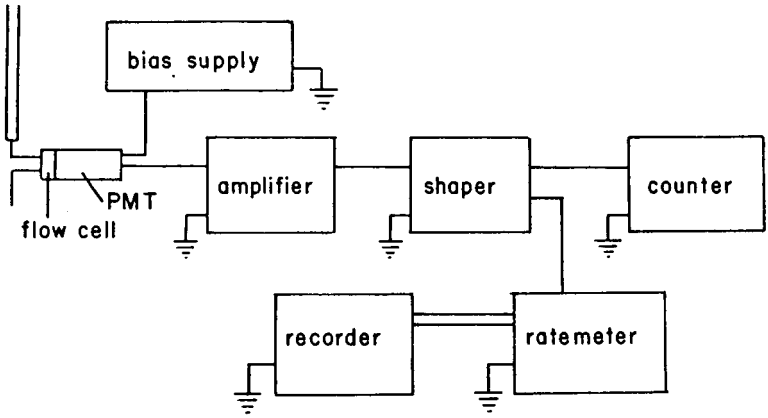


Fig. 1. Block diagram showing major components of CPA detector system. PMT = Photomultiplier tube.

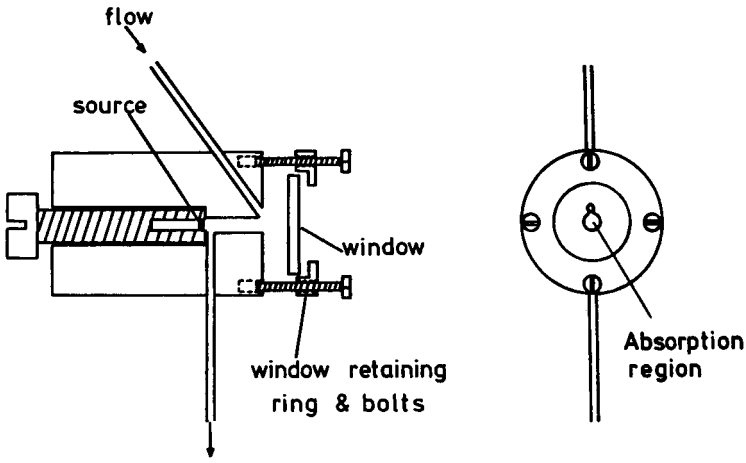


Fig. 2. Detail of CPA flow cell.

The interference filters used to select the wavelength monitored were supplied by Anachem (254-nm and 280-nm Altex filters) or loaned by A.C.S. (210-nm filter).

The quartz windowed photomultiplier tube (EMI type 9804QB) was operated with a grounded photocathode and an anode potential of 1400 V. An emitter follower preamplifier, built onto the tube base, was designed to allow pulses with half-widths of *ca.* 100 nsec to be passed to a fairly conventional nuclear pulse counting system[5] consisting of the following modules: Model 2110, timing filter amplifier; Model 1433, discriminator; Model 1776, scaler counter–timer; and Model 1481L, ratemeter; all obtained from Canberra Instruments. The ratemeter output signal (0–100 mV analogue) was passed through a low-pass filter to the chart recorder, and in most cases the filter was operated with a cut-off frequency of 0.2 Hz.

The technique of pulse counting is probably not the most desirable way of using the Cerenkov photon absorption detector, as many of the pulses detected correspond to multiphoton events. However the technique has been adopted for these preliminary experiments because of the low cost of obtaining precisely quantified data with apparatus already available in our laboratory[5].

The experimental detector system was connected to the outlet of a conventional liquid chromatography system. Columns of Spherisorb ODS and Spherisorb-amino were used in lengths and particle sizes as described below. Eluting solvents of methanol, acetonitrile (Fisons, HPLC grade), acetic acid and distilled water were used. In most cases the elution rate was adjusted to be 1 ml min^{-1}. Samples of a variety of materials were made up in solvents of methanol or water (as appropriate). As our purpose here is limited to demonstrating the detector operation, no specific effort was made to obtain especially purified materials; most of the compounds used were of standard laboratory reagent grade.

Samples were loaded onto the columns using a Rheodyne Model 7125 injection valve fitted with a 20-μl injection loop. This loop could be partially filled using a syringe, so that where calibrations of detector response *vs.* sample loading are presented, the different sample loadings have been calculated from the volume of a single solution injected into the loop.

In operation the prototype CPA detector produces a chart record showing a signal proportional to the number of pulses per second detected by the photomultiplier system. This number is simultaneously displayed on a digital ratemeter.

When photon absorbing material passes through the flow cell the recorded rate is lowered, and the chart record shows a chromatogram of conventional appearance. Quantitative detector responses reported below are given in terms of the difference between the number of pulses per second detected when only eluting solvent is present in the flow cell and the minimum number of pulses per second detected as the absorbing component elutes. This somewhat unorthodox way of presenting the results was chosen for the same reason that pulses per second were used in the early stages of β-induced fluorescence development[5], namely that this allows direct comparisons to be made between different radioactive sources and different flow cells without the risk of confusion arising through the variation of other instrumental parameters.

RESULTS

A selection of test materials was passed through the chromatograph fitted with

TABLE I

RESPONSES OF CPA DETECTOR WITH 254-nm FILTER TO TEST SAMPLES

Flow-rate *ca.* 1 ml min^{-1}, solvent methanol.

Sample	*Amount* (μg)	*Peak response* (sec^{-1})
Carbazole	1	4430
Ascorbic acid	2	1300
Salicyclic acid	2	650
Indole	2	4900
Aniline	2	1750
9-Amino acridine	2	3350
Chlorobenzene	2	2100
p-Dichlorobenzene	2	2300
4-Chlorotoluene	2	2800

a 15 × 0.5 cm 5-μm Spherisorb ODS column using methanol as solvent. When solvent alone is passing through the CPA detector the pulse count rate recorded through the 254-nm filter was *ca.* 8100 sec^{-1}. The detector response obtained for each test material is given in Table I.

Further examples of detector operation were obtained by recording analogue chromatograms for a number of mixed samples. Fig. 3 shows the separation of

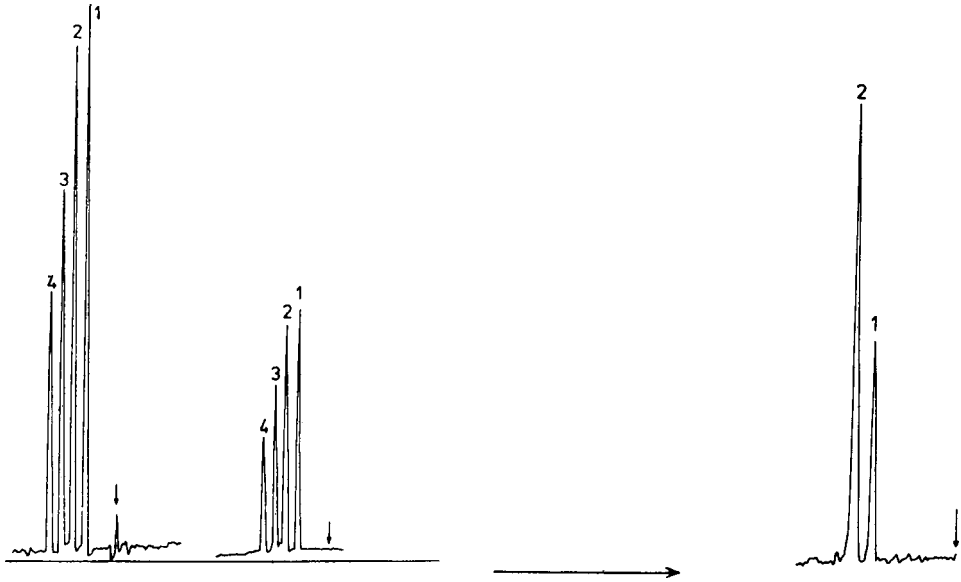


Fig. 3. CPA detection of 20 μg each of four phthalates eluted from a Spherisorb ODS column using acetonitrile–water (60:40) eluent. Peaks: 1 = dimethyl phthalate; 2 = diethyl phthalate; 3 = diallyl phthalate; 4 = diisopropyl phthalate. Left-hand trace is gain × 2.

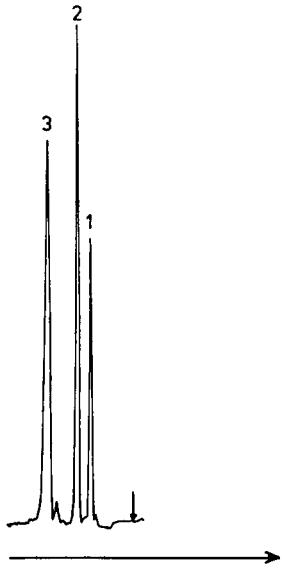
Fig. 4. CPA detection of Bendiocarb (1) and Carboryl (2) eluted from a Spherisorb ODS column using acetonitrile–water (35:65) eluent.

dimethyl-, diethyl-, diallyl-, and diisopropyl-phthalates (20 μg of each) using a 25 × 0.5 cm 10-μm Spherisorb ODS column eluted with a acetonitrile–water (60:40) mixture.

Fig. 4 shows the separation of Bendiocarb (13.5 μg) and Carboryl (8 μg) using the same column eluted with acetonitrile–water (35:65) solvent.

Fig. 5 shows the separation of anthracene, naphthalene and biphenyl (2 μg of each) using a 15 × 0.5 cm 5-μm Spherisorb ODS column eluted with methanol–water (80:20).

Fig. 6 shows the separation of vitamin A and vitamin D_2 (palmitates) using the 10-μm Spherisorb ODS column eluted with methanol.



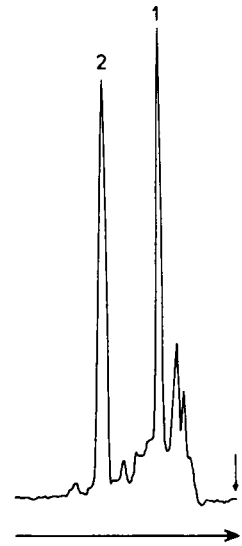


Fig. 5. CPA detection of naphthalene (1), biphenyl (2), and anthracene (3) eluted from a Spherisorb ODS column using methanol–water (80:20) eluent.

Fig. 6. CPA detection of vitamin D_2 palmitate (1) and vitamin A palmitate (2), eluted in methanol from a Spherisorb ODS column.

Fig. 7 shows the variation of detector response (as percentage absorption) with sample size for Carbazole at small loadings (up.to 1 μg), using the 10-μm Spherisorb ODS column eluted with methanol. The chromatographic peaks corresponding to each sample size are shown in Fig. 8.

A limited number of samples which do not absorb strongly at 254 nm were passed through the CPA detector fitted with a 210-nm filter. The detector responses are shown in Table II. In these cases the eluent was water and the photon count-rate recorded with water flowing through the detector was *ca.* 5600 sec^{-1}. [Note that the 210-nm filter passed only 8% of incident 210 nm radiation, whereas the 254-nm filter passed 17% of the incident 254-nm radiation.]

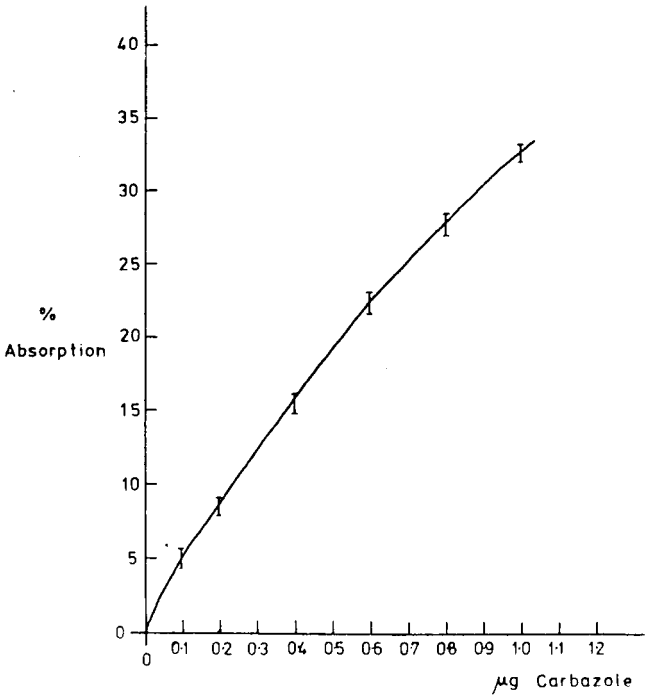


Fig. 7. Variation of CPA detector response (% absorption with sample size for Carbazole eluted in methanol).

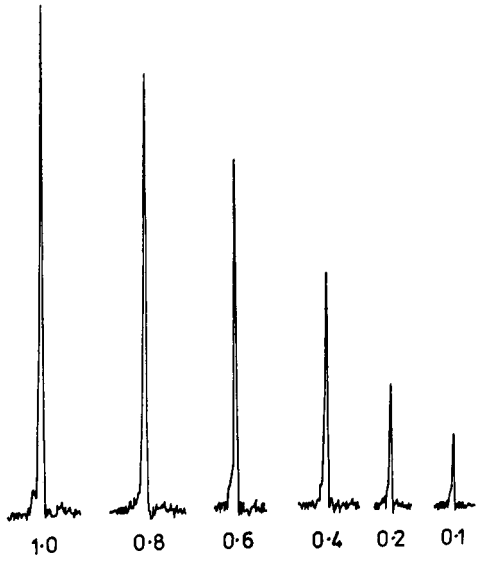


Fig. 8. Chromatographic peaks corresponding to the responses recorded in Fig. 7.

TABLE II

RESPONSES OF CPA DETECTOR WITH 200-nm FILTER TO TEST SAMPLES

Sample	*Amount (μg)*	*Peak response (sec^{-1})*
Potassium nitrate	2	4220
	0.2	2600
Sodium nitrite	2	4430
	0.02	300
Sodium bromide	2	3350

DISCUSSION

The results presented above clearly demonstrate that the experimental CPA detector may be used for the detection of a variety of materials which absorb UV light —certainly between 210 and 254 nm. The radioactive source used in these experiments appeared to be secure, in that no radioactivity was detected in any of our collected eluents. The system therefore appears to offer an attractive alternative to lamp-operated UV absorption detectors. Clearly there are a number of areas in which improvements still have to be made before the system can be regarded as a practical alternative to conventional UV detectors.

The first of these is the linearity of response, which, as the results of Fig. 7 show, is currently rather poor. We believe that the main reason for the distorted response of the experimental system is the pulse-counting technique chosen for signal detection. Recording the number of photons detected (rather than the number of pulses) should allow the detector to follow Beers' law more precisely, and we are modifying the experimental system accordingly. The second major area where improvement is essential is sensitivity. We anticipate a significant improvement in sensitivity when the detector system has been modified to record photon numbers rather than pulse numbers. However a further improvement should also be obtained by simply using a radioactive source of greater activity, such as 10 mCi rather than the 1 mCi used in this work.

The advantages inherent in the CPA detector compared with conventional UV detectors will become particularly attractive as the improvements outlined above are introduced. For example, the radioactive source decays at a precisely known rate and produces photons without short- or medium-term intensity flucutuations. The system is operational as soon as the electronics are switched on —there is no "warm up" time— and the power requirements are sufficiently small that portable, battery-operated detectors could be readily produced. Cerenkov radiation covers a wide range of wavelengths, certainly from below 200 nm to the visible region, so that a variable-wavelength or variable-filter system has considerable potential as a relatively low-cost detector for a very wide variety of materials.

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INFLUENCE OF ELUTION TEMPERATURE AND POLARITY OF DIFFERENT STATIONARY LIQUIDS ON THE RESOLUTION OF THE DIASTEREOMERS OF NORPRISTANE, PRISTANE AND PHYTANE*

H. BORWITZKY**,* and G. SCHOMBURG
Max-Planck-Institut für Kohlenforschung, Kaiser Wilhelm-Platz 1, 433 Mülheim/Ruhr (G.F.R.)
(First received November 5th, 1981; revised manuscript received January 8th, 1982)

SUMMARY

It is shown that glass capillaries of high separation efficiency and optimized selectivity are necessary to resolve the diastereomers of the C_{18}–C_{20} acyclic isoprenoid alkanes. In accordance with theory, relatively low column temperatures favour maximum selectivities (relative net retention times). Therefore high phase ratios of tailor-made glass capillaries are one of the prerequisites. In addition, the resolution can be more easily achieved with Carbowax 20M or even more polar stationary liquids than with non-polar ones.

INTRODUCTION

The determination of norpristane [2,6,10-trimethylpentadecane (1)], pristane [2,6,10,14-tetramethylpentadecane (2)] and phytane [2,6,10,14-tetramethyl-hexadecane (3)] (Fig. 1) is of importance in organic geochemical analysis in drawing certain conclusions about the diagenesis and maturation of natural oil and related products[1]. In this context Maxwell and co-workers published partial separations of the diastereomers of pristane[2–5] and phytane[6]. It is noteworthy that these authors achieved their separations of the non-polar alkanes only on capillaries (100 m × 0.25 mm and 100 m × 0.2 mm) coated with the polar stationary liquids BDS (butanediol succinate polyester), DEGS (diethylene glycol succinate) and DEGS–PEGS (polyethylene glycol succinate) (3:1) mostly in isothermal runs[2–4,6], but also in temperature-programmed[5] runs at comparatively low elution temperatures between 40 and 100°C. A capillary column (100 ft. × 0.01 in.) coated with the non-polar solvent Apiezon L proved to be insufficient[2]. Incomplete separations of the pristane and phytane diastereomers can be deduced from an environmental study by Grob[7]. In view of previous separations of diastereomeric alkanes up to C_{13} by capillary GC[8,9] we decided to optimize the separations of the above-mentioned diastereomeric alkanes.

* From the thesis of H.B., University of Bochum, Bochum, July 1981.

** Present address: Milchwirtschaftliche Untersuchungs- und Versuchsanstalt, Hirnbeinstrasse 10, D-8960 Kempten, G.F.R.

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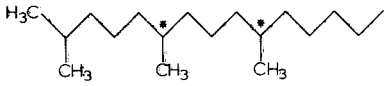
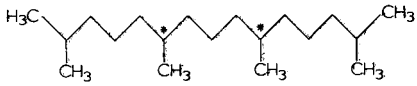
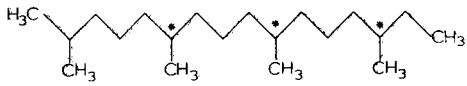
Formula and name		Number of possible stereochemical isomers	Number of possible peaks on non-chiral stationary liquids
	Norpristane	4	2
	Pristane	3	2
	Phytane	8	4

Fig. 1. Structural formulae and some stereochemical details of norpristane, pristane and phytane.

THEORY

Detailed treatments of the relevant theory can be found in text-books by Purnell[10] and Littlewood[11]. According to basic thermodynamics the isothermal separation of compound 1 from compound 2 can be represented by:

$$\Delta G_1 - \Delta G_2 = \Delta\Delta G \quad (1)$$

$$= RT \ln (K_2/K_1) \quad (2)$$

$$= \Delta H_v - T\Delta\Delta S \quad (3)$$

By substituting $K = k'\beta$ and $k'_2/k'_1 = \alpha$ we finally get:

$$\ln \alpha = \frac{\Delta H_v}{RT} + \frac{\Delta\Delta S}{R} \quad (4)$$

ΔG_1, ΔG_2 = Gibbs free energies of compounds 1 and 2
$\Delta\Delta G$ = difference of Gibbs free energies of separated compounds 1 and 2
K_1, K_2 = equilibrium constants of compounds 1 and 2 between the stationary liquid and the mobile gas phase
β = phase ratio of the gas chromatographic (GC) column
k'_1, k'_2 = capacity ratios of separated compounds 1 and 2
α = net retention time of compound 2 relative to compound 1
ΔH_v = difference of heats of vaporization of compounds 1 and 2 from the stationary into the mobile gas phase
$\Delta\Delta S$ = difference of entropy changes caused by the transition of compounds 1 and 2 from the stationary into the mobile gas phase

Strictly, eqn. 4 holds only if both the stationary and the mobile phase are pure compounds and if the isothermal partition of the solutes between the two phases is not influenced by adsorption. The first requirement was met exactly, when Kováts'[12] hydrocarbon $C_{87}H_{176}$ was used as stationary phase, whereas the polymers OV-101 and Carbowax 20M can only be considered as approximately pure substances. The second assumption was easily fulfilled, since branched, saturated hydrocarbons are

non-polar and hardly suffer from adsorption on active support surfaces. In conclusion, eqn. 4 describes the basic chromatographic experience that resolutions of most compounds increase with decreasing temperature. If ln α in eqn. 4 is plotted against $1/T$, ΔH_v can be derived from the slope and $\Delta\Delta S$ from the intercept of this graph with the y-axis. In Tables II–IV we have listed only the ΔH_v values for the temperature range under investigation. Due to the limited number of data points and temperature range, determinations of the $\Delta\Delta S$ values by extrapolation were not tried.

Under isothermal conditions, the net retention time of a solute is directly proportional to the mass of the stationary liquid in the column. Because glass capillaries contain much lower amounts of stationary liquid than packed columns, an increase in the relative net retention time, α, and thus selectivity can be more easily realized by lowering the elution temperatures with open tubular columns than with packed columns. This fact is overlooked by many chromatographers.

EXPERIMENTAL

Glass capillaries (85, 100 and 140 m × 0.27 mm I.D.) were drawn from alkali glass tubes (Schott, Mainz, G.F.R.), treated with gaseous HCl and HF (the latter highly diluted in nitrogen by a two-stage process), deactivated, coated by the mercury-plug method and tested as described previously by Schomburg and co-workers[13,14]. The performance and operation of these columns is given in Table I. Hydrogen was exclusively used as carrier gas with an average linear velocity of 0.35 m/sec (minimum height equivalent to a theoretical plate, $HETP_{min.}$).

TABLE I

PERFORMANCE AND OPERATION OF GLASS CAPILLARY COLUMNS FOR THE SEPARATION OF THE DIASTEREOMERS OF NORPRISTANE, PRISTANE AND PHYTANE

	Column I	*Column II*	*Column III*
Dimensions	140 m × 0.27 mm	100 m × 0.27 mm	85 m × 0.27 mm
Stationary phase	$C_{87}H_{176}$	OV-101 (dimethylpolysiloxane)	Carbowax 20M (polyethylene glycol)
Number of theoretical plates at	295,000	217,000	131,000
k' value	20.4	17.4	38.4
Carrier gas	Hydrogen	Hydrogen	Hydrogen
Linear velocity (m/sec)	0.35	0.35	0.35
Column temperatures	100–230°C, 0.8°C/min		
Isothermal mode	130, 150, 180°C	120, 130, 150°C	90, 100, 120°C

Because our minute supplies of standard compounds had been used up, we used a distillation cut (250–330°C, 1 bar) of natural oil rich in paraffins, kindly provided by Messrs. Herold and Kegler, Deutsche Shell, Hamburg, G.F.R. Pristane and phytane were identified as constituents of this distillation fraction by their characteristic elution behaviour from columns coated with dimethylpolysiloxanes as stationary phase. Secondly we compared their Kováts' indices obtained from column II with those of standard compounds obtained in earlier experiments[15] from a 100-m

capillary column (stationary phase dimethylpolysiloxane DC 200). Thirdly we confirmed our identification of norpristane, pristane and phytane by glass capillary GC–mass spectrometry (MS). These experiments were made with another 50-m column coated with a relatively thick film of 550 nm of dimethylpolysiloxane OV-101, in order to obtain mass spectra of adequate intensity of the highly branched alkanes. The capillary column was connected to a Varian CH 7A magnetic field mass spectrometer in the open split mode[16,17]. The numerous mass spectra of acyclic isoprenoid alkanes compiled by Holzer *et al.*[18] and Johns *et al.*[19] were used for comparison. Norpristane was also confirmed as a constituent of the petroleum cut investigated by comparing its Kováts' indices obtained from column II with a value calculated by Schomburg's method[9] of index increments of first and second order.

RESULTS AND DISCUSSION

Fig. 2 shows an overview (a) and the relevant part (b) of a temperature-programmed run of norpristane (1), pristane (2) and phytane (3) obtained from capillary I (stationary phase Kováts'[12] hydrocarbon $C_{87}H_{176}$). A low heating rate of 0.8°C/min was chosen, because in temperature-programmed GC the resolution increases with decreasing heating rate[20]. Better resolutions of the three diastereomeric pairs were achieved with column I under isothermal conditions (Fig. 2c and d, retention data in Table II). The maximum selectivity, expressed by the relative net retention time, α, was obtained from column I at an elution temperature of 130°C. Under these conditions a retention time of 380 min for the baseline-separated phytane diastereomers was obtained and extreme care had to be taken not to overload the column. Therefore, we suppose that Maxwell *et al.*[2] failed to resolve the pristane diastereomers with their Apiezon L capillary because the elution temperature of 160°C was too high, and not because of the nature of Apiezon L which mainly consists of high-molecular-weight hydrocarbons and methyl phenyl ethers[21]. This assumption is strongly supported by the retention data compiled in Table II. Here an increase of the relative net retention time, α, from 1.0045 to 1.0085 for the pristane diastereomers is observed, if the elution temperature of column I is lowered from 180°C to 130°C.

Table III compiles the chromatographic data obtained from column II (stationary liquid dimethylpolysiloxane OV-101). In comparison to Table II, slightly lower α values are observed for the pristane and phytane diastereomers at 150 and 130°C. With column II we achieved a slightly lower oven temperature of 120°C, resulting in an increase of all α values. The relative net retention time, α, of the pristane diastereomers of 1.0095 at 120°C even supersedes the α value of 1.0085 for the same separation obtained from column I (stationary phase $C_{87}H_{176}$) at 130°C. This increase can also be seen in Fig. 3a where, according to eqn. 4, ln α of the diastereomers of pristane is plotted against $1/T$ for the stationary phases $C_{87}H_{176}$ hydrocarbon, dimethylpolysiloxane OV-101 and polyethylene glycol Carbowax 20M of capillary columns I, II and III. The corresponding graphs for the separated phytane diastereomers are plotted in Fig. 3b. Additionally, the differences of the heats of vaporization, ΔH_v, of the diastereomers of pristane and phytane were calculated from the slopes of these graphs (Tables II–IV). Thus the strongest temperature dependence of the separation of the pristane and phytane can be observed on column II (stationary phase dimethylpolysiloxane OV-101).

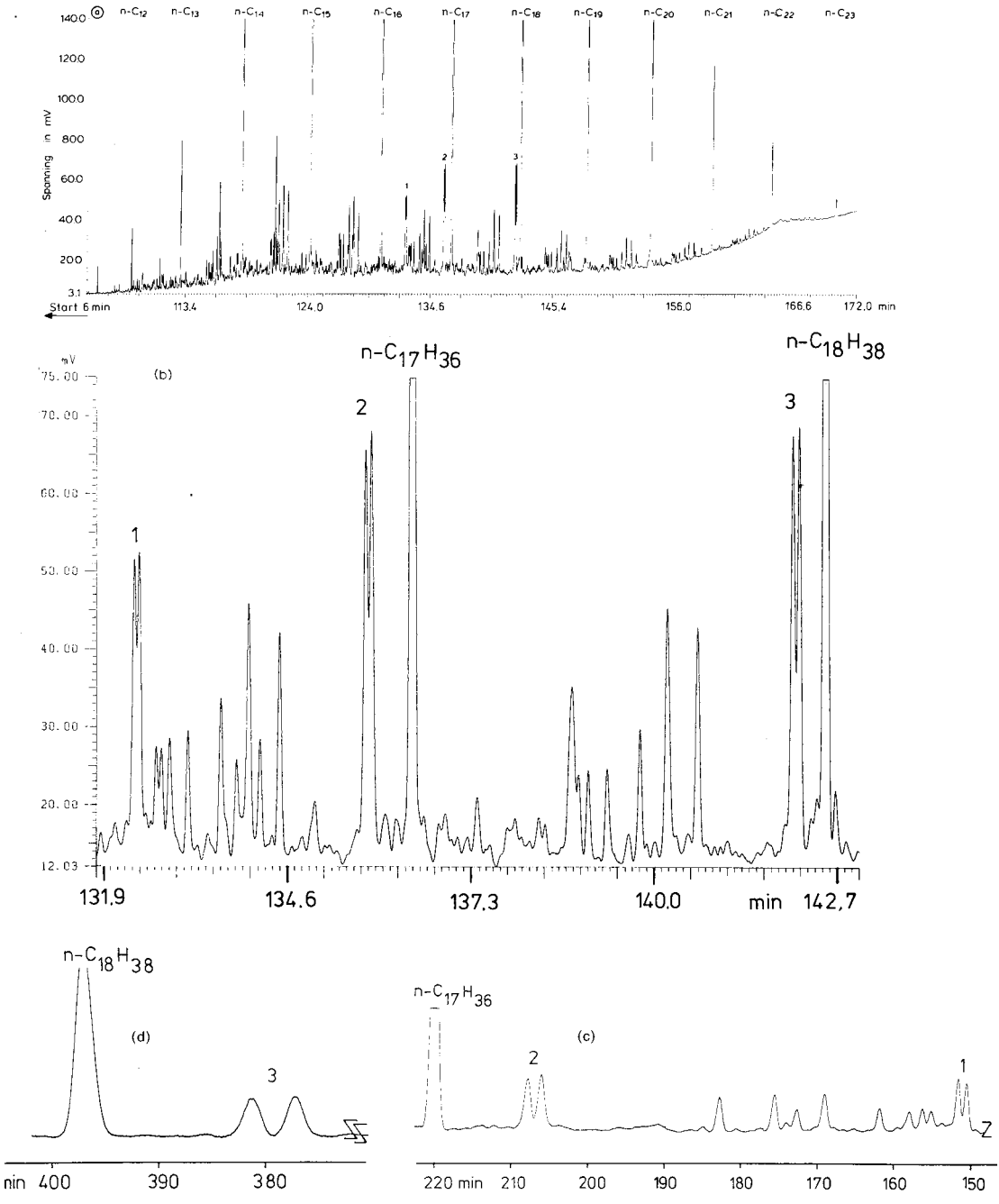


Fig. 2. Analysis of diastereomers of norpristane (1), pristane (2) and phytane (3) in a petroleum cut. a, Overall chromatogram. Column: 140 m × 0.27 mm I.D. Stationary liquid: $C_{87}H_{176}$. Temperature: 100–230°C, 0.8°C/min. Carrier gas: hydrogen, 0.35 m/sec at 230°C. b, Relevant part. Temperature: 132–143°C, 0.8°C/min. Other conditions as in a. c, Isothermal elution of norpristane (1) and pristane (2). Temperature: 130°C. Other conditions as in a. d, Isothermal elution of phytane (3) as in c.

TABLE II

RETENTION DATA OF THE DIASTEREOMERS OF PRISTANE AND PHYTANE OBTAINED FROM COLUMN I (STATIONARY LIQUID $C_{87}H_{176}$)

	Diastereomers of pristane			*Diastereomers of phytane*		
Column temperature, T (°C)	130	150	180	130	150	180
$1/T$ (K^{-1})	0.002481	0.002363	0.002207	0.002481	0.002363	0.002207
α	1.0085	1.0074	1.0045	1.0116	1.0092	1.0063
ln α	0.008464	0.007373	0.004490	0.01153	0.009158	0.006280
ln $\alpha = 14.73/T\ (K^{-1}) - 0.028$ Correl. coeff. 0.985				ln $\alpha = 19.14/T\ (K^{-1}) - 0.036$ Correl. coeff. 0.9996		
$\Delta\Delta G$ (J/mol)	28.45	25.94	16.92	38.78	32.22	23.66
ΔH_v (J/mol)	123.2 between 130 and 180°C			160.3 between 130 and 180°C		
Kováts' indices		1688.8	1689.2		1791.7	1792.6
Kováts' indices		1690.2	1690.2		1793.3	1793.9
ΔI (IE)		1.4	1.0		1.6	1.3
Resolution	1.051			1.301	1.225	

TABLE III

RETENTION DATA OF THE DIASTEREOMERS OF PRISTANE AND PHYTANE OBTAINED FROM COLUMN II (STATIONARY LIQUID OV-101)

	Diastereomers of pristane			*Diastereomers of phytane*		
Column temperature, T (°C)	120	130	150	120	130	150
$1/T$ (K^{-1})	0.0025436	0.0024805	0.0023632	0.0025436	0.0024805	0.0023632
α	1.0095	1.0081	1.0055	1.0112	1.0098	1.0070
ln α	0.009429	0.008044	0.005474	0.01112	0.009709	0.006937
ln $\alpha = 21.92/T - 0.046$ Correl. coeff. 0.99999				ln $\alpha = 23.63/T - 0.049$ Correl. coeff. 0.999999		
$\Delta\Delta G$ (J/mol)	30.82	26.96	19.26	36.35	32.54	24.41
ΔH_v (J/mol)	182.2 between 120 and 150°C			193.2 between 120 and 150°C		
Kováts' indices	1707.7	1708.4		1809.5	1810.6	
	1709.4	1710.0		1811.6	1812.4	
ΔI	1.7	1.6		2.1	1.8	
Resolution	1.177	0.872		1.253	1.025	

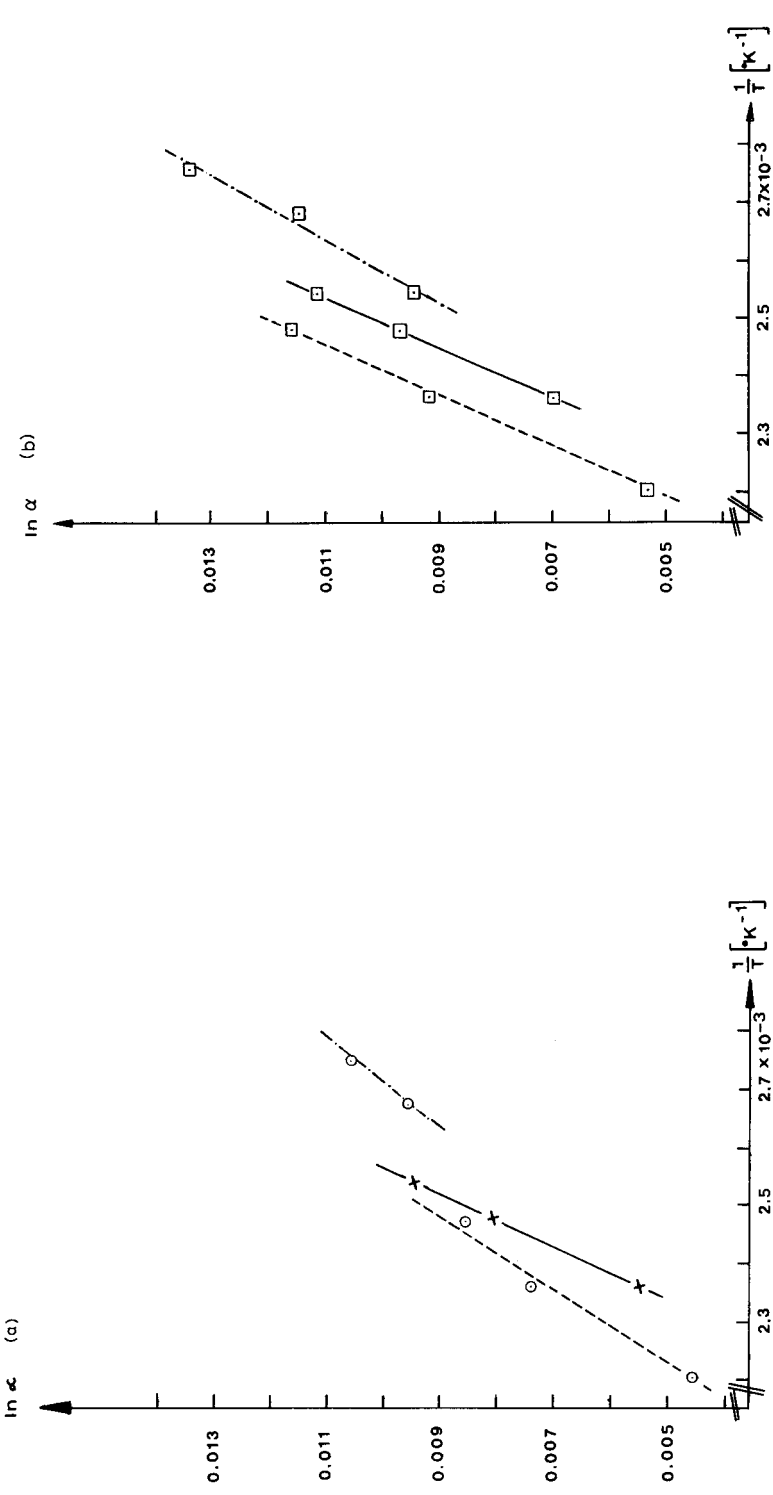


Fig. 3. Dependency of ln α of the pristane (a) and phytane (b) diastereomers on $1/T$ on glass capillaries coated with stationary liquids of different polarity: ——, dimethylpolysiloxane OV-101; – – –, $C_{87}H_{176}$ hydrocarbon; – · – · –, polyethylene glycol Carbowax 20M.

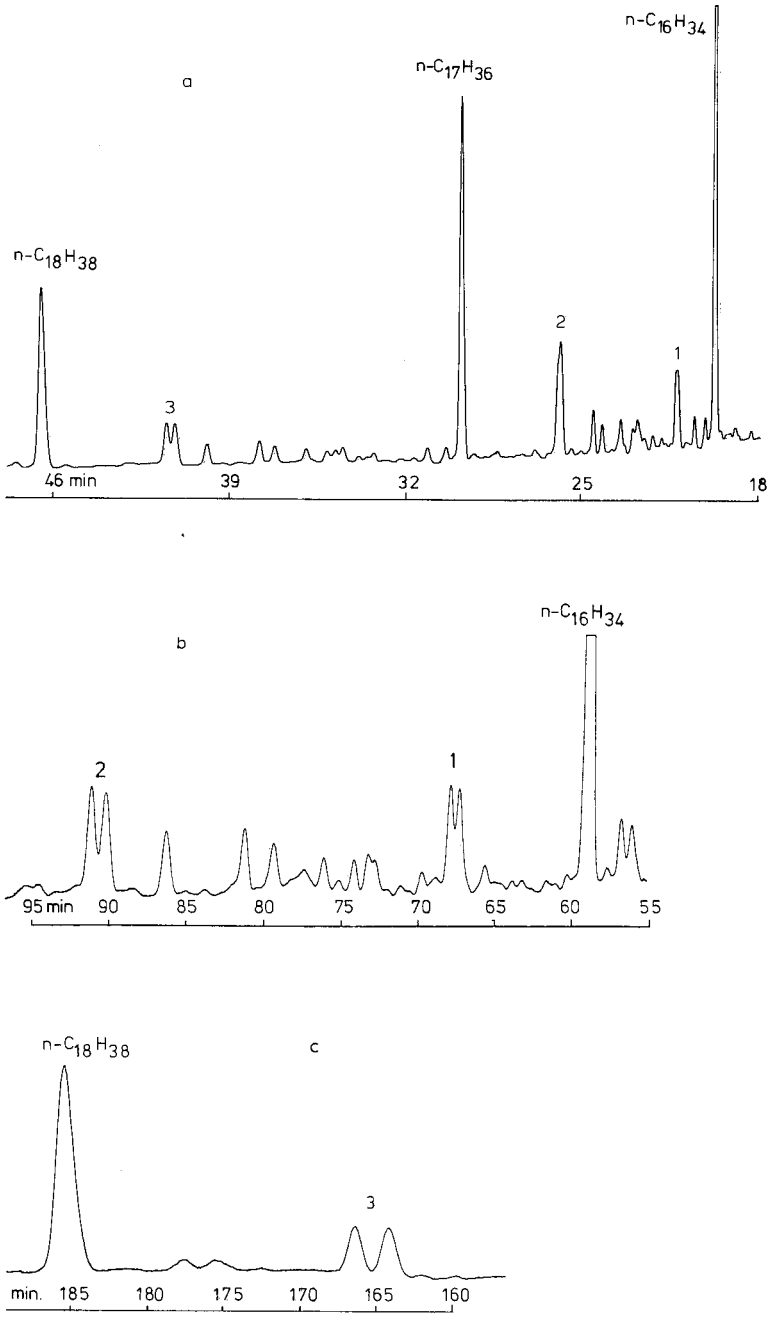


Fig. 4. Isothermal analysis of the diastereomers of norpristane (1), pristane (2) and phytane (3) on the polar stationary liquid Carbowax 20M. a, No resolution at elevated temperature. Column: 85 m × 0.27 mm I.D. Temperature: 120°C. Carrier gas: hydrogen, 0.35 m/sec. b, Greatly improved resolution of norpristane (1) and pristane (2) at decreased elution temperature of 90°C. c, Greatly improved resolution of phytane (3) at decreased elution temperature of 90°C.

TABLE IV

RETENTION DATA OF THE DIASTEREOMERS OF PRISTANE AND PHYTANE OBTAINED FROM COLUMN III (STATIONARY LIQUID CARBOWAX 20M)

	Diastereomers of pristane			*Diastereomers of phytane*		
Column temperature, T (°C)	90	100	120	90	100	120
$1/T$ [K^{-1}]	0.002754	0.002680	0.002544	0.002754	0.002680	0.002544
α	1.0106	1.0095		1.0135	1.0115	1.0095
ln α	0.0105	0.009503		0.01241	0.01144	0.009440
				ln $\alpha = 18.36/T - 0.037$ Correl. coeff. 0.986		
$\Delta\Delta G$ (J/mol)	31.71	29.48		40.48	35.49	30.86
ΔH_v (J/mol)				152.7 between 90 and 120°C		
Resolution	0.84	0.75		1.161	1.072	0.79

Fig. 4a–c illustrates the increase of selectivity when the elution temperature of column III (stationary liquid Carbowax 20M) is decreased from 120°C to 90°C. The corresponding retention data are compiled in Table IV. While at 120°C the diastereomers of norpristane (1) and pristane (2) are not at all resolved (Fig. 4a), a decrease of just 30°C shows greatly improved separations (Fig. 4b and c). The α values of pristane and phytane increase to 1.0106 and 1.0135, respectively, thus superseding all other α values obtained from the non-polar columns I and II at higher temperatures (compare also Fig. 3a and b). This clearly suggests that the separations of the diastereomers under investigation are mostly favoured by relatively very low elution temperatures. This can be more easily achieved with polar stationary phases because of the decreased solubility of non-polar isoprenoid alkanes in polar solvents. By switching from column I (stationary liquid $C_{87}H_{176}$) to column III (stationary liquid Carbowax 20M) the retention times of the phytane diastereomers are roughly halved from 380 to 165 min. In spite of this progress, the maximum number 2^{n-1} (n = number of chiral centres in a molecule) of possible peaks which can be theoretically obtained on non-chiral stationary phases could only be achieved in the case of norpristane and pristane. In the case of phytane with its three chiral centres, however, four enantiomeric pairs and thus four peaks can be expected in theory, whereas we obtained only two peaks in spite of highly efficient glass capillaries (compare Table I). This lack may be explained by the thermal energy of the molecules which is too high for the desired separation even at a column temperature of 90°C. Furthermore, it is interesting to note that pristane and phytane are eluted from columns I and III (stationary phases $C_{87}H_{176}$ and Carbowax 20M) before the n-alkanes C_{17} and C_{18} respectively, whereas on column II (stationary liquid OV-101) pristane and phytane follow these n-alkanes (compare Kováts' indices in Tables II and III, and Figs. 2a–d and 4a–c). We must finally recognize that our previous general statement[22] "selectivity changes for better relative volatilities cannot be successfully used in the case of weak intermolecular interaction of low polarity compounds like hydrocarbons" is not always correct.

As mentioned before, very high phase ratios of GC columns favour low elution temperatures and thus greatly improved selectivities. In this context we would like to expand Grob's question[23] "Are We Using the Full Range of Film Thicknesses in Capillary-GLC?" to "Are We Using the Full Range of Film Thicknesses and of Phase Ratios in Capillary-GLC to Obtain Improved Selectivities at Lower Temperatures?". This question is especially relevant for the elution of thermally sensitive solutes.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON DYNAMICALLY MODIFIED SILICA

III*. MODIFICATION OF SILICA WITH LONG-CHAIN AND SYMMETRICAL QUATERNARY AMMONIUM COMPOUNDS

STEEN HONORÉ HANSEN
Department of Organic Chemistry, Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)
PER HELBOE*
National Board of Health, Drug Standardization Laboratory, 378 Frederikssundsvej, DK-2700 Brønshøj (Denmark)
and
ULLA LUND
Danish Civil Defence, Analytical-Chemical Laboratory, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)
(First received October 7th, 1981; revised manuscript received January 15th, 1982)

SUMMARY

The influence of the nature of quaternary ammonium compounds on retention in high-performance liquid chromatography on dynamically modified silica was investigated. Adsorption isotherms were determined on bare silica (LiChrosorb Si 60) for four alkyltrimethylammonium bromides and two symmetrical tetraalkylammonium bromides, each containing 15–21 carbon atoms. It was found that only the long-chain quaternary ammonium ions are adsorbed on to the silica surface in appreciable amounts and that the affinity increases with increasing number of carbon atoms in the alkyl chain. The maximum amount that can be adsorbed per gram of silica is of the same order of magnitude for each of the four long-chain quaternary ammonium compounds. This amount, however, is reached at lower concentrations in the eluent the longer is the alkyl chain.

The retention of five test compounds was determined over the whole concentration range investigated for each of the modifying agents. The surfactant concentration that causes maximum retention for four of five test compounds coincides with its critical micellar concentration. The retention mechanisms and the influence of the type of the modifying agent on selectivity are discussed, and compared with published results on related experiments on chemically bonded stationary phases.

* For Part II, see ref. 2.

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INTRODUCTION

The use of bare silica dynamically modified by adding surfactants such as cetyltrimethylammonium (CTMA) bromide to the eluent in high-performance liquid chromatography (HPLC) has been discussed in recent publications[1,2]. It was stated that a major advantage offered by the dynamic coating approach over the use of chemically bonded phases is that only slight variations in selectivity for different brands of column material occur.

The aim of this work was to investigate the adsorption of different kinds of quaternary ammonium ions on to the silica surface and their influence on the retention of various test compounds.

EXPERIMENTAL

Apparatus

UV–visible measurements were made on a rebuilt[3] Beckman Model DU spectrophotometer.

Breakthrough volumes were measured using a liquid chromatograph consisting of a Gynkotek Model 600 pump and an Optilab Multiref 902 differential refractometer detector. The detector response and the trace of a 1-ml siphon counter were recorded on a Kipp & Zonen Model BD-8 recorder.

Chromatographic testing of the individual systems was performed on a Waters liquid chromatograph consisting of a 6000 A pump, a 710 A WISP autoinjector, a 440 ultraviolet absorbance detector (254 nm), a 730 data module and a 720 system controller, or on a liquid chromatograph consisting of an Altex Model 110 solvent metering pump, a Pye-Unicam LC–UV detector (operated at 254 nm) and a Rheodyne Model 7120 injection valve. Chromatograms were recorded on a Kipp & Zonen Model BD-8 recorder. Retention data were collected on the Waters 730 data module or on a Hewlett-Packard Model 3353 A laboratory data system.

Procedures

Determination of the amounts of quaternary ammonium compounds adsorbed on to the column by the breakthrough method was performed as described previously[2].

Determination of the surfactants adsorbed on to the column using an elution method was performed by eluting the previously equilibrated column with 100 ml of methanol–0.05 *M* phosphoric acid (1:1). Surfactant concentrations were measured, after appropriate dilution, using the previously described procedure[2], or by liquid chromatography using the liquid chromatograph described for the breakthrough experiment, the LiChrosorb Si 60 column and 2.5 m*M* CTMA in methanol–water–0.2 *M* phosphate buffer (pH 6.0 or 5.0) (70:25:5) as the eluent. The results were corrected for the dead volume of the column.

The critical micellar concentrations (CMC) were determined by solubilizing 4,4′-diethoxyazobenzene in the eluents. By plotting the absorbance at 375 nm of the saturated solutions against the concentrations of the surfactant two straight lines can be constructed, the intersection of which is equivalent to the CMC.

Chromatography

All experiments were performed on 120 × 4.6 mm I.D. columns from Knauer (Berlin, G.F.R.), packed by the dilute slurry technique with LiChrosorb Si 60 (5 μm) (E. Merck, Darmstadt, G.F.R.). The eluent was methanol–water–0.2 *M* phosphate buffer (pH 7.5) (50:45:5) with the addition of various kinds and concentrations of quaternary ammonium compounds. During chromatography the column was guarded by a silica pre-column situated between the pump and the injection device. Following each adsorption experiment the column was brought to its initial status by eluting with methanol–0.05 *M* phosphoric acid (1:1) and finally with methanol.

Chemicals

4,4′-Diethoxyazobenzene was prepared by coupling diazotized 4-ethoxyaniline to phenol followed by ethylation using diethyl sulphate; the crude product was recrystallized from methanol–water. Stearyltrimethylammonium (STMA) bromide was prepared from stearylamine by dimethylation with formaldehyde–formic acid[4] and subsequent methylation of the tertiary amine with methyl bromide[5]. Dodecyltrimethylammonium (DTMA) bromide and tetradecyltrimethylammonium (TTMA) bromide were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical-reagent grade from E. Merck.

RESULTS AND DISCUSSION

Adsorption isotherms

The adsorption of four alkyltrimethylammonium compounds, DTMA, TTMA, CTMA, and STMA, and of two symmetrical tetraalkylammonium compounds, tetrabutylammonium (TBA) bromide and tetrapentylammonium (TPA) bromide, was investigated at concentrations ranging from 0 to 50 m*M*. The upper value represents an increase relative to that previously investigated[2] for CTMA, in order to establish for each component the limit of the amount adsorbed. Measurements of the amounts adsorbed at high concentrations of the surfactant-type compounds were not possible by means of the previously described breakthrough technique. At these concentrations it was found that the buffer capacity of the eluent was insufficient to maintain a constant pH, as protons on the surface of the silica were exchanged for surfactant ions. This gave rise to a decrease in the pH, which at low concentrations of surfactant ions was so small (0.2 pH units for 2.5 m*M* CTMA[2]) that it caused no problems. At higher concentrations larger shifts were measured (2.5 pH units for 25 m*M* CTMA); thus the ionization of the silanol groups is affected considerably. The ability to adsorb surfactant ions is temporarily decreased, giving rise to a partial breakthrough. The early breakthrough was observed from *ca.* 10 m*M* for the four surfactants, and the amounts adsorbed at concentrations above 10 m*M* were determined by the elution method.

Fig. 1 shows the six adsorption isotherms. It appears that the structure of the quaternary ammonium compounds rather than the number of carbon atoms present is of vital importance for the amount adsorbed. The two symmetrical compounds TBA and TPA are adsorbed to only a minor extent, and only a small difference between the two compounds, which differ by four carbon atoms, is seen. For the alkyltrimethylammonium compounds at lower concentrations the affinity to silica

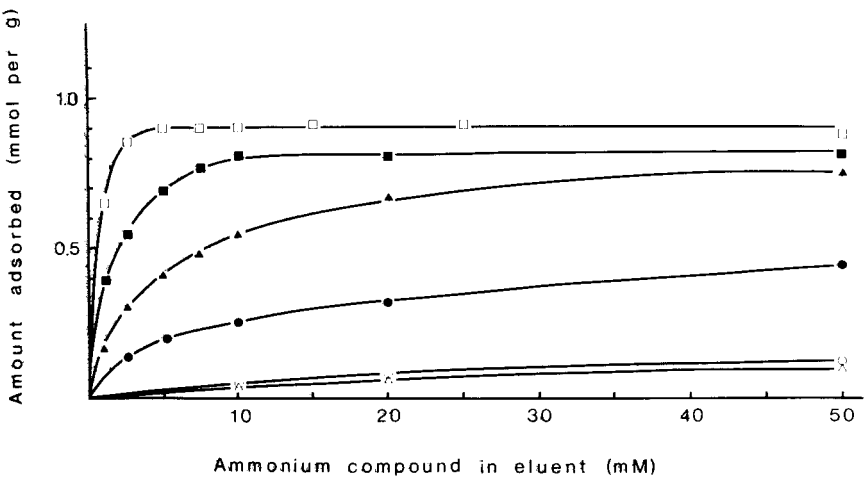

Fig. 1. Relationship between the concentration of quaternary ammonium compound and the amounts adsorbed per gram of silica. ○, TBA; △, TPA; ●, DTMA; ▲, TTMA; ■, CTMA; □, STMA. Eluent: methanol–water–0.2 *M* phosphate buffer (pH 7.5) (50:45:5) plus quaternary ammonium compounds at the indicated concentrations.

increases with increasing carbon number of the alkyl chain. The maximum amount adsorbable, however, seems to be of the same order of magnitude for STMA, CTMA and TTMA, indicating that the limitation is due mainly to steric reasons. The maximum is *ca.* 0.9 mmol/g, corresponding to *ca.* 2 μmol/m^2 (calculated from the surface area per gram of silica as stated by the supplier). For DTMA only *ca.* 0.45 mmol/g is adsorbed at a concentration of 50 m*M* but the curve is still rising. The degree of coverage of the silica surface attainable is comparable to that achieved by derivatizing with long-chain alkylsilanes[6] and no indication of the formation of a double layer or a multi-layer is found.

Retention mechanism

For the chromatographic testing of the individual systems a test mixture containing pyridine, phenethylamine, benzene, phenol and benzoic acid was used. Figs. 2a–d show the capacity factors (k') for four of the test substances as functions of the concentration of the individual quaternary ammonium compounds. The retention of the fifth compound, pyridine, was largely unaffected by the type and concentration of modifying agent, as was previously found[2] for CTMA.

When significant amounts of an alkyltrimethylammonium compound are adsorbed on to the silica surface, neutral compounds such as benzene are chromatographed according to a reversed-phase partition mechanism, acidic compounds such as benzoic acid by reversed-phase ion-pair partition and phenol by an intermediate mechanism as shown earlier for CTMA[2]. As was found in the adsorption experiments, only a monolayer of surfactant seems to be present. This supports the statement that the retention of acidic solutes is due to ion-pair partition and not ion exchange as has been reported to take place in chromatography with surfactants on chemically bonded phases[7]. The previously reported results with CTMA were found for concentrations ranging from 0 to 6 m*M*, whereas the experiments underlying Fig.

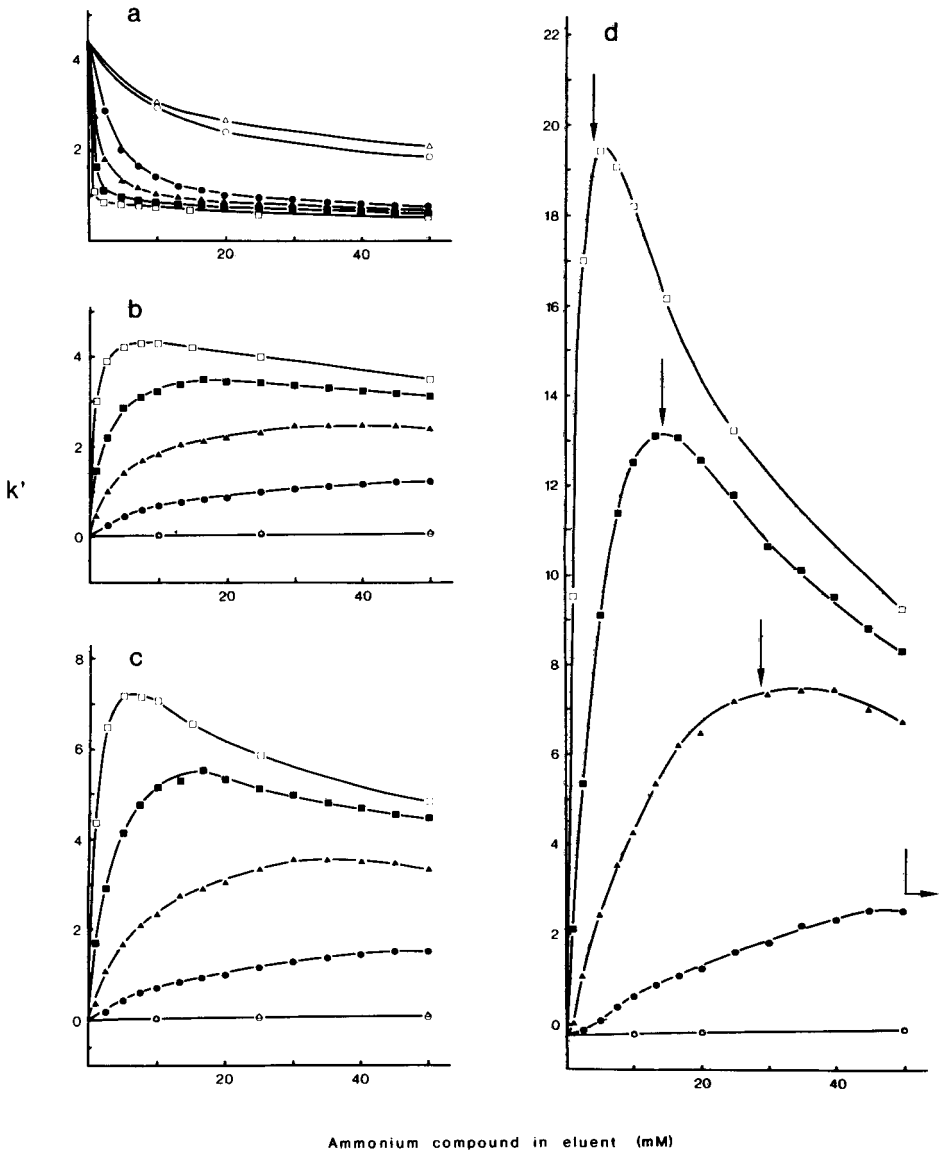

Fig. 2. Relationship between the concentration of quaternary ammonium compound in the eluent and k' for four of the test substances: (a) phenethylamine; (b) benzene; (c) phenol; (d) benzoic acid. Symbols and eluent as in Fig. 1. The arrows in (d) indicate the estimated critical micellar concentrations (see Experimental).

2 were extended to 50 m*M*. At higher concentrations, for all four surfactants the retention of the neutral and acidic solutes exhibit a distinct maximum. In chromatography on chemically bonded phases it has recently been shown[8,9] that the retention of acidic solutes is influenced similarly by both symmetrical and surfactant-type quaternary ammonium compounds. Although it has been claimed that micelle forma-

tion (which is possible for the surfactant-type compounds only) had no influence, other workers[10,11] have found that for alkyl sulphates the maximum retentions of various solutes coincide with the CMC values. In the work presented here (*cf.*, Fig. 2) the maximum retentions of both acidic and neutral solutes show a high degree of coincidence with the CMC values found for the four surfactants (57, 27, 16 and 3.3 mm, for DTMA, TTMA, CTMA and STMA, respectively), indicating that micelle formation apparently increases the hydrophobicity of the eluent.

The two symmetrical compounds, TBA and TPA, affect the retention of the neutral and acidic compounds to only a minor extent. This is in accordance with the above-mentioned fact that only minor amounts of the said compounds are adsorbed on to the silica surface and thus reversed-phase partition is not possible. A certain decrease in the retention of phenethylamine is seen, which indicates that the symmetrical quaternary ammonium compounds do influence the ion-exchange process of the basic solute but to a much smaller extent than do the surfactant-type compounds.

We investigated whether any effect on the retention could be observed on adding a symmetrical pairing ion to an eluent containing a surfactant-type ion of the same charge. Table I shows the retention of benzene, phenethylamine, phenol and

TABLE I

INFLUENCE OF THE ADDITION OF TBA ON RETENTION

Eluent: 2.5 m*M* CTMA in methanol–water–0.2 *M* phosphate buffer (pH 7.5) (50:45:5) plus TBA at the stated concentrations.

Solute	*Concentration of TBA (mM)*				*Decrease in k′ from 0 to 20 mM (%)*
	0	*5*	*10*	*20*	
Phenethylamine	0.68	0.67	0.66	0.64	5.9
Benzene	0.95	0.91	0.92	0.87	8.4
Phenol	1.06	1.02	1.01	0.96	9.4
Benzenesulphonic acid	1.19	1.08	1.04	0.92	22.7
Sorbic acid	1.57	1.36	1.32	1.18	24.8
Benzoic acid	1.63	1.50	1.44	1.29	20.9

three different acids when various concentrations of TBA are included in the eluent containing CTMA (2.5 m*M*). It appears that the TBA causes a decrease for all solutes, the decrease being most pronounced for the anionic compounds. Owing to an ion-exchange mechanism, TBA replaces a small part of the CTMA adsorbed on to the silica, resulting in a less hydrophobic surface and thus in a general decrease in retention. To some extent, however, TBA also forms ion pairs with the anions, but the hydrophobicities of these ion pairs are lower than those of the ion pairs between CTMA and the anions, thus leading to a further decrease in the retention of the acidic solutes.

Alkyl chain length

In reversed-phase HPLC on chemically bonded supports it has been demonstrated[12,13] that the retention increases linearly with increasing amounts of bonded

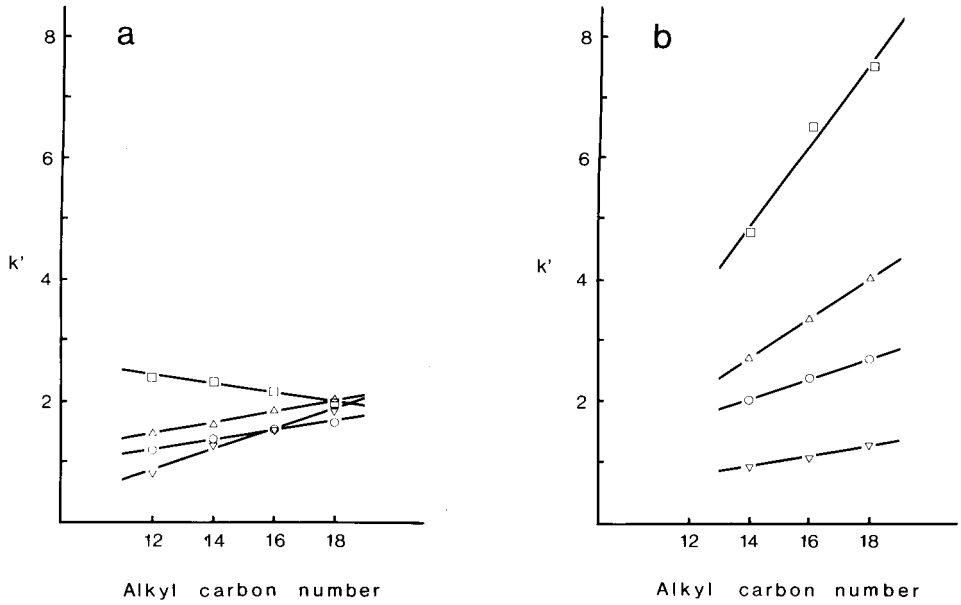

Fig. 3. Relationship between alkyl chain length (carbon number) and k' for four of the test substances at two different levels of coverage: (a) 0.4 mmol of surfactant per gram and (b) 0.6 mmol of surfactant per gram. k' values calculated from Figs. 1 and 2. ○, Benzene; △, phenol; ▽, phenethylamine; □, benzoic acid.

phase, *i.e.*, there is a linear relationship between retention and alkyl chain length if the degree of coverage is kept constant. Figs. 3 a and b show the influence of the chain length on retention of four of the test compounds at two levels of adsorbed surfactant. It appears that the effect of increasing chain length at both levels is an increased retention of benzene and phenol, both of which are chromatographed mainly according to a reversed-phase partition mechanism. The retention of benzoic acid decreases with increasing chain length at low coverage (0.4 mmol/g), whereas the opposite is seen at a higher coverage (0.6 mmol/g). This may be explained by the fact that the surfactant concentrations needed to achieve the 0.4 mmol/g coverage are very low for the longer chain compounds (*cf.*, Fig. 1), and thus the possibility of ion-pair formation is drastically decreased with increasing chain length of the surfactant. For phenethylamine an increase in retention is found with increasing chain length of the surfactant, most pronounced at low coverage of the silica. Also in this instance the concentration effect is of vital importance as the ion-exchange mechanism, partly responsible for the retention of cationic compounds, is greatly influenced by the concentration of the concurrent ion.

The influence on retention of the degree of coverage for each of the four surfactants is shown in Fig. 4 for benzene, which is the only compound in the mixture that is assumed to be chromatographed by a pure reversed-phase partition mechanism, *i.e.*, without any ion-exchange or ion-pair effects. The linear relationship that appears is in accordance with what has been demonstrated between retention and carbon content for chemically bonded phases[13].

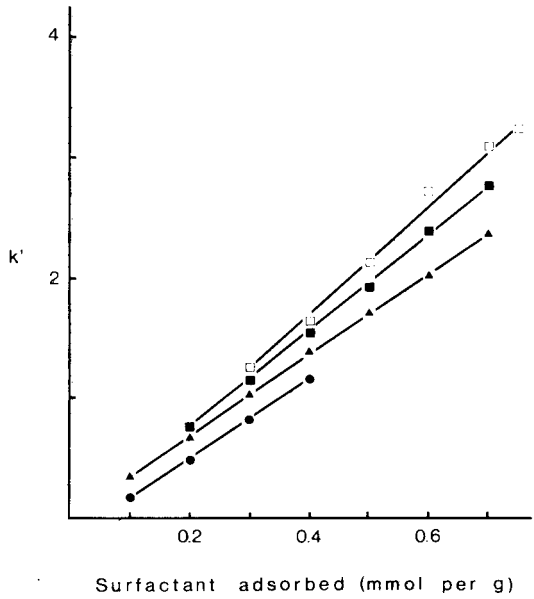

Fig. 4. Relationship between amount of surfactant adsorbed and retention of benzene. k' values calculated from Figs. 1 and 2. ●, DTMA; ▲, TTMA; ■, CTMA; □, STMA.

Selectivity

A change in selectivity in the separation of a mixture of compounds, when using the dynamically modified silica approach, may be accomplished in several ways. Changing the chain length of the quaternary ammonium compound has a pronounced effect (*cf.*, Fig. 3), the effect being different at different concentration levels. If a change in selectivity between ionic solutes of opposite charge is wanted, a change in the concentration of surfactant is to be preferred (*cf.*, Fig. 2). If a change in retention of compounds in a mixture without affecting the selectivity is wanted, a change in the accessible surface area of the column is a possibility. This can be achieved by using a different column length or by changing to another brand of silica[2].

Apart from the nature and concentration of the surfactant, the whole composition of the mobile phase is of vital importance for the selectivity, *e.g.*, the nature and concentration of the organic modifier and buffer components, the pH and the ionic strength. Work is in progress to clarify the influence of these parameters.

CONCLUSION

It has been demonstrated that the affinity to bare silica of quaternary ammonium compounds depends greatly on the structure and the size of the ions. Symmetrical quaternary ammonium ions are adsorbed to only a minor extent and have little influence on the retention of acidic and neutral solutes. For long-chain alkyltrimethylammonium compounds it was found that the maximum amounts adsorbable are of comparable magnitude but, owing to increased affinity to the silica, this amount is reached at lower concentrations the longer is the alkyl chain. The reten-

tions of neutral and acidic solutes for increasing surfactant concentration reach a maximum that coincides with the critical micellar concentration for the individual surfactants. This is in accordance with what has been found previously for chemically bonded supports.

ACKNOWLEDGEMENT

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PREDICTION OF PEPTIDE RETENTION TIMES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DURING LINEAR GRADIENT ELUTION

TATSURU SASAGAWA*
Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)
TSUNEO OKUYAMA
Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, 2-1-1 Fukazawa, Setagaya-ku, Tokyo (Japan)
and
DAVID C. TELLER
Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)
(First received December 1st, 1981; revised manuscript received January 20th, 1982)

SUMMARY

The retention behavior of 100 peptides was studied during high-performance liquid chromatography on a C_{18} column using aqueous trifluoroacetic acid as the mobile phase and acetonitrile as the mobile phase modifier in a linear gradient elution system. Retention times of the peptides were linearly related to the logarithm of the sum of Rekker's constants (R. F. Rekker, *The Hydrophobic Fragmental Constant*, Elsevier, Amsterdam, 1977, p. 301) for the constituent amino acid. Assuming this relationship, the best fit constants for this system were computed by non-linear multiple regression analysis. Using the new constants, it is possible to predict retention times for a wide variety of peptides at any slope of linear gradient, if the amino acid composition is known. It also enables accurate prediction of the retention time of peptides, whose amino acid composition is not known, after an analytical run with an alternate gradient.

INTRODUCTION

The introduction of a volatile eluent system[1,2] in reversed-phase high-performance liquid chromatography has greatly simplified the isolation of pure peptides from complex mixtures. However, selection of chromatographic conditions for purification of a given peptide is still found by time-consuming trial and error methods. *A priori* knowledge of the retention time of a given peptide would simplify the selection of chromatographic conditions. Calculations of peptide R_F values which are based on the amino acid composition were already reported by Knight[3] and Pardee[4]. Similar works using high-performance liquid chromatography were also reported[5–7]. O'Hare and Nice[6] noticed that the retention time of small peptides (< 15 residues) is linearly

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TABLE I

COMPARISON OF PREDICTED AND OBSERVED RETENTION TIMES

The retention times were predicted from eqn. 2, where A', C' were reported in Fig. 2. D'_j was reported in Table II (column 5, 6). The predicted retention times in parentheses were calculated from weighted fit parameters.

No.	*Peptide**	*Retention time (min)*		
		Observed	*Predicted*	
1	GGG	1.8	1.3	(1.9)
2	PG	2.5	1.3	(2.3)
3	ARKM*	2.8	1.0	(2.0)
4	TEEQ	5.0	4.7	(5.2)
5	GL-NH_2	5.3	5.5	(5.2)
6	Ac-AAA	6.1	6.5	(6.8)
7	MTAK	6.5	8.0	(6.4)
8	NLC*	6.6	7.0	(6.9)
9	MARKM*	7.5	10.4	(9.3)
10	MAR	7.8	5.3	(7.2)
11	YK	8.0	6.0	(7.5)
12	TPGSR	8.1	7.6	(7.3)
13	KYE	8.2	7.7	(9.4)
14	GY	8.5	9.9	(8.8)
15	TEAEMK	9.2	10.7	(10.3)
16	EY	9.6	9.3	(9.1)
17	HLK	9.8	12.9	(10.6)
18	FK	9.9	11.4	(10.8)
19	IRE	10.3	12.2	(11.7)
20	PL	10.3	13.8	(11.2)
21	IAE	10.9	13.1	(10.9)
22	GF	11.5	14.1	(11.9)
23	KMKDTDSEEE	11.5	9.9	(13.7)
24	AFR	12.0	13.5	(12.9)
25	DIAAK	12.0	11.7	(13.1)
26	QIAE	12.0	14.2	(11.0)
27	NIPC*	12.4	11.5	(10.6)
28	ASEDLK	13.0	11.9	(12.7)
29	EAFR	13.5	14.5	(14.3)

30	FDR	13.8	12.6	(12.8)
31	VFDKDGNGY	14.8	19.0	(19.7)
32	FKE	15.0	12.5	(12.5)
33	KVFGR	15.6	15.5	(15.6)
34	SLGQNPTEAE	15.8	17.4	(16.9)
35	GW	16.3	15.5	(15.3)
36	MIRE	16.5	16.9	(16.3)
37	SHPETLEK	16.7	19.2	(17.5)
38	HGLDNYR	17.0	18.0	(17.6)
39	WY-NH_2	17.1	16.6	(17.4)
40	Ac-ADQL	18.2	16.6	(17.8)
41	LFK	18.2	19.4	(16.2)
42	IAEFK	19.5	20.7	(19.6)
43	ADIDGDGQVNYEE	19.8	21.1	(22.4)
44	VFDKDGNGYI	20.2	19.0	(19.7)
45	ISAAELR	20.3	21.0	(20.1)
46	FESNFNTQATNR	20.3	19.0	(19.4)
47	ELGTVMR	21.2	21.0	(19.2)
48	GHHEAELK	21.3	18.9	(17.2)
49	WWC*NDGR	21.4	22.1	(24.9)
50	LQDMINE	22.0	21.0	(20.7)
51	FVQMMTAK	22.5	23.6	(22.8)
52	WWC*	23.5	23.4	(24.5)
53	QIAEFK	23.8	21.3	(20.9)
54	Ac-ADQLTEEQIAE	24.0	25.7	(26.0)
55	RSLGQNPTEAELQDM*	24.0	25.2	(25.1)
56	MIREADIDGDGQVNYEE	24.8	27.6	(29.7)
57	FLTMMAR	25.1	25.3	(24.9)
58	VDADGNGTIDFPE	25.3	24.5	(23.8)
59	HVMTNLGEK*LTDEEVDEM*	25.7	28.1	(27.7)
60	LGTVMRSLGQNPTEAE	25.8	27.5	(27.4)
61	SALLSSDITASVNC*	26.0	26.4	(25.9)
62	NTDGSTDYGILQINSR	26.9	27.6	(27.9)
63	VEADVAGHGQDILIR	26.9	29.0	(28.7)
64	FLTMMARKMKDTDSEEE	27.0	29.1	(30.7)
65	LRHVMTNLGEK*LTDE	27.0	24.7	(24.8)
66	VTVPLVSDAEC**R	27.3	27.0	(26.1)
67	VFDKDGNGYISAAELR	27.5	28.5	(30.1)
68	AFRVFDKDGNGYISAAE	28.6	29.3	(31.2)

(Continued on p. 332)

TABLE I *(continued)*

No.	*Peptide**	*Retention time (min)*		
		Observed	*Predicted*	
69	LRHVMTNLGEK*LTDEEVDE	28.6	29.6	(30.8)
70	VFDKDGNGYISAAEL	29.0	28.5	(29.5)
71	GYSLGNWVC**	29.1	29.1	(29.6)
72	IREADIDGDGQVNYEEFVQM*	29.2	28.8	(30.5)
73	Ac-ADQLTEEQIAEFK	29.2	27.7	(28.4)
74	EAFSLFDKDGDGTITTK	30.0	31.4	(31.5)
75	ALELFR	30.2	25.4	(24.6)
76	AFSLFDKDGDGTITTKE	30.4	31.4	(31.5)
77	HVMTNLGEK*LTDEEVDEMIR	33.5	31.0	(32.5)
78	NKALELFRKDIAAKYKELGYQG	34.2	34.0	(37.3)
79	PGYPGVYTEVSYHVDWIK	34.8	34.5	(36.7)
80	DDYGADEIFDSMIC**AGVPEGGK	35.9	34.8	(37.1)
81	EADIDGDGQVNYEEFVQMMTAK	37.2	31.2	(33.5)
82	INEVDADGNGTIDFPEFLTM*	37.5	33.9	(34.1)
83	KDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK*LTDEEVDEM*	37.8	40.6	(45.8)
84	IILHENFDYDLLDNDISLLK	38.5	38.1	(40.8)
85	ASSTNLKDILADLIPKEQARIKTFRQQHGNTVVGQITVDM*	39.0	40.3	(43.7)
86	HGVTVLTALGAILK	40.5	32.1	(30.6)

87	Ac-ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMR	42.1	41.3	(44.6)
88	SQLSAAITALNSESNFARAYAEGIHRTKYWELIYEDC**M*	42.3	42.9	(47.5)
89	Ac-ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVM*	42.8	41.4	(45.4)
90	SLGQNPTEAELQDMINEVDADGNGTIDFPEFLTM	44.0	39.0	(42.3)
91	YLEFISEAIIHVLHSR	45.0	36.5	(38.2)
92	MARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK*LTDEEVDEMIREADIDGDGQVN YEEFVQMMTAK	45.5	46.0	(53.8)
93	NGLAGPLHGLANQEVLVWLTQLQKEVGKDVSDEKLRDYIWNTLNSGRVVPGYGHAVLRKTDPRYTC** QREFALKHLPHDPM*	45.5	50.3	(53.8)
94	VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEM*	45.6	47.2	(54.1)
95	SLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMAR	45.8	39.8	(43.6)
96	KMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK*LTDEEVDEMIREADIDGDGQVNYEE FVQMMTAK	46.2	45.5	(53.1)
97	VDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK*LTDEE VDEMIREADIDGDGQVNYEEFVQMMTAK	48.3	50.1	(58.8)
98	FKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKD TDSEEEIREAFRVFDKDGNGYISAAE	48.9	50.7	(59.0)
99	KASEDLKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGN FGADAQGAM*	50.0	49.6	(56.2)
100	Ac-ADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTID FPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEK*LTDEEVDEMIREADI DGDGQVNYEEFVQMMTAK	51.0	56.0	(66.7)

* M* = Homoserine or its lactone; C* = aminoethylcysteine; C** = carboxymethylcysteine; K* = trimethyllysine.

related to the sum of Rekker's constants[8] (which are based on the partition coefficients of free amino acids between water and octanol) for the constituent amino acids in a linear gradient elution system. Meek[7] reported a similar relation for small peptides, and extended the method to numerical analysis of the retention constants of amino acids. However, using their methods, we observed many discrepancies in the predicted retention order in a volatile eluent system and have reinvestigated procedures to predict those retention times.

EXPERIMENTAL

Materials

Almost all peptides were obtained by either enzymatic or cyanogen bromide degradation of calmodulin[9,10], lysozyme, citrate synthase[11], myoglobin, or crayfish trypsin[12]. Other oligopeptides were purchased from the indicated sources: Pro–Gly, Pro–Leu, Gly–Leu–NH_2, Gly–Tyr, Glu–Tyr, Lys–Tyr–Glu(Cyclo Chemical); Gly–Gly–Gly (Hoffmann-La Roche); Gly–Phe (California Biochemical Research); Gly–Trp (Mann Research Labs.); Trp–Tyr–NH_2 (Vega Biochemicals). The μBondapak C_{18} column (30 × 0.4 cm) was a product of Waters Assoc. Acetonitrile was obtained from Burdick & Jackson. Trifluoroacetic acid (Pierce) was used after distillation.

Methods

Retention times were measured on a μBondapak C_{18} column using a Varian Model 5000 liquid chromatograph. The mobile phase was 0.1% trifluoroacetic acid (pH 2) and the mobile phase modifier was acetonitrile containing 0.07% trifluoroacetic acid. The flow-rate was 2.0 ml/min. The concentration of the mobile phase modifier was increased linearly from 0 to 60% over 60 min (1%/min). The elution was monitored by absorption at 216 nm, and retention times were measured from the time at injection to that at the center of the eluting peak. Regression analyses were done either by a VAX computer or by a PDP-12 computer with a floating point processor. Matrix inversion was performed using double precision arithmetic.

RESULTS

Retention behavior of peptides

Retention times of 100 peptides tested are listed in Table I. The observed retention times (t_{Ri}) were plotted against $\sum_j D_j n_{ij}$, where D_j is the retention constant of amino acid j (Table II), and n_{ij} is number of residues of amino acid j in peptide i. Using Meek's constants as D_j the plot (Fig. 1a) gave a poor correlation (correlation coefficient 0.78). However, using Rekker's constants (where values for hydrophilic amino acids were slightly modified, Table II) as D_j the plotted data indicated an exponential relationship (Fig. 1b). Therefore, the data were fitted to a function of the form:

$$t_{Ri} = A \ln (1 + B \sum_j D_j n_{ij}) + C \qquad (1)$$

TABLE II

RETENTION CONSTANTS (D_j) OF AMINO ACIDS

The numbers in parentheses represent the number of amino acids used for each calculation

Amino acid	*Rekker's*	*Rekker's (modified)*	*Meek's*	*Present study (D'_j)*	
				Non-weighted	*Weighted*
Tryptophan	2.31	2.31	18.1(7)	35.8(12)	2.34(12)
Phenylalanine	2.24	2.24	13.9(18)	31.4(86)	1.71(86)
Isoleucine	1.99	1.99	11.8(4)	27.4(95)	1.38(95)
Leucine	1.99	1.99	10.0(13)	26.4(129)	1.34(129)
Tyrosine	1.70	1.70	8.2(16)	21.0(43)	1.23(43)
Methionine	1.08	1.08	7.1(11)	14.5(64)	0.85(64)
Proline	1.01	1.01	8.0(13)	7.9(33)	0.48(33)
Valine	1.46	1.46	3.3(6)	7.4(89)	0.38(89)
Threonine	−0.26	0.10	1.5(9)	7.4(111)	0.12(111)
Histidine	−0.23	−0.10	0.8(6)	8.8(38)	0.34(38)
Alanine	0.53	0.53	−0.1(8)	2.4(139)	0.13(139)
Glutamine	−1.09	0.20	−2.5(5)	3.2(59)	0.36(59)
Glutamic acid	−0.07	0.20	−7.5(4)	2.7(198)	0.27(198)
Glycine	0.00	0.10	−0.5(20)	4.0(134)	0.22(134)
Serine	−0.56	0.10	−3.7(11)	1.1(62)	0.18(62)
Arginine	–	−0.10	−4.5(10)	0.0(73)	0.26(73)
Aspartic acid	−0.02	0.10	−2.8(7)	− 0.1(165)	0.10(165)
Asparagine	−1.05	0.10	−1.6(6)	−11.3(71)	−0.45(71)
Lysine	0.52	−0.10	−3.2(9)	− 3.1(98)	0.05(98)
Carboxymethylcysteine	–	0.10	–	32.5(5)	1.57(5)
Homoserine	–	0.10	–	12.3(13)	0.23(13)
Aminoethylcysteine	–	−0.10	–	4.3(5)	0.31(5)
Trimethyllysine	–	−0.10	–	−38.1(9)	−1.38(9)
Acetyl-	–	0.00	3.9(1)	12.4(6)	0.81(6)
Amide-	–	0.00	5.0(8)	−13.2(2)	−0.56(2)

by using the method of least squares, where the modified Rekker's constants were used for D_j. The best fit for B was evaluated by plotting the correlation coefficient (of t_{Ri} and $\ln [1 + B \sum_j D_j n_{ij}]$) against B. The maximum correlation coefficient (0.97) was estimated when B was 1.1. The intercept (C) and the slope (A) were −5.6 and 13.4 respectively.

Calculation of retention constants

Assuming an equation of the form:

$$t_{Ri} = A' \ln (1 + \sum_j D'_j n_{ij}) + C' \qquad (2)$$

new retention constants (D'_j) and constants A', C' were computed from the observed retention times by non-linear multiple regression analysis[13]. As initial values for D'_j, modified Rekker's constants were multiplied by 1.1; for A' and C', 13.4 and −5.6 were used respectively. Several variations of curve fitting were applied to the data. By

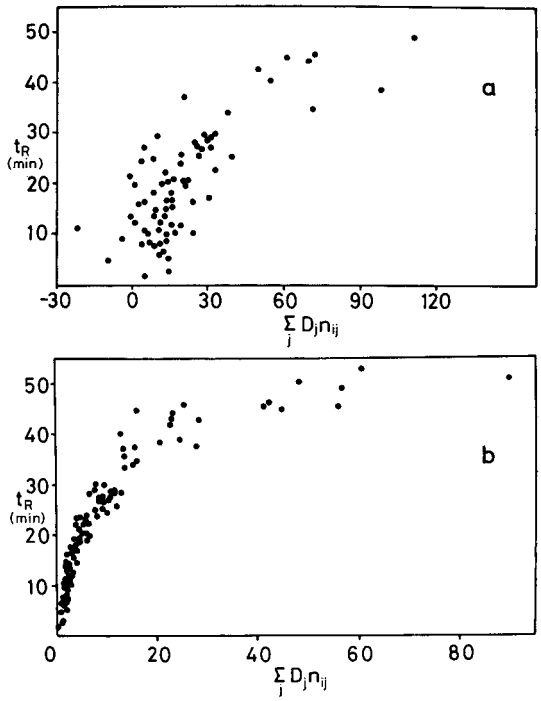

Fig. 1. Dependence of retention time (t_R) on amino acid composition. The observed retention times (Table I) were plotted against $\sum_j D_j n_{ij}$, were D_j is the retention constant of amino acid j (Table II), and n_{ij} is number of amino acid residues j in peptide i. (a) Meek's constants were used for values of D_j. Peptides which contain amino acids whose retention constants were not reported were excluded from the plot. The linear correlation coefficient was 0.78. (b) Modified Rekker's constants were used for values of D_j.

straightforward least squares analysis without weights, the parameters D'_j (Table II, column 5) obtained for the amino acids were rather different from those of Meek[7] or Rekker[8]. It was also found that the correlation coefficient of observed and predicted retention times was rather insensitive to the retention constants D'_j. For this reason, and because we wished to have a more uniform percentage deviation of observed and predicted retention times, weighted least squares were performed. The weights used were $1/N_i^2$ where N_i is the number of amino acids in the peptide. The results of this calculation are quite satisfactory. The retention constants (Table II, column 6) were of similar magnitude to those reported by other workers (Table II, columns 2, 3). The unweighted correlation of observed and predicted retention times is 0.984 while the weighted correlation is 0.982 (Fig. 2). The mean percent deviation of retention times is only 9 percent. The predicted retention times for each peptide in this study from both unweighted and weighted curve fitting methods are reported in Table I for comparison with observed values. Fig. 2 shows the plot of t_{Ri} as a function of $\ln(1 + \sum_j D'_j n_{ij})$ for both unweighted (a) and weighted (b) least squares parameters. The graphs are both linear with correlation coefficients of 0.98.

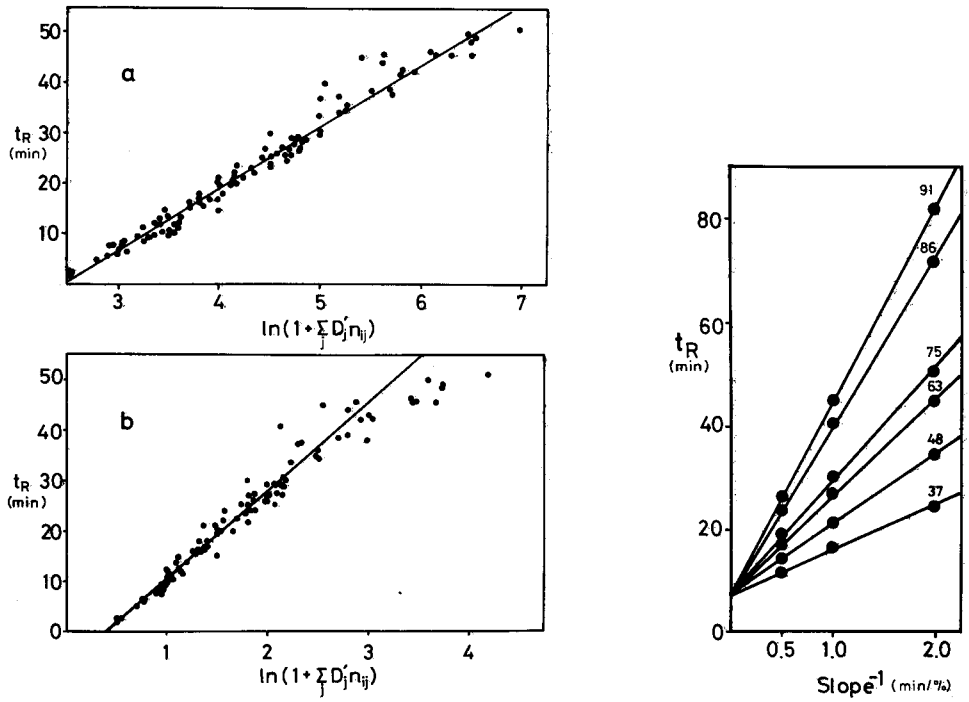

Fig. 2. Relationship between retention time (t_R) and ln $(1 + \sum_j D'_j n_{ij})$. The observed retention times (t_{Ri}) were plotted against ln $(1 + \sum_j D'_j n_{ij})$, where D'_j is the calculated retention constants either by unweighted or weighted curve fitting. (a) Unweighted fit retention constants were used for D'_j. The intercept and the slope of the straight line was -30.3 and 12.4, respectively. The correlation coefficient was 0.984. The mean percent deviation of retention time was 9.9%. (b) Weighted fit retention constants were used for D'_j. The intercept and slope of the straight line were -7.04 and 13.6, respectively. The weighted correlation coefficient was 0.981. The mean percent deviation of retention time was 9.2%.

Fig. 3. Plot of retention time (t_R) against the inverse of the slope (m) of the gradient. Retention times of six peptides (the number of the peptide corresponds to that in Table I) in different gradients were plotted against the inverse of the slope (m) of the gradient.

Effect of slope of gradient on retention time

Retention times of six different peptides were measured in the same elution system but using three different gradients, between 0.5 and 2.0%/min. The retention times (t_{Ri}) were plotted against the inverse of the slope (m) of the gradient (Fig. 3). Late eluting peptides gave straight lines which have a common intercept (0,7). Therefore, it should be possible to estimate the variation of t_{Ri} with m by equation (3):

$$t_{Ri} = F_i(1/m) + 7 \tag{3}$$

where F_i is the slope of the straight line and is characteristic for a given peptide. By combination of equations (2) and (3) one may express the retention time of a peptide in any linear gradient (0.5 to 2%/min) as

$$t_{Ri} = (1/m) \times [A' \ln (1 + \sum_j D'_j n_{ij}) + C' - 7] + 7 \tag{4}$$

TABLE III

COMPARISON OF PREDICTED AND OBSERVED RETENTION TIMES

Numbers correspond to those in Table I. The retention times were predicted by using non-weighted parameters

No.	*Peptide*	*Retention time (min)*			
		Slope (2%/min)		*Slope (1%/min)*	
		Predicted	*Observed*	*Predicted*	*Observed*
94	Myoglobin residues 1–55	26.4	26.0	45.8	45.6
99	Myoglobin residues 56–131	27.5	28.3	47.9	50.0
78	Myoglobin residues 132–153	20.1	20.6	33.2	34.2

The predictive utility of equation (4) was tested on cyanogen bromide peptides of myoglobin. The results are presented in Table III.

DISCUSSION

The object of this study has been the development of a method capable of predicting retention times for a wide range of peptides in a linear gradient elution system. Peptides of known sequence have been examined using aqueous trifluoroacetic acid as mobile phase. Both Meek[7] and O'Hare and Nice[6] reported a linear relation between t_R values of small peptides and the sum of their amino acid retention constants. However, the present study clearly shows an exponential relationship (Fig. 1b). Apparent discrepancy between the previous methods and ours may simply be due to a difference in the range of peptides investigated. In his system, Meek only tested small peptides (<29 residues long), and thus obtained data which approximated a linear relation. Our data also look linear during the first 20 min of elution. Non-linear eqn. 2 accurately describes the dependence of retention time on the amino acid composition for a wider range of peptide size. An example is given in Table IV in order to demonstrate the applicability of the above method. The reason underlying the exponential relation remains to be explained. Assuming the empirical eqn. 2, we have computed a set of retention constants for amino acids both with and without attaching weights on the observations. As was expected, both aromatic and aliphatic amino acids make large positive contribution to retention, and the relative degree of the contribution is almost the same as Meek's constants. However, some differences were found between our constants and Meek's constants (correlation coefficient between our constants and Meek's constants[7] was 0.816) which probably explain the poor correlation (Fig. 1a) of his prediction and our data. Neutral and acidic amino acids in our system showed a small positive contribution except for aspartic acid and asparagine. Meek, however, assigned negative values for almost all of these amino acids, and an especially large negative value for glutamic acid. The positive contribution of glutamic acid or glutamine in our system was clearly illustrated by the retention order in the following sets of peptides: YK < KYE, FK < FKE, AFR < EAFR, IAE < QIAE, IAEFK < QIAEFK. The apparent discrepancies between our constants and Meeks' may simply be due to a difference in the eluent system. We used trifluoroacetic acid (pH 2) in the mobile

TABLE IV

COMPARISON OF DATA FROM LITERATURE WITH PREDICTED RETENTION TIMES

Data were from O'Hare and Nice[6]. Retention times were predicted using equation 2 and the non-weighted retention constants for amino acids of Table II. Due to the different solvent system and the presence of pyroglutamic acid and cystine, it was necessary to fit four new constants. The slope and intercept of the straight line were 8.30 and −14.73, respectively. The retention constants for pyroglutamic acid and cystine were found to be −12.2 and −27.3, respectively. The correlation coefficient was 0.81 and the mean percent deviation of retention time was 12.0%.

Peptides	*Number of residues*	*Retention time (min)*	
		Predicted	*Observed*
Arginine vasotosin	9	12.8	12.0
Lysine vasopressin	9	13.1	13.0
Arginine vasopressin	9	13.9	14.0
ACTH 5-10	6	22.5	17.0
Diphenylalanine	2	20.2	18.0
ACTH 1-18	18	25.8	18.5
Met-enkephalin	5	21.7	19.0
Oxytosin	9	18.6	19.5
ACTH 4-10	7	23.8	20.5
ACTH 1-24	24	28.8	21.5
α-Endorphin	16	27.0	22.0
Leu-enkephalin	5	22.9	22.0
Insulin A(bovine)	21	22.2	22.0
Angiotensin II	8	24.3	23.0
Neurotensin	13	25.1	24.5
α-Melanotropin	13	26.4	26.0
Bombesin	14	25.0	26.0
RNAase	124	36.7	27.5
Triphenylalanine	3	23.5	28.0
Gastrin I	17	29.1	28.5
Substance P	11	26.0	29.0
Substance P 4-11	8	25.7	30.0
ACTH 1-39 (human)	39	32.8	30.5
ACTH 18-39	22	27.8	30.5
ACTH 34-39	6	24.2	31.0
Somatostatin	14	24.6	32.0
Insulin (bovine)	51	34.7	32.0
ACTH 1-39 (porcine)	39	33.5	33.0
Insulin B (bovine)	30	32.6	33.5
β-Endorphin (ovine)	31	31.3	34.0
β-Lipotropin (human)	91	38.4	34.5
Calcitonin (human)	32	31.7	34.5
Cytochrom C	104	40.1	35.0
Glucagon	29	31.7	36.0
Tetraphenylalanine	4	25.9	36.5
Calcitonin (salmon)	32	29.4	37.0
Lysozyme	129	40.4	37.5
Myoglobin	153	45.7	45.0
Melittin	25	32.3	46.0

phase, and Meek used perchloric acid (pH 2.1). However, it also should be noted that he calculated his 26 constants using only 25 peptides, which may be too small a sample for accurate estimation of the constants. During course of present study, Meek[14] revised his amino acid retention constants. Correlation coefficients between our constants and Meek's new constants are 0.844 (phosphate system), 0.821 (perchlorate system). The contribution of basic amino acids was small except for trimethyllysine. It is not so simple to explain the contribution of basic amino acids, because they will also interact with residual silanol.

For large peptides, their conformations, besides their amino acid composition, may also contribute to their retention times. This information was incorporated in the analysis by weighting each observation. The weighted fit constants predict retention times more accurately for small peptides. Probably the retention constants obtained by using weighted analysis reflect more realistic constants.

The effect of the slope of the gradient on the retention time is clearly illustrated in Fig. 3, where, for each peptide tested, a steeper gradient eluted the peptide earlier. Thus retention times were linearly related to the inverse of the slope. The intercept (7 min) corresponds closely to the delay time for the gradient front to reach the sample injection point.

These studies were carried out with a single C_{18} column to establish the empirical relationship between amino acid composition and elution time. Extension of these studies to other C_{18} columns and different elution system (using phosphate at both neutral and acidic pH values) have been recently completed, and will be reported elsewhere.

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ESTIMATION OF MOLECULAR WEIGHTS OF PEPTIDES BY DETERMINATION OF HEIGHT EQUIVALENT TO A THEORETICAL PLATE IN SIZE-EXCLUSION CHROMATOGRAPHY

YOSHIHIDE SHIOYA, HISANOBU YOSHIDA* and TERUMI NAKAJIMA
Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734 (Japan)
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SUMMARY

The analysis of the flow-rate required to obtain the optimum height equivalent to a theoretical plate, HETP, has been used to estimate the molecular weight, MW, of peptides in size-exclusion chromatography. A straight line is obtained when log MW is plotted against the flow-rate which gives the optimum HETP. This relationship holds even for peptides which adsorb to column packings. The typical quantity of peptide required is 1 nmol and analysis time was less than 2 h. The method is applied to peptides of 200–10,000, and the precision is $\pm 20\%$. The method may be applicable to the analysis of MW for all classes of compounds.

INTRODUCTION

The estimation of the molecular weight, MW, of proteins can be performed by either size-exclusion chromatography[1–4] or by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis[5]. However, these methods cannot always be applied for peptides of small MW. Gel electrophoresis is not suitable because of too large a pore size. Many column packing materials have been developed for size-exclusion chromatography, but there are inherent difficulties in their use to estimate small MW. Because ionic, hydrophobic interactions between solutes and column packing materials become more pronounced in the separation, the estimation of low MWs is not easily performed from the elution volume. Addition of salts, organic solvents or detergents to prevent anomalous elution in size-exclusion chromatography is not always satisfactory.

However, in a size-exclusion analysis, some chromatographic factors such as Eddy and longitudinal diffusion and the time to reach equilibrium may be MW dependent, and the diffusion or equilibrium should occur more rapidly for substances of small MW. If this holds true, the flow-rate to obtain the optimum height equivalent to a theoretical plate, HETP, should depend on the MW of the solute considered. In this paper we describe the relationship between the MW of the solute and the flow-rate required to obtain the optimum HETP.

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EXPERIMENTAL

Chemicals

Bovine γ-globlin, bovine serum albumin, cytochrome c, insulin, insulin A and B chain S-sulphonate, melittin and gramicidine J were obtained from Sigma (St. Louis, MO, U.S.A.), glucagon was from Calbiochem (Los Angeles, CA, U.S.A.) and secretin was a gift from Dr. S. Tachibana (Eisai Pharmaceutical Ltd., Tokyo, Japan). The peptides mentioned above were of natural origin; other peptides were synthetic. ACTH was a gift from Dr. R. Matsueda (Sankyo Pharmaceutical Ltd., Tokyo, Japan), and insulin C-peptide and vasoactive intestinal peptide (VIP) were gifts from Dr. N. Yanaihara (Shizuoka College of Pharmaceutical Sciences, Shizuoka, Japan). Other synthetic peptides were obtained from Protein Research Foundation (Osaka, Japan). The peptides studied are listed in Table I. Other chemicals including solvents for chromatography were obtained from Yoneyama Pharmaceutical Ltd. (Osaka, Japan). All were used without further purifications.

Size-exclusion chromatography

The high-performance liquid chromatographic (HPLC) system used was a TSK 805 with a TSK GEL G2000SW size-exclusion column (10 μm, 7.5 × 600 mm; Toyo Soda Ltd., Tokyo, Japan). Post-column detection of peptides was carried out by the *o*-phthalaldehyde (OPA) method according to Benson and Hare[6]. The fluorometer used was a FLD-1 (Shimadzu, Kyoto, Japan). The typical quantity of peptide needed for one chromatogram was about 0.2 nmol. The void volume was determined from the elution volume of Blue Dextran.

RESULTS AND DISCUSSION

Elution of peptides from the size-exclusion column

Over 50 peptides (MW 200–10,000, as shown in Table I) were tested to determine whether there were good relationships between MW and elution volumes (capacity factors), using some elution solvents. As shown in Table II, the elution volumes (capacity factors) of the peptides differed from one solvent to another. In the tested solvent systems, 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS eluted peptides from the column in approximate order of MW as shown in Fig. 1. However, two groups of peptides were eluted in unexpected positions. One group adsorbed to the resin and eluted later than expected, and contained Gly–Trp, angiotensin I and II and insulin A and B chain S-sulphonate. The second group were eluted faster than expected, and contained melittin and mastoparan. The reason for the faster elution was not clear. Thus the estimation of the MW of unknown peptides from simple size-exclusion chromatography is not promising.

Relationship of MW and the rate of attainment of equilibrium

Using model peptides of approximately the same MW but of different elution volumes from the size-exclusion column, the relationships between the flow-rate and the HETP were obtained as shown in Fig. 2. Clearly, when the MW of the peptides were approximately the same, the flow-rates required to obtain optimum HETP were

TABLE I

THE PEPTIDES TESTED

Substance	*MW*	*Log MW*	*Isoelectric point*	*Hydrophobicity**	*Biological activity*
1 γ-Globulin	160,000	5.20			–
2 Albumin	70,000	4.85	4.0		–
3 Cytochrome *c*	12,384	4.09	10.0		+
4 Insulin	5750	3.76	5.0	36.1	+
5 ACTH	4500	3.65	4.7		+
6 Glucagon	3550	3.55	6.0	8.5	+
7 Insulin C-peptide	3306	3.52	6.1	13.5	?
8 Vasoactive intestinal peptide (VIP)	3325	3.52	6.4	15.1	+
9 Insulin B chain S-sulphonate	3040	3.48	3.8	22.7	–
10 Secretin	2956	3.47	9.0	9.9	+
11 Melittin	2848	3.45	6.2	17.5	+
12 α-Endorphin	1684	3.23		6.9	+
13 Insulin A chain S-sulphonate	1653	3.22	6.4	13.4	–
14 Somatostatin	1638	3.21	6.3	10.7	+
15 Mastoparan	1479	3.17	6.8	14.6	+
16 Substance P	1348	3.13	10.9	7.9	+
17 Vespakinin	1344	3.13	9.6	10.7	+
18 Met–Lys–bradykinin	1319	3.12	9.4	8.6	+
19 Physalaemin	1266	3.1	7.1	9.0	+
20 Angiotensin I	1297	3.1	6.8	8.9	+
21 Gramicidin J	1369	3.14	7.2	13.4	+
22 Lys–bradykinin	1188	3.07	11.9	7.5	+
23 Bradykinin potentiator B	1181	3.07	8.5	9.6	+
24 Bradykinin	1060	3.03	9.4	7.0	+
25 Angiotensin II	1046	3.02	7.4	7.1	+
26 Val^1–Thr^6–bradykinin	1017	3.01	8.3	8.7	+
27 Angiotensin III	931	2.97	6.9	7.2	?
28 Eledoisin related peptide	707	2.85	9.6	7.8	+
29 Glutathione oxidized	613	2.79	5.9	0.0	?
30 [Leu^5]-enkephalin	556	2.74	7.2	5.9	+
31 Tuftsin	501	2.70	8.7	1.3	+
32 $(Gly)_6$	360	2.56	6.0	0	–
33 Liver cell growth factor	340	2.53	7.0	0.3	+

(Continued on p. 344)

TABLE I *(continued)*

Substance	*MW*	*Log MW*	*Isoelectric point*	*Hydrophobicity**	*Biological activity*
34 Reduced glutathione	307	2.49	5.9	0.0	?
35 $(Gly)_5$	303	2.48	6.0	0.0	–
36 Gly–Gly–Arg	288	2.46	8.3		–
37 Gly–Gly–His	269	2.43	5.8	– 0.2	–
38 $(Glu)_2$	275	2.44	3.2	– 2.2	–
39 His–Leu	268	2.43	5.8	1.8	–
40 Gly–Gly–Leu	245	2.42	6.0	1.9	–
41 $(Gly)_4$	246	2.39	6.0	0.0	–
42 Leu–Gly–Gly	245	2.39	6.0	1.9	–
43 Gly–Trp	260	2.42	6.0	2.3	–
44 Gly–Phe	222	2.35	5.7	2.2	–
45 Gly–His	212	2.33	5.8	– 0.2	–
46 Gly–Lys	203	2.31	7.4	0.5	–
47 $(Gly)_3$	189	2.28	6.0	0.0	–
48 Gly–Leu	188	2.27	6.0	1.9	–
49 Leu–Gly	188	2.27	6.0	1.9	–
50 $(Gly)_2$	132	2.12	6.0	0.0	–

* Relative lipophilicities of the side chains according to Rekker[9], excluding Arg and Orn residues.

also the same, even so the optimum HETP value for each peptide was different due to different elution mechanisms. Peptides, which adsorbed to the column and were eluted later showed smaller HETP. Fig. 3 shows the relationship between MW and the flow-rate required to obtain optimum HETP for the model peptides. It is clear that the optimum flow-rate is inversely and linearly related to log MW. The relationship holds even for peptides which were eluted from the column at unexpected positions from the viewpoint of size-exclusion chromatography. This relationship was also true when the elution solvent was a simple phosphate–saline, although the retention volumes were larger for many peptides due to strong adsorptive effects and the time required for one analysis was increased.

Estimation of the MW

First a working plot of the relationship between log MW and the optimum flow-rate is drawn using at least three peptides of different MW on the column being used, as follows. By use of three flow-rates, ranging from 0.2 to 1.0 ml/min, each HETP is calculated. As HETP $= A + B/u + Cu$ (refs. 7 and 8), where u is the flow-rate and A, B and C are constants for each peptide under the same analytical conditions including temperature, from three HETP values from different flow-rates, A, B and C can be calculated. For the optimum HETP, the flow-rate should be $\sqrt{B/C}$.

The optimum flow-rate for the sample peptide is then determined by the same procedure.

From the working plot obtained in the first step and the optimum flow-rate for the sample obtained in the second step, the MW of the sample can be estimated.

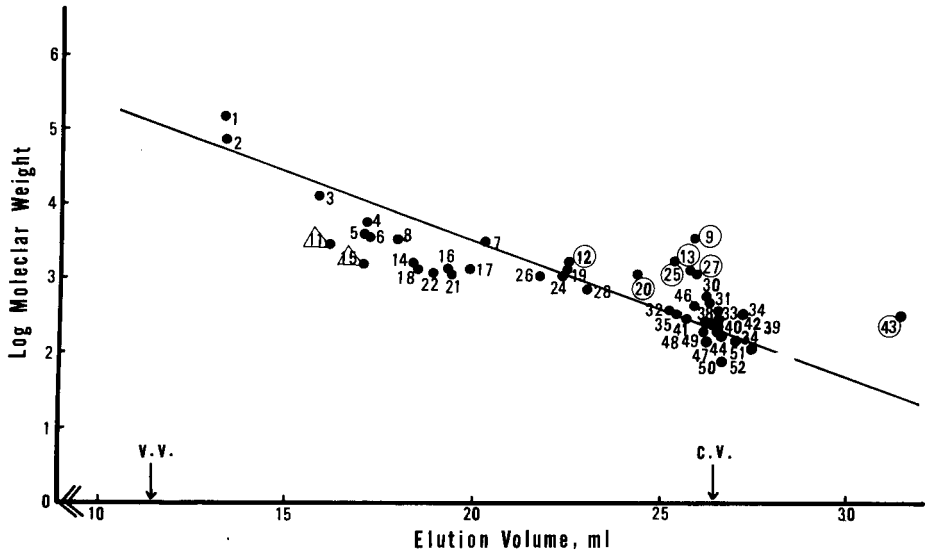

Fig. 1. The relationship between log MW and elution volumes of peptides. Column: TSK GEL G2000SW (7.5 × 600 nm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. Flow-rate: 0.9 ml/min. V.V. = Void volume; C.V. = column volume. Numbers correspond to the peptides in Table I; 51 = norleucine; 52 = glycine; ○ and △, see Fig. 3.

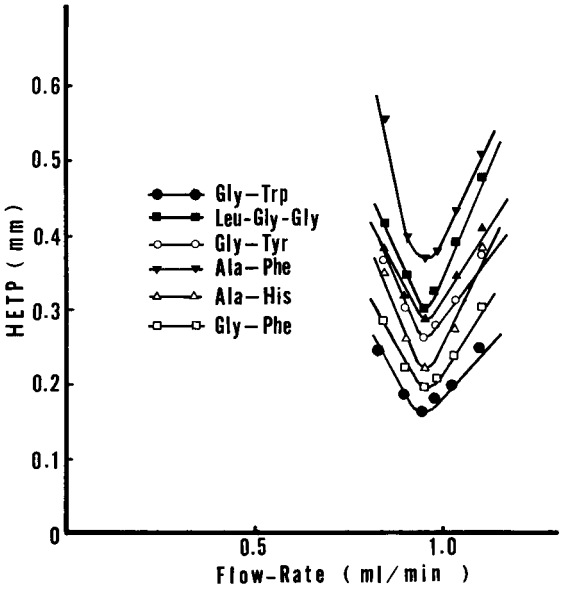

Fig. 2. The relationship between flow-rate and HETP. Column: TSK GEL G2000SW (7.5 × 350 mm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. ▲—▲, $(Gly)_4$.

TABLE II

CAPACITY FACTORS OF PEPTIDES ON TSK GEL G2000SW WITH SEVERAL SOLVENTS

PB = 0.15 *M* Phosphate buffer; NaCl = 1 *M* NaCl; MeOH = 20% methanol; MC = 20% methyl cellosolve; Brij = 1% Brij-35; Triton = 1% Triton X-100; SDS = 1% sodium dodecyl sulphate.

Substance	*Distilled water*	*PB*	*PB NaCl*	*PB NaCl MeOH*	*PB NaCl MC*	*PB NaCl Brij-35*	*PB NaCl MeOH Brij-35*	*PB NaCl MeOH Triton*	*PB NaCl MC Triton*	*PB NaCl MC SDS*
3 Cytochrome	0.28	0.80	0.59	0.90	0.78	0.64	0.80	0.90	0.76	0.40
16 Substance P	0.48	0.97	3.28	2.26	1.25	3.01	1.18	1.22	1.27	0.68
20 Angiotensin I	3.0	0.96	2.16	1.04	1.19	2.20	1.15	1.21	1.27	1.13
30 [Leu^5]–enkephalin	0.50	1.04	1.82	1.05	1.25	1.76	1.16	1.22	1.28	1.33
40 Gly–Gly–Leu	0.90	0.99	1.20	1.31	1.26	1.16	1.16	1.23	1.27	1.33
51 Norleucine	0.48	0.99	1.25	1.04	1.35	1.32	1.27	1.33	1.35	1.38

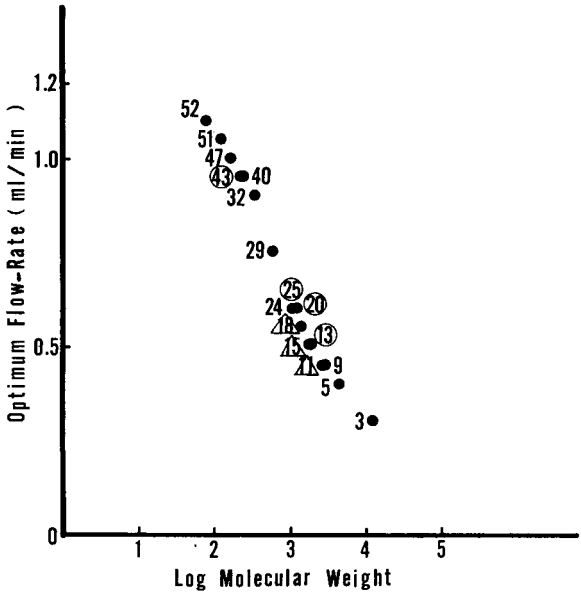

Fig. 3. The relationship between log MW and flow-rate required to obtain optimum HETP. Column: TSK GEL G2000SW (7.5 × 600 mm). Elution: 0.15 *M* phosphate buffer (pH 7.4) containing 1 *M* NaCl, 20% methyl cellosolve and 1% SDS. Temperature: 22°C. Numbers correspond to the peptides in Table I. ○, Peptides adsorbed to the column and eluted later than expected by size-exclusion chromatography with the same solvent; △, peptides eluted faster than expected by size-exclusion chromatography with the same solvent. Numbers correspond to the peptides in Table I; 51 = norleucine; 52 = glycine.

Accuracy of the method

Using the same size-exclusion column, the results of the MW estimation of some model peptides by the present method and by the conventional method are presented in Table III. The present method is superior than the conventional method, and is suitable to estimate MW in the range of 200–10,000 with an error of ±20%. The amount of sample peptide needed is a few nmol.

TABLE III

MW DETERMINATIONS BY THE DESCRIBED METHOD AND BY THE CONVENTIONAL METHOD

Substance	*MW*	*Present method**	*Conventional method***
Gly–Trp	260	260	25
$(Gly)_6$	360	400	320
Bradykinin	1060	1200	1000
Angiotensin I	1250	1300	400
Mastoparan	1480	1500	6310
Melittin	2850	3000	10,000
ACTH	4500	4500	5620

* By a calibration curve for the relationship between log MW and the optimum flow-rate.
** By a calibration curve for the relationship between log MW and the elution volume.

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APPLICATION OF FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY TO THE ANALYSIS OF UNDERIVATIZED DRUGS

L. L. PLOTCZYK
Hewlett-Packard Company, Route 41, Avondale, PA 19311 (U.S.A.)
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SUMMARY

The feasibility of using fused-silica capillary chromatography for the routine analysis of several common drugs is illustrated. Considerations for optimizing on-column and splitless injection are discussed as part of a study of system discrimination and reproducibility. The peak shape of polar solutes is improved through the use of a binary solvent and non-extractable stationary phase columns. Cold on-column injection with cross-linked polysiloxane deactivated columns produced linear quantitation from 1 to 100 ng with precisions of 0.1–2% for selected anticonvulsant drugs.

INTRODUCTION

Capillary chromatography was originally used as a qualitative technique for the high-resolution analysis of petroleum-based substances. Advances in instrument design and column technology have led to the quantitative analysis of not only relatively inert hydrocarbons, but also more polar solutes including phenols, amines, and mercaptans[1,2]. The development of the cold on-column injection technique and non-extractable stationary phase columns has enabled capillary chromatography to expand into more diverse application areas. One area is the analysis of underivatized drugs. Growth into biological or pharmaceutical drug related areas will be spurred by the high sensitivity, selectivity and speed offered by fused-silica column gas chromatography.

Methods using glass column chromatography for the biological analysis of several important drugs, steroids, and prostaglandins were recently reviewed[3,4]. Only marginal success has been experienced with these techniques due to problems with column surface activity. These problems can be minimized using sample derivatization procedures and column leaching techniques. The inertness and high-temperature stability of commercially available fused-silica columns eliminates the need for derivatization, for many samples, while providing enhanced sensitivity. Nanogram quantities of several drugs and their metabolites may be resolved on a single stationary phase.

Quantitative use in such areas as therapeutic drug monitoring, dosage phar-

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macokinetics and forensic drug screening requires an investigation into the linearity and reproducibility of the method. In this study, several underivatized anticonvulsant drugs were selected to test factors affecting column selection and optimization of the chromatographic system. The fused-silica capillary gas chromatography of common alkaloids, barbiturates, analgesics and tricyclic antidepressants is illustrated.

EXPERIMENTAL

The analysis was performed on a Hewlett-Packard (Avondale, PA, U.S.A.) 5880A gas chromatograph equipped with splitless and dedicated on-column injection ports and a 7672A automatic liquid sampler. The injection volume was 1 μl. A flame ionization detector was used with nitrogen make-up gas at 45 ml/min, air at 400 ml/min and hydrogen at 35 ml/min. On-column syringes were prepared by fitting Hamilton 701-RN gas-tight syringes with fused-silica needle stock (100 $\times$ 0.14 mm I.D.). Siloxane deactivated cross-linked and gum phase SE-54* fused-silica columns were obtained from Hewlett-Packard. Distilled-in-glass methanol and toluene were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.).

RESULTS AND DISCUSSION

Two fused-silica capillary systems were defined for the purpose of this study. They include splitless injection on gum phase columns and cold on-column injection on non-extractable cross-linked columns. The first system is composed of instrumentation and column technology which is routinely available and has the advantage of automation. By comparison, technology for both the on-column injection and non-extractable columns only recently became available. Their combination, however, results in a state-of-the-art system specifically designed for accurate and reproducible quantitation. The degree of activity present in each of these systems was investigated along with factors affecting their optimization.

Splitless injection on gum phase columns

Splitless injection of polar solutes requires a careful choice of both solvent and inlet temperature. Considerations for solvent selection should include the ability to dissolve the sample, to provide a good "solvent effect" and to be compatible with the column stationary phase. In general, the "solvent effect" occurs when the solute encounters a steadily increasing stationary phase film (solvent plus stationary phase) at the head of the column. Molecules at the front of the solute band are slowed to a greater extent than those at the rear, creating an effective reconcentration of the solute[5,6]. Conditions for an optimized "solvent effect" require that the solvent be soluble in the stationary phase. Since primarily non-polar stationary phases are currently available with fused-silica columns, the usual capillary solvents are non-polar.

Several typical capillary solvents, including hexane, toluene and methylene chloride, were tested and found to lack sufficient solvent strength to dissolve the drug sample. More polar solvents, such as methanol and ethyl acetate, will dissolve most

* SE-54 is a registered trademark of General Electric.

common drugs but will also strip or extract the thin layer of column stationary phase. The result is a steadily decreasing stationary film thickness, at the front of the column and a subsequent increase in column activity. Secondly, the use of polar solvents will not provide an optimized "solvent effect". The low solubility of the solvent in the stationary phase leads to an ineffective reconcentration and poor polar solute peak shape.

Jenkins[7] recently proposed a technique to improve polar solute peak shape by co-injection of a low-polarity secondary solvent. A plug of an immiscible non-polar solvent is drawn into the syringe barrel followed by the sample plug. A second plug of the secondary non-polar solvent is drawn into the syringe after the sample. The three liquid plugs are separated by air spaces. During the "solvent effect", the polar solvent is essentially encapsulated at the front and rear by a non-polar solvent–stationary phase film. A more efficient reconcentration results in an improvement in solute peak shape. Co-injections of methanol–hexane, methanol–cyclohexane, and acetonitrile–benzene were investigated.

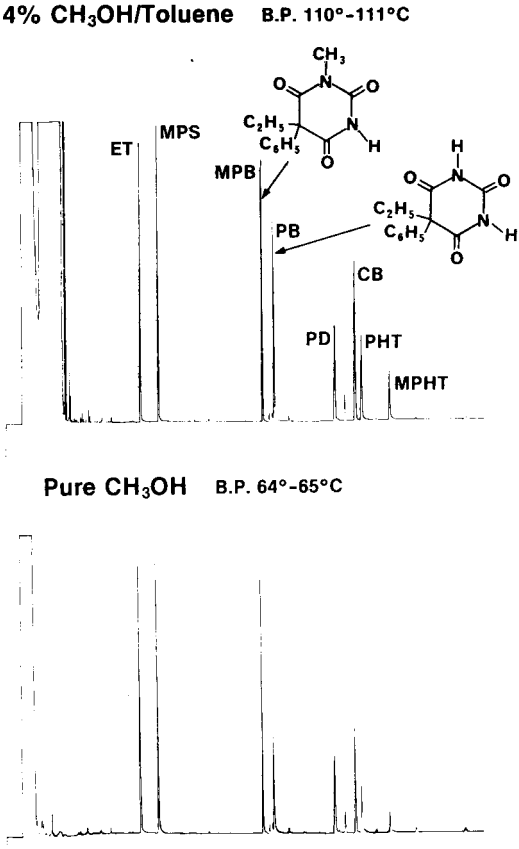

Fig. 1. Solvent selection in the splitless sampling of anticonvulsant drugs at 20 ng per drug. Column: SE-54 gum (25 m × 0.32 mm I.D.), β = 450; oven profile: 45°C for 1 min, 15°/min to 240°C. ET = ethosuximide; MPS = methyl-propylsuccinimide; MPB = mephobarbital; PB = phenobarbital; PD = primidone; CB = carbamazepine; PHT = phenytoin; MPHT = methyl phenytoin.

A second technique to improve polar solute peak shape is the use of a low percentage polar solvent in a miscible, higher boiling and inert non-polar solvent. A binary mixture of 1 to 10% methanol in toluene eliminates the need for co-injection while providing a means for dissolving the drug sample, optimizing the solvent effect and minimizing column deterioration. Fig. 1 shows a splitless injection of the anticonvulsants at 20 ng per drug using methanol (b.p. 64°C) as the solvent. A suitable solvent effect is obtained at an initial oven temperature of 10–30°C below the boiling point. However, the low relative response of the reactive secondary amine, phenobarbital, *versus* the methylated tertiary amine, mephobarbital, suggests that solvent extraction of the SE-54 gum phase has occurred. Phenobarbital adsorption to newly exposed surface silanol groups may account for the loss. This is supported by the observation that repeated injections produces steadily declining phenobarbital recoveries.

A binary solvent of 4% methanol in toluene restores the relative response of the phenobarbital peak. Toluene (b.p. 110°C) is a suitable choice for the secondary solvent since it is both miscible with methanol and shows less tendency to extract the

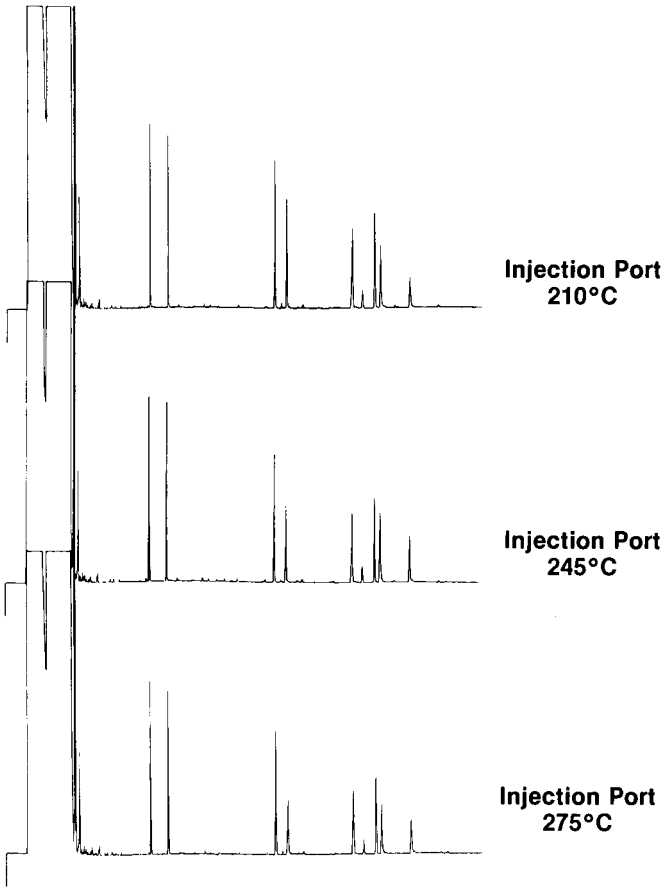

Fig. 2. Effect of injection port temperature on splitless sampling of anticonvulsant drugs at 4.8 ng per drug.

non-polar gum phase. Peak splitting or other forms of peak irregularities may occur with the use of binary solvents or co-injected solvents if liquid–liquid partitioning and/or zones of differing phase ratios are formed during the solvent effect. Co-injections will be particularly prone to these effects since the use of relatively immiscible solvents creates a multi-phase system. These problems are avoided with the binary solvent if the initial oven temperature is far enough below the boiling points of the miscible solvent pair or some form of secondary cooling is used. A value of 20°C below the boiling point of methanol was adequate for a methanol–toluene mix of 1–10% with splitless injection. There was no evidence of peak splitting over a range of initial oven temperatures near the boiling point of methanol. The formation of a single phase "solvent effect" may account for these observations.

Thermal lability of the underivatized drug is minimized in splitless sampling by operating at the lowest inlet temperature possible. Increasing thermal degradation with unnecessarily high inlet temperatures is evidenced by the declining phenobarbital response (Fig. 2). Typically, an inlet temperature of 200–250°C will be sufficient. Lower inlet temperatures than are used with either split or packed column injection are possible due to an increased residence time in the inlet liner and a longer volatization time.

TABLE I

SPLITLESS REPRODUCIBILITY OF ANTICONVULSANTS (4.8 ng PER DRUG)

HP 7672A automatic liquid sampler; 1-μl injections; purge activation time, 0.5 min.

	Area counts							
	ET	*MPS*	*MPB*	*PB*	*CB*	*PD*	*PHT*	*MPHT*
	27.54	27.82	28.22	18.56	24.08	26.55	23.71	16.37
	26.20	26.74	27.19	18.18	23.49	26.16	23.75	15.59
	24.18	24.72	24.70	16.76	21.07	23.36	19.63	13.82
	24.78	25.13	25.32	16.90	21.25	23.57	19.42	13.20
	25.00	25.36	25.92	17.80	22.38	24.92	18.87	14.85
	27.19	27.87	29.16	20.70	25.94	28.53	20.63	17.63
	24.61	24.97	25.07	15.67	21.49	24.09	21.26	12.68
Mean	25.64	26.09	26.51	17.79	22.81	25.31	21.04	14.87
S.D.	1.33	1.36	1.71	1.61	1.79	1.87	2.00	1.78
Relative S.D. (%)	5.20	5.22	6.45	9.05	7.85	7.41	9.51	11.99

Reproducibility of splitless sampling on a gum phase SE-54 column was tested with the aid of an HP 7672A automatic liquid sampler. Results appear in Table I. Deviations in peak area are caused by both reversible and irreversible adsorption phenomena to active sites present in the fused silica inlet liner and column. In addition, sample may be lost at the moment of injection by back flow against the inlet septum or syringe needle as a result of the flash vaporization technique.

Relative standard deviations in absolute area of 5–12% were typical for the anticonvulsant drugs at 4.8 ng per drug. Quantitation in the splitless mode will benefit from the use of internal standards but will be limited to those analyses which tolerate precisions in the order of 5%. Into this category fall many of the clinical methods

which routinely use internal standards and often report therapeutic concentrations to within 10%.

On-column injection with cross-linked columns

Sample losses associated with the splitless inlet are eliminated with the cold on-column injection technique. Sample is introduced onto the capillary column as a liquid aerosol with the aid of a specifically designed inlet and fused-silica syringe[8,9]. Reconcentration of the solutes occurs via the "solvent effect" when the initial oven temperature is equal to or near the boiling point of the solvent.

An on-column injection of the anticonvulsants with an SE-54 gum phase column is shown in Fig. 3. Improvement in the relative response of phenobarbital, carbamazepine, and phenytoin is evident when compared to splitless injection (see Fig. 1). Improved accuracy with on-column *versus* splitless injection is apparent for polar and thermally labile solutes. Optimization of the "solvent effect" for the methanol–toluene mix occurred at an initial oven temperature between the boiling points of the solvent pair. A value of 75°C was chosen because it is slightly above, but near, the boiling point of methanol yet below the boiling point of toluene.

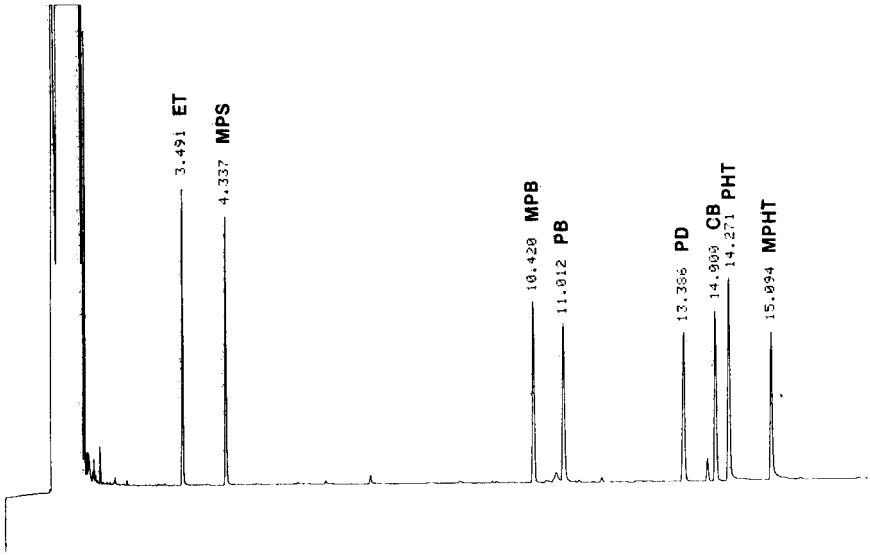

Fig. 3. On-column injection of anticonvulsants at 4.8 ng per drug. Column: SE-54 gum (25 m × 0.32 mm I.D.), β = 450; solvent: methanol–toluene (4:96); oven profile: 75°C for 0.5 min, 10°C/min to 260°C.

Effects of stationary phase solvent extraction will be even more severe than with splitless injection since the liquid is placed directly on the column. The use of a binary solvent will reduce these effects but will not eliminate them. Columns compatible with the on-column injection of polar solvents may be prepared by a process known as cross-linking. The technique was first described by Noll[10] in 1960 and later by Madani and Chambaz[11], Blomberg *et al.*[12], and Grob *et al.*[13]. The process involves the addition of peroxides to the gum phase to initiate *in situ* cross-linking. Methylene linkages are formed in the case of the methylsilicone OV-1*, and a combination of methylene and vinyl linkages in the methyl-phenylsilicone phases. When

coated onto a siloxane deactivated fused silica surface, columns are produced which exhibit inertness, high thermal stability and low solvent extractability.

Column selection for the analysis of underivatized drugs is further aided by the use of column test mixtures. The fused silica surface is inherently acidic in nature due to the presence of surface silanol groups ($pK_a = 6.5$). Drugs, such as the anticonvulsants, which are normally considered "acidic" may appear "basic" or even "neutral" relative to the silanol groups. Substantial adsorption of the drug would occur with an undeactivated surface. The analysis of both acidic and moderately basic drugs will be improved through deactivation of the fused silica. Column test mixtures are a useful means of defining the level of surface activity remaining after deactivation and coating of the stationary phase.

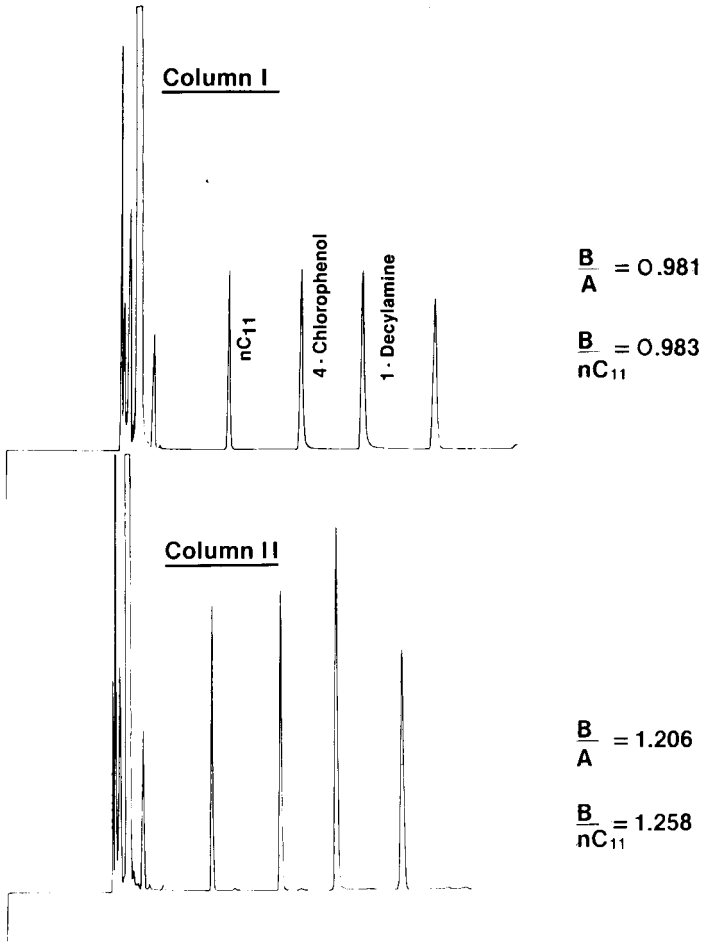

Fig. 4. Test mixture comparison of two cross-linked SE-54 siloxane deactivated columns (25 m × 0.32 mm I.D.), $\beta = 150$. The higher base to acid ratio of column II indicates a better level of deactivation resulting in decreased reversible and irreversible adsorption. Column II is preferred for the analysis of moderately polar drugs.

* OV-1 is a registered trademark of Ohio Valley.

TABLE II

RESPONSE FACTOR REPRODUCIBILITY OF ANTICONVULSANTS (10 ng PER DRUG; $n = 6$)

Area ratio	*Column I*	*Column II*
	Mean ± S.D. (Rel. S.D.)	*Mean ± S.D. (Rel. S.D.)*
ET/n C_{16}	0.5965 ± 0.0169 (2.83)	0.6307 ± 0.0009 (0.14)
MPB/n C_{16}	0.4721 ± 0.0135 (2.86)	0.4972 ± 0.0019 (0.37)
PB/n C_{16}	0.4215 ± 0.0092 (2.18)	0.4679 ± 0.0046 (0.99)
PHT/n C_{16}	0.5132 ± 0.0193 (3.77)	0.5520 ± 0.0095 (1.72)

Results (Fig. 4) of a column test mixture containing equal amounts of an inert hydrocarbon (undecane), an organic acid (4-chlorophenol), and an organic base (1-decylamine) are compared for two cross-linked polysiloxane deactivated columns. The increased amount of tailing evident in column I indicates a lower level of siloxane deactivation. Adsorption phenomena are minimized on column II which yields a higher base to acid (B/A) and base to hydrocarbon (B/n C_{11}) ratio. Column II will be more suitable for the analysis of moderately polar drugs.

This hypothesis is verified by the response factor reproducibility shown in Table II. In this case, hexadecane has been added as an internal standard to a solution containing 10 ng of each anticonvulsant drug. For ten manual injections, the more

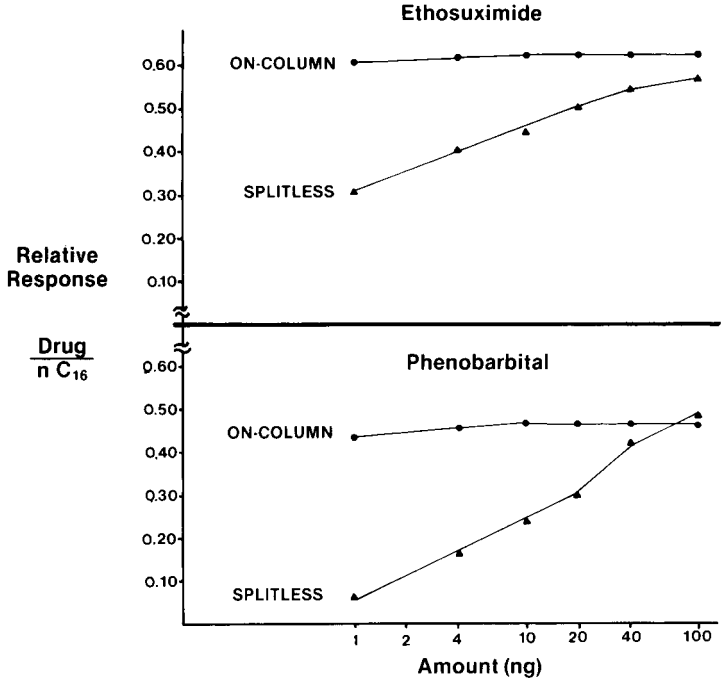

Fig. 5. Linearity of selected anticonvulsant drugs under splitless *versus* on-column injection. Inlet effects are the major contribution to discrimination occurring from 1 to 100 ng per drug. Column effects will predominate for the on-column injection at less than 1 ng. Column: cross-linked SE-54 (column II); same conditions as in Fig. 4.

inert column (column II) exhibits a slightly higher relative response and much lower relative standard deviation. Improvement in accuracy and precision is due to decreased irreversible and reversible adsorption effects.

Variations in relative response with the amount of drug injected were used as a measure of discrimination occurring with on-column *versus* splitless sampling (Fig. 5). To reduce the effects of discrimination which may be occurring on the column itself, the same cross-linked column was used for both injection techniques. The result is a comparison of the relative activities of the two sampling systems, including syringe effects and inlet adsorption phenomena. Non-linearities in the splitless data are due to the chemically active inlet system and the use of a vaporizing injection technique.

Cold on-column injection yields a linear response from 1 to 100 ng of drug with

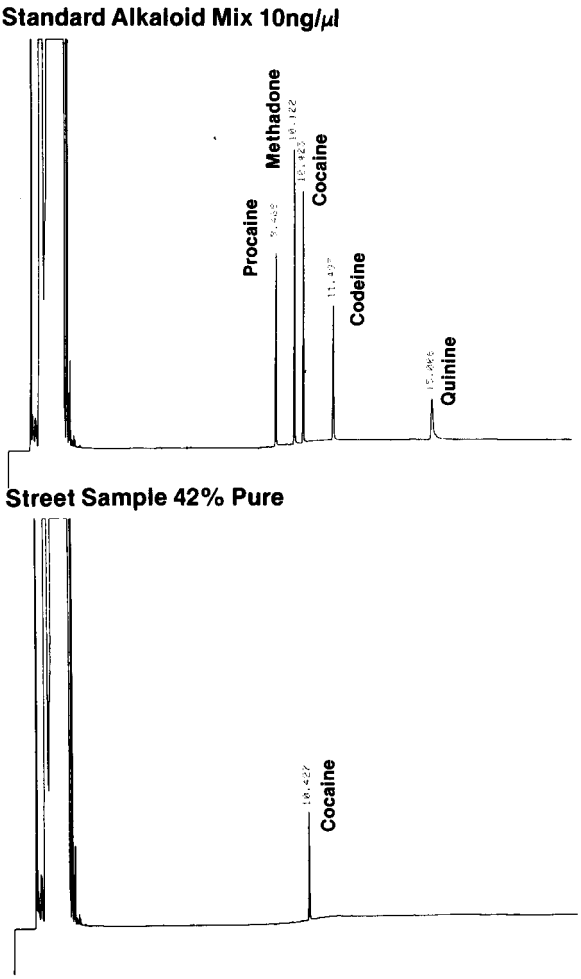

Fig. 6. Identification and quantitation of cocaine with on-column injection. Column: cross-linked SE-54 (25 m × 0.32 mm I.D.), β = 150; oven profile: 80°C for 0.5 min, 20°/min to 280°C; solvent; methanol–toluene (1:99).

reproducibilities of 0.1–2 % at the 10-ng level. Precisions in this range indicate that the technique may be useful for the accurate quantitation of underivatized drugs, in relatively pure samples, without the need for internal standards or multilevel calibration. The on-column injection of serum, or other complex biological samples, will still benefit from the use of internal standards as a check on extraction efficiency in the sample clean-up.

Applications

Forensic screening of "street" drug samples is one application of this chromatographic method. Fig. 6 shows the quantitative and qualitative analysis of cocaine. The "street" sample is dissolved in methanol–toluene (1:99). Sugars and other undissolved additives are centrifuged and separated. The remaining solution is injected on-column. An alkaloid mix containing 10 ng/μl of drug is injected as an external standard. With just two injections, the unknown drug is both identified via its retention time and determined to be 42 % pure. Retention indexing or mass spectral identification could also be employed to support the results.

An on-column injection of a serum extract is shown in Fig. 7. A single extraction was performed on 100 μl of human serum from a patient on phenytoin therapy for the treatment of epilepsy. The serum was buffered to pH 6, extracted into chloroform, dried, reconstituted with 25 μl of the binary solvent and 1 μl was injected on-column. The amount of co-extracted substances could be reduced and column life lengthened through the use of a back extraction in the sample preparation sequence. With splitless injection, non-volatile material will remain in the replaceable inlet liner.

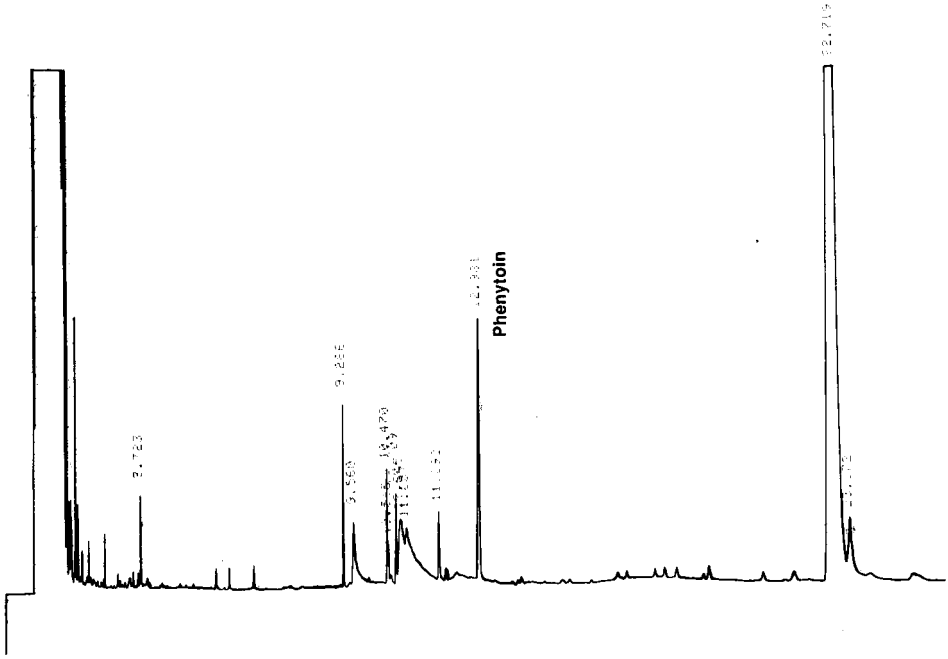

Fig. 7. On-column injection of a human serum extract. Sample: 100 μl of serum with a reported phenytoin concentration of 10.9 μg/ml. Serum was buffered to pH 6, extracted into chrloroform, dried, reconstituted with 25 μl of the binary solvent and 1 μl was removed for injection. Column: cross-linked SE-54 (25 m × 0.32 mm I.D.), $\beta = 150$; solvent: methanol–toluene (4:96); oven profile: same as in Fig. 8.

Dirtier samples will be more easily tolerated and sample preparation time reduced with splitless injection. Quantitation with splitless injection, however, should only be done with the use of internal standards and multi-level calibration.

CONCLUSION

Fig. 8 summarizes several types of drugs which have been analyzed by fused-silica capillary chromatography. Underivatized anticonvulsants, alkaloids, barbiturates, and tricyclic antidepressants have been combined into a single drug screen. The high resolution offered by this technique makes a single stationary phase suitable for multi-drug identification. The use of the on-column injection technique with non-extractable siloxane deactivated stationary phases can lead to the linear and reproducible trace quantitation of selected members from each of these drug families.

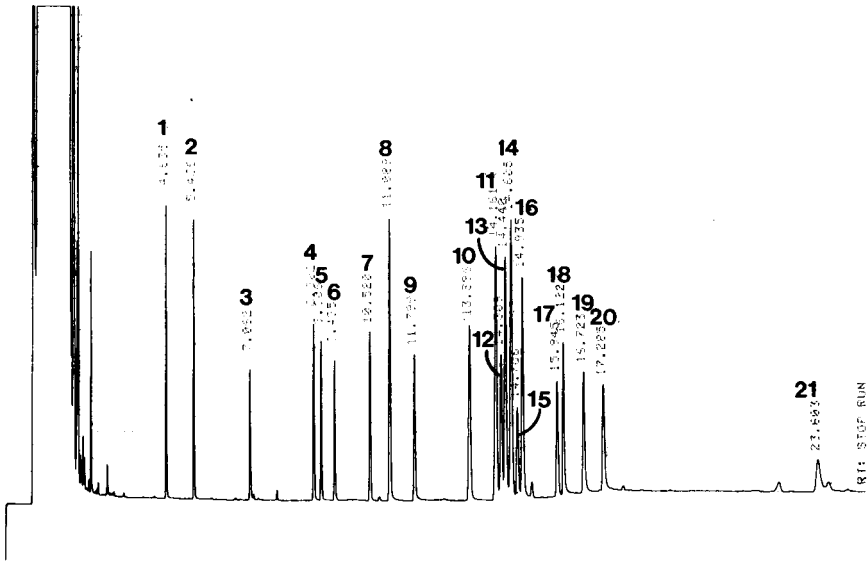

Fig. 8. Underivatized drug screen with on-column injection of 5 ng per drug; solvent: methanol–toluene (4:96); oven profile: 75°C for 0.5 min, 10°/min to 200°C, 5°/min to 210°C, 15°/min to 280°C; column: cross-linked SE-54 (25 m × 0.32 mm I.D.), β = 150. 1 = Ethosuximide; 2 = methyl-propylsuccinimide; 3 = barbital; 4 = amobarbital; 5 = pentobarbital; 6 = secobarbital; 7 = mephobarbital; 8 = phenobarbital; 9 = procaine; 10 = methadone; 11 = amitriptyline; 12 = cocaine; 13 = imipramine; 14 = nortriptyline; 15 = primidone; 16 = desipramine; 17 = carbamazepine; 18 = phenytoin; 19 = codeine; 20 = methylphenytoin; 21 = quinine.

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GAS CHROMATOGRAPHIC DETERMINATION OF LOWER FATTY ACIDS IN GASEOUS SAMPLES VIA CONVENTIONAL *IN SITU* DERIVATIZATION OF THE STRONTIUM SALTS CATALYSED BY POLY(CROWN ETHER)

KEIICHI KIMURA*, MICHITAKA SAWADA and TOSHIYUKI SHONO
Department of Applied Chemistry, Faculty of Engineering, Osaka University, Yamada-oka, Suita, Osaka 565 (Japan)
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SUMMARY

Poly(crown ether)-catalysed derivatization of lower fatty acids with *p*-bromophenacyl bromide or pentafluorobenzyl bromide has been used for their determination in gaseous samples by gas chromatography (employing either flame-ionization or electron-capture detection). Strontium hydroxide was employed as a base for the derivatization, taking advantage of the fact that a column packed with strontium hydroxide-coated glass beads is very suitable for preconcentration of the fatty acids from ambient air. *In situ* derivatization of lower fatty acids, preconcentrated on the glass beads as their strontium salts, proceeded nearly quantitatively in acetonitrile. Traces of lower fatty acids in artificial sample gases could be determined successfully using this conventional derivatization followed by direct injection of the reaction mixture into a gas chromatograph.

INTRODUCTION

Air pollution caused by traces of lower fatty acids has been well documented. High-sensitivity procedures for their determination are necessary, as the fatty acids generally have very low threshold values for perception. A popular method of determining fatty acids in the environment is chromatography including both gas and liquid chromatography, which must be preceded by preconcentration and chemical derivatization[1]. However, the derivatization reaction does not necessarily proceed rapidly or quantitatively. One of the most notable successes in this field was the use of crown ethers as phase-transfer catalysts of the derivatization reactions[2–4]. Although the derivatization techniques were greatly improved by the use of crown ethers, some problems still remained, namely interference of the crown ethers themselves on the gas chromatogram and their toxicity. A polymer containing a crown ether in the side chain, *i.e.* a poly(crown ether) such as I, which is non-volatile and much less toxic than low-molecular-weight crown ethers, has been tested for its usefulness as a catalyst for the derivatization of fatty acids to their *p*-bromophenacyl esters[5]. The poly(crown ether) did not interfere with the gas chromatogram of the fatty acid

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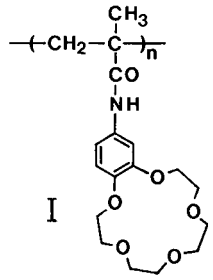


I

esters, unlike the volatile crown ethers which did so severely. By this method trace amounts of fatty acids and phenols in environmental aqueous samples have been successfully determined[6].

Another important factor in the derivatization of acids contained in the atmosphere, which normally contains a very low level of lower fatty acids, is effective preconcentration prior to analysis. Alkali metal hydroxides can be employed for this purpose, but the hydroxides are usually very hygroscopic, thus making it difficult to preconcentrate traces of gaseous fatty acids from a large quantity of sample gas. Weak bases such as sodium furancarboxylate do not absorb them very fast. Thus, a column packed with glass beads coated with strontium hydroxide[7] has been developed as a convenient tool for the preconcentration of lower fatty acids in sample gases.

Crown ethers could also be expected to catalyse reactions between strontium salts of fatty acids and derivatizing reagents, as they are known to form complexes with the strontium cation. This prompted us to combine the preconcentration of lower fatty acids by the column containing strontium hydroxide-coated glass beads with the poly(crown ether)-catalysed derivatization of the acids to their esters. This paper concerns the poly(crown ether)-catalysed derivatization of the strontium salts of lower fatty acids with *p*-bromophenacyl bromide or pentafluorobenzyl bromide prior to their determination by gas chromatography [employing either flame-ionization detection (FID) or electron-capture detection (ECD)], and the *in situ* derivatization of fatty acids preconcentrated by strontium hydroxide-coated glass beads from artificial sample gases.

EXPERIMENTAL

Chemicals

The poly(crown ether) (I) was synthesized according to the procedure described elsewhere[8]. Internal standards for gas chromatography, *p*-bromophenacyl isocaproate and pentafluorobenzyl isocaproate, were prepared by esterification of potassium salt of isocaproic acid with the corresponding derivatizing reagents[9]. *p*-Bromophenacyl bromide was recrystallized from light petroleum. Pentafluorobenzyl bromide, acetonitrile, and various fatty acids (acetic, propionic, *n*- and isobutyric and *n*- and isovaleric acids) were purified by distilliation. Strontium hydroxide was of analytical-reagent grade.

TABLE I

GAS CHROMATOGRAPHIC CONDITIONS

Detector	*Temperature (°C)*			*Carrier gas*	*Flow-rate (ml/min)*
	Injection port	*Column*	*Detector*		
FID	250	200	250	N_2	30
ECD	200	130	250	He	30

Apparatus

The gas chromatographs employed were Shimadzu GC-3BF (with FID) and Yanaco G-2800 (with ECD) instruments. Glass columns, 2.1 m × 3 mm, packed with 4% OV-17 on Gas-Chrom Q, and 2.0 m × 3 mm, packed with 5% PEG-HT on Chromosorb W AW were employed for the FID and ECD chromatographs, respectively. The injection port, column, and detector temperatures are summarized in Table I, which includes details of carrier gas and flow-rate.

A glass column (70 × 15 mm I.D.), packed with 2 g of strontium hydroxide-coated glass beads (1%; 20–30 mesh) was employed for preconcentration of the fatty acids in the sample gas.

Procedure

An aqueous solution (0.1 ml) of fatty acid [$3 \cdot 10^{-2}$ to $3 \cdot 10^{-5}$ *M* ($3 \cdot 10^{-4}$ to $3 \cdot 10^{-6}$ *M* for ECD system)] and strontium hydroxide ($5 \cdot 10^{-2}$ *M*) was carefully evaporated. To the residue was added 1 ml of acetonitrile solution containing *p*-bromophenacyl bromide [($2 \cdot 10^{-2}$ *M*) ($1.2 \cdot 10^{-6}$ *M* pentafluorobenzyl isocaproate for ECD system)]. In this case of *in situ* derivatization of strontium salts of fatty acids on glass beads, the procedure was as follows. Traces of lower fatty acids were preconcentrated by passing 20 l of sample gas through the column at a flow-rate of 1 l/min. To the glass beads was then added 1 ml of an acetonitrile solution containing *p*-bromophenacyl bromide, poly(crown ether) and the internal standard (the same solution as above). The mixture was allowed to reflux with vigorous stirring. An aliquot (2 µl for the FID system, 0.2 µl for the ECD system) of the reaction mixture was then injected directly into the gas chromatograph.

RESULTS AND DISCUSSION

Strontium salts of fatty acids have not often been utilized for crown ether-catalysed esterifications, unlike potassium salts. In order to determine the reactivity behaviour of the fatty acid strontium salts and to optimize the reaction conditions, derivatization of the isolated strontium salts with *p*-bromophenacyl bromide was carried out in the presence of a large excess of of strontium hydroxide prior to determination of the fatty acids on the FID gas chromatograph. Acetic, propionic, *n*- and isobutyric and *n*- and isovaleric acids, which are very important in air pollution, were selected as fatty acids. Acetonitrile was employed as reaction solvent; this has often been found to be a good solvent for solid–liquid phase-transfer reactions catalyzed by crown ethers. The results of studies on the effect of reaction time and

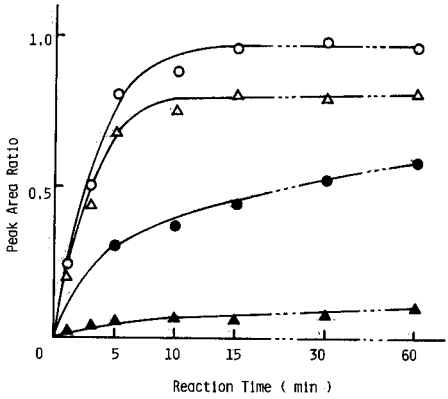


Fig. 1. Time dependence of peak-area ratio on derivatization of fatty acids with *p*-bromophenacyl bromide (FID system). [Acid] = $3 \cdot 10^{-3}$ *M* (0.1 ml). Acids: acetic (△); *n*-valeric (○); acetic, without crown ether (▲); *n*-valeric, at room temperature (●).

temperature on the ratio of peak areas of the fatty acid ester to the internal standard in the gas chromatogram are shown in Fig. 1 for the reactions of acetic and *n*-valeric acid strontium salts with *p*-bromophenacyl bromide. This esterification in acetonitrile seems to proceed rather fast under reflux, but very slowly at room temperature. A large excess of strontium hydroxide does not seem to interfere with the reaction. Since reaction hardly occurs in the absence of poly(crown ether), as anticipated, the crown ether should be present in the reaction system. In our previous work[5], the potassium salts of fatty acids were found to be very reactive with *p*-bromophenacyl bromide in the presence of poly(crown ether), and the reaction proceeded almost quantitatively even at room temperature. This difference in reactivity between the strontium and potassium salts of fatty acids may be derived from the fact that the poly(crown ether) can complex with potassium cations more easily than with strontium cations irrespective of their similar ionic radii. In the poly(crown ether)-catalysed derivatization of

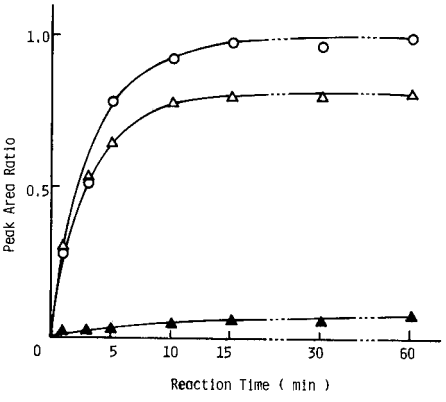


Fig. 2. Time dependence of peak area ratio on derivatization of fatty acids with pentafluorobenzyl bromide (ECD system). [Acid] = $3 \cdot 10^{-4}$ *M* (0.1 ml). Acids: acetic (△); *n*-valeric (○); acetic, without crown ether (▲).

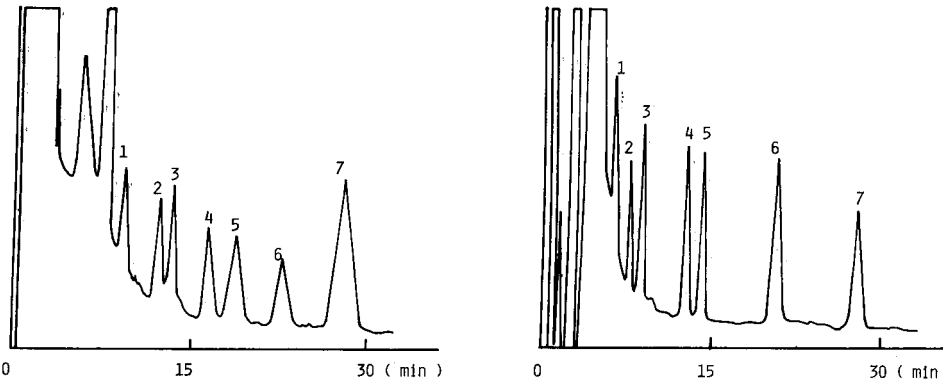


Fig. 3. Typical gas chromatogram of a mixture of fatty acid *p*-bromophenacyl esters (FID system). Acids: 1 = acetic; 2 = propionic; 3 = isobutyric; 4 = *n*-butyric; 5 = isovaleric; 6 = *n*-valeric; 7 = isocaproic (internal standard).

Fig. 4. Typical gas chromatogram of a mixture of fatty acid pentafluorobenzyl esters (ECD system). Acids: 1 = acetic; 2 = propionic; 3 = isobutyric; 4 = *n*-butyric; 5 = isovaleric; 6 = *n*-valeric; 7 = isocaproic (internal standard).

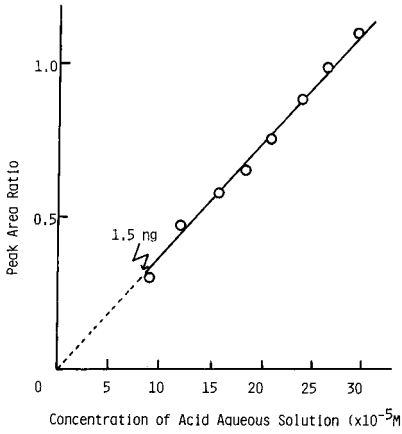
strontium salts of acetic and *n*-valeric acids, the peak area ratio in the gas chromatogram approached a constant value within 15 min under reflux conditions. The results for the other fatty acids employed in this study were essentially the same. It is, however, recommended that the reaction mixture is allowed to reflux for 30 min in order to achieve nearly quantitative derivatization of the fatty acids.

In order to detect traces of fatty acids as their esters with a higher sensitivity than FID, one may be forced to use ECD, and for this purpose the fatty acids should be derivatized with ECD-sensitive reagents such as pentafluorobenzyl bromide[10]. However, the derivatization of strontium salts of fatty acids with pentafluorobenzyl bromide should be carried out under much lower concentrations of fatty acid and derivatizing reagent than the above-mentioned FID systems because of the higher sensitivity of the ECD gas chromatograph. The lower fatty acids were nevertheless found to be derivatized with pentafluorobenzyl bromide almost quantitatively under the same conditions (reaction time and temperature) as employed for the *p*-bromophenacyl bromide system (Fig. 2).

Figs. 3 and 4 show typical chromatograms of a mixture of fatty acids which had been successfully derivatized with *p*-bromophenacyl bromide for the FID system and pentafluorobenzyl bromide for the ECD system. Excellent separation was obtained, and the calibration plots gave straight lines over a wide concentration range, the lowest detection limit of which was at the ng level for the *p*-bromophenacyl bromide system and at the pg level for the pentafluorobenzyl bromide systems (Figs. 5 and 6).

Attempts were made to carry out *in situ* derivatization of lower fatty acids preconcentrated by strontium hydroxide-coated glass beads and to apply this method to the determination of traces of acetic, propionic, *n*-butyric and *n*-valeric acids. Several sample gases having an acid concentration at the ppb* level were prepared by

* Throughout this article, the American billion (10^9) is meant.



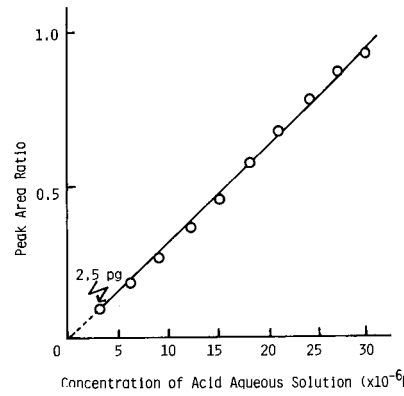


Fig. 5. Calibration plots for *n*-valeric acid–*p*-bromophenacyl bromide system (FID system) at the lowest concentration range.

Fig. 6. Calibration plots for *n*-valeric acid–pentafluorobenzyl bromide system (ECD system) at the lowest concentration range.

injecting an aliquot of fatty acid into a polyethylene bag with a microsyringe, followed by shaking of the bag. Traces of fatty acid contained in a 20-l bag were preconcentrated by a column packed with strontium hydroxide-coated glass beads. The glass beads were then placed in an acetonitrile solution containing *p*-bromophenacyl bromide, poly(crown ether) and the internal standard. The mixture was refluxed with vigorous stirring for 30 min, and then the fatty acid in the sample gas was determined by direct injection of 2 μl of the reaction mixture without further treatment. Table II shows the results of the *in situ* derivatization of some lower fatty acids followed by their gas chromatographic determination. The calculated and found amounts of fatty acid in the polyethylene bag are in good agreement with acceptable negative errors of only a few per cent, due to experimental errors in preparing the

TABLE II

DETERMINATION OF LOWER FATTY ACIDS IN ARTIFICIAL SAMPLE GASES

Acid	*Calculated amount (ng)*	*Found amount (ng)*	*Error (%)*
Acetic	72.0	71.1	−1.3
	36.0	34.9	−3.1
	14.4	13.6	−5.6
Propionic	88.8	86.9	−2.1
	44.4	42.0	−5.4
	17.8	16.1	−9.6
n-Butyric	106	105	−0.9
	52.8	49.2	−6.8
	21.1	19.3	−8.5
n-Valeric	122	119	−2.5
	61.2	58.3	−4.7
	24.5	22.9	−6.5

sample, *i.e.* incomplete vaporization of the fatty acid during injection into the bag. The table again suggests that the process of preconcentration of the fatty acids as their strontium salts on the strontium hydroxide-coated glass beads followed by their derivatization by *in situ* esterification is nearly quantitative. Let us consider the lowest *n*-valeric acid concentration in a sample gas determined in this study. For 25 ng of *n*-valeric acid contained in 20 l of sample gas, the acid concentration is 0.27 ppb, which is lower than its threshold value for perception (0.62 ppb). Thus, this *in situ* derivatization technique for fatty acids combined with their preconcentration using strontium hydroxide-coated glass beads can be utilized for practical purposes.

In our previous work[5], the preconcentration of lower fatty acids in sample gases was achieved by bubbling the gas into concentrated solutions of potassium carbonate. A small amount of the solution was evaporated to dryness, and then the isolated potassium salts of the fatty acid were derivatized with *p*-bromophenacyl bromide via a poly(crown ether)-catalysed solid–liquid phase-transfer reaction. Isolation of the potassium salts is time consuming, and also requires precautions to prevent the solution from bumping during evaporation. In order to eliminate this process, attempts were made at a liquid–liquid phase-transfer reaction between the potassium carbonate solution containing the preconcentrated lower fatty acids and a water-immiscible organic solution containing the derivatizaing reagent and the poly(crown ether); however these failed, probably because of the slowness of the liquid–liquid phase-transfer reaction. The *in situ* derivatization technique developed in this study does not require a tedious isolation process, and this is also a great advantage for the practical application of this technique.

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DETERMINATION OF FATTY ACIDS IN AIRBORNE PARTICULATE MATTER, DUST AND SOOT BY MASS CHROMATOGRAPHY

AKIO YASUHARA* and KEIICHIRO FUWA
Division of Chemistry and Physics, National Institute For Environmental Studies, Yatabe-machi, Tsukuba-gun, Ibaraki 305 (Japan)
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SUMMARY

Extracts from airborne particulate matter, dust taken from an air filter and some soots were fractionated by alumina column chromatography. The solution eluted with ethanol–acetic acid (9:1) contained fatty acids and other polar substances. After heating the solution with a small amount of sulphuric acid, the esters of the fatty acids formed were extracted and separated from other polar substances by column chromatography. Identification and quantification were performed by mass chromatography at the molecular ion masses of the esters. Fatty acids from octanoic to tetratriacontanoic acid were determined in these samples and dotriacontanoic, tritriacontanoic and tetratriacontanoic acids were found for the first time.

INTRODUCTION

Organic components in airborne particulate matter are very complex and much information is available regarding the extractable compounds, although most of the investigations have been directed towards to analysis of polynucelar aromatic hydrocarbons such as benz[*a*]anthracene and benzo[*a*]pyrene because of their carcinogenic properties. Recently, some attention has been paid to polynuclear heteroaromatic compounds. Higher fatty acids in environmental samples such as airborne particulate matter are very interesting components as regards their origin and toxicity. Laseter and Valle[2] showed that many free fatty acids are contained in fungal spores, which constitute a significant proportion of the airborne particulate matter[3]. It is not known whether direct contact of fatty acids on respiratory tissue has any detrimental effects, but some epoxides of unsaturated fatty acids have been found to be carcinogenic[4].

Analysis of higher fatty acids in airborne particulate matter has usually been performed by successive procedures of Soxhlet extraction, reverse extraction with an alkaline aqueous solution, neutralization, extraction, esterification with diazomethane and gas chromatography–mass spectrometry (GC–MS). Cautreels and Van Cauwenberghe[5] determined linear chain fatty acids (dodecanoic acid to triacontanoic acid) in airborne particulate matter sampled in Antwerp by the procedures described above.

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Also, they found that the separation of acidic and neutral components was not complete. Cautreels and Van Cauwenberghe[6] measured major fatty acids (C_{12} and C_{14}–C_{22}) in crude extracts from airborne particulate matter by methylation with diazomethane and mass chromatography at *m/z* 74, omitting several extraction and separation steps in order to avoid time-consuming procedures and manipulation losses of the products. However, the analysis of minor fatty acids was very difficult using this method.

Airborne particulate matter sampled near the Chacaltaya Cosmic Ray Laboratory in Bolivia was analysed by Cautreels and Van Cauwenberghe[7] in order to establish the natural background level of fatty acids and other components. Lunde *et al.*[8] detected C_7–C_{29} normal fatty acids (except C_{26}) and many branched-chain acids in rain and snow sampled in Norway. The distribution of organic pollutants containing fatty acids between airborne particulate matter and the corresponding gas phase has been investigated[9]. The results showed that lower fatty acids are more abundant in the gas phase and higher acids, from docosanoic acid up, are not detected in the gas phase. Some alternation of the distribution factors is observed for the even and odd carbon numbered fatty acids. This effect is known for several physico-chemical parameters of these compounds.

In this study, octanoic to tetratriacontanoic acids were determined by a combination of column chromatography and GC–MS and dotriacontanoic acid to tetratriacontanoic acid were found for the first time.

EXPERIMENTAL

Sample collection

Airborne particulate matter was collected with a Hi-Volume sampler, Model HVS-500, made by Sibata Chemical Apparatus Mfg. Co., near a bypass road at Ohomiya, Japan. A glass-fibre filter, 110 mm in diameter, was used to collect the sample by drawing the ambient air through it at a flow-rate of 610 l/min for 6 h.

As another airborne particulate matter sample, accumulated dust on the air conditioner filter used at the National Institute for Environmental Studies (Ibaraki, Japan) was used.

Soot was collected at the top of the chimney of a furnace in Hyogo Prefecture where firewood is burnt every day. Soot obtained by combustion of soybean oil and petroleum oil was prepared in the laboratory.

Sample extraction and separation procedure

Airborne particulate matter (33.9 mg), dust taken from a filter (15.61 g), soot from a chimney (13.57 g), soot from the combustion of soybean oil (6.70 mg) and soot from the combustion of petroleum oil (136.9 mg) were extracted with benzene (200 ml) in a Soxhlet apparatus for over 24 h. The extracts were concentrated with a vacuum rotary evaporator at room temperature and then column chromatography was carried out as shown in Fig. 1. The solution eluted with ethanol–acetic acid (9:1) was mixed with concentrated sulphuric acid (0.5 ml) and heated under reflux for 4 h. This solution was concentrated with a vacuum rotary evaporator to a third of its volume, then poured into an aqueous solution of sodium hydrogen carbonate. The ethyl esters of the fatty acids formed were extracted three times with dichloromethane

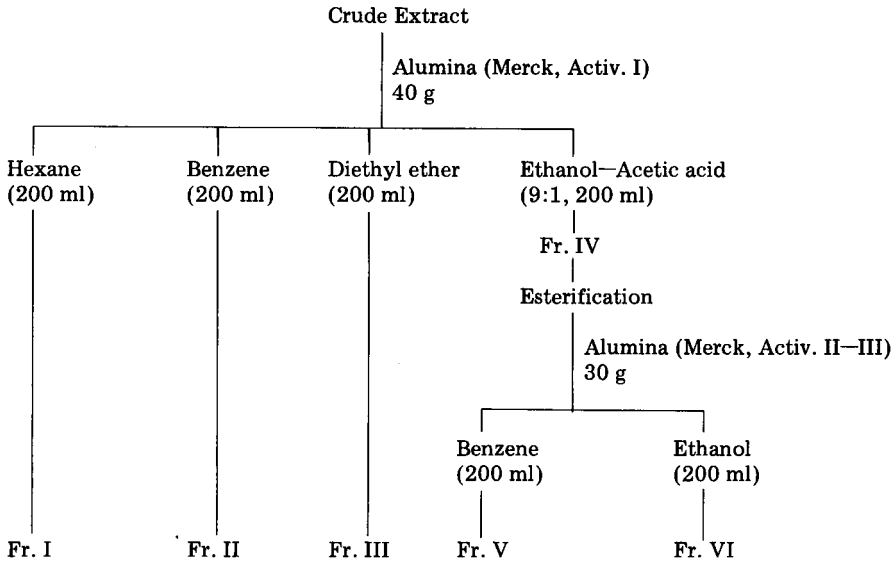


Fig. 1. Column chromatographic separation procedures.

(*ca.* 100 ml) and after concentration were chromatographed again as shown in Fig. 1. The corresponding fraction (V) was concentrated to *ca.* 1 ml for GC–MS and an internal standard (pyrene) amounting to 1 μg per millilitre of the solution was added.

Benzene, hexane, diethyl ether, ethanol and dichloromethane were distilled in all-glass apparatus from pesticide-grade solvents obtained from Wako (Osaka, Japan). Alumina was washed with diethyl ether in a Soxhlet apparatus for 15–20 h and then heated at 200°C under vacuum for 3–5 h before use. Acetic acid and sulphuric acid were of "super special" grade for trace analysis.

Gas chromatography–mass spectrometry

Mass spectra were measured with a JEOL Model JMS-DX 300 mass spectrometer connected with a JEOL GCG-05 gas chromatograph and a JEOL JMA-2000 mass data analysis system and stored on a 2.4-MW disk. The glass column (1 m × 3 mm I.D.) was packed with Chromosorb W coated with 1% OV-17. The column temperature was set at 70°C for 2 min, increased to 300°C at 4°C/min, then held at 300°C until completion of analysis. The injector temperature was 340°C and the helium carrier gas flow-rate was 40 ml/min. The separator temperature for GC–MS was 250°C (the same results were obtained at higher temperatures). The mass spectrometric conditions were as follows: ionizing source pressure, $1 \cdot 10^{-6}$–$2 \cdot 10^{-6}$ Torr; ionizing source temperature, 200°C; ionizing current, $3 \cdot 10^{-4}$ A; ionizing energy, 70 eV; accelerating voltage, 3 kV; scan range, m/z 10–600; scan speed, 1.3 sec per scan; scan interval, 3.0 sec. Identification was carried out by mass chromatography at the molecular ion masses of each fatty acid ethyl ester. Standard solutions were prepared for ethyl esters of C_{10}, C_{12}, C_{14}, C_{16} and C_{18} fatty acids. Quantification was performed by comparison of peak heights between each ester and the internal standard in the mass chromatogram. Compounds whose standard solutions were absent were tentatively analysed using calibration graphs of other esters, as follows:

C_8–C_{10} acid esters: calibration graph of ethyl decanoate;
C_{11}, C_{12} acid esters: calibration graph of ethyl dodecanoate;
C_{13}, C_{14} acid esters: calibration graph of ethyl tetradecanoate;
C_{15}, C_{16} acid esters: calibration graph of ethyl hexadecanoate;
C_{17}–C_{34} acid esters: calibration graph of ethyl octadecanoate.

RESULTS AND DISCUSSION

Column chromatography is a more desirable technique than using several extraction steps for the systematic separation of organic substances in airborne particulate matter, because with the latter technique separation is not complete and because it is almost impossible to fractionate neutral components. Fraction I in Fig. 1 contained aliphatic hydrocarbons; fraction II, polynuclear aromatic hydrocarbons and weakly polar compounds; fraction III, medium polar compounds such as phthalic acid esters; and fraction IV, strongly polar components. Alcohols such as methanol and ethanol could not elute fatty acids from the column, but a mixture of an alcohol and acetic acid (9:1) was successfully used to elute them. Ethanol was a better solvent than methanol because the solubility of fatty acids in methanol is lower than that in ethanol and because the esterification yield is higher in ethanol than in methanol, although Hill *et al.*[10] found that methanol is more effective than cyclohexane for the extraction. By esterification and rechromatography, fraction V was composed of esters of fatty acids.

TABLE I

RECOVERY OF OCTADECANOIC ACID

Run No.	*Recovery (%)*
1	59.5
2	58.0
3	60.4
4	59.4
Mean (%)	59.4
Standard deviation (%)	1.0
Coefficient of variation (%)	1.7

Table I shows the overall results of the recovery test for octadecanoic acid using Soxhlet extraction, a first column chromatographic step, esterification and a second column chromatographic step. The mean recovery was relatively low, but the standard deviation was very small. Therefore, all subsequent analytical values were corrected by a recovery factor of 59.4%. Blank values were negligible in the overall analytical procedures.

Mass chromatogram plots were measured at m/z 88 (a McLafferty rearrangement ion), at the molecular ion masses of every fatty acid ethyl ester and at m/z 202 (molecular ion mass of pyrene internal standard). The precision of the mass chromatographic measurements is shown in Table II for five esters. The coefficients of variation were slightly higher in mass chromatography than in gas chromatography, but

TABLE II

PRECISION OF MEASUREMENT BY MASS CHROMATOGRAPHY

Solutions to be tested were prepared at concentrations of *ca.* 35 μg/ml for all acids.

Run No.	*Measured concentration (μg/ml)*				
	C_{10} *acid*	C_{12} *acid*	C_{14} *acid*	C_{16} *acid*	C_{18} *acid*
1	32.5	36.5	37.0	37.2	43.0
2	41.0	32.7	33.0	33.2	36.4
3	46.0	38.0	38.0	34.7	38.0
4	36.0	33.0	32.7	30.5	34.8
5	37.8	33.0	34.9	31.6	37.0
Mean (μg/ml)	38.7	34.6	35.1	33.4	37.8
Standard deviation (μg/ml)	5.1	2.4	2.4	2.6	3.1
Coefficient of variation (%)	13	7	7	8	8

mass chromatography has a higher selectivity and sensitivity than gas chromatography. Although selected ion monitoring (SIM) has a greater sensitivity than mass chromatography, it was not effective in practice because the number and mass range of the selected ions in each run were strictly limited in the magnetic-type mass spectrometer used. Also, in mass chromatography the sensitivity of this instrument was satisfactory and it was very convenient to confirm entire mass spectra of peaks because all data in the full range were stored. Detection limits were 10, 5, 5, 2 and 1 ng for decanoic, dodecanoic, tetradecanoic, hexadecanoic and octadecanoic acid, respectively.

Mass chromatograms of ethyl esters of fatty acids in airborne particulate

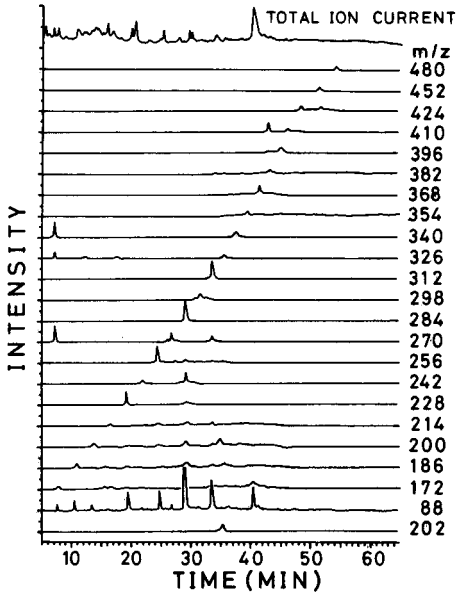
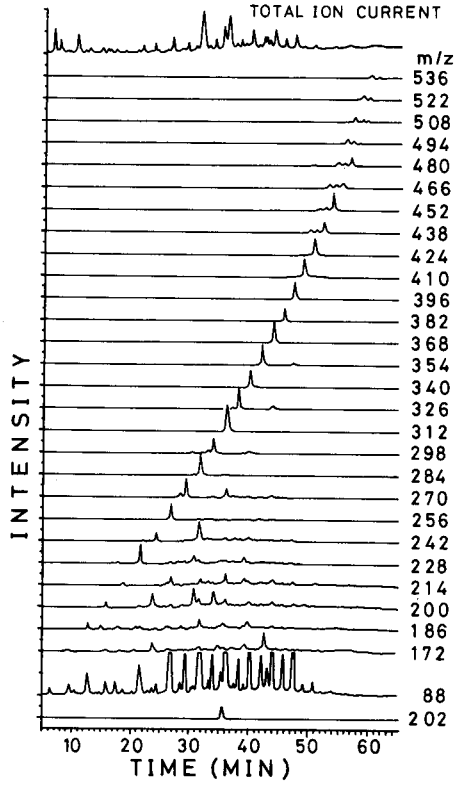


Fig. 2. Mass chromatograms of fatty acid (ethyl esters) in airborne particulate matter.



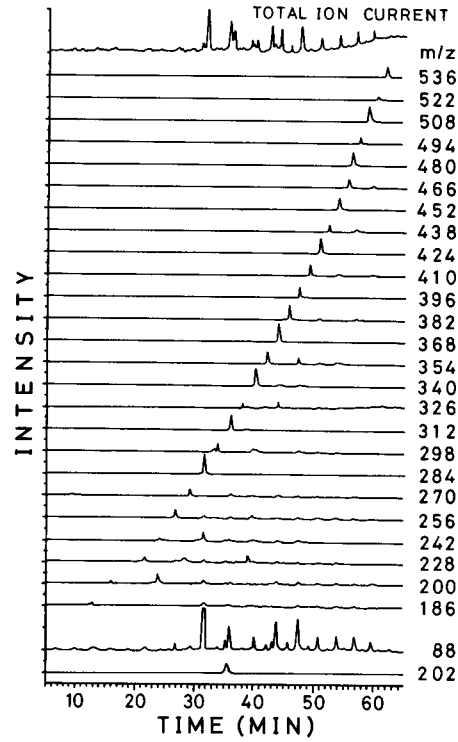


Fig. 3. Mass chromatograms of fatty acid ethyl esters in dust taken from an air conditioner filter.

Fig. 4. Mass chromatograms of fatty acid ethyl esters in soot obtained from a chimney.

matter, dust taken from the filter and soot from the chimney are shown in Figs. 2, 3 and 4, respectively. Each chromatogram intensity was suitably magnified in order to make the peaks distinct. Normal and branched-chain fatty acid ethyl esters were clearly observed. Esters of normal acids appeared in constantly increasing order of retention time and esters of branched-chain acids were eluted faster than those of the corresponding normal acids. The structures of the branched-chain fatty acids were not investigated in detail.

It was observed in the mass spectra measured with this instrument that the relative intensity of molecular ions in the normalized mass spectra of esters increased with increasing molecular weight. Therefore, esters of fatty acids of high molecular weight were easily detected by mass chromatography at the molecular ion masses, although it was relatively difficult to detect them by mass chromatography at the McLafferty ion (*m/z* 88). Intensity ratios of molecular ions with respect to the McLafferty ion are shown in Fig. 5. There was a clear distinction between even and odd carbon numbered fatty acid ethyl esters.

The concentration patterns of the C_8–C_{34} fatty acids were dependent on the origins of the samples. Fig. 6 shows the concentration patterns obtained in this study. Air particulate matter and soot from the combustion of soybean oil had fatty acids at

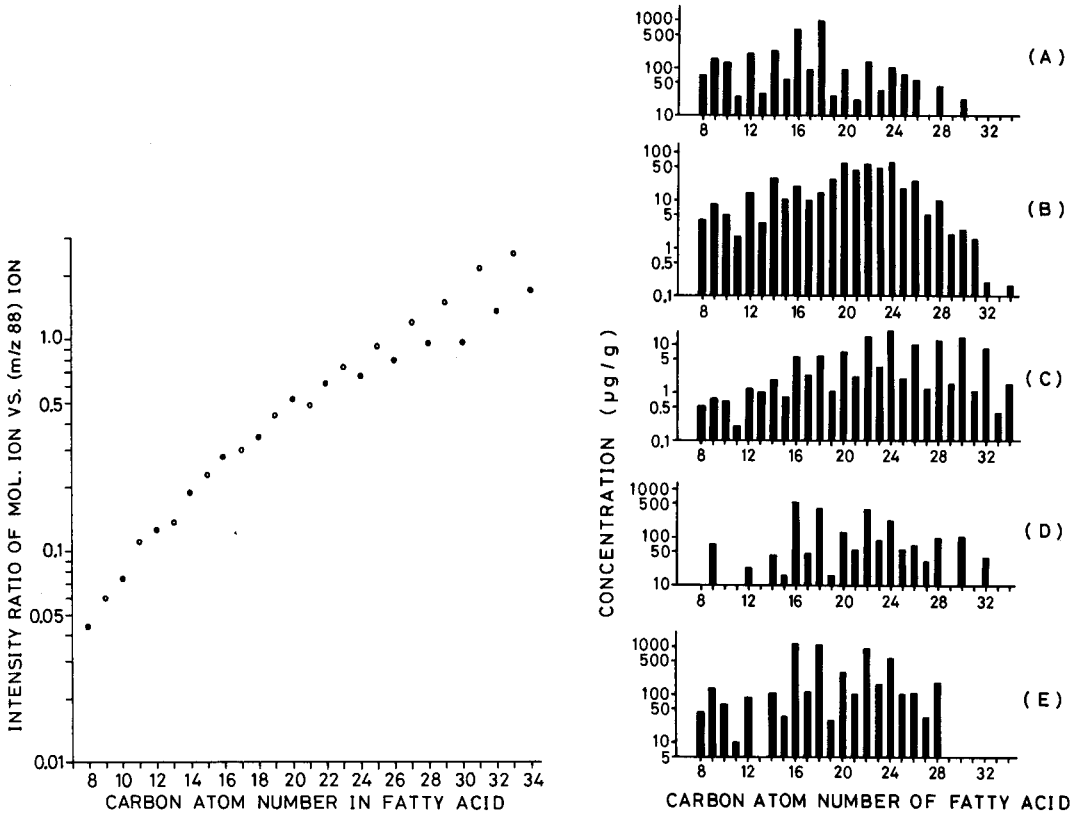


Fig. 5. Intensity ratios of molecular ions with respect to the McLafferty ion in mass spectra of fatty acid ethyl esters. ●, Fatty acids possessing an even carbon number; ○, fatty acids possessing an odd carbon number.

Fig. 6. Concentration patterns of fatty acids. (A) Airborne particulate matter; (B) dust taken from an air conditioner filter; (C) soot obtained from a chimney; (D) soot obtained by combustion of petroleum oil; (E) soot obtained by combustion of soybean oil.

similar concentrations. Contents of fatty acids in the dust taken from the filter were fairly low compared with those in airborne particulate matter, probably because the dust contained a significant amount of soil. It was interesting that the concentrations of fatty acids were extremely low in the soot from the chimney compared with the soot obtained by combustion of petroleum oil or soybean oil. The reason may be the disappearance of the acids during long-term heating of the soot in the chimney. It was characteristic that the concentration patterns were significantly different between airborne particulate matter and the dust taken from the filter. The origins of the fatty acids in the two samples might be different. Octadecanoic acid was the most abundant acid in airborne particulate matter. Hexadecanoic acid was the most abundant acid in the soot obtained by combustion of petroleum oil or soybean oil, and tetracosanoic acid in the dust taken from the filter or soot from the chimney. Fatty acids above hentriacontanoic or nonacosanoic acid could not be detected in airborne particulate matter or soot obtained by combustion of soybean oil because the amount of the

samples was inadequate. Dotriacontanoic, tritriacontanoic and tetratriacontanoic acids were detected for the first time in the dust taken from the air conditioner filter, soot obtained from the chimney and soot obtained by combustion of petroleum oil.

The concentrations of fatty acids in airborne particulate matter were comparable to the values reported by other investigators[7]. In all instances except for the dust taken from the filter, even carbon numbered fatty acids were always present in considerably higher concentrations than the odd carbon numbered acids.

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GAS–LIQUID CHROMATOGRAPHIC ANALYSES

IV. GLASS CAPILLARY GAS CHROMATOGRAPHY OF METHYL AND CHLOROMETHYL MONOCHLORO ESTERS OF ALIPHATIC C_5-CARBOXYLIC ACIDS

ILPO O. O. KORHONEN
Department of Chemistry, University of Jyväskylä, Kyllikinkatu 1–3, SF-40100 Jyväskylä 10 (Finland)
(Received December 4th, 1981)

SUMMARY

The gas chromatography of methyl and chloromethyl esters of pivalic, 2-methylbutyric, isovaleric and valeric acid and certain of their monochloro derivatives was studied. Separation of the combined mixtures of methyl and chloromethyl esters was better on Carbowax 20M than on SE-30. The retention times of esters with substituents adjacent (*i.e.* at C-2) to the carboxyl group appear to be sensitive to column polarity, particularly in the case of the chloromethyl esters. The retention order and relative retention times of compounds are discussed.

INTRODUCTION

Earlier studies have reported the gas chromatographic (GC) separations of mixtures with a wide range of chain lengths of methyl and chloromethyl monochloro esters of aliphatic *n*-carboxylic acids on Carbowax 20M glass capillary columns[1–5]. Also the GC of higher chlorinated methyl propionates[6,7] and methyl butyrates[7,8] has been studied.

Although numerous papers have been published on the GC of aliphatic carboxylic acids and their derivatives, few published chromatographic data are available for lower branched-chain methyl esters[9–14] and studies on their chlorinated derivatives have not yet been reported.

This paper describes a GC study of methyl, chloromethyl and the corresponding monochloro esters of pivalic, 2-methylbutyric, isovaleric and valeric acid. The separations of combined mixtures of both esters were studied on a Carbowax 20M glass capillary column and on a vitreous-silica SE-30 wall-coated open tubular (WCOT) column. Retention times of derivatives were compared by separating the mixtures of methyl and chloromethyl esters of each acid under the same operating conditions.

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EXPERIMENTAL

Apparatus

GC analyses were carried out on a Varian Model 2400 gas chromatograph, adapted for glass capillary work, and on a Perkin-Elmer Model Sigma 3 instrument. The former was equipped with a glass capillary column (50 m × 0.30 mm I.D.) prepared in our laboratory (drawn from soda-glass, etched with hydrogen chloride gas and coated with a 3% Carbowax 20M stationary phase). The latter chromatograph was equipped with a vitreous-silica SE-30 WCOT column (25 m × 0.22 mm I.D.) supplied by Scientific Glass (North Melbourne, Australia). The operating conditions for both columns were as follows: injector and flame-ionization detector temperatures 230 and 250°C, respectively; nitrogen carrier gas flow-rates 1 ml/min; splitting ratios *ca.* 1:20. The column temperatures for the analyses are shown in Figs. 1–3.

The samples were purified using a Perkin-Elmer Model 800 instrument, adapted for preparative work, on a 6 m × 9.5 mm O.D. aluminium tube packed with 10% Carbowax 20M on Chromosorb W (60–80 mesh). Appropriate temperatures were used, with a nitrogen flow-rate of 120 ml/min.

Samples

Methyl pivalate (1), methyl 2-methylbutyrate (3), methyl isovalerate (9) and methyl valerate (13) were obtained via treatment of the corresponding acids with thionyl chloride and methanol. Valeric acid and isovaleric acid were commercial products (Fluka, Buchs, Switzerland); 2-methylbutyric acid and pivalic acid were prepared by a general method[15] employing the carboxylation of the corresponding Grignard reagent.

The methyl esters of chlorinated acids were obtained as follows: methyl 2-chloro-2-methylbutyrate (4), methyl 2-chloroisovalerate (10) and methyl 2-chlorovalerate (14) by esterification of the corresponding acid chlorides[16] with methanol; methyl *erythro*-3-chloro-2-methylbutyrate (5), methyl 3-chloroisovalerate (11) and methyl 3-chlorovalerate (15) from α,β-unsaturated methyl esters[17] (methyl *trans*-2-methyl-2-butenoate and methyl 3-methyl-2-butenoate were prepared from the corresponding commercial acids (E. Merck, Darmstadt, G.F.R.) with hydrogen chloride[18]; methyl chloropivalate (2), methyl *threo*-3-chloro-2-methylbutyrate (6), methyl 2-chloromethylbutyrate (7), methyl 4-chloro-2-methylbutyrate (8), methyl 4-chloroisovalerate (12) and methyl 4-chlorovalerate (16) by isolation from the reaction mixtures of monochloro esters obtained by chlorinating[1] the parent esters (1, 3, 9 and 13) with chlorine; methyl 5-chlorovalerate (17) from commercial acid (E. Merck) by appropriate esterification method.

Chloromethyl pivalate (18), chloromethyl 2-methylbutyrate (20), chloromethyl isovalerate (26) and chloromethyl valerate (30) were prepared from the corresponding acid chlorides and paraformaldehyde in the presence of a trace amount of zinc chloride[19]. Chloromethyl monochloro isomers (19, 21–25, 27–29 and 31–34) were obtained by chlorination[20] of the corresponding starting materials (18, 20, 26 and 30) in the liquid phase with chlorine.

The purities of the separately prepared esters were checked by GC and when required the products were purified by preparative gas–liquid chromatography. The

structures of compounds were confirmed by ^1H nuclear magnetic resonance and mass spectrometry. The mixtures of the isomeric chloromethyl monochloro esters were analysed by GC and GC–mass spectrometry and products were identified as described earlier[20]. The crude chlorination mixtures of chloromethyl esters were used for the GC analyses.

RESULTS AND DISCUSSION

The retention times of positional isomers of equivalent monochloro esters increase continuously as the chlorine substituent is separated from the carbonyl group, 2-chloro isomers always being eluted first[1–5]. By separating the mixtures of closely related compounds, the order of elution on a non-polar column such as SE-30

TABLE I

ABSOLUTE AND RELATIVE RETENTION TIMES FOR METHYL, CHLOROMETHYL AND METHYL MONOCHLORO ESTERS OF ALIPHATIC C_5-CARBOXYLIC ACIDS

Conditions as shown in Fig. 1.

Peak	*Compound (methyl ester = Me; chloromethyl ester = Cl–Me)*	*Column*				
		Carbowax 20M		*SE-30*		
		*Time**	*RRT***	*Time**	*RRT***	*RRT****
1	Me pivalate	4.18	1.00	3.55	1.00	0.85
18	Cl–Me pivalate	5.73	1.37	8.40	2.37	1.47
2	Me chloropivalate	6.74	1.61	10.45	2.94	1.55
3	Me 2-methylbutyrate	4.34	1.00	4.70	1.00	1.08
4	Me 2-chloro-2-methylbutyrate	6.03	1.39	9.69	2.06	1.61
5	Me 3-chloro-2-methylbutyrate (*erythro*)	6.91	1.59	10.68	2.27	1.55
20	Cl–Me 2-methylbutyrate	7.20	1.66	11.07	2.36	1.54
6	Me 3-chloro-2-methylbutyrate (*threo*)	7.56	1.74	11.60	2.47	1.53
7	Me 2-chloromethylbutyrate	8.51	1.96	12.72	2.71	1.49
8	Me 4-chloro-2-methylbutyrate	9.30	2.14	13.68	2.91	1.47
9	Me isovalerate	4.36	1.00	4.74	1.00	1.09
10	Me 2-chloroisovalerate	6.83	1.57	10.55	2.23	1.54
11	Me 3-chloroisovalerate	6.55	1.50	9.49	2.00	1.45
26	Cl–Me isovalerate	7.70	1.77	11.07	2.34	1.44
12	Me 4-chloroisovalerate	10.20	2.34	14.22	3.00	1.39
13	Me valerate	4.60	1.00	5.91	1.00	1.28
14	Me 2-chlorovalerate	7.70	1.67	12.15	2.06	1.58
15	Me 3-chlorovalerate	9.15	1.99	13.05	2.21	1.43
30	Cl–Me valerate	9.37	2.04	13.63	2.31	1.45
16	Me 4-chlorovalerate	10.13	2.20	14.05	2.38	1.39
17	Me 5-chlorovalerate	15.60	3.39	18.04	3.05	1.16

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

** Relative retention times for unchlorinated methyl esters taken as 1.00.

*** Relative retention times for compounds on Carbowax 20M taken as 1.00.

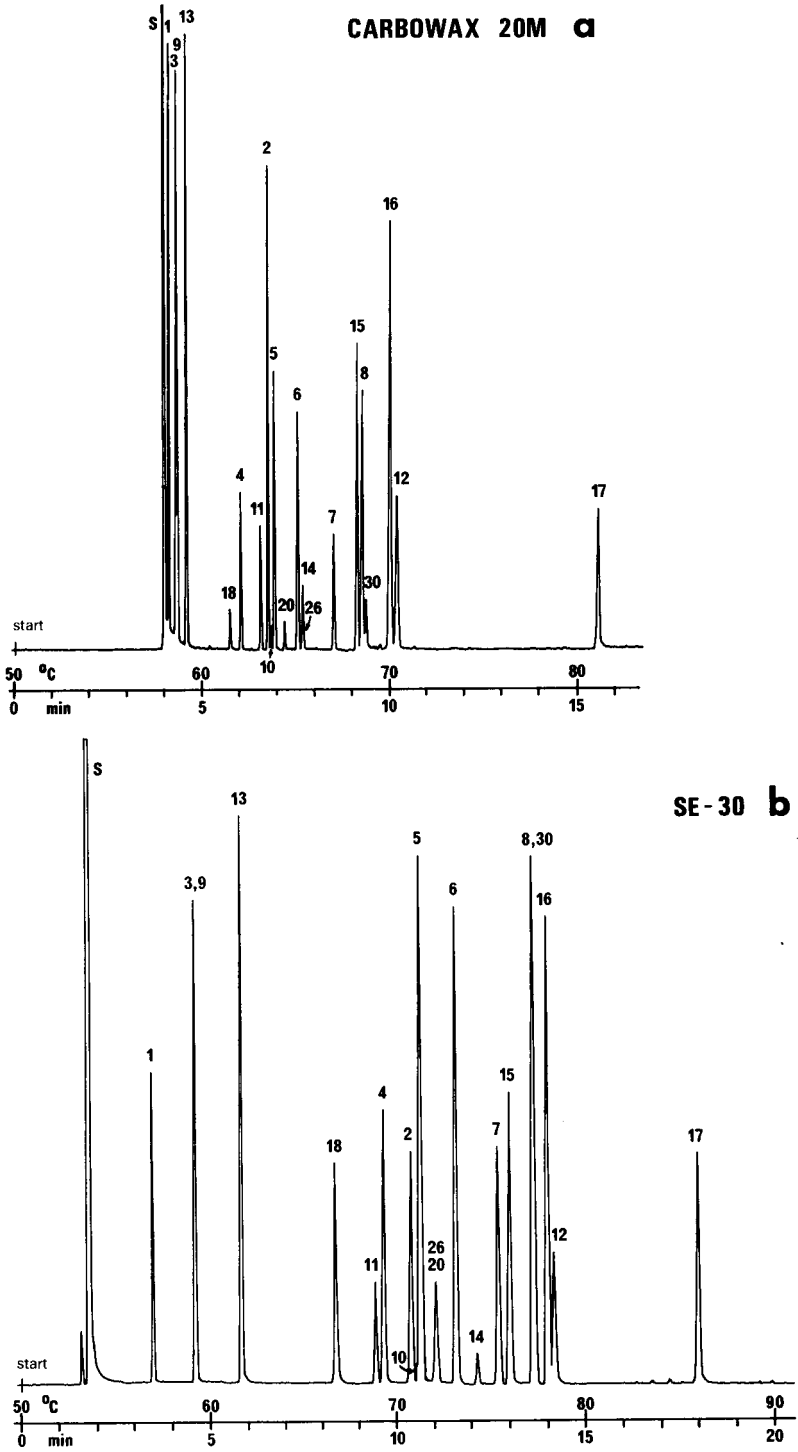

Fig. 1. Chromatogram of the mixture of methyl, chloromethyl and methyl monochloro esters of aliphatic C_5-carboxylic acids analysed on Carbowax 20M (a) and on SE-30 (b). Temperature programme: 50°C at 2°C/min. S = solvent; peaks identified in Table I.

is largely determined by the boiling point of the esters. However, on a polar column such as Carbowax 20M the order in which the compounds appear is greatly influenced by their structure.

Fig. 1 illustrates the separation of methyl, chloromethyl and methyl monochloro esters of aliphatic C_5-carboxylic acids analysed on Carbowax 20M and SE-30. Table I gives the identification of peaks and the absolute and relative retention times for the compounds. All retention times were measured from sample injection and are tabulated relative to unchlorinated methyl esters = 1.00. The retention is also expressed as the ratios of the retention times of the compounds on SE-30 divided by the times on Carbowax 20M.

Silica WCOT columns are more efficient than glass capillary columns. The analysis time of the mixture on SE-30 was longer than on Carbowax 20M under the same operating conditions used, in spite of the double length of the latter. However, separation of the compounds was better on a polar column, only methyl 2-chlorovalerate (14) and chloromethyl isovalerate (26) and also partly methyl 2-methylbutyrate (3) and methyl isovalerate (9) overlapping. The latter compounds 3 and 9 were coincident on SE-30 owing to their similar boiling points, 116 and 117°C, respectively. Obviously the methyl substituent adjacent to the carboxyl group (*i.e.* at C-2) has the stronger effect on the polarity of the compound than the substituent farther away causing the separation of compounds 3 and 9 on Carbowax 20M. Methyl 2-chloroisovalerate (10) was eluted as a shoulder with methyl *erythro*-3-chloro-2-methylbutyrate (5), and methyl 4-chloro-2-methylbutyrate (8) and chloromethyl valerate (30) fully overlapped giving a broadened peak on SE-30.

The elution order of the compounds on the polar and non-polar column was with one exception the same: methyl 3-chloroisovalerate (11) was eluted from the SE-30 column before methyl 2-chloro-2-methylbutyrate (4), the samples being eluted on the Carbowax 20M column in the opposite order.

The polar column also provided a more efficient separation of chloromethyl esters as may be seen from Fig. 2. The identification and absolute and relative retention times of various peaks are given in Table II. Like the corresponding methyl esters, chloromethyl 2-methylbutyrate (20) and chloromethyl isovalerate (26) were coincident on SE-30, whereas the compounds were fully separated on Carbowax 20M. Chloromethyl pivalate (19) and chloromethyl 2-chloroisovalerate (27) overlapped on both columns, 27 being eluted as a shoulder with 19 on SE-30. In addition, chloromethyl 4-chloro-2-methylbutyrate (25) and chloromethyl 3-chlorovalerate (32) were coincident on Carbowax 20M and chloromethyl *threo*-3-chloro-2-methylbutyrate (23) and chloromethyl 2-chlorovalerate (31) on SE-30, 32 and 31 being eluted as shoulders.

The elution order of isomeric monochloro esters studied in previous papers[3–5] has always been the same, independent of the polarity of the column. In this work, however, as may be seen from Fig. 2a, chloromethyl 2-chloroisovalerate (27) left the Carbowax 20M column before chloromethyl 3-chloroisovalerate (28). Chloromethyl 2-chloro esters (21, 27 and 31) are less polar than the other isomers having relatively short retention times on the polar column (Table II). The same effect has been reported earlier with chloromethyl monochloro esters of aliphatic *n*-carboxylic acids[5].

Like the corresponding methyl isomer, chloromethyl 3-chloroisovalerate (28) has a shorter retention time on SE-30, being eluted before chloromethyl chloropi-

TABLE II

ABSOLUTE AND RELATIVE RETENTION TIMES FOR CHLOROMETHYL AND CHLOROMETHYL MONOCHLORO ESTERS OF ALIPHATIC C_5-CARBOXYLIC ACIDS

Conditions as in Fig. 2.

Peak	*Chloromethyl ester of*	*Column*						
		Carbowax 20M			*SE-30*			
		*Time**	*RRT***	*RRT*[§]	*Time**	*RRT***	*RRT****	*RRT*[§]
18	Pivalic acid	5.19	1.00	1.23	7.01	1.00	1.35	2.01
19	Chloropivalic acid	11.97	2.31	2.05	14.31	2.04	1.20	1.70
20	2-Methylbutyric acid	6.03	1.00	1.47	8.85	1.00	1.47	2.04
21	2-Chloro-2-methylbutyric acid	10.35	1.72	1.89	13.58	1.53	1.31	1.70
22	3-Chloro-2-methylbutyric acid (*erythro*)	12.45	2.06	2.04	14.70	1.66	1.18	1.71
23	3-Chloro-2-methylbutyric acid (*threo*)	13.05	2.16	2.02	15.28	1.73	1.17	1.66
24	2-Chloromethylbutyric acid	14.30	2.37	2.03	16.17	1.83	1.13	1.64
25	4-Chloro-2-methylbutyric acid	15.06	2.50	2.02	16.88	1.91	1.12	1.63
26	Isovaleric acid	6.29	1.00	1.35	8.88	1.00	1.41	2.07
27	2-Chloroisovaleric acid	11.98	1.90	1.91	14.40	1.62	1.20	1.67
28	3-Chloroisovaleric acid	12.19	1.94	2.01	13.90	1.57	1.14	1.78
29	4-Chloroisovaleric acid	16.05	2.55	2.19	17.38	1.96	1.08	1.62
30	Valeric acid	7.32	1.00	1.57	10.39	1.00	1.42	1.98
31	2-Chlorovaleric acid	12.78	1.75	1.98	15.21	1.46	1.19	1.60
32	3-Chlorovaleric acid	15.08	2.06	2.08	16.39	1.58	1.09	1.62
33	4-Chlorovaleric acid	15.90	2.17	2.08	17.21	1.66	1.08	1.59
34	5-Chlorovaleric acid	19.21	2.62	1.94	19.72	1.90	1.03	1.51

* Absolute retention times (min) measured from Fig. 2a and b.

** Relative retention times for unchlorinated chloromethyl esters taken as 1.00.

*** Relative retention times for compounds on Carbowax 20M taken as 1.00.

[§] Relative retention times for the corresponding methyl esters taken as 1.00, determined from the mixtures of methyl and chloromethyl esters of the same acids (*e.g.* Fig. 3a and b).

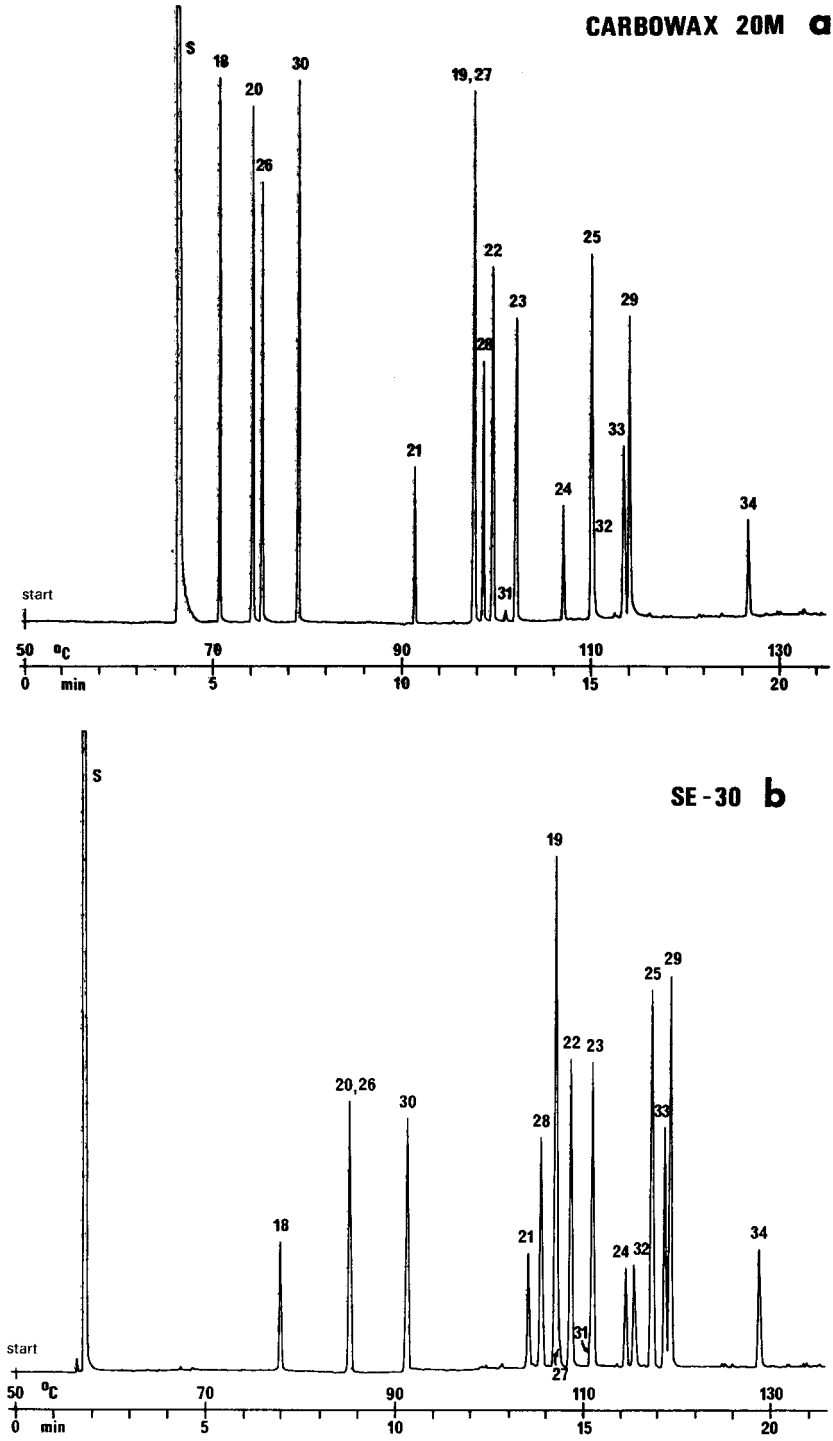

Fig. 2. Chromatogram of the mixture of chloromethyl and chloromethyl monochloro esters of aliphatic C_5-carboxylic acids analysed on Carbowax 20M (a) and on SE-30 (b). Temperature programme: 50°C at 4°C/min. S = solvent; peaks identified in Table II.

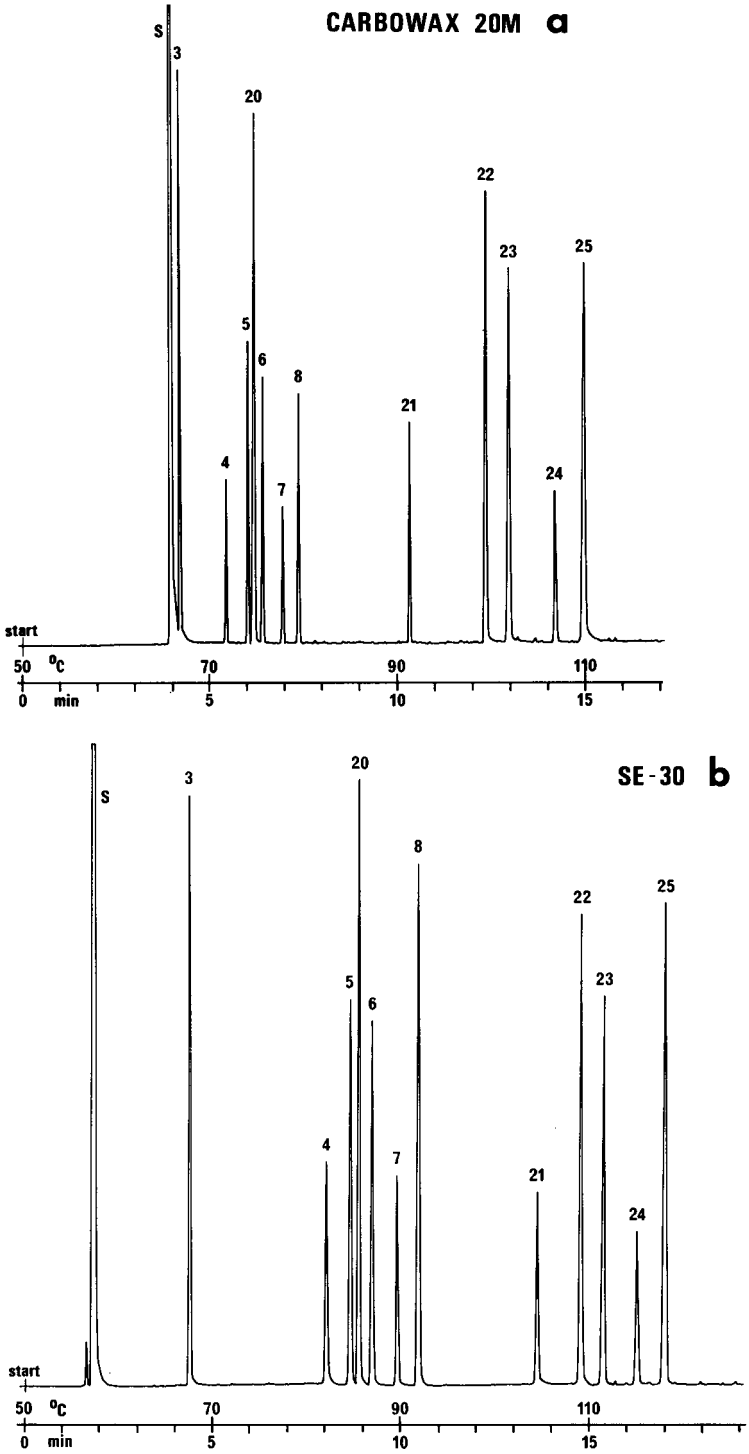

Fig. 3. Chromatogram of the mixture of methyl and chloromethyl 2-methylbutyrates and their monochloro derivatives analysed on Carbowax 20M (a) and on SE-30 (b). Temperature programme: 50°C at 4°C/min. S = solvent; peaks identified in Tables I and II.

valate (19). Also, chloromethyl 3-chlorovalerate (32) left the non-polar column before chloromethyl-4-chloro-2-methylbutyrate (25).

To compare the elution times, GC separations of the mixtures of methyl and chloromethyl esters of the same acids were performed under the same operating conditions. The results are presented in Table II and tabulated relative to the corresponding methyl esters = 1.00. The gas chromatograms of the mixture of methyl and chloromethyl 2-methylbutyrates are illustrated in Fig. 3. It can be seen that the highest values, *ca.* 2.0, for the unchlorinated chloromethyl esters are observed on SE-30, whereas on Carbowax 20M these are the lowest ones, from 1.2 to 1.6. The relative retention times of isomeric monochloro esters vary little on SE-30, while on the other hand, the 2-chloro and ω-chloro isomers give rise to the greatest disparities on Carbowax 20M.

As can be seen from Tables I and II, the relative retention times, relative to the corresponding esters on Carbowax 20M, of compounds on SE-30 are with one exception (methyl pivalate, 1) greater than on Carbowax 20M under the operating conditions used. The values for chlorinated methyl and chloromethyl esters vary from 1.6 to 1.2 and from 1.3 to 1.0, respectively, the 2-chloro isomers giving always the highest and the ω-chloro compounds the lowest values.

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ANALYSIS OF METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS IN PLANT MATERIAL

FALK IMMO SCHÄFERS and KARL HERRMANN*
Institut für Lebensmittelchemie der Universität Hannover, Wunstorfer Strasse 14, D-3000 Hannover 91 (G.F.R.)
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SUMMARY

A method is described for the extraction and purification of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids from plant material. The esters were analyzed as trimethylsilyl derivatives by glass capillary gas chromatography (OV-1, OV-73, Dexsil 300) and gas chromatography–mass spectrometry. The method has been applied to analysis of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids in vegetables and potato peels.

INTRODUCTION

In plants, phenolic acids occur ubiquitously and in various forms. Fruits and vegetables chiefly contain esters of hydroxycinnamic acids with quinic acid or D-glucose besides smaller concentrations of hydroxybenzoic acid compounds. In contrast, methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids have been discovered in nature only in a few cases, with the exception of methyl salicylate and methyl gallate[1]. Bohlmann and co-workers[2–6] isolated, amongst other natural substances, methyl and ethyl caffeate, methyl 4-coumarate and methyl ferulate from some plants.

Phenolic acids are important constituents of plants. The antioxidative and antimicrobial effect of hydroxycinnamic and hydroxybenzoic acids is well known[7]. Gallic acid esters are used in the food industry as antioxidants and methyl, ethyl and propyl 4-hydroxybenzoates (PHB esters) as preservatives. Hydroxycinnamic and hydroxybenzoic acids are further known as growth regulators[8,9] and in the resistance of cultivated plants against pathogenic microorganisms[10,11], especially chlorogenic acid. Chlorogenic acid is easily oxidized enzymatically by phenol oxidases, which for example causes brown discoloration of light fruits, fruit juices and wines.

In this laboratory Sontag *et al.*[12] separated the hydroxycinnamic acid esters by high-performance liquid chromatography (HPLC). It seemed important to develop a method for qualitative and quantitative analysis of the esters of the frequently occurring phenolic acids including the hydroxybenzoic acid esters. Schulz and Herrmann[13,14] employed gas–liquid chromatography for the determination of phenolic

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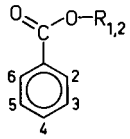
TABLE I

HYDROXYBENZOIC ACID ESTERS



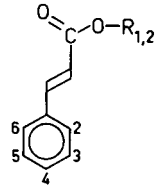
R_1= $-CH_3$
R_2= $-CH_2-CH_3$

Substituents	*Trivial name*	*Source*
2-OH	Methyl salicylate	E. Merck (Darmstadt, G.F.R.)
	Ethyl salicylate	Aldrich-Europe (Nettetal, G.F.R.)
4-OH	Methyl 4-hydroxybenzoate	Riedel de Haen (Seelze, G.F.R.)
	Ethyl 4-hydroxybenzoate	Roth (Karlsruhe, G.F.R.)
2,5-di-OH	Methyl gentisate	–
	Ethyl gentisate	
3,4-di-OH	Methyl protocatechuate	–
	Ethyl protocatechuate	Nipa Ltd. (Pontypridd, Great Britain)
3,4,5-tri-OH	Methyl gallate	Roth
	Ethyl gallate	E. Merck
3-OCH_3, 4-OH	Methyl vanillate	Aldrich-Europe
	Ethyl vanillate	Nipa Ltd.
3,5-di-OCH_3, 4-OH	Methyl syringate	Nipa Ltd.
	Ethyl syringate	

acids, after hydrolysis, as trimethylsilyl (TMS) derivatives. Methyl and ethyl esters of TMS-hydroxybenzoic and -hydroxycinnamic acids are suitable for GLC because they are more volatile than the free acids (TMS derivatives) and because they allow one to distinguish naturally occurring hydroxyl groups from methoxy groups. So we chose capillary GLC in addition to HPLC because of its high performance and to develop

TABLE II

HYDROXYCINNAMIC ACID ESTERS



R_1= $-CH_3$
R_2= $-CH_2-CH_3$

Substituents	*Trivial name*
4-OH	Methyl 4-coumarate
	Ethyl 4-coumarate
3,4-di-OH	Methyl caffeate
	Ethyl caffeate
3-OCH_3, 4-OH	Methyl ferulate
	Ethyl ferulate
3,5-di-OCH_3, 4-OH	Methyl sinapate
	Ethyl sinapate

another chromatographic system for the alkyl esters of the frequently occurring phenolic acids. Another advantage of glass capillary GLC is the possibility to use a mass spectrometer, a highly specific detector.

EXPERIMENTAL

Some of the esters are not commercially available. These esters were synthesized by esterification of the acid with the corresponding alcohol and characterized by melting point and GLC and mass spectrometric (MS) data. MS data of some hydroxycinnamic acid methyl esters (TMS derivatives) have been reported by Horman and Viani[15].

Evaporations were performed in a rotary vacuum evaporator at a temperature not higher than 40°C.

Sample extraction

Fresh plant material (10 g) was homogenized with 25 ml acetonitrile for about 5 min in a centrifuge tube using an Ultra-Turrax (Janke & Kunkel, Staufen i. Br., G.F.R.). The Ultra-Turrax was washed with 25 ml acetonitrile. The two acetonitrile fractions were combined and centrifuged for about 5 min at 4300 *g*. The supernatant was filtered through glass wool into a 250-ml round-bottomed flask. The residue was re-extracted once by homogenizing with 25 ml acetonitrile, washing the Ultra-Turrax and centrifuging. The acetonitrile was evaporated, leaving an aqueous solution.

Preparation of polyamide columns

Polycaprolactam powder (MN-Polyamid SC-6, 0.05–0.16 mm; Macherey, Nagel & Co., Düren, G.F.R.) was suspended in water–methanol (1:1). After about 3 h the suspension was poured into a tube (250 × 14 mm I.D., with a G2-frit and a stopcock) to a height of 150 mm. To remove soluble polyamide components, the column was washed with 50 ml methanol–25% aqueous ammonia (990:10), 50 ml water–acetic acid (998:2) and 50 ml water.

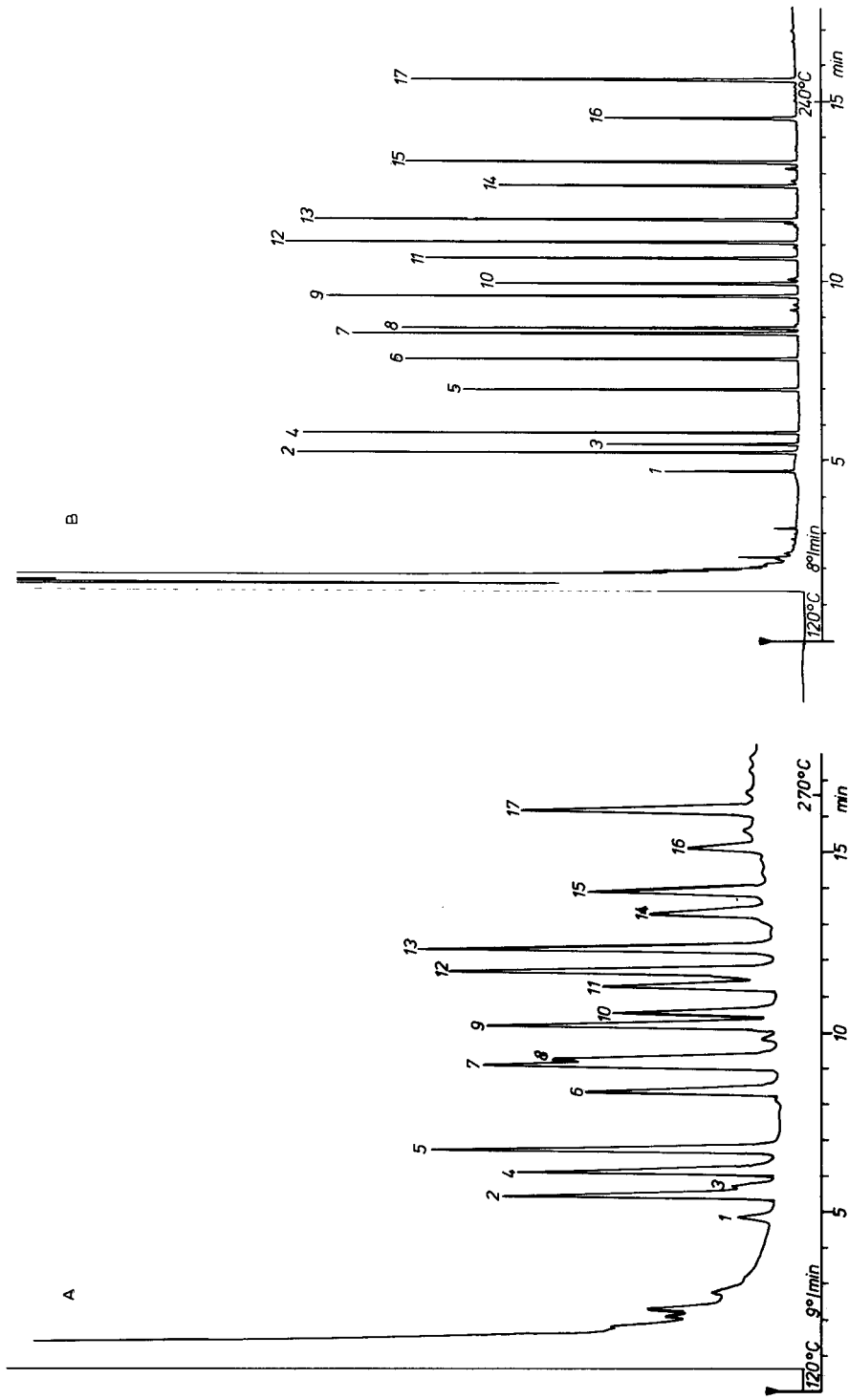
The aqueous plant extract was placed on top of the polyamide column by rinsing the flask three times with 5 ml hot water. The column was washed with 100 ml water to remove carbohydrates, salts and other undesired compounds. Elution was performed with 30 ml methanol. The eluate was collected when methanol first reached the end of the column, producing visible streaks.

Liquid–liquid extraction

For further clean-up, extraction with diethyl ether was useful. The water-containing eluate was adjusted to pH 5 and extracted twice by shaking with 20 ml diethyl ether. The ether solutions were combined, dried over sodium sulphate and filtered into a conical flask.

Derivatization and quantification

After adding 500 μl internal standard solution [tricosane and 3-*tert*.-butyl-4-hydroxyanisole (BHA), each 20 mg per 100 ml hexane], the sample was evaporated to dryness. About 0.4 ml N,O-bis(trimethylsilyl)acetamide (BSA) were added and the


A
B
120°C
9°/min
270°C
240°C
8°/min
min
5
10
15

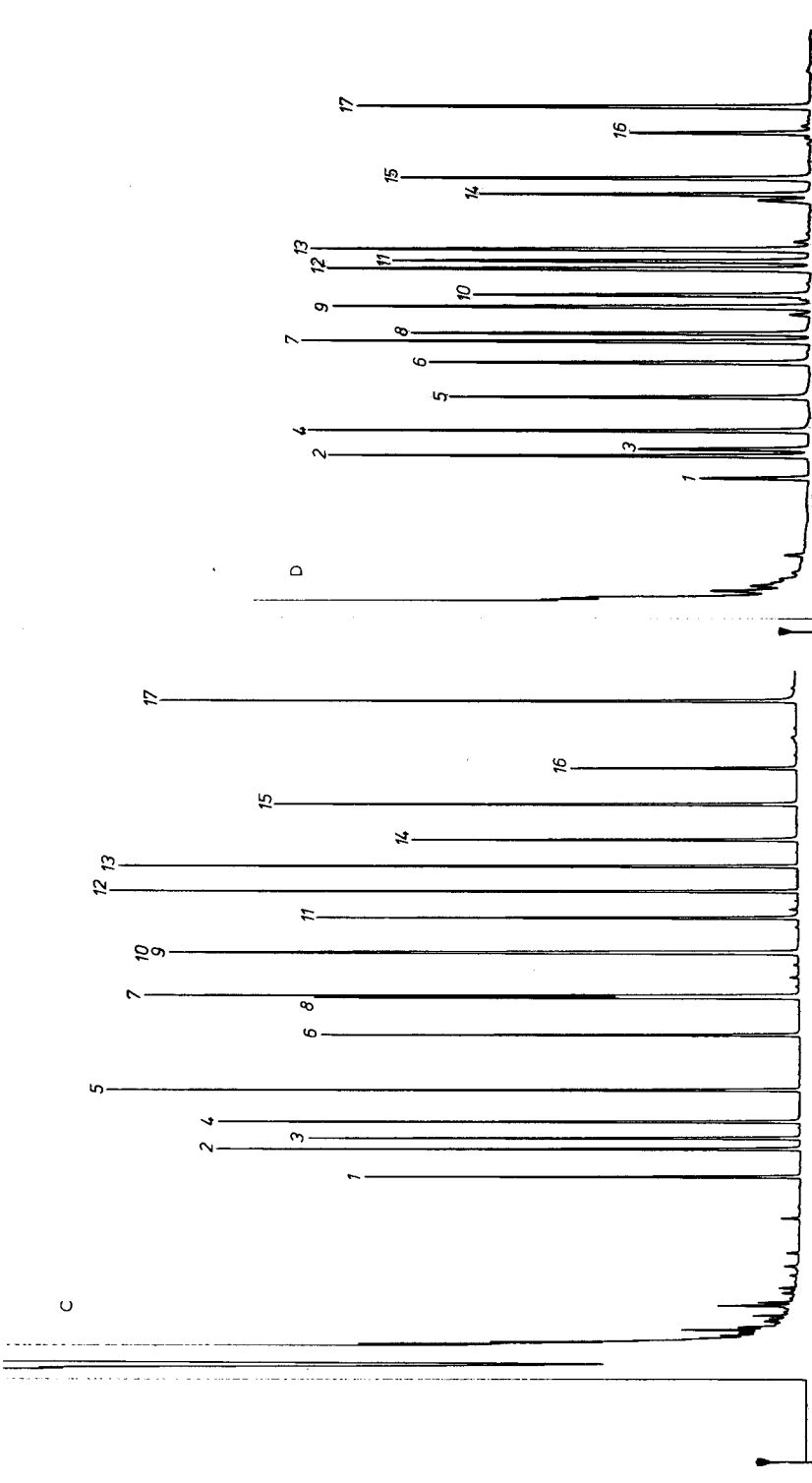


Fig. 1. Chromatograms of methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids (TMS derivatives). Comparison of packed column (A) and glass capillary column is with some different stationary phases (B–D). A: packed column (3 m × 4 mm), Carlo Erba 2150 with flame ionization detector (FID), 1% SE-52 on Gas-Chrom Q (80–100 mesh); injector and detector, 275°C; chart speed, 10 mm/min; carrier gas (N_2) flow-rate, 36 ml/min. B–D: glass capillary columns; Carlo Erba 2150/FID; splitting ratio, 1:20; injector and detector, 250°C; chart speed, 10 mm/min; B, OV-73, wall-coated open tubular column (WCOT) (20 m × 0.26 mm I.D.), carrier 0.8 bar N_2; C, OV-1, WCOT (33 m × 0.26 mm I.D.), carrier 0.95 bar N_2; D, Dexsil 300, WCOT (15 m × 0.29 mm I.D.), carrier 0.45 bar N_2. Peaks: 1 = methyl salicylate; 2 = methyl 3-hydroxybenzoate; 3 = ethyl salicylate; 4 = methyl 4-hydroxybenzoate; 5 = BHA; 6 = methyl vanillate; 7 = methyl gentisate; 8 = ethyl vanillate; 9 = methyl protocatechuate; 10 = methyl syringate; 11 = ethyl 4-coumarate; 12 = methyl gallate; 13 = ethyl gallate; 14 = ethyl ferulate; 15 = ethyl caffeate; 16 = ethyl sinapate; 17 = tricosane.

flask was sealed with a polyethylene stopper followed by heating on an oil-bath. Silylation was complete after 60 min at 70°C. Tricosane (C_{23}) was selected as internal standard, because it is unaffected by silylation and column deactivation. The ratio BHA/C_{23} was used to check on the efficiency of silylation.

A reference solution of phenolic acid ester TMS derivatives, containing 100 μg per 0.4 ml of each ester and the same amount of internal standard, was analysed under the same GLC conditions. A computing integrator SP4100 (Spectra-Physics, Santa Clara, CA, U.S.A.) was used for calculations.

Gas–liquid chromatography

For separation, glass capillary GLC was performed because of its high resolution (Fig. 1). Fig. 1A shows the chromatogram of a standard solution of the esters

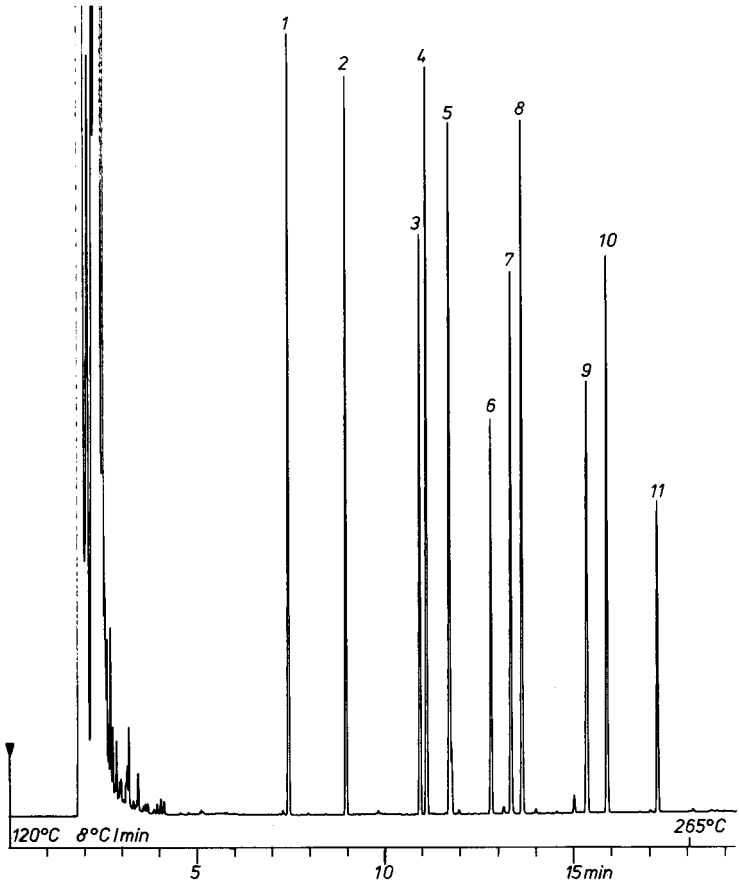


Fig. 2. Chromatogram of phenolic acids (TMS derivatives) on OV-73 (WCOT, 28 m × 0.26 mm I.D.). Conditions: Carlo Erba 2150 with FID; carrier, 0.95 bar N_2; injector and detector, 250°C; chart speed, 10 mm/min. Peaks: 1 = salicylic acid; 2 = 4-hydroxybenzoic acid; 3 = vanillic acid; 4 = gentisic acid; 5 = protocatechuic acid; 6 = syringic acid; 7 = 4-coumaric acid; 8 = gallic acid; 9 = ferulic acid; 10 = caffeic acid; 11 = sinapic acid.

separated on a packed column and Fig. 1B–D the same mixture separated on glass capillaries with different stationary phases.

The sensitivity of TMS-phenolic acids to alkaline supports and glasses has been reported by Schulz and Herrmann[14]. This sensitivity to alkaline and insufficiently deactivated glass capillaries may be the reason for the statement of Verzele and Sandra[16] that some separations succeed better on packed columns than on capillaries. As an example they mentioned the separation of persilylated plant phenolic acids. We succeeded in separating TMS-phenolic acids on a glass capillary without any loss (Fig. 2).

The sensitivity to insufficiently deactivated glass capillaries caused problems initially as it was not possible to buy a column suitable for analyzing TMS-phenolic acid esters. Therefore, we prepared glass capillary columns using exclusively borosilicate glass. A slightly modified procedure, based on that described by Grob[17], involving acidic leaching, flushing, dehydration, persilylation with tetraphenyldimethyldisilazane (TPDMDS)[18] and static coating[19–21], gave excellently deactivated capillary columns for use up to 340°C. Column evaluation was performed according to Donike[22] because this is a temperature-programmed test at elevated temperatures using fatty acid TMS esters. Fig. 1B shows the chromatogram of fifteen methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids separated on a glass capillary column produced as described.

TABLE III

RELATIVE RETENTION TIMES OF METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS ON THREE STATIONARY PHASES RELATED TO TRICOSANE (C_{23}) AND 3-*tert.*-BUTYL-4-HYDROXYANISOLE (BHA)

Compound	*OV-1*		*OV-73*		*Dexsil 300*	
	BHA	C_{23}	*BHA*	C_{23}	*BHA*	C_{23}
Methyl salicylate	0.686	0.214	0.737	0.286	0.643	0.209
Ethyl salicylate	0.809	0.254	0.852	0.331	0.814	0.266
Methyl 4-hydroxybenzoate	0.872	0.272	0.902	0.350	0.929	0.301
BHA	1.000	0.313	1.000	0.388	1.000	0.324
Ethyl 4-hydroxybenzoate	1.050	0.329	1.047	0.407	1.146	0.374
Methyl vanillate	1.270	0.396	1.235	0.479	1.372	0.445
Ethyl vanillate	1.467	0.460	1.354	0.526	1.523	0.494
Methyl gentisate	1.493	0.465	1.381	0.536	1.569	0.512
Methyl protocatechuate	1.536	0.479	1.394	0.541	1.569	0.509
Ethyl gentisate	1.660	0.521	1.475	0.573	1.675	0.546
Methyl syringate	1.664	0.519	1.530	0.594	1.745	0.569
Ethyl protocatechuate	1.725	0.541	1.532	0.595	1.815	0.589
Methyl 4-coumarate	1.732	0.540	1.589	0.617	1.824	0.591
Ethyl syringate	1.916	0.601	1.512	0.665	1.983	0.647
Ethyl 4-coumarate	1.916	0.601	1.722	0.668	2.004	0.649
Methyl gallate	2.104	0.656	1.784	0.693	2.089	0.665
Methyl ferulate	2.142	0.667	1.892	0.735	2.131	0.695
Ethyl gallate	2.244	0.704	1.894	0.735	2.275	0.738
Ethyl ferulate	2.364	0.741	2.019	0.784	2.404	0.779
Methyl caffeate	2.374	0.740	2.061	0.800	2.478	0.809
Ethyl caffeate	2.579	0.808	2.176	0.845	2.587	0.844
Methyl sinapate	2.592	0.809	2.237	0.868	2.709	0.879
Ethyl sinapate	2.785	0.873	2.388	0.927	2.880	0.939
C_{23}	3.208	1.000	2.534	1.000	3.073	1.000

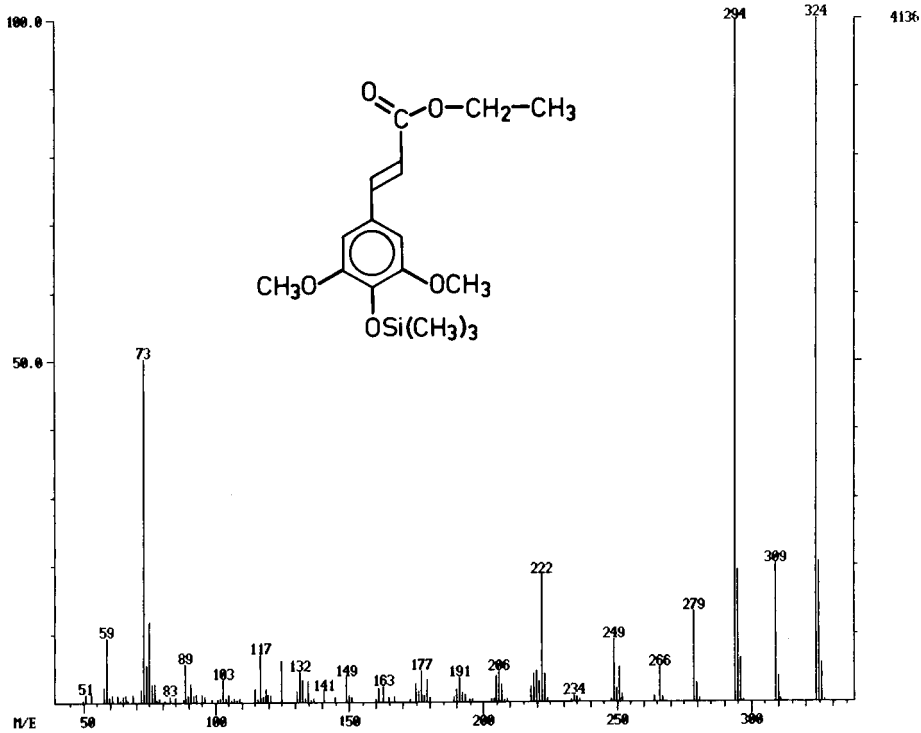


Fig. 3. Mass spectrum of ethyl sinapate TMS derivative.

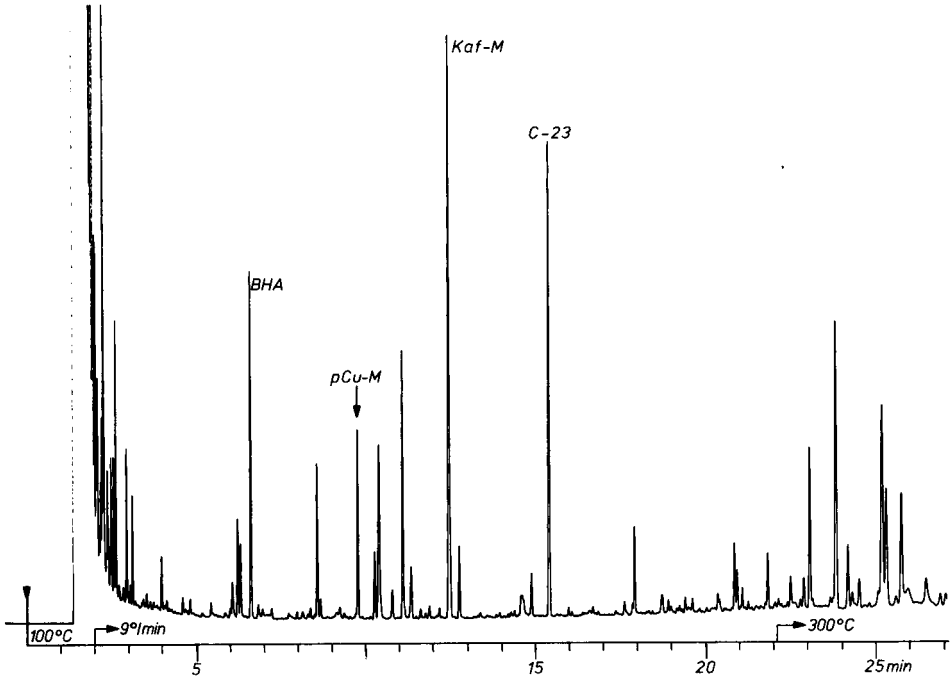


Fig. 4. Chromatogram of extract from fresh potato peelings. Peaks: pCu-M = methyl-4-coumarate; Kaf-M = methyl caffeate.

RESULTS AND DISCUSSION

Gas–liquid chromatography

In Table III the relative retention time data for 22 methyl and ethyl esters of phenolic acids (TMS derivatives) are listed. Relative retention time was related to both tricosane and BHA for three stationary phases, OV-1, OV-73 and Dexsil 300. The retention behaviour on different stationary phases is shown in Fig. 1B–D. Methyl and ethyl esters of salicylic acid cannot be determined with the described extraction method because of their great volatility and these esters are not retained on the polyamide column during washing with water. Methods for determination of methyl and ethyl salicylate have been described for example in refs. 23–25.

Mass spectrometry

The mass spectra of silylated methyl and ethyl esters of hydroxybenzoic and hydroxycinnamic acids show molecular ion peaks of high relative intensity (Fig. 3) if the ion source temperature is not too high (130°C). The compounds would therefore be readily amenable to single-ion monitoring (SIM). Often the ion $(CH_3)_3Si^+$, *m/e* 73, constitutes the base peak, especially when the ion source temperature exceeds 130°C.

The fragmentation pattern of the methyl and ethyl esters of the hydroxybenzoic acids is similar to that of the methyl esters of TMS-cinnamic acids discussed by Horman and Viani[15]. The esters with two neighbouring hydroxyl groups and TMS substituents on the aromatic ring show simple spectra. Besides the molecular ion peak

TABLE IV

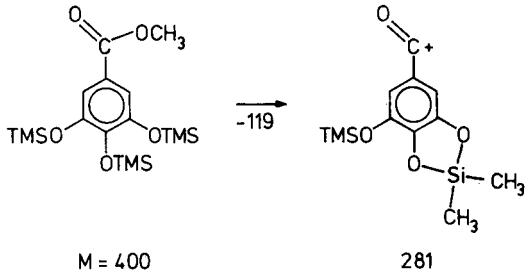
MASS SPECTRAL DATA FOR THE METHYL AND ETHYL ESTERS OF HYDROXYBENZOIC AND HYDROXYCINNAMIC ACIDS (TMS DERIVATIVES)

Data in *m/e*, relative intensities in parentheses. M = Molecular ion; BP = base peak. Conditions: Finigan 4023 gas chromatography–quadrupole mass spectrometry system with open-split interface[26]; interface, 250°C; ion source, 130°C; electron beam energy, 70 eV; ionizing current, 300 μA.

Compound	*M*	*BP*	*Other characteristic ions*
Methyl 4-coumarate	250(100)	250(100)	235(66),219(40),203(39)
Ethyl 4-coumarate	264(100)	264(100)	219(53),249(35),203(30),192(37)
Methyl ferulate	280(72)	72(100)	250(97),217(85)
Ethyl ferulate	294(100)	294(100)	264(92)
Methyl caffeate	338(50)	219(100)	
Ethyl caffeate*	352(22)	73(100)	219(66)
Methyl sinapate	310(78)	280(100)	
Ethyl sinapate	324(100)	324(100)	294(97)
Methyl salicylate*	224	59(100)	209(88)
Ethyl salicylate*	238	195(100)	223(44)
Methyl 4-hydroxybenzoate*	224(62)	209(100)	193(28)
Ethyl 4-hydroxybenzoate*	238(18)	73(100)	193(32)
Methyl vanillate*	254(32)	224(100)	239(56),193(62)
Ethyl vanillate*	268(26)	73(100)	253(48),193(56)
Methyl gentisate*	312	297(100)	267(24)
Ethyl gentisate*	326	73(100)	311(17),239(45)
Methyl protocatechuate*	312(18)	193(100)	
Ethyl protocatechuate*	326(12)	193(100)	
Methyl syringate*	284(24)	254(100)	269(50),223(39)
Ethyl syringate*	298(12)	73(100)	283(18),268(43)
Methyl gallate*	400(6)	73(100)	281(15)
Ethyl gallate*	414(7)	73(100)	281(34)

* Varian/MAT 44S gas chromatography–quadrupole mass spectrometry system modified with a Carlo-Erba split 76; interface, 220°C; ion source, 220°C; electron beam energy, 70 eV; ionizing current, 300 μA.

there are only two other peaks in the spectrum of methyl gallate for example, *m/e* 73 and *m/e* M − 119. M − 119 is of high intensity and seems to be formed by cleavage of the methyl ester group, one TMS group and another methyl group, forming a ring of high stability resulting in an intensive peak at *m/e* 281.



M = 400 281

Application

The method described in this paper was applied successfully to the qualitative and quantitative analysis of methyl and ethyl esters of phenolic acids in vegetables, for example, and potato peels (Fig. 4). The results will be reported elsewhere[27].

ACKNOWLEDGEMENTS

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SEPARATION AND QUANTIFICATION OF BASIC HYDROXYCINNAMIC AMIDES AND HYDROXYCINNAMIC ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MICHEL PONCHET*
Station de Botanique et de Pathologie Végétale, I.N.R.A., B.P. 78, 06602 Antibes (France)
JOSETTE MARTIN-TANGUY
Station de Physiopathalogie Végétale, I.N.R.A., B.V. 1540, 21034 Dijon (France)
and
ALAIN POUPET, ANTOINE MARAIS and DANIEL BECK
Station de Physiopathologie Végétale, I.N.R.A., B.V. 1540, 21034 Dijon (France)
(Received December 29th, 1981)

SUMMARY

A method for the separation of basic hydroxycinnamic amides and hydroxycinnamic acids by high-performance liquid chromatography is described. N-*p*-Coumaryl-, N-caffeyl- and N-ferulylputrescine, N-*p*-coumaryl-, N-caffeyl- and N-ferulylspermidine and *p*-coumaric, caffeic, ferulic and sinapic acids were chromatographed on a μBondapak C_{18} reversed-phase column (particle size 9 μm) with different methanol–water gradients as the mobile phase. It is possible with this high-resolution and reproducible method to assay biological samples containing more than 10^{-5} *M* of hydroxycinnamic amides, using either *p*-coumaric or ferulic acid as the internal standard: this is demonstrated for tobacco extracts.

INTRODUCTION

Hydroxycinnamic amides (HCA) have been observed in higher plants. HCA are hydroxycinnamic acid (HC)–amine conjugates with an amide bond between them, and they occur in basic (water-soluble) and neutral (water-insoluble) forms. In the basic form, only aliphatic amines (di- and polyamines) are linked with HC.

Numerous basic HCA have been found in plant tissues (ferulylputrescine[1–5], caffeylputrescine[4–10], *p*-coumarylputrescine[4] and caffeylspermidine[10]), but more recently several workers have reported that the different basic HCA are closely related to the flowering process and to sexual organogenesis in different botanical species[11], in *Nicotiana tabacum* sp.[12–14], in *Zea mays*[15] and in *Araceae* species[16].

Moreover, results obtained in the study of the interaction between tobacco and tobacco mosaic virus (TMV) strongly suggest that basic HCA have an antiviral effect and are synthesized after virus infection as a protective mechanism[17,18]; in addition, virus particles are nearly absent from meristems, sex organs and seeds[19], where basic

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HCA accumulate in large amounts. Some HCA derivatives, *viz.*, glycocinnamoylspermidines, have been considered as a new class of antibiotics[20].

Previously reported procedures for the separation of basic HCA, such as differential extraction, thin-layer chromatography, paper chromatography and the use of cation-exchange resins, were not suitable for rapid assays because of their low resolution, long duration and lack of sensitivity.

The development of rapid and reproducible techniques for the separation, quantification and identification of these metabolites is essential in order to elucidate the molecular mechanism of their action.

In this paper we report an efficient method using reversed-phase high-performance liquid chromatography (HPLC), which allows the rapid and reproducible separation of basic HCA.

EXPERIMENTAL

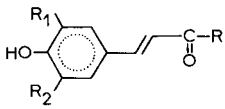
Apparatus

All experiments were performed on a Model 200/6/4/GM solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), using a U6K injector (Waters Assoc.). The column used was reversed-phase μBondapak C_{18} (particle size 9 μm; 30 cm × 3.9 mm I.D.; Waters Assoc.).

To protect the analytical column, a small and easily replaceable precolumn (2.3

TABLE I

STRUCTURES OF HYDROXYCINNAMIC ACIDS AND HYDROXYCINNAMIC ACID DERIVATIVES



Compound	*R*	R_1	R_2	*Supplier*
p-Coumaric acid	OH	H	H	Sigma (St. Louis, MO, U.S.A.)
Caffeic acid	OH	OH	H	
Ferulic acid	OH	OCH_3	H	
Sinapic acid	OH	OCH_3	OCH_3	
p-Coumarylputrescine	$HN(CH_2)_4NH_2$	H	H	Rhône-Poulenc
Caffeylputrescine	$HN(CH_2)_4NH_2$	OH	H	
Ferulylputrescine	$HN(CH_2)_4NH_2$	OCH_3	H	
p-Coumarylspermidine	$HN((CH_2)_7NH)NH_2$	H	H	–*
Caffeylspermidine	$HN((CH_2)_7NH)NH_2$	OH	H	–**
Ferulylspermidine	$HN((CH_2)_7NH)NH_2$	OCH_3	H	–*
Chlorogenic acid	$OC_7O_5H_{11}$	OH	H	Fluka (Buchs, Switzerland)
p-Coumarylquinic acid	$OC_7O_5H_{11}$	H	H	–***

* Purified from mature *Zea mays* seeds

** Purified from *Nicotiana tabacum* var. *Samsun* pistils.

*** Purified from apple fruits.

cm × 4 mm I.D.) filled with μBondapak C_{18} Porasil (Waters Assoc.) was used. Basic HCA and HC were detected on a Schoeffel spectrophotometer (monitored at 310 nm).

Recording and integration were carried out on ICAP 50-EI 510 (Delsi, France). Samples were injected with the use of a 25-μl Precision Sampling syringe (Precision Sampling Co., Baton Rouge, LA, U.S.A.) and chromatography was performed at ambient temperature (flow-rate 2 ml/min).

Reagents and standards

Methanol and acetic acid (R.P. Normapur) were obtained from Prolabo (Paris, France). Pic A (tetrabutylammonium phosphate) was purchased from Waters Assoc. The water used was deionized, redistilled twice and stored in dark containers.

The solvents were filtered under vacuum through a 5-μm Millipore Mitex LS type filter and degassed before use.

Details of the standards are given in Table I. Hydroxycinnamylspermidines were purified from plant tissues and synthetic hydroxycinnamylputrescines were given by Rhône-Poulenc Industries, France.

Preparation of standards

Concentrated standard stock solutions were prepared by direct weighing and dissolution in methanol (HC) or methanol–water (1:1) (HC derivatives), then stored at −20°C.

The concentration of the working standards generally ranged from 10^{-4} to $5 \cdot 10^{-4}$ *M* (for an injection volume of 15 μl), whereas those of stock solutions were 10^{-2} *M*; working standards were obtained from the stock solutions by dilution with methanol–water (1:1).

HPLC procedure

The gradient system consisted of absolute methanol (solvent A) and water–1% acetic acid–1.5% Pic A (solvent B), and the mobile phase obtained was run at a constant flow-rate. Acetic acid and Pic A (as ion-pairing reagent) were necessary to reduce retention times and to bring significant improvements in peak shape and resolution. This is because HCA have basic (amine) and acidic (phenolic) functions, which interfere with the free silicic acid poles of the packing material, to which no C_{18} carbon chain was added.

Linearity

Linearity was tested by injecting various volumes of standard solutions.

Sample preparation

Freshly harvested plant tissues (10 g fresh weight) were homogenized in methanol (100 ml) in a Sorwall Omnimixer, then filtered. The pellet was washed twice with methanol (100 ml), then discarded.

The filtered methanol extract was evaporated to 5 ml at 40°C under vacuum, diluted with water (50 ml) and treated with ethyl acetate (three 50-ml volumes) to remove neutral substances. The ethyl acetate fraction was dicarded. The aqueous fraction was concentrated to dryness and dissolved in methanol–water (1:1) (5 ml/g fresh material weight).

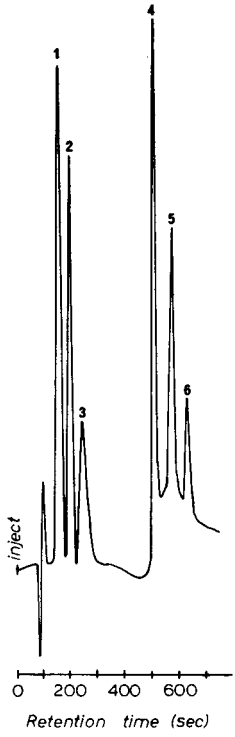


Fig. 1. Chromatogram of a mixture of six basic HCA. Column: μBondapak C_{18}, 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1% acetic acid, 1.5% Pic. A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated with A–B (10:90) for 10 min before injection. Peaks: 1 = caffeylputrescine; 2 = *p*-coumarylputrescine; 3 = ferulylputrescine; 4 = caffeylspermidine; 5 = paracoumarylspermidine; 6 = ferulylspermidine.

The solution was filtered through a 5-μm Mitex LS Millipore filter before injection.

RESULTS

Separation of basic HCA

Preliminary studies, using isocratic conditions with various proportions of water in methanol, showed that putrescine and spermidine derivatives required two different mobile phases to be chromatographed well. Moreover, for each group, isocratic conditions resulted in good resolution but the last peak (*i.e.*, ferulic acid derivative) spread over 2 min and did not allow reproducible quantitation. To obtain a good peak shape, a gradient (with an increasing proportion of methanol) was necessary and a convex gradient gave a more efficient resolution than a linear one.

The presence of Pic A as an ion-pairing reagent brought significant improvements: it reduced the retention time by almost half and it gave excellent symmetrical pointed peaks, whereas omission of Pic A caused trailing peaks.

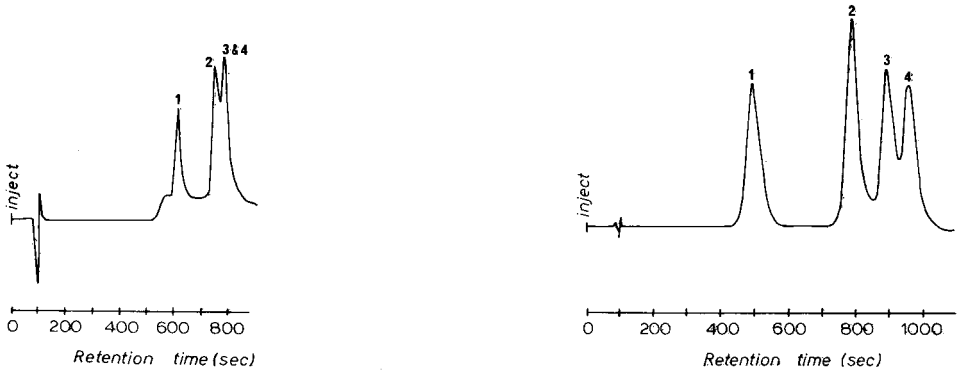


Fig. 2. Chromatogram of a mixture of HC. Column: μBondapak C_{18}, 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1% acetic acid, 1.5% Pic A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated with A–B (10:90) for 10 min before injection. Peaks: 1 = caffeic acid; 2 = paracoumaric acid; 3 = ferulic acid; 4 = sinapic acid.

Fig. 3. Chromatogram of a mixture of HC. Conditions and peaks as in Fig. 2, except mobile phase: solvent A (methanol)–solvent B (water with 2% acetic acid, no Pic A), convex gradient A–B (20:80) to A–B (30:70) for 10 min. The column was equilibrated with A–B (20:80) for 10 min before injection.

The best results were obtained with the solvent programme convex gradient 10% solvent A to 35% solvent A (in solvent B) for 5 min. Under these conditions, the separation of a mixture of basic HCA is effective, as shown in Fig. 1. Basic HCA were separated in the following order: caffeyl-, paracoumaryl-, ferulylputrescine, caffeyl-, *p*-coumaryl- and ferulylspermidine.

Using a detector sensitivity of 0.02 a.u.f.s., the minimum detectable amount of HCA was about 10 ng. Linearity was satisfactory up to 50 ng. Increasing the sensitivity would permit the detection of smaller amounts, but such an improvement would require additional precautions with respect to solvent purity and other conditions because rapid gradients are very detrimental to baseline stability when high sensitivity is employed.

The recovery was about 95% for the different compounds when the same sample was injected several times. The retention times were constant, the variations not exceeding 1%.

Separation of HC

Incomplete separation of HC occurred under the conditions outlined above (Fig. 2) and caffeic acid had the same retention time as ferulylspermidine. As free HC have not been found in plant tissues, either *p*-coumaric, ferulic or sinapic acid could be used as the internal standard for HCA assay.

When the gradient conditions were modified, as specified in Fig. 3, the separation of HC could be achieved in the following order: caffeic, *p*-coumaric, ferulic and sinapic acids (Fig. 3). The separation of HC could be helpful in the analysis of hydrolysed fractions containing HC derivatives, especially HCA.

Basic HCA separation was developed for the study of these compounds in tobacco extracts (HCA accumulated in ovaries during flowering and HCA formed in

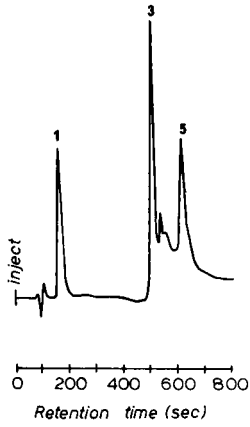


Fig. 4. Chromatogram of an extract of *Nicotiana tabacum* c.v. *Samsun* n.n. pistils (from non-bloomed flowers). Column: μBondapak C_{18}, 9 μm (30 cm × 3.9 mm I.D.). Flow-rate: 2 ml/min. Room temperature. Peak detection: 310 nm. Pressure: 3100–3500 p.s.i. Mobile phase: solvent A (methanol)–solvent B (water with 1 % acetic acid, 1.5 % Pic A), convex gradient A–B (10:90) to A–B (35:65) for 5 min. The column was equilibrated for 10 min before injection. Peaks: 1 = caffeylputrescine; 2 = ferulylputrescine; 3 = caffeylspermidine; 4 = unknown; 5 = caffeyl-3-quinic acid (chlorogenic acid); 6 = *p*-coumarylquinic acid.

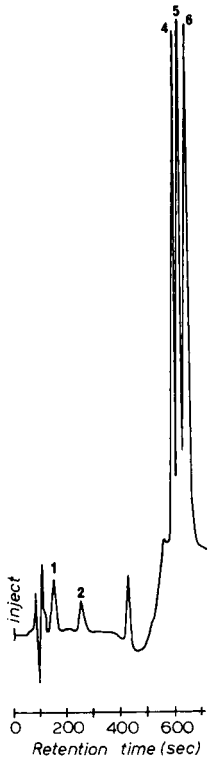


Fig. 5. Chromatogram of an extract of TMV-infected vegetative *Nicotiana tabacum* c.v. *Xanthi* n.c. leaves (48 h after inoculation). Conditions and peaks as in Fig. 4.

leaves during the hypersensitive reaction of *Nicotiana tabacum* c.v. *Xanthi* n.c. to TMV).

Basic HCA in non-purified tobacco extracts

All of the conditions were as in Fig. 1.

Fig. 4 shows the separation profile obtained from the analysis of *Nicotiana tabacum* c.v. *Samsun* n.n. pistils. Caffeylputrescine and caffeylspermidine were the main basic HCA accumulated in this sex organ, with concentrations close to 0.9 mg/g fresh material weight for caffeylputrescine and 1.5 mg/g fresh material weight for caffeylspermidine.

Fig. 5 illustrates the separation of basic HCA synthesized in the leaves of TMV-infected non-flowering *Nicotiana tabacum* c.v. *Xanthi* n.c. at the beginning of infection (48 h after inoculation with an inoculum of 0.1 mg/ml TMV). Ferulylputrescine and caffeylputrescine were formed and reached concentrations of about 0.1 mg/g fresh material weight. All of these compounds were absent from the fully extended vegetative leaves under normal growing conditions and from the control leaves inoculated with water.

Peak 5 (620 sec, Figs. 4 and 5) contained caffeylquinic acid (chlorogenic acid) and peak 6 (650 sec, fig. 5) *p*-coumarylquinic acid. Chlorogenic acid appeared in the peak area of ferulylspermidine and interfered with the *p*-coumarylspermidine peak area, so that sample purification was necessary to improve the separation and the quantitation of these compounds. The use of Amberlite CG-50 (H^+) resin (Serva, Heidelberg, G.F.R.) allows the separation of quinic esters from basic HCA and is thus the most suitable technique. The identity of peaks 5 and 6 was based on results obtained both by injection of individual standards and of a pair of standards (varying the concentration of one standard in the mixture each time).

Table II summarizes retention times of the various HC and HC derivatives (for chromatographic conditions, see Fig. 1).

DISCUSSION

This paper demonstrates the ability of a C_{18} reversed-phase column to separate basic HCA and HC by HPLC. HPLC provides a highly sensitive (the minimum detectable amount of HCA is *ca.* 10 ng, or *ca.* 50 pmole), rapid and reproducible method for the determination and quantitation of basic HCA.

This technique allows the separation of these compounds from non-purified plant extracts or simply purified extracts [prior clean-up with Amberlite CG-50 (H^+) resin]. Detection of peaks at 310 nm is very helpful in eliminating UV absorption of all co-extractable compounds able to interfere at lower wavelengths (254 or 270 nm); for example, aromatic amines (tyramine, dopamine, tryptamine) have almost the same retention time as caffeylputrescine but do not absorb at 310 nm.

HC such as *p*-coumaric and ferulic acids can be used as internal standards for quantitation of basic HCA.

The proposed method promises to be very useful for the study of these compounds with respect to the flowering process and to virus resistance.

TABLE II

RETENTION TIMES OF THE DIFFERENT HC AND HC DERIVATIVES

Column: μBondapak C_{18} (9 μm), 30 cm × 3.9 mm I.D. Flow-rate: 2 ml/min. Wavelength: 310 nm. Mobile phase: Water with 1% acetic acid and 1.5% Pic A–methanol, 90:10 to 65:35 for 5 min.

Compound	*Retention time (sec. ± 5 sec)*
Caffeylputrescine	160
p-Coumarylputrescine	200
Feruloylputrescine	255
Caffeylspermidine	515
p-Coumarylspermidine	585
Caffeyl-3-quinic acid (chlorogenic acid)	620
Caffeic acid	620
Feruloylspermidine	640
p-Coumarylquinic acid	650
p-Coumaric acid	750
Ferulic acid	780
Sinapic acid	785

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ANALYSIS OF LIPOPOLYSACCHARIDES BY METHANOLYSIS, TRIFLUOROACETYLATION, AND GAS CHROMATOGRAPHY ON A FUSED-SILICA CAPILLARY COLUMN

KLAUS BRYN* and ERIK JANTZEN
Statens Institutt for Folkehelse, Postuttak Oslo 1 (Norway)
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SUMMARY

A gas chromatographic method for simultaneous analysis of fatty acids and sugars of lipopolysaccharides (LPS) has been developed. The sample (1 mg or less) is methanolyzed at 85°C overnight in 2 *M* HCl in methanol. The released methyl esters and methyl glycosides are trifluoroacetylated and chromatographed on a methylsilicone-impregnated fused-silica capillary column. This column resolves all ordinary LPS sugars and fatty acids, and quantitative analysis is possible, including 2-keto-3-deoxyoctanoic acid (KDO), glucosamine and heptoses. 3,6-Dideoxyhexoses show some thermal degradation at 85°C during methanolysis, but this can be overcome by lowering the temperature to 37°C. For KDO the higher temperature similarly causes some degradation, but a reproducible response factor was found. The method appears to be useful for analysis of purified LPS as well as a means for monitoring for LPS content during purification of bacterial antigens of different kinds.

INTRODUCTION

Bacterial lipopolysaccharides (LPS, endotoxins) are encountered in almost all Gram-negative bacteria, and play an important role in bacterial infections. This group of highly complex structures have therefore been extensively studied; they have been shown to evoke a wide variety of biological responses in the host and are reported to affect almost all organ systems[1,2].

The most studied LPS are isolated from strains of *Salmonella*. The general structure is composed of three chemically different regions[3]. A lipophilic moiety (lipid A) consists of a disaccharide of glucosamine with one acid-labile and one acid-stable phosphate group, and hydroxylated and non-hydroxylated fatty acids in ester and amide linkages. Lipid A is ketosidically linked to a core polysaccharide consisting of 2-keto-3-deoxyoctanoic acid (KDO) and L-glycero-D-mannoheptose, both partly phosphorylated, together with several hexoses and hexosamines. Some LPS (rough, R) only contain lipid A and a biosynthetically completed or uncompleted core polysaccharide, but most LPS are of S type (smooth), and these have an additional region (O-chain) that is a polymerized oligosaccharide.

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Owing to the complexity of LPS, with a variety of constituents and types of linkage, several different methods are commonly utilized for compositional analysis. These comprise separate colorimetric assays of KDO, hexosamine, heptose and neutral sugars, and thin-layer chromatography (TLC) or gas chromatography (GC) of sugars and fatty acids. It was therefore of interest to develop a rapid and simple method where most constituents could be quantitated simultaneously. Previously[4,5] we have described a GC technique for bacterial identification which was based on methanolyzed and trifluoroacetylated bacterial cells. In this communication we report a similar analytical approach for determination of LPS constituents, including the use of a fused-silica capillary column.

EXPERIMENTAL

Chemicals

Solvents of analytical grade were distilled before use. Methanolic HCl (2 *M*) was obtained by bubbling dry HCl gas (Fluka, Buchs, Switzerland) into dry methanol until saturation, and subsequent dilution. Most LPS preparations and the N-(3-hydroxymyristyl)glucosamine standard were gifts from O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, G.F.R. (*Salmonella abortus equi*; *S. typhi*; *S. minnesota*, Ra to Re). A LPS preparation from *Actinobacillus actinomycetemcomitans* was received from J. Jonsen and I. Olsen, University of Oslo, Norway. LPS of *Neisseria elongata* and *Yersinia enterocolitica* were our own preparations. All LPS samples were extracted and purified according to Westphal *et al.*[6] by the hot phenol procedure, followed by dialysis and ultracentrifugation. The K-13 polysaccharide of *Escherichia coli*[7] was obtained from K. Jann. Max-Planck-Institut für Immunbiologie. Type I polysaccharide of *Klebsiella*[8] was a gift from J. Eriksen, University of Oslo.

Fatty acid methyl ester standards were from Applied Science Labs. (State College, PA, U.S.A.). Ethanolamine, monosaccharides, disaccharides, mannan, and cerebroside were from Sigma (St. Louis, MO, U.S.A.).

Methanolysis and trifluoroacetylation

LPS preparations (0.1–1 mg) were suspended in 2 *M* HCl in methanol (1 ml) in teflon-lined screw-capped vials and kept at 85°C for 18 h[4,5]. Methanolysates were concentrated to dryness by nitrogen or on a rotary evaporator at room temperature. Trifluoroacetyl (TFA) derivatives were then formed by adding 50 μl of 50% trifluoroacetic anhydride (Merck, Darmstadt, G.F.R.) in acetonitrile (Merck) and heating to boiling for *ca.* 2 min. After 10 min at room temperature, the reaction mixture was diluted to 10% trifluoroacetic anhydride and injected onto the gas chromatograph. Derivatized samples could be stored for several months at −20°C in sealed capillaries.

Gas chromatography

The GC analyses were carried out on a Hewlett-Packard 5710 chromatograph equipped with a flame ionization detector and a Hewlett-Packard 18740B capillary column control. The fused-silica capillary column (25 m × 0.2 mm I.D., methylsilicone (SE-30) stationary phase, Hewlett-Packard, Avondale, PA, U.S.A.) was operated in splitless mode and with a carrier gas (helium) flow-rate of 1.5 ml min^{-1}.

Column oven temperature was held for 4 min at 90°C and then programmed at 8°C min^{-1} to 250°C. Peak areas and retention times were recorded by a Hewlett-Packard 3390A recorder-integrator.

Peak identification

Generally the constituents were identified by comparison of retention times with those of methanolyzed standards. In addition, the identities were confirmed by mass spectrometry (MS) using a Hewlett-Packard 5992A gas chromatograph–mass spectrometer instrument equipped with a glass capillary column (25 m × 0.5 mm, CP Sil 5 (methyl silicone), Chrompack, Middelburg, The Netherlands). The ionization conditions used were 70 eV at 170°C.

Heptose was identified by GC and MS of two mutant R form LPS that contained (Rd) or was devoid (Re) of heptose, as reported earlier[9]. Several specific fragments were recorded, *e.g. m/e* 591 (M − TFAO), 531 (M − TFAO and CH_3OCHO), 445 (M − CH_3O and 2 TFAO), and also the general fragment of *m/e* 157 ($CH_3OCHOTFA$).

KDO peaks were recognized by analysis of methanolysates of *Escherichia coli* polysaccharide-K-13, a ribose-KDO polymer[7]. The same KDO peaks were also recorded for methanolysates of *Salmonella* LPS. Verification of identity was performed by MS, where characteristic fragments of M − 31 (loss of CH_3O) and M − 59 (loss of $COOCH_3$) were observed (to be published).

RESULTS AND DISCUSSION

Methanolysis

The fatty acids of LPS are usually liberated by saponification using aqueous methanol and NaOH or KOH, followed by methyl ester formation and GC. Analysis of the various monosaccharide constituents is more problematic, and several different assays are required for reliable identification and quantitation. For example, at least three different hydrolytic conditions are commonly utilized to avoid degradation of the most labile sugars and to accomplish complete cleavage of all glycosidic linkages of an oligosaccharide chain.

Methanolic HCl is usually considered to be a mild and effective reagent for cleaving oligosaccharide linkages[10,11]. Sugar–phosphate linkages are normally not cleaved and hence phosphorylated LPS constituents are not represented in the chromatograms. Otherwise, it is our experience that most glycosidic linkages are quantitatively broken and that most commonly occurring sugars are stable at the conditions used. Dideoxy sugars represent an exception in being partly degraded. A quantitative GC analysis of these fragile sugars requires methanolysis at a more moderate temperature (unpublished).

KDO, another common and fragile LPS constituent, can be determined by the presented method. However, a certain percentage of this sugar commonly occurs in phosphorylated form, and only the unphosphorylated moieties will be detected. During methanolysis of KDO at 85°C, an extensive conversion between different ring-forms takes place. After completed reaction, the two most abundant peaks (Fig. 1) constitute 70% of total KDO with a low coefficient of variation (below 5%), and these peaks can apparently be used for quantitation of unphosphorylated KDO.

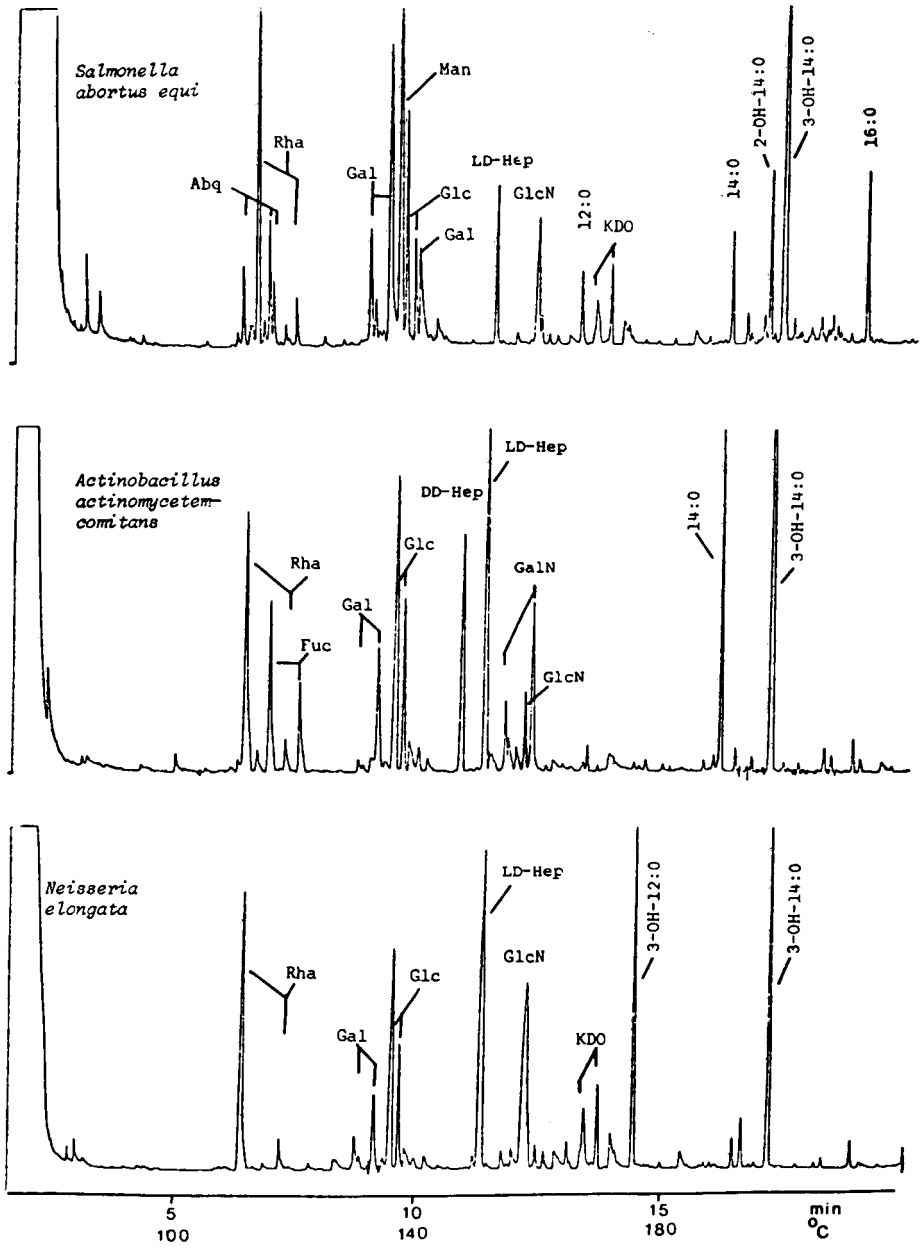

Fig. 1. Chromatograms of three LPS preparations after methanolysis and trifluoroacetylation. Conditions of sample preparation and chromatography are given in the text. Abbreviations: Abq, abequose; Rha, rhamnose; Fuc, fucose; Gal, galactose; Man, mannose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GlcN, 2-deoxy-2-aminoglucose; KDO, 2-keto-3-deoxyoctanoate; 12:0, dodecanoate; 3-OH-12:0, 3-hydroxydodecanoate; 14:0, tetradecanoate; 3-OH-14:0, 3-hydroxytetradecanoate; 16:0, hexadecanoate.

The fact that several peaks are obtained for each monosaccharide makes the chromatograms of many LPS rather complex. When a capillary column is used, the peaks are normally well resolved (Fig. 1), and the specific peak pattern of a sugar is a useful aid for its identification. The ratio of the various ring-forms was found to be relatively stable under the conditions used (Table I) and in agreement with previous reports[10,12]. The quantitation of a certain sugar may thus be based on one or more selected peaks.

TABLE I

GAS CHROMATOGRAPHIC DATA OF SOME COMMONLY OCCURRING LPS MONOSACCHARIDES AS TRIFLUOROACETYLATED METHYLGLYCOSIDES*

Component	*Retention times (min)** and peak distribution (%)*				*Source*
Ethanolamine	*3.08*				Synthetic
	100				
Abequose***	*6.14*	*6.66*	*6.76*		
	45	23	32		LPS of *S. abortus equi*
Tyvelose***	*5.43*	*6.39*	*6.52*		
	70	15	15		LPS of *S. typhi*
Rhamnose	*6.40*	*7.22*			
	88	12			LPS of *S. abortus equi*
	86	14			LPS of *N. elongata*
Fucose	*5.00*	*6.67*	*6.92*	*7.51*	
	6	8	55	30	LPS of *A. actinomycetemcomitans*
	10	18	51	22	*Klebsiella* type 1 polysaccharide
Ribose	*6.89*	*7.15*	*7.24*	*8.31*	
	8	13	16	63	LPS of *S. minnesota* Ra[§]
	12	6	16	66	*E. coli* K-13 polysaccharide
Glucose	*8.87*	*9.47*	*9.62*		
	1	72	27		LPS of *S. abortus equi*
	7	66	27		LPS of *S. typhi*
	3	69	28		Lactose
	–	73	27		Cellobiose
	–	75	25		Cerebroside
Galactose	*8.74*	*9.14*	*9.67*		
	13	60	27		LPS of *S. abortus equi*
	13	62	25		LPS of *S. typhi*
	9	64	28		Lactose
Mannose	*9.35*	*9.98*			
	91	9			LPS of *S. abortus equi*
	95	5			Mannan
L-Glycero-D-mannoheptose		*11.36*			
		100			LPS of *S. abortus equi*
		100			LPS of *N. elongata*
D-Glycero-D-mannoheptose		*10.89*			
		> 80			LPS of *A. actinomycetemcomitans*
		> 80			LPS of *Y. enterocolitica*
Glucosamine		*12.22*			
		100			LPS of *S. minnesota* Ra and Re
		100			N-Acetylglucosamine[§§]
		100			N-(3-OH-14:0)-glucosamine

(Continued on p. 410)

TABLE I *(continued)*

Component	*Retention times (min)** and peak distribution (%)*			*Source*
Galactosamine		*11.72*	*12.36*	
		28	72	N-Acetylgalactosamine §§
2-Keto-3-	*12.26*	*13.38*	*13.71*	
deoxyoctanoate	17	32	39	LPS of *S. abortus equi*
(KDO) §§§	17	30	41	LPS of *S. typhi*
	18	37	29	LPS of *S. minnesota* Ra
	16	28	39	LPS of *S. minnesota* Re
	14	39	34	LPS of *N. elongata*
Neuraminic acid		*15.53*		
		100		N-Acetylneuraminic acid

* Conditions of methanolysis were 2 *M* HCl in methanol, 85°C, 18 h. Trifluoroacetylation was performed by heating to boiling with 50% trifluoroacetic anhydride in acetonitrile. Column: fused silica (25 m × 0.2 mm I.D.) coated with SE-30. Carrier gas (He) flow-rate 1.5 ml min^{-1}. Temperature program: 90°C for 4 min, 8° min^{-1} to 250°C.

** Retention times (italicized) given are normalized relative to a $C_{12:0}$ retention time of 13.10 min.

*** Abequose and tyvelose are partially degraded during methanolysis at standard conditions. Recorded figures for distribution are as obtained by mild methanolysis (2 *M* HCl, 37°C, 90 min).

§ "Leaky" Ra mutant (O. Lüderitz, private communication) originating from a ribose-containing T1 strain[19].

§§ Free-N-unacylated hexosamines give a distinctly different pattern.

§§§ Peak distribution of KDO varies with conditions of methanolysis, and two minor additional peaks are commonly found.

Derivatization

Quantitation of monosaccharides by GC is often performed as alditol acetates[13,14] or as silylated methylglycosides[15,16]. Zanetta *et al.*[10], and others[12,17], have amply demonstrated the applicability of trifluoroacetylated (TFA-derivatized) methylglycosides for GC of sugars from complex glyco-conjugates. In spite of these reports such derivatives are apparently still considered to be unstable and unsuitable in practical use[18].

As illustrated in Fig. 1, the trifluoroacetate derivatives of the monosaccharides are very volatile, and all except the peaks of KDO elute before dodecanoate ($C_{12:0}$). Accordingly, when the retention times (Tables I and II) and the individual response factors (Table III) are known, a considerable number of LPS constituents liberated by methanolysis can be determined simultaneously in a rapid and simple manner. Some loss of the more volatile fatty acid methyl esters (*e.g.* $C_{12:0}$) may occur during the evaporation step for removal of the methanolic HCl. Quantitative yields of these esters therefore require separate analysis including extraction into hexane[5].

Molar response

Molar response factors have been determined relative to 3-hydroxymyristic acid (TFA-derivatized), a widely occurring LPS constituent (Table III). A direct measure of the response ratio between fatty acids and monosaccharides was obtained by methanolysis and GC of synthetic N-(3-hydroxy-myristyl)-glucosamine, which shows structural similarity to lipid A. The relative response of the individual fatty acids was determined from standards. Similarly the molar response values of the

TABLE II

RETENTION TIMES OF COMMON LPS FATTY ACIDS AS (O-TRIFLUOROACETYLATED) METHYL ESTERS

Conditions as in Table I.

Chain length	*Retention time (min)*		
	Unhydroxylated	*2-Hydroxy*	*3-Hydroxy*
C_{10}	9.53	–	11.34
C_{12}	13.10	14.17	14.46
C_{13}	–	–	15.91
C_{14}	16.20	16.98	17.25
C_{15}	17.62	–	18.55
C_{16}	18.98	19.55	19.74
C_{18}	21.45	21.91	22.05

TABLE III

COMPOSITIONAL ANALYSIS OF THREE LIPOPOLYSACCHARIDES

See Table I, Fig. 1 and text for experimental details.

Component	*Molar response**	*Molar ratio*			
		S. abortus equi		*N. elongata*	*A. actino-mycetemcomitans*
		GC value	*Calc. from ref. 21*		
Abequose	0.1**	10	11.9	–	–
Rhamnose	0.38	12.0	12.0	4.2	5.1
Fucose	0.38	–	–	–	6.9
Mannose	0.42	12.2	12.0	–	–
Galactose	0.40	13.1	12.6	1.7	3.7
Glucose	0.44	4.1	2.8	4.4	7.8
Glucosamine	0.44	1.5	3.0	5.2	1.1
Galactosamine	0.44	–	–	–	4.3
LD-Glyceromannoheptose	0.55	1.1	3.0	4.8	5.0
DD-Glyceromannoheptose	0.55	–	–	–	3.1
KDO	0.46	2.7	3.6	4.7	–
$C_{12:0}$	0.87	0.7		–	–
$C_{14:0}$	1.00	0.5		–	2.0
$C_{16:0}$	1.12	0.4		0.2	–
3-OH-$C_{12:0}$	0.88	–		2.0	–
2-OH-$C_{14:0}$	1.00	0.4		–	–
3-OH-$C_{14:0}$	1.00	3.8		2.1	6.0

* Relative to 3-OH-$C_{14:0}$ (TFA-derivatized).

** Response value inaccurate owing to degradation, see text.

monosaccharides were calculated on the basis of methanolysis and GC of various disaccharides, LPS preparations, polysaccharides and cerebroside (Table I). Molar response factors of heptose and KDO were determined by analysis of a series of nineteen preparations of S and R forms of *Salmonella* LPS of known composition. All these LPS preparations were found to exhibit KDO and 3-hydroxymyristic acid in a constant ratio, corresponding to approximately 3 moles per polysaccharide chain.

The relative amount of heptose showed dissimilarities among the nineteen *Salmonella* LPS preparations examined, presumably owing to variation in the degree of phosphorylation. However, in all cases the number of heptose moieties recorded was in accordance with the expected number of unphosphorylated heptose moieties in LPS[20].

Applications

The chromatograms of three LPS preparations are shown in Fig. 1. Generally, all peaks are well resolved and simultaneous determinations could be performed of individual hexoses, deoxy- and dideoxy-hexoses, heptoses, hexosamines and KDO, as well as of fatty acids. With a few exceptions, the quantitative data obtained (Table III) show good agreement with values expected for *S. abortus equi*[20,21]. A low yield of glucosamine was obtained (1.5 mole compared with 2 moles expected). *Salmonella* LPS has one glucosamine in the polysaccharide chain, and two phosphorylated moieties in the lipid A part, one of which is phosphorylated in the 1-position, and hence acid-labile. The reason for the low yield of glucosamine is not known, but it was observed also in S form LPS of *S. typhi*, in contrast to all R form LPS tested.

Previous analysis of *A. actinomycetemcomitans* LPS[22] detected two unidentified sugars. One of these, as indicated in Fig. 1, had chromatographic properties identical with D-glycero-D-mannoheptose, occurring also in LPS of *Yersinia enterocolitica*[23]. The ratio between the two heptoses in *A. actinomycetemcomitans* is 3 to 2, in accordance with the data of Kiley and Holt[22]. No KDO could be observed by GC of this LPS, which may indicate that all KDO moieties, if present, are phosphorylated. For LPS of *N. elongata*, the third example in Fig. 1, no analytical data have been published.

In addition to its usefulness in structural elucidations, this simple GC method is valuable for monitoring during purification of LPS. It may presumably also be of advantage for detection of LPS in bacterial vaccines and other bacteriological materials.

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PRECISION AT THE LOW PICOGRAM LEVEL IN THE ANALYSIS OF DERIVATIZED IODOTHYRONINE STANDARDS BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

JEFFREY A. CORKILL, MARKUS JOPPICH, ALBERT NAZARETH and ROGER W. GIESE*
Department of Medical Chemistry in the College of Pharmacy and Allied Health Professions, and Institute of Chemical Analysis, Northeastern University, Boston, MA 02115 (U.S.A.)
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SUMMARY

Previously an acceptable precision (coefficient of variation less than 10%) could be obtained only part of the time for the peak areas of derivatized iodothyronine standards, even relative to that of a structurally close internal standard, 3,5-diiodo-3′,5′-dibromothyronine, when these compounds were analyzed at the low picogram level by capillary gas chromatography with electron-capture detection. This problem has now been overcome, primarily by introducing a new design, material and conditioning of a direct vapor-injector insert. An extensive analysis under practical operating conditions of derivatized 3,5-di-, 3,5,3′-tri- and 3,5,3′,5′-tetraiodothyronine at the 5–10-pg level, involving several changes in the injection equipment and several days of injections, now gives within-day coefficients of variation ranging from 2.7 to 7.7%, 1.5 to 5.3% and 1.8 to 5.2%, respectively, for the relative peak areas of these compounds.

INTRODUCTION

In earlier work, we reported that capillary gas chromatography with fused-silica columns and electron-capture detection (GC–ECD) can be used to analyze derivatized iodothyronine standards at the pg level under both isothermal and temperature-programming conditions[1,2]. It was found that this analysis was especially facilitated by the use of short columns and high column flow-rates.

In this current paper, we present further improvements of the GC–ECD system for this analysis, mainly involving the injection step. We have shortened the length of the direct vapor injector that we used previously, changed it from glass to quartz, and modified the pre-treatment and conditioning of this injector. These and certain other changes have allowed us consistently to obtain a good precision within-day for the temperature-programmed analysis of derivatized iodothyronines at the low picogram level under practical operating conditions.

The results reported here are significant not only for the potential of capillary GC–ECD to be used for trace analysis of the thyroid hormones and their metabolites

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in biological samples, but also for the analysis of similarly difficult analytes by this approach. The derivatized iodothyronines are unusual solutes for analysis by GC–ECD due to their high molecular weights (up to 1183), polar composition and content of labile iodine atoms. Thus, the ability to establish good precision consistently at the low picogram level for the analysis of these compounds by capillary GC–ECD indicates an expanding role for this method in biological analysis.

EXPERIMENTAL

Instrumentation

The instrument was a Model 3740 gas chromatograph fitted with a quartz direct vapor-injection insert (0.9 mm capillary bore, 11 cm length), which was cleaned and silanized (as indicated below) before installation; a fused-silica capillary column (10 m × 0.25 mm I.D., DB5; J & W Scientific); a constant-current, pulse-modulated, ^{63}Ni electron-capture detector, and a pressure regulator. A Varian Model 101 data system was used. The injector and detector temperatures were 300 and 320°C, respectively. The column was initially held at 200°C for 4 min, and then its temperature was raised at a rate of 30°C min^{-1} to a final temperature of 275°C, which was held for 2 min. The carrier and make-up gas was nitrogen, with flow-rates (measured at room temperature and uncorrected) of 5.0, 5.0, and 5.0 cm^3 min^{-1} in the column, at the detector insert base, and at the detector base, respectively. The septum-purge flow-rate was in the range of 2 cm^3 min^{-1} per 5 p.s.i. column-head pressure. Injections into the gas chromatograph were made with silanized 10-μl syringes, type 1701N (Hamilton Co.), fitted with a type 26S needle.

Treatment of inserts

The quartz inserts were cleaned, silanized and conditioned as follows: (1) soak in warm (50–60°C) nitric acid overnight, and rinse with water; (2) soak in warm, 6 *N* hydrochloric acid overnight, sonicate in this same solution for 10 min, and pull through 200 ml each of boiling water and methanol; (3) dry at 240°C overnight under high vacuum; (4) place on a hot-plate and solution-silanize with a warm solution of 5% each of hexamethyldisilazane (HMDS) and trimethylchlorosilane in toluene (freshly prepared), involving three changes of this solution in the interior volume of the insert over a 0.5-h period; (5) wash with warm toluene, methanol, and toluene, and dry at 240°C overnight under high vacuum; (6) vapor silanize as described previously[3]; (7) either store sealed in a desiccator until use, or install into the gas chromatograph; (8) obtain stable base frequencies in the gas chromatograph in 50°C increments up to a column temperature of 300°C, then return the column temperature to 200°C; (9) inject a mixture of the derivatized iodothyronines, approximately 250–400 pg of each, in 1 μl of toluene under the above temperature-programming conditions; (10) reduce the pressure to 5 p.s.i., and the oven temperature to 150°C, and slowly inject (over 5 sec) 2 μl of 5% HMDS solution in toluene with the needle not fully inserted (by 2 cm) into the insert, followed by temperature programming at 20°C min^{-1} up to 300°C with a 12-min hold; and (11) combine equal volumes of the solutions used in steps (9) and (10), and inject 2 μl of this mixed solution of HMDS and derivatized iodothyronines using the conditions in step (10), and then repeat step (9). The column was kept at 170°C during overnight periods between analyses, at a

flow-rate of about 2 cm^3 min^{-1}, without any changes in the injector or detector temperatures. Each morning, the GC–ECD was reconditioned according to steps 9 to 11.

The N,O-diheptafluorobutyryl methyl ester derivatives of 3,5-diiodothyronine (T_2), 3,5,3′-triiiodothyronine (T_3), 3,5-diiodo-3′,5′-dibromothyronine (Br_2T_2), and 3,5,3′,5′-tetraiodothyronine were prepared as described elsewhere[4].

The other experimental details were as defined previously[1].

Although we observed a linear range in our previous work of 0.4 to 700 pg for the analysis of derivatized iodothyronine standards by capillary GC–ECD, along with a detection limit of 30 fg[1], several parameters in the system remained to be more fully characterized and optimized, particularly in regard to the injection step.

Syringes

Two types of syringes were used for injecting the samples into the GC–ECD in our previous work, a Hamilton type 701N (fixed needle) syringe, and a type 1701RN (removable needle) syringe; only the latter has a PTFE-tipped plunger. We now prefer to use a type 1701N syringe (fixed needle, PTFE-tipped plunger). This syringe is less prone to blockage from septum particles (for undefined reasons) than the 1701RN syringe, is less subject to contamination than this latter syringe (probably because the 1701RN syringe has a more complex internal construction), and is less subject to the leakage problems of the latter RN syringe during cleaning and injection. The problem of leakage with the type 1701RN syringe increased with continued use, and involved the hub of the syringe.

Pneumatics

We have employed both a flow regulator and pressure regulator to control the carrier gas in our GC–ECD instrument, and favor the pressure regulator for the temperature-programmed analysis of our solutes. This latter regulator, as opposed to the flow regulator, gives a much shorter delay time when its setting is changed. This faster response of the pressure regulator may explain the more constant retention times that it provided for our solutes. Variation in retention times related to the pneumatics could arise from leaks at the septum during the injection step, leaks at the graphite–metal–glass connections in the system caused by repeated temperature programming, and flow-rate changes in the column accompanying this temperature programming. The pressure regulator, because of its faster response, may have given more constant retention times by responding more rapidly or consistently to these carrier-gas variations.

Injection conditions and technique

The performance of the injection step in capillary GC can be affected by several subtle parameters, such as smooth *vs.* jagged column edge[5], injection speed[6], hot *vs.* cold needle injection[7], and needle-residence time[8]. We examined all of these factors in our temperature-programmed analysis of derivatized iodothyronines, and did not observe any major changes in the resultant chromatograms. This included the solute-peak shapes, chromatographic response and short-term (five injections) precision in these chromatograms, which never changed by more than 25% (although the solvent peak is considerably broader when a slow injection is carried out). When lindane and

aldrin were injected under isothermal conditions, hot-needle injections gave peak heights as much as 1.7 times higher than those obtained by injection with the cold-needle technique.

The use of a longer needle (3 in. as opposed to the customary 2 in.) was also examined, potentially to minimize any back-flashing, and reduce the exposure of the sample vapor to the walls of the injector insert. However, the result was a reduced response (about 2-fold) for our solutes, with no improvement in short-term precision, perhaps due to increased exposure of the sample components to the outer syringe-needle surface during the injection step.

Injection inserts

Our standard practice previously was to replace the glass insert in our injector whenever a change in peak shape was observed for our solutes that could not be traced to other causes. The average lifetime of the inserts was 2 to 4 weeks when derivatized iodothyronines were analyzed above the 100-pg level. However, when we began repeated injections of these solutes at the 5–10-pg level, we observed inconsistent performance from one insert to another, even when apparently equivalent inserts were cleaned and silanized together before installation. Some inserts performed equivalently (in terms of chromatographic response, peak shapes, and insert lifetime) whether 5–10-pg, or 100-pg or larger amounts of the derivatized iodothyronines were injected repeatedly. Other inserts gave a good performance only for injections of these solutes in amounts above 100 pg. For these latter inserts, significantly decreased responses, or even distorted chromatograms, were observed for the injection of low picogram amounts of these solutes. In all instances, satisfactory peak shapes and responses were observed for the injection of low picogram amounts of lindane and aldrin. These latter substances are relatively inert as solutes when analyzed by GC–ECD.

In some of the distorted chromatograms, we observed a mixture of sharp and broad solute peaks, suggesting that the cold trapping of the solutes during the injection step might be occurring on two types of adsorption sites. Since the injector in our instrument is partly exposed to the column oven, it was likely that cold trapping of the solutes was occurring in both the lower part of the injector insert, and at the top of the column, during the injection step. Glass-lined metal inserts are available from the manufacturer for the injector on this instrument, and these types of inserts were tried in an effort to maintain the overall injection insert at a high temperature, including the part that is exposed to the lower temperature of the column oven during the injection step. Although the initial performance of these inserts was satisfactory, the structure and presence of the metal sleeve limited the strength of the nitric acid cleaning conditions that could be employed. The final result was that we were unable to re-use these inserts once they had been contaminated with 3 to 4 weeks of injection residues in the instrument.

Based on this experience, we shortened the injector insert, changed it from glass to quartz, and subjected it to more extensive silanizing and conditioning steps, as described under Experimental. We then proceeded to analyze the derivatized iodothyronines repeatedly with temperature programming by GC–ECD. Overall, we made 217 analytical injections of these solutes at the 5–10-pg level, and 10 such injections at the 0.5–1.0-pg level, averaging 15 injections per day, and comprising 4

successive inserts and 15 days of injections. The resultant data were calculated as follows for the 5–10-pg level. First, we determined the ratios of the peak areas and heights for derivatized T_2, T_3 and T_4 relative to the corresponding areas and heights for the internal standard, derivatized Br_2T_2. Then we averaged these values on each day. The resultant within-day means and coefficients of variation (C.V.) were grouped in terms of the 4 inserts employed, and the lowest and highest mean and C.V. values in each group were selected for presentation here, as seen in Table I.

TABLE I

PRECISION OF PEAK RATIOS FOR ANALYSIS OF DERIVATIZED IODOTHYRONINES AT THE 5–10-pg LEVEL

Insert/days in use/total No. of injections per insert	*Within-day mean values and C.V. values for peak ratios**					
	Range of area values (and C.V., %) for each insert			*Range of height values (and C.V., %) for each insert*		
	T_2/Br_2T_2	T_3/Br_2T_2	T_4/Br_2T_2	T_2/Br_2T_2	T_3/Br_2T_2	T_4/Br_2T_2
A/2/38	0.80–0.81 (3.8 –7.7)	0.72–0.73 (4.0 –5.3)	1.24** (2.2)	0.83–0.84 (7.7 –9.3)	0.86–0.87 (6.6 –8.8)	(0.80)** (3.9)
B/6/98	0.88–0.98 (2.6 –6.9)	0.73–0.83 (1.9 –4.8)	1.11–1.22 (1.8 –5.2)	0.86–1.04 (4.7 –7.6)	0.84–0.95 (3.6 –7.0)	0.70–0.81 (4.2 –6.4)
C/5/72	0.84–0.86 (2.7 –6.3)	0.79–0.81 (1.5 –2.8)	1.20–1.25 (2.1 –3.7)	0.75–0.83 (3.4 –6.6)	0.88–0.91 (2.9 –5.8)	0.72–0.91 (2.5 –4.5)
D/2/9	0.73 (4.3)	0.70 (3.7)	1.21 (2.0)	0.69 (5.3)	0.75 (7.1)	0.80 (4.0)

* On each day, an aliquot of the same stock solution (kept at 4°C) of derivatized iodothyronine standards in toluene (228, 204, 253 and 437 ng/ml of derivatized T_2, T_3, Br_2T_2 and T_4, respectively), was diluted with toluene to the 5 to 10 pg/μl level. This working solution was kept at room temperature and injected throughout the day.

** Outlying mean peak-area and peak-height values of 1.60 and 1.05 were observed for T_4 on the first day of the experiment, apparently due to adventitious contamination of this solution with derivatized T_4, since this ratio persisted for this working solution on the second day, whereas a fresh working solution gave an acceptable ratio for T_4/Br_2T_2.

Precision at the 5–10-pg level

The main point to be drawn from the data in Table I is that an acceptable level of precision (C.V. values ranging from 1.5 to 7.7% for peak-area ratios, and from 2.5 to 9.3% for peak-height ratios) is established for this analysis on any given day, irrespective of the degree of variation that develops in the mean peak ratios when an insert is changed, or between days for a given insert. Changes in the mean values for the peak areas and heights arising between analyses conducted with different inserts, and determined for each derivatized iodothyronine, range from 13 to 34%, and from 27 to 51%, respectively. (For example, 0.98 − 0.73/0.73 = 34% as the change in the mean peak-area ratios for derivatized T_2). Between-day variations for a given insert were smaller, ranging from 9 to 14% for the mean peak-area ratios, and 13 to 26%

for the mean peak-height ratios. Closer analysis of the original data shows random variation to be present as opposed to any drift in the values on a given day. Assuming that these variations arise from active sites in the GC–ECD system, we see progressive differences in these sites when the results are compared day-to-day, and then insert-to-insert.

The precision for the relative peak areas is better than the precision for the relative peak heights by an absolute difference of about 1–2% overall. Considering that the C.V. values for the peak heights occasionally are actually lower than the corresponding C.V. values for the areas, the main conclusion on this aspect is that only slightly better precision is obtained by measuring peak areas (with the data system employed) as opposed to peak heights (manually) in this analysis.

Performance of the internal standard

It is next interesting to compare the relative monitoring of the three solutes, T_2, T_3 and T_4, by Br_2T_2, the internal standard (all derivatized). From a structural standpoint, Br_2T_2 would seem to be most similar to T_4, but the retention of Br_2T_2 is closest to that of T_3. Consistent with this, the overall variations in peak ratios and in C.V. values, either for areas or heights, are comparable for T_3 and T_4, but somewhat higher for T_2. Thus, a more appropriate internal standard for T_2 may allow closer monitoring of this solute.

Precision at the sub-pg level

For the ten analyses conducted at the 0.5–1.0-pg level, which involved five injections each on separate days (one day with insert C, and one with D), the C.V. values for the mean peak-area ratios ranged from 8.0 to 9.6% with insert C, and from 5.1 to 5.5% with insert D (data not shown in Table I). Thus, an acceptable level of precision is obtained, at least initially, at this lower solute level as well. A representative chromatogram from one of these analyses is shown in Fig. 1.

The corresponding values for the mean peak-area ratios of these solutes at the 0.5–1.0-pg level were 0.78, 0.88 and 1.13 for T_2, T_3 and T_4, respectively, with insert C, and, correspondingly, 0.62, 0.62 and 1.30 with insert D. These mean values differ from the mean values for the peak-area ratios established on the same day at the 5–10-pg level for these compounds by -7, $+11$ and -6% for T_2, T_3 and T_4, respectively, with insert C, and by -15, -10 and $+8$% in a corresponding manner with insert D on the other day involved. This is consistent with our prior conclusion that the linear range for the derivatized iodothyronines with the current equipment and techniques is 0.4 to 700 pg, with some indications of reproducible non-linearity (agreement among triplicate injections) near the level of 0.4 pg (see ref. 1). It further now appears that this non-linearity, although consistent within-day, tends to vary, at least for analyses conducted with different inserts prepared according to the current method.

Detector make-up flow-rates

The manufacturer recommends a total flow-rate of 30 $cm^3\ min^{-1}$ in the GC–ECD that we use. Consistent with this recommendation, we observed the lowest baseline noise level under this condition, as obtained for example, by column, column make-up, and detector make-up flow-rates of 5.0, 20.0,, and 5.0 $cm^3\ min^{-1}$, respec-

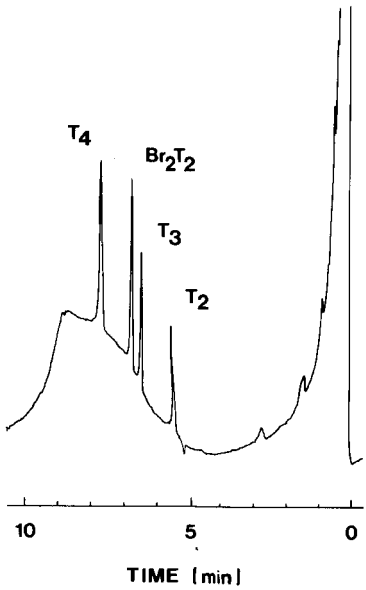

Fig. 1. Analysis of derivatized T_2, T_3, Br_2T_2 and T_4 at the 0.5 to 1.0-pg level by capillary GC–ECD with temperature programming; attenuation 80.

tively. However, similar settings of 5.0, 10.0, and 5.0 $cm^3\ min^{-1}$ gave a 2-fold higher response with only a 25 % increase in noise, and settings of 5.0, 5.0 and 5.0 $cm^3\ min^{-1}$ provided an additional 20 % higher response with only a 30 % further increase in noise (measured at attenuation 2). The latter as opposed to the initial conditions were selected for the experimental work reported in this paper, since baseline noise was not evident at the attenuation value of 128 generally employed here, while a 50 % reduction in carrier-gas consumption was realized.

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DETECTION AND IDENTIFICATION OF VOLATILE ORGANIC COMPOUNDS IN BLOOD BY HEADSPACE GAS CHROMATOGRAPHY AS AN AID TO THE DIAGNOSIS OF SOLVENT ABUSE

J. D. RAMSEY*
Toxicology Unit, Department of Chemical Pathology, St. George's Hospital, Blackshaw Road, London SW17 0QT (Great Britain)
and
R. J. FLANAGAN
Poisons Unit, Guy's Hospital, St. Thomas' Street, London SE1 9RT (Great Britain)
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SUMMARY

A gas chromatographic method has been developed for the detection and identification of some volatile organic compounds in whole blood, plasma or serum. After incubation of the sample (200 μl) together with the internal standard solution in a sealed vial, a portion of the headspace is analysed using a 2-m glass column packed with 0.3% (w/w) Carbowax 20M on Carbopack C, 80–100 mesh. The column oven, after a 2-min isothermal period, is programmed from 35 to 175°C at 5°/min and held for 8 min. The effluent is monitored by both flame-ionisation and electron-capture detection, and peak assignment is by means of retention time and relative detector response.

The method has proved applicable to the detection of bromochlorodifluoromethane, *n*-butane, carbon tetrachloride, chlorobutanol, cryofluorane (Halon 114), dichlorodifluoromethane (Halon 12), ethyl acetate, halothane, isobutane, isopropanol, isopropyl nitrate, methyl ethyl ketone, propane, tetrachloroethylene, toluene, 1,1,1-trichloroethane, 2,2,2-trichloroethanol, trichloroethylene and trichlorofluoromethane (Halon 11) in blood specimens obtained from patients suspected of abusing these agents.

INTRODUCTION

The diagnosis of poisoning by volatile organic compounds such as solvents, aerosol propellants and anaesthetics is often aided by either the past medical history or circumstantial evidence such as the discovery of empty containers, the presence of traces of glue on the patient's clothing or the odour of chemicals on the breath. However, in the absence of such evidence, serious diagnostic problems may occur since the clinical features of acute poisoning with this type of compound are by no means definitive. These features range from ataxia and drowsiness to coma and res-

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piratory depression with in some instances direct cardio- or hepatotoxicity[1].

The introduction of gas chromatographic techniques for the detection, identification and measurement of ethanol[2,3] prompted the application of similar methods to compounds such as toluene, benzene, acetone, isopropanol and methyl ethyl ketone in blood specimens from poisoned patients[4,5]. More recently, some workers have used headspace analysis with packed columns operated isothermally and flame-ionisation detection (FID)[6–8] for the analysis of a variety of compounds, although others have used capillary columns[9], headspace mass spectrometry[10] or gas chromatography–mass spectrometry[11].

The system described in the present work was developed to facilitate the rapid detection and identification of a wide range of volatile substances in blood specimens at the concentrations attained in patients acutely poisoned with these agents. The method employs headspace sample preparation together with a programmed analysis using a column packed with 0.3% (w/w) Carbowax 20M on Carbopack C and split FID–electron-capture detection (ECD).

EXPERIMENTAL

Internal standard solution

The internal standards, 1,1,2-trichloroethane and ethylbenzene, were both obtained from BDH, Poole, Great Britain. Each batch of material was tested for the presence of interferences (notably 1,1,1-trichloroethane and toluene, respectively) by gas chromatography before use. Approximately 50 mg of each standard were weighed into 50-ml glass volumetric flasks containing outdated blood-bank whole blood. After thorough mixing, 1.0 ml of the stock 1,1,2-trichloroethane solution and 2.5 ml of the ethylbenzene solution were diluted to 100 ml with glass-distilled water containing sodium azide (approximately 100 mg) to give the working internal standard solution. This solution, if stored in 1-ml portions at −20°C, was found to be stable for at least 6 months.

Gas chromatography

The instrument used was a Perkin-Elmer F17 gas chromatograph fitted with a liquid carbon dioxide subambient accessory and dual-pen recorder. The injection port was fitted with a "septum swinger" (Perkin Elmer 045-0496), and the injection/detector block was maintained at 275°C. The column oven, after a 2-min isothermal period, was programmed at 5°/min from 35 to 175°C and held for 8 min. A 2 m × 2 mm I.D. glass column was packed with 0.3% (w/w) Carbowax 20M on Carbopack C, 80–100 mesh (Supelco, obtained from Atlas-Bioscan, Canvey Island, Great Britain), and was conditioned according to the manufacturer's recommendations. The carrier-gas (nitrogen) flow-rate was approximately 30 ml/min, and the column effluent was monitored using both FID and ECD with a stainless-steel splitter system giving a split ratio of 9:1. The hydrogen and oxygen (FID) inlet pressures were 10 and 14 p.s.i., respectively, and the ECD purge (nitrogen) flow-rate was 30 ml/min. The ECD was used at a pulse setting of 6 at range 1, attenuation 64, and the FID at range 1, attenuation 8.

Instrument calibration

A qualitative standard mixture (Table I) was prepared daily by adding the appropriate volume of the gaseous components to an evacuated 125-ml gas-sampling bulb (Supelco 2-2146). Air was admitted to atmospheric pressure and a portion (25 μl) of the mixture of the liquid and solid components (Table II) was added. The liquid–solid components mixture was stored in a glass-stoppered vessel at −20°C and was stable for at least 3 months.

The qualitative standard mixture was chromatographed daily prior to sample analyses. From 0.01 μl ("Nanojector", Precision Sampling, obtained from Atlas Bio-scan) to 1.0 μl (SGE 1.0-μl glass syringe) were injected onto the gas chromatographic

TABLE I

QUALITATIVE STANDARD MIXTURE

For details of preparation, see text.

	Volume added to gas sampling bulb (μl)
Bromochlorodifluoromethane	20*
Dichlorodifluoromethane	750*
Trichlorofluoromethane	20*
Liquid–solid stock mixture (see Table II)	25

* Volume of gas phase at atmospheric pressure.

TABLE II

LIQUID AND SOLID COMPONENTS STOCK MIXTURE

Compound	*Amount*
Acetone	20 ml
Methyl ethyl ketone	20 ml
Carbon tetrachloride	200 μl
Chlorobutanol	10 mg
Chloroform	1.5 ml
Ethanol	20 ml
Ethyl acetate	20 ml
Ethylbenzene	10 ml
n-Hexane	40 ml
Isopropyl nitrate	1 ml
Methanol	15 ml
Methyl isobutyl ketone	5 ml
Isopropanol	20 ml
Tetrachloroethylene	100 μl
Toluene	10 ml
1,1,1-Trichloroethane	1 ml
1,1,2-Trichloroethane	2 ml
2,2,2-Trichloroethanol	50 μl
Trichloroethylene	1 ml

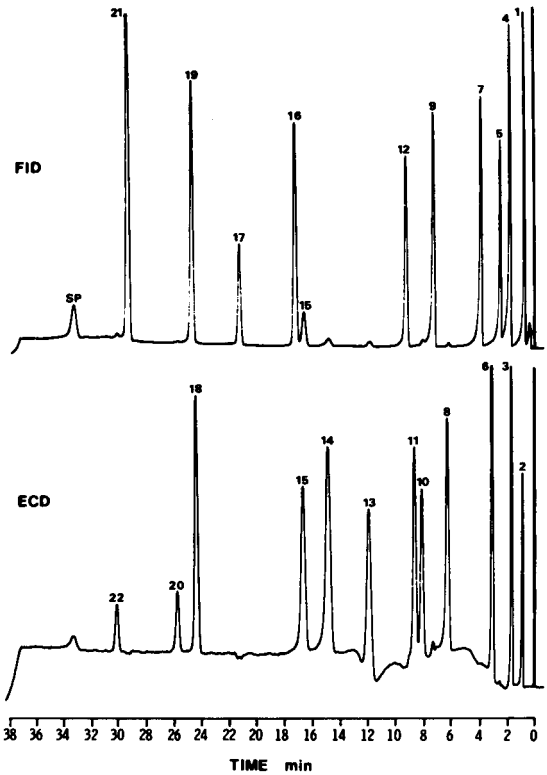

Fig. 1. Chromatogram obtained on analysis of the qualitative standard mixture; 0.75-μl injection. GC conditions: column 2 m × 2 mm I.D. glass packed with 0.3% (w/w) Carbowax 20M on Carbopack C (80–100 mesh); carrier gas flow 37 ml/min; temperature program: 2 min at 35°C, 5°/min to 175°C and held for 8 min. Peaks: 1 = methanol, 2 = dichlorodifluoromethane, 3 = bromochlorodifluoromethane, 4 = ethanol, 5 = acetone, 6 = trichlorofluoromethane, 7 = isopropanol, 8 = chloroform, 9 = methyl ethyl ketone, 10 = 1,1,1-trichloroethane, 11 = carbon tetrachloride, 12 = ethyl acetate, 13 = isopropyl nitrate, 14 = trichloroethylene, 15 = 1,1,2-trichloroethane, 16 = *n*-hexane, 17 = methyl isobutyl ketone, 18 = tetrachloroethylene, 19 = toluene, 20 = 2,2,2-trichloroethanol, 21 = ethylbenzene, 22 = chlorobutanol, SP = "Septum peak" (see text).

column, and the retention times of the components were measured manually from the injection point. The analysis of this mixture is illustrated in Fig. 1, and the retention times of these and some other compounds of interest are given in Table III.

Sample preparation

Internal standard solution (100 μl) was added using a disposable 1.0-ml plastic syringe to a 7-ml nitrogen-filled vial (Schubert, Portsmouth, Great Britain) sealed with a crimped-on teflon-silicone disc (Phase Separations, Queensferry, Great Britain). The vial was maintained at 65°C in a heating block. After 15 min, a portion (400 μl) of the headspace was taken using a warmed (approximately 40°C) gas-tight glass syringe (SGE) and injected onto the gas chromatographic column.

Subsequently, the sample (whole blood, plasma or serum) (200 μl) was added to the vial using a disposable syringe and, after 15 min, a further portion (400 μl) of

the headspace was taken for analysis. After sample injection, the gas-tight syringe was purged by removing the plunger and sucking ambient air through the barrel and needle of the syringe using a vacuum pump.

RESULTS AND DISCUSSION

The detection and identification of volatile organic compounds in specimens of body fluids poses unusual analytical problems in clinical and forensic toxicology. A wide variety of compounds may be encountered in such analyses ranging from, for example, the polyhalogenated hydrocarbon dichlorodifluoromethane (Halon 12, b.p. $-30°C$) to the aromatic hydrocarbon xylene (b.p. approximately 140°C), and simultaneous exposure to or abuse of more than one solvent is not infrequent. In addition, many compounds undergo transformation to metabolites which are usually more polar than the parent compound. The widespread occurrence of organic solvents in the environment (not least in that of analytical laboratories) must also be considered.

The aim of this study was thus to develop a practical method to assist in the diagnosis of solvent or aerosol abuse using readily available clinical specimens. It was clear that such a method should be capable of detecting the presence of a wide range of compounds and possess adequate selectivity and sensitivity. Such a technique should be simple, reliable and use easily available apparatus wherever possible, and should also permit the subsequent quantitative analysis of the compounds of interest.

Choice of gas chromatographic column and conditions

Initially, it was thought that a capillary system would prove ideal, but several disadvantages to the use of such systems became apparent. An inlet splitter system was needed in order to obtain reproducible retention times, and this had the effect of reducing the overall sensitivity of the method as compared to the system finally chosen even taking into account the enhanced peak height/peak area ratio obtainable with capillary columns. An additional problem lay in obtaining adequate retention of some of the more volatile compounds studied, for example the low molecular weight hydrocarbons and the halons. Some of these difficulties could probably be overcome by "trapping" the headspace components of interest using a solid adsorbent followed by desorption onto the chromatographic system, but only at additional cost and complexity. Another practical consideration was the much greater expected life of packed columns, especially when the repeated injection of 400-μl volumes of wet oxygen-containing headspace was contemplated.

Other packed columns considered included a range of Carbopack materials modified with different stationary phases and more conventional materials such as Tenax and Porasil. However, none of these materials gave such good resolution of the compounds of interest as the system chosen. An additional factor was that relatively polar materials such as ethanol and acetone gave rise to almost symmetrical peaks on the modified Carbopacks (*cf.* Fig. 1) in contrast to the results obtained on the other materials. However, where additional information or confirmation of compound identity was required, retention indices obtained on columns prepared using either OV-1 or Carbowax 20M were available from a variety of sources, and these data are also given in Table III.

TABLE III

RETENTION AND OTHER DATA OF THE COMPOUNDS STUDIED

Abbreviations: DNA = datum not available. NIM = not in Merck Index.

*Compound**	*Retention time (mm)***	*Electron capture response****	*Kovats retention index* §		*Boiling point (°C)* §§	*Molecular weight*	*Merck index No.**
			OV-1	*Carbowax 20M*			
Methane	1.5	0	100	100	−161	16.0	5809
Ethane	1.5	0	200	200	−88	30.1	3649
Ethylene	1.5	0	DNA	DNA	−104	28.1	3728
Nitrous oxide	1.5	1	DNA	DNA	−88	44.0	6473
Cyclopropane	3.0	0	DNA	DNA	−33	42.1	2753
Propane	3.0	0	300	300	−42	44.1	7591
Propylene	3.0	0	310	DNA	−48	42.1	7638
Acetaldehyde	3.5	0	372	695	21	44.1	27
Ethylene oxide	3.5	0	400	680	11	44.1	3738
Methanol	3.5	0	491	883	65	32.0	5814
Vinyl chloride	4.0	2	440	505	−14	62.5	9645
Dichlorodifluoromethane (Halon 12)	4.5	2	305	DNA	−30	120.9	3038
Methyl formate	4.5	0	499	761	32	60.1	5942
Ethyl chloride	5.5	2	447	668	12	64.5	3713
Acetonitrile	7.0	0	455	1010	82	41.1	56
1-Chloro-2,2,2-trifluoroethane	7.5	1	375	665	7	118.5	NIM
Bromochlorodifluoromethane (BCF)	8.0	2	405	DNA	−4	165.4	NIM
Isobutane	8.0	0	370	DNA	−12	58.1	NIM
1-Butene	9.0	0	390	415	−6	56.1	1502
Methyl iodide	9.0	2	515	806	43	142.0	5955
Dichloromethane	9.5	2	515	917	40	84.9	5932
Ethanol	9.5	0	421	919	79	46.1	211
Cryofluorane (Halon 114)	10.0	2	361	DNA	4	170.9	2599
n-Butane	11.5	0	400	400	−1	58.1	1497
Acetone	12.5	1	469	819	57	58.1	52
Nitromethane	12.5	2	565	1154	101	61.0	6433
Carbon disulphide	13.5	1	524	745	47	76.1	1817
Trichlorofluoromethane (Halon 11)	15.0	2	484	605	24	137.4	9320

Acrylonitrile	16.0	0	590	1010	77	53.1	127
1,3-Dioxolane	17.0	0	635	935	74	74.1	NIM
Ethyl formate	18.0	0	545	920	53	74.1	3743
Methyl acetate	18.5	0	513	796	57	74.1	5884
Vinylidene chloride	18.5	2	515	715	32	97.0	9647
Methylal	19.5	0	505	740	42	76.1	5888
Propionitrile	19.5	0	580	1015	97	55.1	7617
Isopropanol	20.0	0	530	908	83	60.1	5069
Bromochloromethane	21.0	2	660	1060	68	129.4	NIM
n-Propyl chloride	21.0	1	570	740	47	78.5	NIM
1,1-Dichloroethane	22.5	2	563	881	57	99.0	3750
Tetrahydrofuran	25.0	0	638	872	66	72.1	8929
1,2-Epoxybutane	27.0	0	600	840	63	72.1	NIM
1,2-Dichloroethylene (mixed isomers)	27.5	2	556	846	55	97.0	85
n-Propanol	27.5	0	571	1033	97	60.1	7630
Ethyl iodide	29.0	2	680	880	72	156.0	3753
Diethyl ether	29.5	0	515	650	35	74.1	3742
Nitroethane	30.0	2	655	1161	115	75.1	6420
Chloroform	31.0	2	605	1024	61	119.4	2120
1,1,2-Trichlorofluoroethane	31.5	2	555	605	46	187.4	NIM
tert.-Butanol	32.0	0	512	875	82	74.1	1526
1,1,1,-Trichlorotrifluoroethane (Halon 113)	32.0	2	530	660	46	187.4	NIM
2,2,2-Trifluoroethanol	32.5	2	580	1135	77	100.0	NIM
1,2-Dichloroethane	35.0	2	631	1051	83	99.0	3733
Dibromomethane	36.5	2	765	1185	97	173.9	5930
Methyl ethyl ketone (MEK)	36.5	1	579	908	80	72.1	5941
Isopropyl formate	39.5	0	567	883	68	88.1	NIM
1,1,1-Trichloroethane	41.0	2	634	886	74	133.4	9316
Enflurane	41.5	2	462	840	57	184.5	3524
n-Pentane	41.5	0	500	500	36	72.2	6913
Epichlorohydrin	42.5	2	730	1205	118	92.5	3536
Halothane	42.5	2	533	858	50	197.4	4455
Carbon tetrachloride	43.0	2	659	886	77	153.8	1821
Cyclohexane	43.0	0	664	726	81	84.2	2728
Dioxane	45.5	0	687	1065	101	88.1	3300
sec.-Butanol	46.5	0	624	1014	100	74.1	1525
2-Chloroethanol	47.0	2	643	1358	129	80.5	3730
Ethyl acetate	47.0	0	596	870	77	88.1	3685
2-Methoxyethanol (methyl cellosolve)	47.0	0	616	1172	124	76.1	5913

(Continued on p. 430)

TABLE III *(continued)*

*Compound**	*Retention time (mm)***	*Electron-capture response****	*Kovats retention index*[§]		*Boiling point (°C)*[§§]	*Molecular weight*	*Merck index No.**
			OV-1	*Carbowax 20M*			
n-Propyl formate	47.0	0	603	916	81	88.1	7647
2-Nitropropane	48.0	2	685	1109	120	89.1	6450
Methyl propionate	48.5	0	639	910	80	88.1	5982
Bromodichloromethane	51.0	2	715	1165	90	163.8	NIM
Isobutanol	51.0	0	619	1076	108	74.1	4981
Cyclohexene	57.0	0	705	811	83	82.1	2732
n-Butyl chloride	57.5	2	642	858	79	92.6	1546
Methyl isopropyl ketone	59.5	1	650	936	93	86.0	NIM
Isopropyl nitrate	60.0	2	693	970	101	105.1	NIM
Chloral hydrate (tailing peak)	62.5	2	695	1015	98	165.4	2033
Methyl cyclopropyl ketone	65.0	1	730	1095	114	84.1	NIM
1-Nitropropane	66.0	2	725	1220	132	89.1	6449
2-Methylbutan-2-ol	67.0	0	636	966	103	88.2	6934
Epibromohydrin	68.0	2	805	1330	135	137.0	NIM
Methylcyclopentane	68.0	0	650	775	72	84.2	NIM
n-Butyl nitrite	68.5	2	608	1220	78	103.1	1570
2-Methylpentane	71.0	0	610	704	72	84.2	NIM
Benzene	74.0	0	660	948	80	78.1	1069
Bromotrichloromethane	74.0	2	810	1070	105	198.3	NIM
n-Propyl iodide	74.0	2	785	975	103	170.0	7651
Trichloroethylene	74.0	2	710	964	87	131.4	9319
Isopropyl acetate	75.0	0	648	896	89	102.1	5066
1,2-Difluorotetrachloroethane (Halon 112)	75.5	2	730	870	93	203.8	NIM
Diethyl ketone	76.0	1	683	965	102	86.1	3102
1,1-Difluorotetrachloroethane	77.0	2	785	1010	92	203.8	NIM
Methyl *n*-propyl ketone	79.0	1	680	1071	102	86.1	5984
2-Mercaptoethanol	81.5	0	795	1855	157[§§§]	78.1	5699
1,1,2-Trichloroethane	82.0	2	748	1240	113	133.4	9317
Diisopropyl ether	83.0	0	594	592	68	102.2	5073
2-Ethoxyethanol	84.0	0	701	1218	135	90.1	3678
Pyrrole	84.0	0	755	1472	130	67.1	7801
Methylcyclohexane	84.5	0	750	781	101	98.2	NIM
Ethylene glycol (tailing peak)	85.0	0	798	DNA	198	62.1	3735

n-Hexane	87.0	0	600	600	69	86.2	4563
n-Butanol	88.0	0	651	1128	117	74.1	1524
Methoxyflurane	88.0	2	701	1124	105	165.0	5866
N-Methylpyrrole	88.0	0	715	1139	115	81.1	NIM
2,3-Pentanedione	88.0	1	681	1044	108	100.1	NIM
Ethyl propionate	90.0	0	679	948	99	102.1	3790
1,2-Dibromoethane	90.5	2	830	1265	131	187.9	3732
n-Propyl acetate	91.5	0	696	950	102	102.1	7629
Methyl *n*-butyrate	93.0	0	723	980	102	102.1	5909
n-Butyl formate (multiple peaks)	97.5	0	701	1020	107	102.1	NIM
Methyl methacrylate	100.0	0	699	1008	100	86.1	5796
Methylpentynol	100.5	0	715	1275	121	98.1	5671
Isoamyl nitrite (amyl nitrite B.P.)	101.5	2	680	1185	98	117.2	5056
3-Methylbutan-1-ol	103.0	0	719	1159	132	88.2	5055
Cyclohexanone	105.0	1	855	1275	156	98.1	2731
Methyl isobutyl ketone	105.5	1	724	1010	117	100.2	5068
1,1,1,2-Tetrachloroethane	106.5	2	870	1255	131	167.9	NIM
Bromoform	107.0	2	469	926	149	252.8	1418
Pyridine (tailing peak)	107.0	0	695	1181	115	79.1	7752
Pentan-1-ol	109.5	0	766	1153	138	88.2	6916
2-Methylpentan-2-ol	111.0	0	725	1160	121	102.2	NIM
2-Methylhex-1-ene	112.0	0	725	740	92	98.0	NIM
Isopropyl propionate	112.5	0	745	948	110	116.2	NIM
Ethyl *n*-propyl ketone	115.0	1	781	1055	125	100.2	NIM
Cyclohexanol	116.0	0	913	1375	161	100.2	2730
Acetylacetone	117.0	1	804	1230	141	100.1	73
n-Butyl iodide	117.0	2	840	1065	130	184.0	1562
Paraldehyde	117.0	0	786	1069	124	132.2	6832
Methyl *n*-butyl ketone	118.5	1	787	1173	127	100.2	5907
Isooctane	120.0	0	725	705	99	114.2	5051
Isobutyl acetate	120.0	0	753	984	118	116.2	4983
Tetrachloroethylene	121.5	2	789	1018	121	165.9	8907
1,2,3-Trichloropropane	122.0	2	910	1455	157	147.4	NIM
n-Amyl formate	124.0	0	772	1070	132	116.2	NIM
Toluene	124.0	0	756	1035	111	92.1	9225
1,1,2,2,-Tetrachloroethane	124.5	2	910	1500	147	167.9	8906
n-Heptane	125.5	0	700	700	98	100.2	4521
n-Butyl acetate	126.0	0	797	1078	125	116.2	1519
γ-Valerolactone	126.5	1	921	1617	218	100.1	NIM

(Continued on p. 432)

TABLE III *(continued)*

*Compound**	*Retention time (mm)***	*Electron-capture response****	*Kovats retention index* §		*Boiling point (°C)* §§	*Molecular weight*	*Merck index No.**
			OV-1	*Carbowax 20M*			
2,2,2-Trichloroethanol	127.5	2	857	1691	151	149.4	9318
1,3-Dichloropropan-2-ol	129.0	2	885	1765	174	129.0	3050
Diacetone alcohol	130.0	1	811	1316	168	116.2	2921
Allyl glycidyl ether	131.0	0	880	1325	154	114.1	NIM
Hexan-2-ol	132.0	0	786	1192	138	102.2	NIM
Chlorobenzene	136.5	1	860	1200	131	112.6	2095
1,1,1-Trichloropropan-2-ol	139.5	2	920	1650	162	163.4	9325
Acetonylacetone	141.0	1	894	1500	188	114.1	57
Cycloheptanone	141.0	1	1010	1495	180	112.2	2727
Di-*n*-propyl ketone	146.0	1	857	1131	144	114.2	3364
2-Ethoxyethyl acetate	146.5	0	872	1305	156	132.2	3679
Ethylbenzene	147.5	0	849	1120	136	106.2	3695
Chlorobutanol	149.0	2	949	1638	167	177.5	2103
2-Octyne	150.0	0	870	1040	138	110.2	NIM
Pyrrolidine	151.0	0	695	1022	89	71.1	7802
Caprylene	152.0	0	779	830	121	112.2	1763
Heptan-2-one	152.0	1	880	1275	152	114.2	4525
α-Pinene (from turpentine)	154.5	0	942	1035	156	136.2	7242
Heptanal	155.0	1	883	1186	153	114.2	4519
Bromobenzene	155.5	2	945	1365	156	157.0	1405
2-Ethylhexyl acetate	156.0	0	1144	1420	199	172.3	6570
Hexachloroethane	158.5	2	1085	1525	189	236.7	4545
Isoamyl acetate	159.0	0	907	1182	142	130.2	4958
n-Octane	159.0	0	800	800	126	114.2	6561

2,5-Dimethylfuran	161.5	1	697	931	94	96.1	NIM
Cumene	162.0	0	905	1162	152	120.2	2615
m-Xylene	166.0	0	863	1147	139	106.2	9743
Styrene	167.0	0	879	1272	145	104.1	8657
Benzaldehyde	171.0	0	947	1502	179	106.1	1057
p-Xylene	171.0	0	860	1140	138	106.2	9743
o-Xylene	172.5	0	884	1191	144	106.2	9743
Ethchlorvynol	176.0	2	1030	1445	173	144.6	3656
Di-*n*-butyl ether	177.0	0	811	969	142	130.2	1557
6-Methyl-5-hepten-2-one	180.0	0	975	DNA	73†	126.0	NIM
o-Chlorotoluene	186.0	1	985	1288	159	126.6	2160
m-Chlorotoluene	187.0	1	960	1291	162	126.6	2159
Octan-4-one	187.0	1	965	1250	163	128.2	NIM
Camphor	191.0	0	1136	1518	204	152.2	1734
Octan-3-one	192.0	1	928	1190	167	128.2	NIM
p-Chlorotoluene	197.0	1	955	1288	162	126.6	2161
Octan-2-one	201.0	1	991	1375	172	128.2	4586
2-Ethylhexan-1-ol	212.0	0	1037	1465	184	130.2	3746
1,4-Dichlorobenzene	213.0	2	994	1495	174	147.0	3030
Octan-1-ol	213.0	0	1083	1513	194	130.2	6563
2,6-Dimethylheptan-4-one (multiple peak)	218.0	0	999	1207	168	142.2	NIM
o-Tolualdehyde	266.0	0	1054	1632	201	120.0	9223
Nonan-5-one	290.0	1	1074	1330	188	142.2	NIM
Nonan-2-one	320.0	1	1093	1478	195	142.2	NIM

* Names of compounds based on those used in the Merck Index (9th Edition), Merck and Co., Rahway, NJ, U.S.A., 1976.

** Retention time (5 mm = 1 min) measured to the nearest 0.5 mm from the injection point on the Carbopack C/Carbowax 20M column system. See text for chromatographic conditions (retention times >180 mm obtained by increasing the final isothermal period).

*** Rated: 0 = nil, 1 = weak, 2 = strong.

§ Compiled from various sources.

§§ At 760 mmHg pressure, except:

§§§ 742 mm.

† 18 mm.

The temperature program used was influenced by the need to retain and resolve relatively volatile compounds such as butane, isobutane, BCF and the halons while completing the analysis of the remaining compounds in a reasonable time and maintaining adequate selectivity. A further consideration was the presence of a compound derived presumably from the septa used in the gas chromatograph which had a retention time of 33.2 min (166 mm) on this program (see figures), and was completely eluted under the conditions chosen. The amount of this compound (thought to contain silicon from mass spectrometric data) present in each analysis could be minimised by the use of the "septum-swinger" but it has proved impossible to eliminate it completely, either by the use of septum purge devices or by heat pretreatment of the septa.

The accurate reproduction of the program used in this work, especially at the low temperature end of the range, is obviously important in the use of the retention data (Table III), and the initial temperature quoted (35°C) has been checked by use of additional instruments. Once the temperatures attained during the program have been standardised, then the carrier-gas flow can be altered if necessary to adjust the retention times to those reported in Table III.

Since many of the commercially available solvents, anaesthetic agents and aerosol propellants show good ECD responses, it was felt that the use of a dual detection system using a split ratio of 9:1 FID/ECD would enhance the sensitivity of the method towards halogenated compounds while not appreciably reducing the sensitivity towards non-electron capturing substances. In practice, those compounds

TABLE IV

REPRODUCIBILITY OF THE RETENTION TIMES OF SOME COMPOUNDS IN THE STANDARD MIXTURE (TABLE I) OVER A 3-MONTH PERIOD

Compound	*Retention time (mm)**			
	n	*Mean*	*S.D.*	*C.V. (%)*
Dichlorodifluoromethane	26	4.3	0.42	9.8
Bromochlorodifluoromethane	26	8.2	0.45	5.5
Ethanol	30	9.5	0.36	3.8
Acetone	29	12.4	0.33	2.7
Trichlorofluoromethane	27	15.2	0.52	3.4
Isopropanol	28	20.1	0.50	2.5
Chloroform	30	31.0	0.62	2.0
Methyl ethyl ketone	27	36.7	0.62	1.7
1,1,1-Trichloroethane	28	40.7	0.68	1.7
Ethyl acetate	22	46.8	0.63	1.3
Isopropyl nitrate	11	60.1	1.03	1.7
Trichloroethylene	29	74.1	1.05	1.4
1,1,2-Trichloroethane	29	82.0	0.92	1.1
n-Hexane	18	86.7	1.54	1.8
Methyl isobutyl ketone	29	105.5	1.34	1.3
Tetrachloroethylene	30	121.5	1.53	1.3
Toluene	30	124.1	1.46	1.2
2,2,2-Trichloroethanol	30	127.7	1.63	1.3
Ethyl benzene	30	147.3	2.12	1.4

* 5 mm = 1 min.

responding fully to the ECD (Table III) only responded to the FID at much higher concentrations despite the split ratio. This was much less marked with some compounds, notably 1,1,2-trichloroethane (Fig. 1), but the relative response still provided a valuable adjunct to the retention time in the assignment of peak identity. Some compounds gave rise to a strong FID response accompanied by a relatively weak ECD response on the system used, and these compounds are also indicated in Table III.

The Carbopack C/Carbowax 20M column system has proved to be reproducible and reliable in routine use over a 12-month period. The inter-assay coefficients of variation (C.V.) of the retention times (all measured manually from the recorder trace) of some of the compounds present in the standard mixture are presented in Table IV. The coefficients were all 2.5% or less for those compounds eluting at retention times of 4 min (20 mm) or longer, although the variation was greater for those compounds eluting at shorter retention times. The standard mixture was chromatographed daily prior to sample analyses in order to ensure correct functioning of the system. However, where confirmation of the assignment of peaks occurring in sample chromatograms not present in the standard mixture was required, an appropriate volume (0.01-μl Pressure-Lok Mini-Injector or 0.1-μl SGE needle-in-plunger syringe) of vapour was injected separately.

Sample collection and storage

Blood was considered to be the specimen of choice for this work for several reasons. Firstly, blood specimens were most easily obtainable, especially from unconscious patients. Secondly, special containers were not required, in contrast to the custom-designed sample bags[12] or Tenax-filled tubes[13] needed when analysing samples of exhaled air. Finally, quantitative analyses carried out on blood were most likely to correlate with the clinical condition of the patient. In the event, most blood specimens sent for analysis during the evaluation of the present method were collected into plastic containers, transported at ambient temperature and stored at 4°C until the qualitative analysis was performed. The specimens were then stored at −20°C until a quantitative analysis was carried out if this was indicated. This procedure has proved satisfactory in routine use and, although no formal stability studies have been performed, repeat analyses of blood specimens containing BCF, Halons 11 and 12, propane, *n*-butane and isobutane, still gave rise to easily identifiable peaks after storage at −20°C for at least 3 months.

Nevertheless, it is prudent to recommend specimen collection into well-stoppered glass bottles [containing sodium fluoride (1%, to inhibit esterase activity) if possible] with a metal faced wad, and rapid (possibly refrigerated) transport and prompt analysis where the inhalation of very volatile materials is suspected. It is essential to ensure that samples of any products thought to have been abused which are to be analysed are packaged separately from biological specimens. It may be noted that many pressure sensitive adhesive tapes contain solvents such as toluene and thus contamination of specimens from this source is possible. It should also be noted that the earlier a clinical suspicion of solvent abuse is raised and specimens obtained and sent for analysis, the greater the likelihood of a positive finding since some inhaled agents may be excreted very rapidly[14].

Sample preparation

Headspace sample preparation was chosen since this required the use of only a small sample volume and a minimum of apparatus and introduced a minimum of contamination onto the chromatographic system. In practice, the only problem observed was that butyl rubber septum caps were found to absorb significant quantities of some solvents after relatively short (15 min) incubation times. The adoption of reusable PTFE faced disks obviated this problem, but at extra cost. This mode of sample preparation has the further advantage of ease of automation should this be contemplated, although the internal standard headspace analysis prior to sample addition would have to be omitted. In the early stages of experience with the technique, this would not be prudent, since by using this procedure any contributions to the sample chromatograms from the vial, cap, syringe or laboratory environment can be monitored.

Interpretation of sample chromatograms

With the exception of the peak derived from the septum used in the gas chromatographic system (see figures), no major peaks were represented on most "blank" chromatograms (Fig. 2). However, three compounds (ethanol, acetone and chloro-

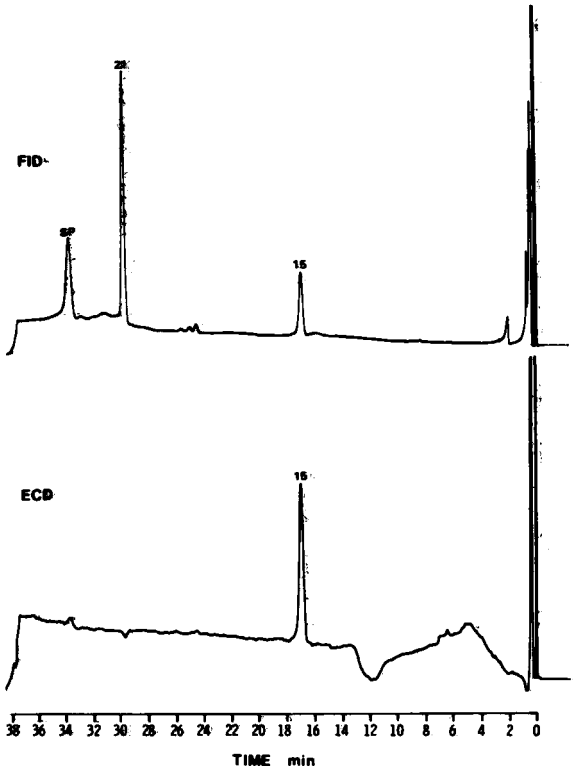

Fig. 2. Chromatogram obtained on analysis of a whole-blood specimen (200 μl) from a volunteer subject to which internal standard solution (200 μl) had been added: 400-μl headspace injection. Peaks: 15 = 1,1,2-trichloroethane and 21 = ethylbenzene (internal standards), SP = "Septum peak" (see text). For chromatographic conditions, see legend to Fig. 1.

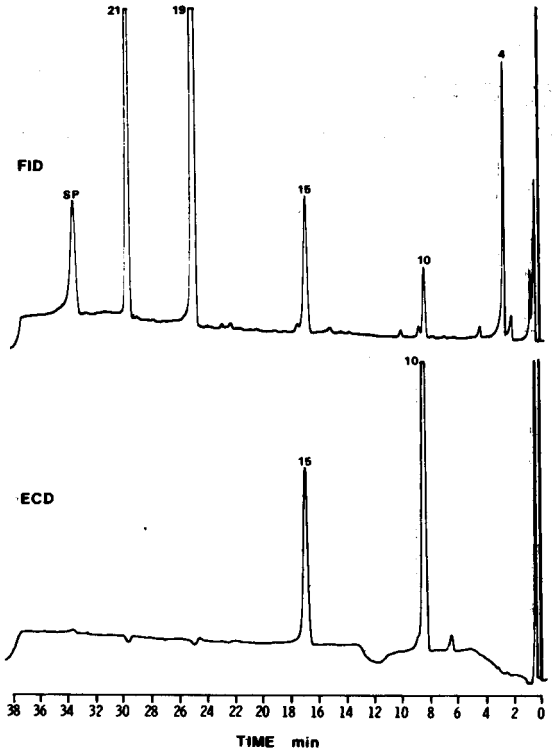

Fig. 3. Chromatogram obtained on analysis of a whole-blood specimen (200 μl) obtained from a patient suspected of abusing "solvents"; 400-μl headspace injection. Peaks: 10 = 1,1,1-trichloroethane, 19 = toluene. See legend to Fig. 1 for identification of remaining peaks and chromatographic conditions. The whole-blood 1,1,1-trichloroethane and toluene concentrations were found on subsequent analysis to be 3.4 and 11.2 mg/l, respectively.

form) were present in many samples, either alone or together with other compounds of interest (*cf.* Fig. 3). Both ethanol and acetone can be products of normal metabolism while ethanol is widely available in alcoholic beverages and both may also be produced by microbial action within the sample[15]. Chloroform (the identification of which has been confirmed by mass spectrometric analysis) was presumed to have arisen in many cases from contamination of the sample, possibly from within the laboratory.

Thus, only when the blood ethanol concentrations (measured by a separate gas chromatographic method) exceeded 0.1 g/l was the presence of this compound reported in clinical samples. Acetone and chloroform present more complex problems since both compounds may occur in preparations which may be abused by inhalation, and thus the presence of either compound was only reported when relatively high concentrations were present and there was circumstantial or other evidence that the compound of interest had been abused. This is illustrated by the case summarised in Fig. 4 where a relatively high concentration of chloroform was present together with tetrachloroethylene in a specimen obtained from a patient suspected of inhaling vapour from a dry-cleaning fluid, and also by the case summarised in Fig. 5 where the patient

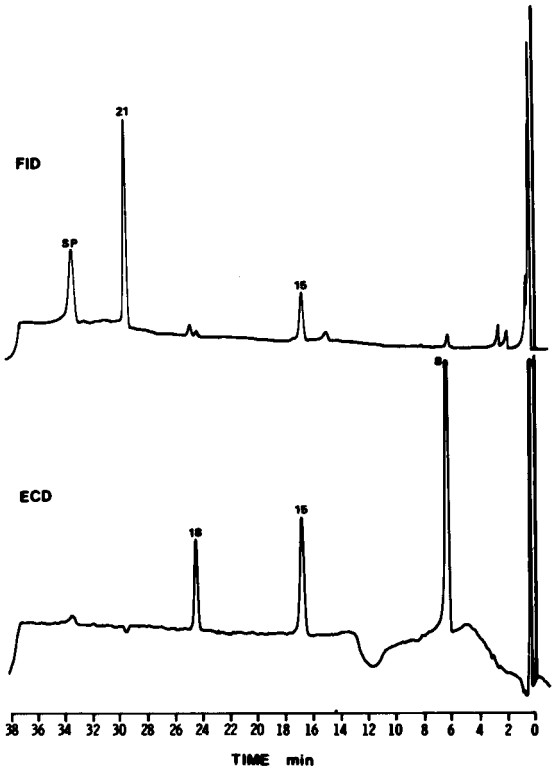

Fig. 4. Chromatogram obtained on analysis of a whole-blood specimen (200 μl) obtained from a patient suspected of inhaling vapour from a dry-cleaning fluid; 400-μl headspace injection. Peaks: 8 = chloroform, 18 = tetrachloroethylene. See legend to Fig. 1 for identification of remaining peaks and chromatographic conditions. The whole-blood tetrachloroethylene concentration was found on subsequent analysis to be 1.4 mg/l.

was suspected of abusing a de-greasing fluid containing trichloroethylene. (*N.B.* This latter case is also of interest since a high concentration of 2,2,2-trichloroethanol, presumably arising from the metabolism of trichloroethylene[16], was present.) The availability of more detailed quantitative information may assist in the interpretation of results where either acetone or chloroform is detected.

The presence of additional compounds derived from the abused agent provided useful corroboratory information in some instances. The presence of propane, isobutane and *n*-butane in a blood specimen from a patient who was suspected on abusing "butane" gas is illustrated in Fig. 6. Similarly, isopropyl nitrate, a stabiliser which has been added to commercial 1,1,1-trichloroethane, has been identified in blood specimens from patients suspected of abusing this latter agent, and methyl ethyl ketone has been identified together with toluene in specimens from patients who had inhaled the vapour from a preparation containing both compounds. When possible the direct analysis of an abused agent by either vapour phase infra-red spectrophotometry or gas chromatography can provide valuable information, and

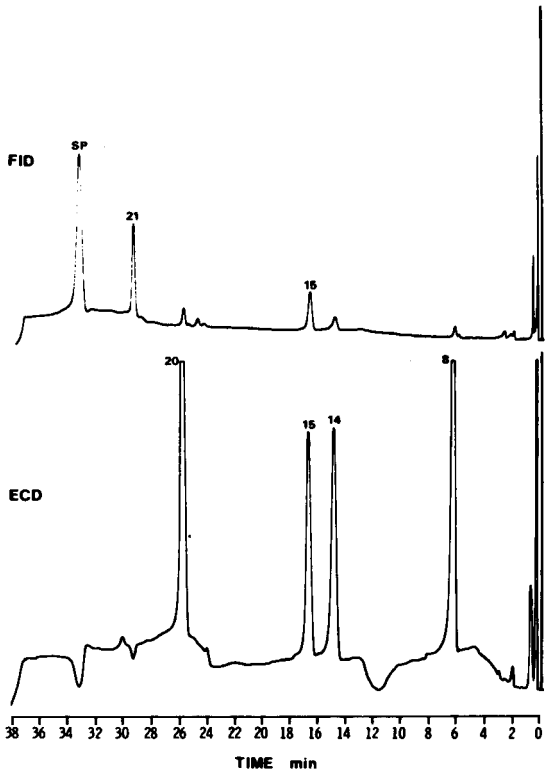

Fig. 5. Chromatogram obtained on analysis of a whole-blood specimen obtained from a patient suspected of inhaling vapour from a de-greasing fluid; 400-μl headspace injection. Peaks: 8 = chloroform, 14 = trichloroethylene, 20 = 2,2,2-trichloroethanol. See legend to Fig. 1 for identification of remaining peaks and chromatographic conditions. The whole-blood trichloroethylene and 2,2,2-trichloroethanol concentrations were found on subsequent analysis to be 9.5 and 77 mg/l, respectively.

the availability of mass spectrometric facilities for the confirmation or identification of unusual or unknown compounds may also be of value.

Limits of sensitivity

Detection limits for individual compounds depended on the appropriate detector response and to a certain extent on volatility both with respect to sample preparation and to elution time on the chromatographic system. The detection limits for some of the compounds encountered in the analysis of blood specimens were approximately 0.1 mg/l for toluene and the trichloro-compounds and 0.01 mg/l for the tetrachloro-compounds (Fig. 7). The availability of a series of clinical and forensic specimens from patients suspected of solvent abuse has proved crucial in the establishment of realistic detection limits for the technique, and it can be seen from the quantitative results summarised in Fig. 8 that in many of these specimens the compounds of interest were present at concentrations several-fold higher than these limits. There is much current interest in the analysis of volatile organic compounds in biological specimens both with respect to the monitoring of environmental pol-

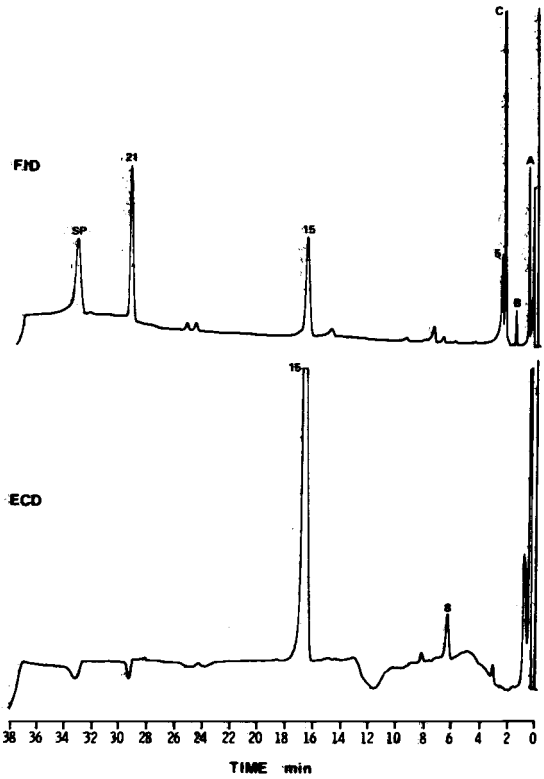

Fig. 6. Chromatogram obtained on analysis of a whole-blood specimen obtained from a patient suspected of inhaling "butane" from a cigarette-lighter refill: 400-μl headspace injection. Peaks: A = propane; B = isobutane, C = *n*-butane. See legend to Fig. 1 for identification of remaining peaks and chromatographic conditions.

lution[17] and to the possible use of such analyses in the diagnosis of disease[18,19]. However, it must be emphasised that the overall sensitivity of the present method is much less than that used in these other procedures, and thus the chromatograms obtained are normally free from interference from endogenous sample components (Figs. 2–7).

The method has proved applicable to the detection of bromochlorodifluoromethane, *n*-butane, carbon tetrachloride, chlorobutanol, cryofluorane, dichlorodifluoromethane, ethyl acetate, halothane, isobutane, isopropanol, isopropyl nitrate, methyl ethyl ketone, propane, tetrachloroethylene, toluene, 1,1,1-trichloroethane, 2,2,2-trichloroethanol, trichloroethylene and trichlorofluoromethane in specimens obtained from patients suspected of the abuse by inhalation or oral ingestion of these agents. In addition, cyclopropane, diethyl ether, paraldehyde and halothane have been detected in blood samples from patients treated with these compounds. However, we have no experience of the application of the technique where the vapours from complex mixtures such as petrol or paraffin have been inhaled. Chronic "petrol sniffing" has been diagnosed by the measurement of blood lead concentrations[20], and detection of the aromatic components could prove useful[21]. The measurement of

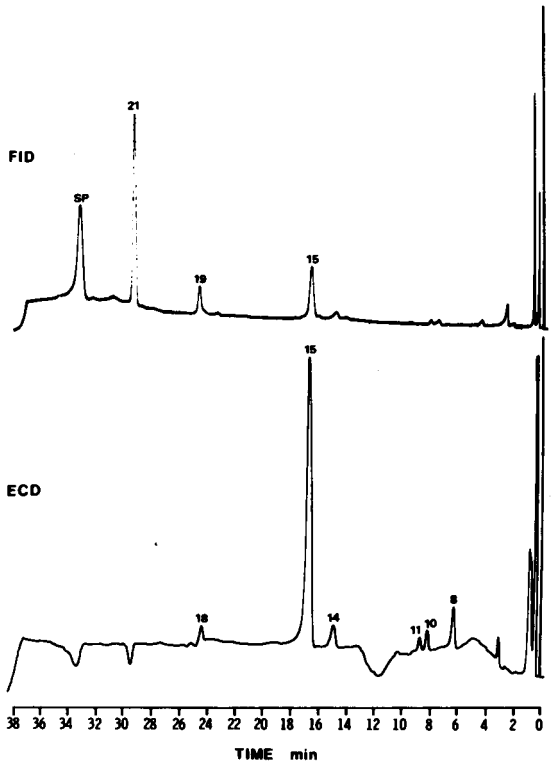

Fig. 7. Chromatogram obtained on analysis of a portion (200 μl) of a synthetic mixture prepared in expired blood-bank whole-blood; 400-μl headspace injection. Peaks: (blood concentrations in brackets), 10 = trichloroethane (105 μg/l), 11 = carbon tetrachloride (16 μg/l), 14 = trichloroethylene (114 μg/l), 18 = tetrachloroethylene (13 μg/l), 19 = toluene (87 μg/l). See legend to Fig. 1 for identification of remaining peaks and chromatographic conditions.

blood aluminium concentrations may provide useful diagnostic information where anti-perspirant aerosol preparations containing aluminum compounds may have been abused. On the other hand, it should be noted that the detection of esters such as ethyl acetate may be limited by blood non-specific esterase activity.

Application to saliva or urine specimens

In view of the lipophilicity and volatility of the compounds studied, it was thought unlikely that analyses of urine and/or saliva for the parent compound(s) would yield useful information. Indeed, the analysis of a number of such specimens taken at the same time as a blood specimen in which one or more of the compounds of interest were detected and identified confirmed this. However, it should be noted that in addition to the detection of metabolites containing the trichloro group[22], urine specimens may be useful in the identification and measurement of compounds such as trichloroacetic acid[23], hippuric[24] and benzoic acids and the cresols[25] (metabolites of toluene), and the toluric acids (metabolites of the xylenes[26]).

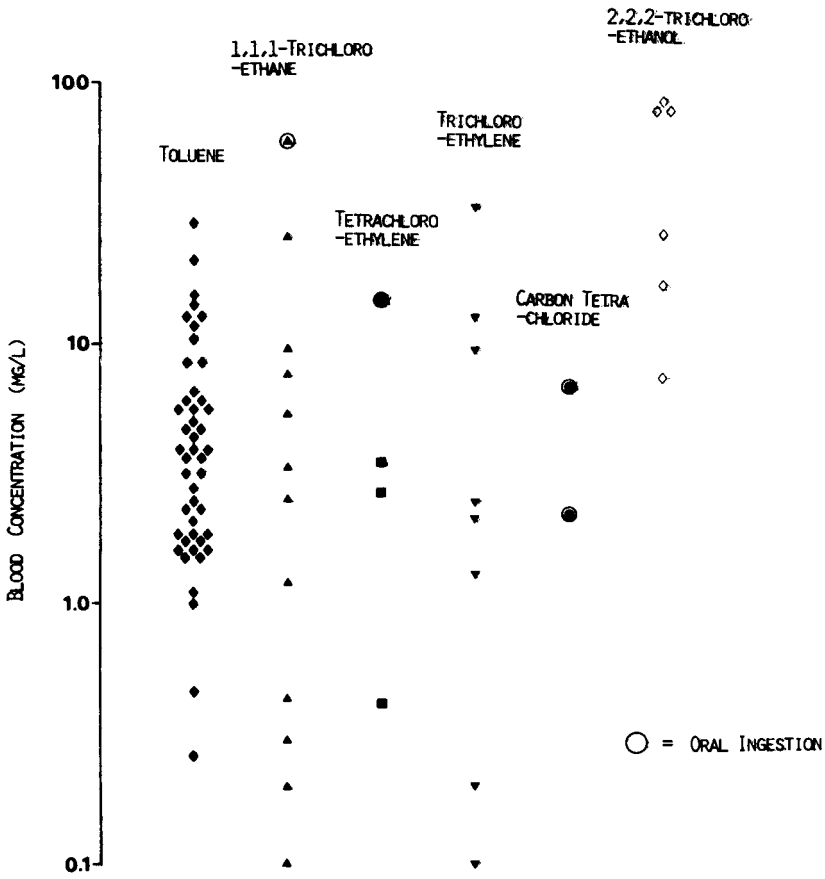

Fig. 8. Summary of quantitative results obtained on specimens analysed initially by the qualitative method.

Analysis of post-mortem tissue

Where forensic examination of tissue specimens was requested, it was found that a simple modification of the method gave satisfactory results. Tissue (approximately 200 mg) was incubated at 65°C for 30 min in a headspace analyser vial with internal standard solution (200 μl) containing a proteolytic enzyme (Subtilisin A; Novo, Windsor, Great Britain; 1 mg)[27]. Subsequently, a headspace sample (400 μl) was taken and analysed as described previously. Reagent blanks were performed in a separate vial.

Application to quantitative analyses

The method has been applied to the quantitative analysis of blood specimens containing some of the less volatile compounds studied (b.p. > 30°C), notably carbon tetrachloride, tetrachloroethylene, toluene, 1,1,1-trichloroethane and trichloroethylene, although it was found that a simple solvent-extraction technique[28] was suitable for the measurement of the blood 2,2,2-trichloroethanol concentrations attained following inhalation of trichloroethylene. Standard solutions at an appropri-

TABLE V

INTERNAL STANDARDS USED IN QUANTITATIVE ANALYSIS

For details of preparation, see text.

Analyte	*Internal standard*
Carbon tetrachloride	Bromodichloromethane
Chlorobutanol	1,1,1-Trichloropropan-2-ol
Halothane	Bromodichloromethane
Tetrachloroethylene	1,1,1,2-Tetrachloroethane
Toluene	Ethylbenzene
1,1,1-Trichloroethane	1,1,2-Trichloroethane
Trichloroethylene	1,1,2-Trichloroethane

ate range of concentrations were prepared gravimetrically in "volatile free" outdated blood-bank whole blood. After addition of the sample or standard and the internal standard solution (Table V) followed by equilibration for 30 min at 65°C, a portion of the headspace was analysed isothermally at an appropriate temperature. Calibration was by means of peak height ratios of analyte to internal standard on the appropriate detection system plotted against analyte concentration. When necessary, blood specimens were diluted with outdated blood-bank whole blood and re-analysed.

A summary of the results of quantitative analyses performed using specimens from patients suspected of abusing these compounds either by inhalation or oral ingestion is summarised in Fig. 8. All of these specimens had been analysed initially using the qualitative procedure. It should be emphasised that these results represent minimum values for the blood concentrations attained in these patients since it was not possible to take special precautions in the collection and transportation of the specimens, as discussed above.

The problems associated with the quantitative analysis of some of the more volatile compounds which have been detected using this procedure, for example butane and the halons, in biological specimens have been discussed[29] and include the need for precautions in the collection and transportation of specimens together with the difficulties inherent in preparing calibration solutions of these compounds.

CONCLUSIONS

The method described here has proved suitable for use in the detection and identification of a wide range of relatively volatile organic compounds in specimens obtained from patients suspected of the abuse of these agents. Only small specimens of blood (200 μl) or tissue (200 mg) are required, and special precautions in the collection and transportation of these specimens are not mandatory in the majority of cases although such precautions are desirable where very volatile materials are concerned. The method has proved to be reproducible and reliable in routine use and requires a minimum of apparatus, but may be readily adapted to automatic operation if required. Finally, the quantitative analysis of a number of the less volatile compounds studied has been accomplished using the same system together with the appropriate calibration solutions.

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ANALYSIS OF INSULIN PREPARATIONS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

L. F. LLOYD* and P. H. CORRAN
Chemistry Section, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB (Great Britain)
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SUMMARY

A reversed-phase high-performance liquid chromatographic system is described for the rapid and complete separation of bovine and porcine insulin from their readily formed monodesamido derivatives under isocratic conditions in the presence of the ion-pairing agent cetrimide. The system is suitable for the direct analysis of formulations of insulins of mixed bovine and porcine origin, and gives satisfactory results with a number of readily available commercial packings. Human insulin is not resolved from porcine in this system, but an alternative system allows the complete separation of all three insulins and their monodesamido derivatives, although acceptable peak shapes were obtained only on a limited number of packings.

INTRODUCTION

A wide variety of insulin preparations, differing in formulation, duration of action and purity, is available for diabetic therapy. At present all materials used in licensed preparations are derived from bovine and porcine pancreases. Although total chemical syntheses of insulin by classical fragment condensation and by stepwise solid-phase methods have been reported[1–4], neither approach has apparently proved commercially attractive, though both have been widely used for the preparation of analogues for bioactivity studies. Two recent methods for the large-scale production of human sequence insulins, one by semi-synthetic modification of the closely related porcine sequence[5] and the other in genetically modified micro-organisms (either producing A and B chains separately for subsequent combination or via proinsulin)[6], appear to be economically feasible, and insulins synthesised by both routes are at present under clinical trial in a number of countries.

The amino acid sequences of all three insulins are very similar (Table I), with variations occurring in only three positions and the maximum difference being three substitutions between bovine and human.

Although insulin preparations may often be required to be labelled with the species of origin, there is at present no officially recognised procedure for satisfac-

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TABLE I

SPECIES-SPECIFIC AMINO ACID RESIDUES IN VARIOUS INSULINS

Species	*Position*			*Composition*	
	A chain		*B chain*	*Thr*	*Ala*
	8	*10*	*30*		
Ox	Ala	Val	Ala	1	3
Pig	Thr	Ile	Ala	2	2
Human	Thr	Ile	Thr	3	1

torily verifying this information where the insulin is of mixed origin. The *British Pharmacopoeia* (1980)[7] includes in the monograph for Insulin a test for species of origin in which the identity is deduced from the content of alanine and threonine following hydrolysis and amino acid analysis. This procedure is not sufficiently sensitive to be applied to the determination of mixtures. However, this information could be obtained for a mixture of porcine and bovine insulin if the A and B chains were separated before the amino acid determination, since bovine A chain contains no threonine and porcine A chain no alanine, but this procedure is both complex and time-consuming.

Several reversed-phase high-performance liquid chromatographic (HPLC) systems for the chromatography of insulins have been described[8–17,24,25], some of which separate native porcine insulin from native bovine. Only two systems[16,24] have been shown to resolve bovine and porcine insulins and their readily formed degradation products, the A_{21} monodesamido derivatives which are found to some extent in most preparations. These forms of insulin possess similar biological potency to the native hormone and consequently no limit is set upon their content in pharmaceutical preparations. Most of the chromatographic systems mentioned above fail to resolve adequately bovine monodesamido-insulin from native porcine, and clearly this may give rise to inaccuracy in results for mixtures in which significant deamidation has occurred. It is desirable that any analytical method for an official specification should be shown to perform satisfactorily on packing materials from different commercial manufacturers. In each case[16,24] where resolution of a mixture of bovine and porcine native and monodesamido-insulins has been published, the separation has been demonstrated only on a single commercial packing.

We describe here a simple isocratic system which achieves this separation, is suitable for the analysis of formulations and gives satisfactory results on a wide range of commercial packings. The introduction of human sequence insulin preparations onto the market means that future systems should be capable additionally of resolving human native and monodesamido-insulin. In our system native human insulin is not resolved from native porcine, and experience suggests that most of the isocratic systems quoted above are unlikely to resolve human insulin from bovine and porcine, particularly when deamidation has occurred, because of inadequate selectivity or efficiency or both. We describe another simple system which resolves all these species and forms of insulin but which gives satisfactory results only on a limited number of the commercial packings examined. A preliminary account of the first system is in press[18].

MATERIALS

Urea AR, ammonium sulphate AR, sodium dihydrogen orthophosphate and cetrimide (cetyltrimethylammonium bromide) were purchased from BDH (Poole, Great Britain), L(+)-tartaric acid from Sigma (Poole, Great Britain). Acetonitrile was obtained from Fisons (Loughborough, Great Britain), or Rathburn Chemicals (Walkerburn, Great Britain), far-UV or 'S' grade. Iodoacetic acid (BDH) was recrystallised from petroleum ether before use. HPLC packings were: Nucleosil 5 μm C_{18} (Camlab, Cambridge, Great Britain); LiChrosorb RP-18 (BDH); Hypersil ODS (Shandon Southern, Runcorn, Great Britain); Spherisorb ODS (Phase Separations Queensferry, Clwyd, Great Britain); Ultrasphere ODS was purchased prepacked from Anachem (Luton, Great Britain) and Zorbax TMS prepacked from Dupont (Hitchin, Great Britain); Sephadex G-25 fine and G-50 superfine were obtained from Pharmacia (Hounslow, Great Britain). Crystalline highly purified native porcine, bovine and semi-synthetic human insulins were a gift of Novo Industries, Copenhagen, Denmark. Monodesamido-insulins were obtained by chromatography of deamidated insulin samples by a modification of published procedures[19]. The purity of insulin samples was evaluated by polyacrylamide gel electrophoresis (PAGE) at pH 8.3 (ref. 26). The European Pharmacopoeia first insulin biological reference preparation (established 1976) was obtained from the European Pharmacopoeia Secretariat, Strasbourg. The fourth international standard for insulin for bioassay NIBSC 58/6 is held at this Institute as is the first international reference preparation of human insulin for bioassay. Purified bovine and porcine proinsulins were purchased from Novo Industries.

APPARATUS

HPLC apparatus was assembled form the following components: Altex 110, Cecil 201 or LDC 720 pumps, Cecil 272, 212 or Hewlett-Packard 1030B variable-wavelength UV monitors, and Shandon or home-made septum injectors or Rheodyne 7125 valve injectors. Columns (10, 15, or 25 cm × 5 mm I.D.) were slurry-packed in propan-2-ol. Amino acid analyses were carried out on an LKB 4102 amino acid analyser.

METHODS

HPLC

System 1. The mobile phase was prepared by mixing 75 volumes of 5 m*M* L-(+)-tartaric acid (or acetic acid)–0.1 *M* ammonium sulphate with 25 volumes of acetonitrile. Sufficient solid cetrimide was then added to give a final concentration of 14 μM. Samples were dissolved in 5 m*M* tartaric acid–14 μM cetrimide or, in the case of neutral formulations, acidified by the addition of 5% v/v acetic acid. Formulations with pH less than 4 (*e.g.* soluble insulin BP) were injected direct. Septum injections were normally carried out with the flow stopped. The column effluent was monitored at either 280 or 225 nm. Columns were stored in mobile phase.

System 2. The column eluant was prepared from 0.1 *M* sodium dihydrogen orthophosphate, adjusted to pH 2 with phosphoric acid, 70 volumes, and acetonitrile,

30 volumes. The column was thermostatted at 45°C with a circulating waterbath. Crystalline samples were dissolved in 50 m*M* HCl, and formulations were treated as described above. Detection was carried out at 210 or 280 nm. Columns were washed with methanol–water (1:1 v/v) after use.

Peak identification. Peaks were collected, neutralised, desalted on a column (20 × 2 cm I.D.) of Sephadex G25 equilibrated with 5 m*M* NH_4HCO_3 lyophilised and rechromatographed by HPLC. Identity was confirmed by co-chromatography with suitable reference compounds or, to confirm that particular peaks were native or desamido-insulin, by PAGE at pH 8.3.

Species identification by amino acid analysis

Formulations were precipitated with an equal volume of 20 m*M* zinc chloride and dried. Crystalline insulin samples (10 mg) were dissolved in 0.5 ml of 1.44 *M* Tris-HCl, pH 8.6. Then 0.6 g of urea and 10 μl of 0.135 *M* EDTA were added, and the insulin was reduced by the addition of 5 μl of 2-mercaptoethanol followed by 4 h incubation at 37°C. Then 67 mg of recrystallised iodoacetic acid in 0.25 ml of 1 *M* NaOH were added dropwise, and the solution was allowed to stand in the dark for 20 min. An equal volume of glacial acetic acid was added, the solution applied to a column (100 × 1 cm I.D.) of Sephadex G-50 superfine, equilibrated with 50% v/v acetic acid, and the peaks were eluted at 4 ml/h with the same solvent. Fractions of 1.5 ml were collected, and the tubes corresponding to the second peak of 280 nm absorption, containing the A chains, were pooled, diluted with water and lyophilised. The lyophilised solid was hydrolysed with 0.2 ml of 6 *M* HCl in a sealed, evacuated tube for 16 h at 110°C, the HCl was removed over NaOH pellets *in vacuo*, and a suitable aliquot (*ca.* 20 nmol) was applied to the amino acid analyser. The purity of the A chain preparation was confirmed by the absence of phenylalanine (present only in the B chains), and the ratio of porcine to bovine insulin was determined by the relative amounts of threonine and alanine.

RESULTS AND DISCUSSION

System 1

When mixtures of bovine and porcine native and monodesamido-insulins were chromatographed on reversed-phase columns with no ion-pairing agent such as cetrimide, bovine monodesamido-insulin was inadequately resolved from porcine native (α 1.06). At high concentrations (0.027 *M*) of cetrimide, porcine and bovine insulin were not resolved from each other, but were well separated from the two unresolved monodesamido derivatives. Over a range of cetrimide concentrations from 2.5 μ*M* to 25 m*M* all four of these compounds were completely resolved from each other (Fig. 1). Over the range 2.5–25 μ*M* cetrimide the absolute and relative retentions of the four peaks changed little, and a cetrimide concentration of 14 μ*M* was chosen for routine use. When reversed phase columns were conditioned with mobile phase containing 14 μ*M* cetrimide the time taken to establish stable retention times was excessive. Accordingly columns were preconditioned with mobile phase containing 0.027 *M* cetrimide. The delay in breakthrough of cetrimide, detected by its ability to extract the dye orange G into chloroform, corresponded to the absorption by the packing of *ca.* 150 mg/g of cetrimide (*i.e. ca.* 30 ml for a 15-cm column). Equilibration with

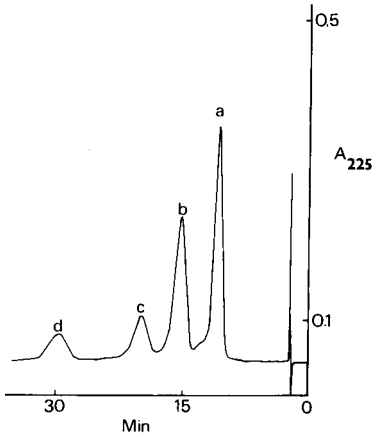


Fig. 1. Chromatogram of the Fourth international standard for insulin. Column, Hypersil ODS; mobile phase, 14 μM cetrimide in acetonitrile plus 0.1 M $AmSO_4$–5 mM tartaric acid pH 3.0 (25:75). Peaks: a, bovine native insulin; b, porcine native insulin; c, bovine monodesamido-insulin; d, porcine monodesamido-insulin.

mobile phase containing 14 μM cetrimide was then continued until retention times had stabilised. Little variation was found between batches of Hypersil ODS and satisfactory results were obtained with LiChrosorb RP-18 and Nucleosil C_{18}.

Reproducibility, recovery and detection limits

The peak height for either bovine or porcine insulin varied linearly with amount injected over the range 0.25–100 μg (correlation factor, 0.9980), and it was possible to detect less than 0.1 % of porcine monodesamido-insulin in a sample of bovine insulin. The detection limit for porcine insulin in bovine was about three times lower (100 μg was the maximum amount injected).

Use for formulations

Under the conditions described, formulations containing protamine, globin and preservatives such as methyl *p*-hydroxybenzoate and phenol may be analysed without special preparation. Protamine and globin are retained on the column indefinitely, and the use of a precolumn is advisable if more than the occasional sample of this type is to be analysed. When an acetonitrile concentration of 24 % was used, phenol and methyl *p*-hydroxybenzoate were eluted well before bovine insulin and did not interfere with the analysis, but in the absence of these preservatives the acetonitrile concentration could be raised to 25 % to allow a more rapid separation.

Comparison with existing methods

The proportions of porcine and bovine insulins present in three insulin samples of mixed origin were determined by the HPLC system described and by separation of A and B chains followed by amino acid analysis as outlined in the Methods section. All three preparations contained substantial amounts of monodesamido-insulins, and the proportions of each species were determined by summing the appropriate peak areas (measured at 280 nm). The results of this comparison are listed in Table II. The

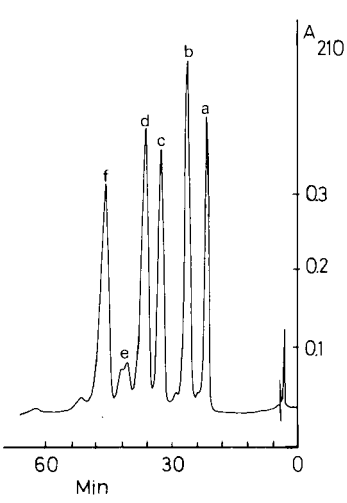
TABLE II

DETERMINATION OF THE PROPORTIONS OF BOVINE AND PORCINE INSULINS IN THREE SAMPLES BY HPLC AND BY ANALYSIS OF A CHAINS

Preparation	*Species*	*Expected content*	*Amino acid analysis*	*HPLC (system 1)*		*Total*	*HPLC (system 2)*		*Total*
				Native insulin	*Monodes-amido-insulin*		*Native insulin*	*Monodesamido-insulin*	
First EP biological reference preparation	Bovine	72	80	71.0	5.0	76	68.2	7.5	75.7
	Porcine	22	20	22.7	1.3	24	22.6	1.7	24.3
Fourth international standard	Bovine	55	55	47.1	7.9	55	45.8	10.1	55.9
	Porcine	45	45	38.4	6.6	45	35.9	8.2	44.1
A commercial bi-phasic preparation	Bovine	70	74	63.0	5.0	68	Same sample not available		
	Porcine	30	26	26.6	5.4	32			

agreement between results obtained by HPLC and by amino acid analysis of A chain preparations is satisfactory, particularly as conventional amino acid analysis typically has a coefficient of variation of *ca.* 5.0%, and small losses of threonine relative to alanine may be expected during hydrolysis. The precision of the HPLC technique seems adequate for a pharmacopoeial method, and the detection of better than 0.1% for one species in another, whether native or desamido, is adequate for all practical purposes.

The chief disadvantage of this chromatographic system is its inability to distinguish human from porcine insulin (α 1.08). Since formulations of human insulin may become generally available in the near future this is a serious weakness and necessitated the development of an alternative system (2) capable of differentiating between porcine and human insulins. For complete resolution of all six compounds a 25-cm length column was employed and an operating temperature of 45°C was necessary to improve the selectivity of the system. The separation of all six compounds: bovine, human, porcine native and monodesamido-insulins is illustrated in Fig. 2. In contrast to the cetrimide system, the order of elution of porcine native and bovine monodesamido-insulin is reversed and porcine insulin is separated from human. The influence of temperature on capacity factor (k') is shown in Fig. 3. Elevation of column temperature caused a corresponding increase in k' values, giving at 45°C almost baseline separation of the peaks. In addition the decrease in viscosity of the eluant reduced the high back-pressure obtained with a 25-cm column and 5-μm packings. Under the conditions used, optimal column performance was always a prerequisite for separation of all six insulins. Some C_{18} packings examined (Li-Chrosorb RP-18, Spherisorb ODS) gave poor insulin peak shapes despite good column



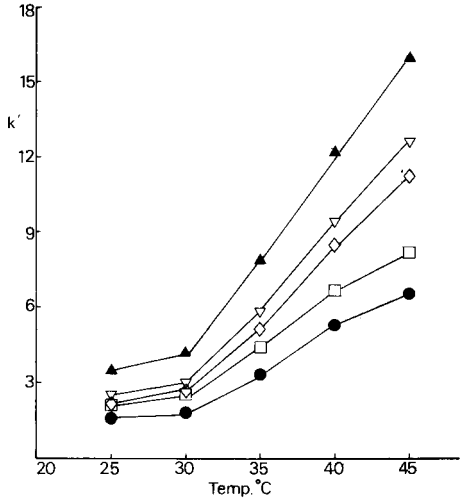


Fig. 2. Separation of bovine, human and porcine native and monodesamido-insulins. Column, Ultrasphere ODS; mobile phase, acetonitrile–sodium phosphate buffer pH 2 (30:70). Peaks: a, bovine native insulin; b, bovine monodesamido-insulin; c, human native insulin; d, porcine native insulin; e, human monodesamido-insulin; f, porcine monodesamido-insulin.

Fig. 3. Effect of temperature on insulin separation. ●, Bovine native insulin; □, bovine monodesamido-insulin; ◇, human insulin; ▽, porcine native insulin; ▲, porcine monodesamido-insulin.

test efficiencies; the most satisfactory results were obtained with Ultrasphere ODS and Hypersil ODS. This HPLC method can also be used to determine the species of origin of crystalline insulins and formulations, unambiguous assignment of species in this case being made by spiking a standard mixture of insulins with the unknown samples.

Detection of insulin-related contaminants in preparations

Occasionally, unidentified minor components were observed in insulin preparations. The relative amounts of these increased with ageing. Samples of formulated and bulk insulins subjected to accelerated degradation at elevated temperatures showed in addition many late-eluting peaks, probably owing to the presence of aggregated forms of insulin. However, we did not observe significant amounts of these components in formulations which had been subjected to normal treatment and which were within the expiry date. Since one of the most important immunogenic contaminants in insulin preparations is proinsulin, we examined the behaviour of purified bovine and porcine proinsulins in both systems (human proinsulin was not available). Bovine proinsulin eluted slightly later than, but was incompletely resolved from, porcine native in system 1, and porcine proinsulin was not eluted from the column. In system 2 bovine proinsulin was eluted slightly earlier than bovine native insulin whereas porcine proinsulin was eluted after porcine monodesamido-insulin.

Prediction of elution order of closely related peptides

Several workers have attempted to establish methods of predicting the retention times and elution order of peptides from a knowledge of their composition[20–23]. While it is unreasonable to expect that these computational procedures may enable the calculation of absolute retention times for different chromatographic systems and packings, one might hope that the relative retentions of the homologous series of peptides provided by the insulins and their monodesamido-derivatives used in this work would be correctly predicted. In fact only one method predicted the elution order in system 2 at 45°C with any accuracy, that of the π values used by Pliška *et al.*[22] (Table III). None of these methods predicted the rather unexpected behaviour of

TABLE III

ELUTION ORDER OF INSULINS IN SYSTEM 2

Insulin	*Actual order*	*Predicted order**			
		Method 1	*Method 2*	*Method 3*	*Method 4*
Bovine native	1	4	2	1	6
Bovine desamido	2	6	5	2=	5
Human native	3	1	1	2=	2
Human desamido	5	3	4	5	1
Porcine native	4	2	3	4	4
Porcine desamido	6	5	6	6	3

* Method 1: computed from relative lipophilicities using Rekker constants[20]. Method 2: calculated from retention coefficients determined by reversed-phase HPLC with NaH_2PO_4 pH 2 mobile phase and acetonitrile gradient[21]. Method 3: calculated from π values as reported by Pliška *et al.*[22]. Method 4: calculated from Σf constants[23].

bovine proinsulin in this system. Other workers[14,24] have also observed that bovine proinsulin is eluted much earlier relative to insulin than might be predicted from amino acid compositions, and that the relative retentions of native and proinsulin vary considerably from system to system, perhaps owing to interactions with residual silanols[14].

That peptides of identical composition but different sequence, or diastereoisomeric peptides differing at one position only, can be separated by reversed-phase HPLC indicates that other, less well understood, structural factors may be involved in retention, and perhaps the more variable C peptide of proinsulin differs sufficiently markedly in conformation under chromatographic conditions to explain the difference in retention observed.

CONCLUSIONS

In analysis of insulins, HPLC has advantages over conventional techniques such as amino acid analysis in that it is fast and sensitive and no sample preparation is necessary. Both systems described can be used for determining the species of origin of formulations as well as bulk insulins. In addition, these systems may be used to assess the purity of insulin preparations, although neither system was found suitable for quantitation of the content of bovine or porcine proinsulins to the very low relative concentration (less than 0.1 %) required in the *British Pharmacopoeia.*

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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SULFUR AMINO ACIDS IN PROTEIN HYDROLYSATES

LARRY N. MACKEY* and TERRI A. BECK
The Procter & Gamble Company, Miami Valley Laboratories, P.O. Box 39175, Cincinnati, OH 45247 (U.S.A.)

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SUMMARY

A rapid, quantitative high-performance liquid chromatographic procedure for the determination of methionine and cystine after oxidation to methionine sulfone and cysteic acid is described. The Dns derivatives of the amino acids are separated by reversed-phase chromatography with a phosphate buffer–acetonitrile gradient and detected by UV absorption at 254 nm. The procedure is validated by confirming the methionine and cystine content of ribonuclease A. The average yields of cysteic acid and methionine sulfone from triplicate analyses of ribonuclease A were 98.1% (±3.3) and 106.1% (±2.4) of the theoretical values, respectively.

INTRODUCTION

Sulfur amino acids are nutritionally limiting in soy protein and other legumes. Because of their low levels and the oxidative instability of cystine and methionine, it is important to develop accurate procedures for their determination.

Due to the lability of cysteine and methionine during acid hydrolysis, these amino acids are more accurately determined after oxidation to cysteic acid and methionine sulfone by performic acid treatment as recommended by Moore[1]. However, the standard cation-exchange chromatographic determination of cysteic acid and methionine sulfone is less than optimal because cysteic acid elutes in the void volume[2,3] and is therefore subject to interferences, and methionine sulfone is difficult to resolve chromatographically from aspartic acid and threonine.

Several investigators have reported poor precision for the analysis of sulfur amino acids using cation exchange chromatographic procedures. Porter *et al.*[4] reported a mean absolute deviation of 17.6% for the interlaboratory determination of methionine in cod muscle, compared to an average deviation of 7.4% for the other amino acids. In gelatin, which has a low methionine content, the mean absolute deviation for methionine was 83.3% compared to 7.5% for the other amino acids. Williams *et al.*[5] reported relative standard deviations of up to 38% for the interlaboratory determination of cystine. Sarwar *et al.*[6] in a recent collaborative study

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reported intralaboratory relative standard deviations of up to 19.4% for the determination of methionine and up to 18% for the determination of cystine.

Standard procedures for amino acid analysis by ion exchange in addition to having high variances for the determination of sulfur amino acids, are time consuming and require expensive specialized instrumentation. Bayer *et al.*[7] and Hsu and Currie[8] have recently described the high-performance liquid chromatographic (HPLC) separation of Dns-amino acids. Quantitative analyses were not reported by Bayer *et al.* Hsu and Currie reported approximately 75% of the theoretical yield for the analysis of various peptides.

A rapid, quantitative analytical procedure and validation data are reported here for the determination of methionine and cystine in protein hydrolysates. These results are much closer to theoretical values and are achieved using standard HPLC instrumentation.

EXPERIMENTAL

Performic acid oxidation

The procedure used was essentially that of Moore[1]. Approximately 0.030 g of protein was treated with 10 ml performic acid (88%, w/w, formic acid–30%, w/v, hydrogen peroxide 9:1, v/v) at 0°C for 18 h. To reduce the excess performic acid, 1.5 ml of cool hydrobromic acid (48%, w/w) was added. The solution was then evaporated to dryness under vacuum.

Acid hydrolysis

To the dry oxidized protein sample, 10 ml of 6 *M* hydrochloric acid was added. The sample was transferred to a vacuum tube (Pierce No. 29564), frozen in a dry ice–acetone bath, evacuated below 10^{-5} MPa and the tube sealed. The sample was then hydrolyzed in an oven at 110°C for 22 h. A 5-ml portion of an aqueous solution containing 0.50 mg/ml 3-aminobutyric acid was added as internal standard, mixed, and the sample was transferred to a 25-ml volumetric flask. The pH was adjusted to 7 ± 1.5 with 50% (w/w) aqueous sodium hydroxide and brought to volume with distilled water.

Dns derivatization

Solutions of sodium carbonate buffer in water (at 0.2 *M*; pH 9.7) and 5 mg/ml Dns chloride (Pierce No. 21755) in acetonitrile–water (70:30) were prepared. Aliquots 100 µl of hydrolyzed sample or standard amino acid mixture were reacted with 1.0 ml of carbonate buffer and 1.0 ml of Dns chloride solution at 55°C in an oven for 10-60 min. If Dns chloride crystals were present after this time, the vial was shaken to dissolve the crystals and reacted in the oven for another 5 min. This step was repeated as long as Dns chloride crystals were still present. The solution was then evaporated under a nitrogen stream to a volume of about 1.5 ml to lower the acetonitrile content. Then 1.0 ml of a 0.5% (w/w) phosphoric acid aqueous solution was added and the pH adjusted to 6.2 ± 0.2 with 1 *M* hydrochloric acid.

If the samples were not chromatographed within 24 h, it was necessary to readjust the pH, because of dissocation of acidified carbonate buffer to carbon dioxide and water with a resulting increase in pH due to evaporation of carbon dioxide.

Chromatographic separation

A Perkin-Elmer Series 3 gradient liquid chromatograph with an LC-65T oven–variable wavelength UV detector and a Sigma 10 Data Station were used. The Dns-amino acids were separated by reversed-phase liquid chromatography on a 25 cm × 4.6 mm I.D. column packed with 5-μm particle size Spherisorb hexyl. A number of chromatographic solvent systems were tried. The solvent system finally used was: (A) acetonitrile–0.02 *M* phosphate buffer pH 6.2 (60:40); (B) acetonitrile–0.02 *M* phosphate buffer, pH 6.2 (5:95). The gradient program consisted of three linear segments: from 7% A to 45% A in 30 min; from 45% A to 60% A in 1 min; from 60% A to 70% A in 7 min. The flow-rate was 1.5 ml/min. The column oven temperature was set at 34°C. Detection was at 254 nm and 0.08 a.u.f.s. Injection volume was 60 μl.

Micro-Kjeldahl analysis of ribonuclease A

Total nitrogen was measured using a mercuric oxide–potassium sulfate catalyst[9].

RESULTS AND DISCUSSION

Quantitation

The relative standard deviations of integrated area ratios for methionine sulfone and 3-aminobutyric acid solutions (whether reacted for 10, 20, 30, 40, 50, or 60 min) was less than 1%. The Dns derivatization reaction proceeded quickly and the Dns-amino acids were stable under the reaction conditions. Thus, samples were derivatized by reacting in an oven at 55°C for 30 min as routine procedure.

Internal standardization with 3-aminobutyric acid was used for quantitative calculations. Using this standard, the linearity of the integrated detector response for cysteic acid and methionine sulfone was measured over the concentration range of 0.02 to 0.20 mg/ml for each of the two compounds. The correlation coefficient (linearity of response) for each sulfur amino acid was greater than 0.998.

To obtain quantitative results, shaking of the mixture was done when needed to insure that Dns chloride crystals did not remain after the reaction. If Dns chloride crystals which formed had not been redissolved, Dns-amino acids would have preferentially adsorbed onto the crystals and changed the solution concentrations of the Dns-amino acids. This would have resulted in relative errors of over 30%.

Optimization of separation

The separation was optimized by characterizing the effect of pH and phosphate buffer concentration on the resolution of the 19 amino acids typically found in protein hydrolysates. A buffer concentration of 0.02 *M* with resulting pH of 6.2 was found to be best. Fig. 1 shows the separation of a Dns derivatized mixture of 20 common amino acids plus Dns-ammonia, Dns chloride, and Dns-sulfonic acid. The chromatographic efficiency required for this separation was approximately 10,000 theoretical plates.

The effect of changes in phosphate buffer concentration at pH 6.2 on the retention times of the Dns-amino acids relative to proline is graphically illustrated in Fig. 2. This graph is useful in a practical sense to optimize the resolution of the Dns-amino acids. If two Dns-amino acids are not adequately resolved in a trial separation

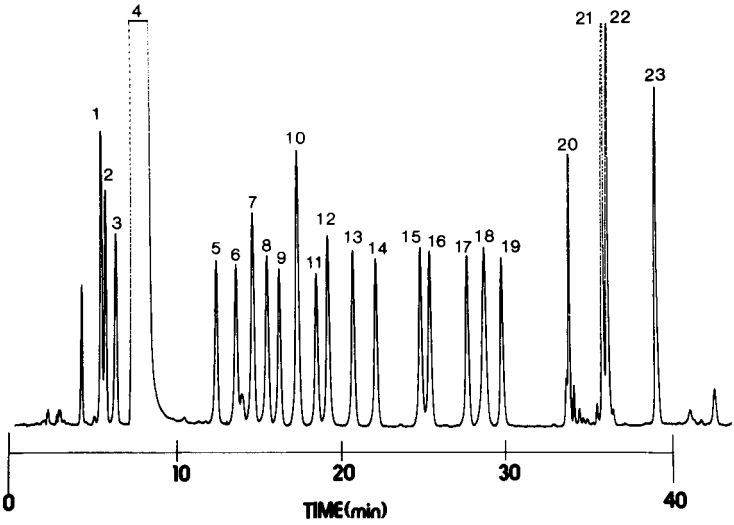

Fig. 1. Separation of Dns-amino acids according to conditions described in text. Peaks: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = Dns-sulfonic acid; 5 = serine; 6 = threonine; 7 = glycine; 8 = alanine; 9 = methionine sulfone; 10 = 3-aminobutyric acid; 11 = arginine; 12 = proline; 13 = valine; 14 = methionine; 15 = isoleucine; 16 = leucine; 17 = phenylalanine; 18 = Dns-chloride; 19 = cystine; 20 = Dns amide; 21 = lysine; 22 = histidine; 23 = tyrosine.

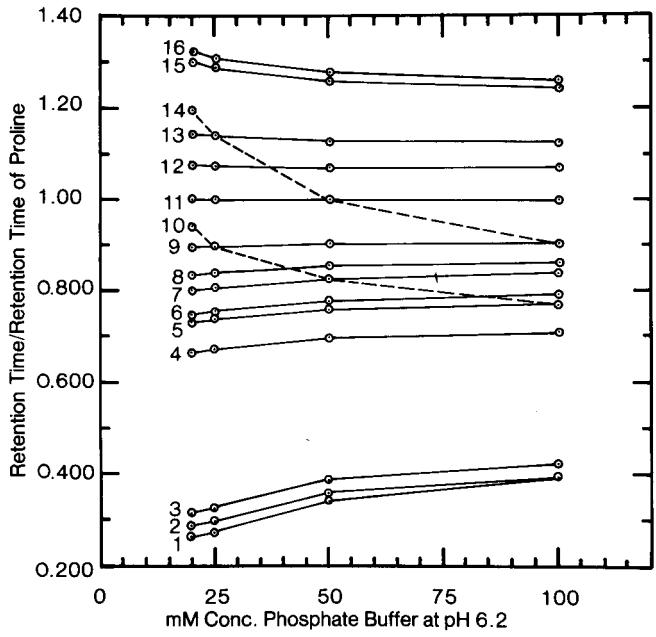

Fig. 2. Effect of buffer concentration on the relative retention times of Dns-amino acids. Plots: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = serine; 5 = threonine; 6 = glycine; 7 = alanine; 8 = methionine sulfone; 9 = 3-aminobutyric acid; 10 = unknown; 11 = proline; 12 = valine; 13 = methionine; 14 = arginine; 15 = isoleucine; 16 = leucine.

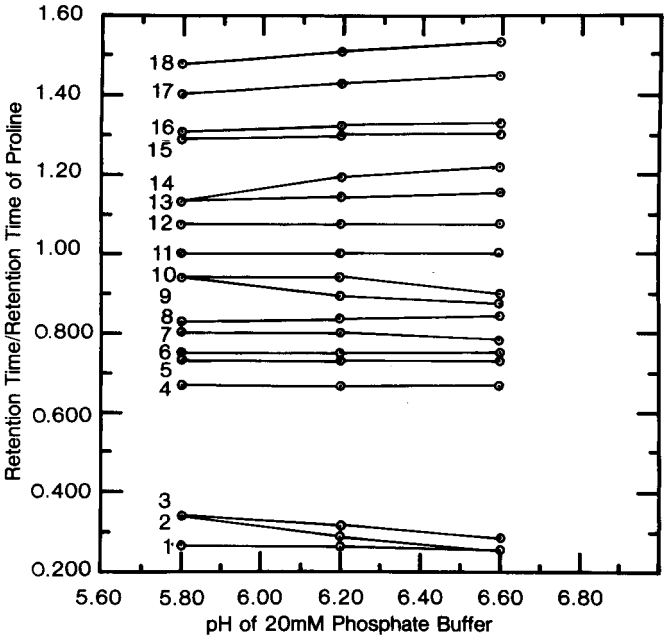

Fig. 3. Effect of pH on relative retention times of amino acids. Plots: 1 = cysteic acid; 2 = aspartic acid; 3 = glutamic acid; 4 = serine; 5 = threonine; 6 = glycine; 7 = alanine; 8 = methionine sulfone; 9 = 3-aminobutyric acid; 10 = unknown; 11 = proline; 12 = valine; 13 = methionine; 14 = arginine; 15 = isoleucine; 16 = leucine; 17 = phenylalanine; 18 = Dns chloride.

run, this graph can be used to determine whether increasing or decreasing the buffer concentration is necessary to improve the chromatographic resolution. Fig. 3 illustrates the effect of changes in pH in a 0.02 *M* phosphate buffer on the relative retention times of the Dns-amino acids. This graph can be used, similarly to the graph in Fig. 2, to optimize separation through pH adjustment.

Detection

Either spectrofluorometric[10] or UV absorption techniques can be used for detection of the eluted Dns-amino acids. In this study, UV detection at 254 nm was used because these detectors are commonly available and the increased sensitivity of spectrofluorometric detection was not needed. The standard amino acid mixture contained approximately $5 \cdot 10^{-7}$ mole/ml of each amino acid, prior to Dns derivatization, and approximately $1 \cdot 10^{-9}$ mole of each amino acid was actually injected onto the column. If sample quantities were limited, detection sensitivity could be improved by about a factor of three by using 220 nm instead of 254 nm as the detection wavelength. Alternatively, detection sensitivity could be improved by at least an order of magnitude by using spectrofluorometric detection.

Validation

To validate the accuracy and precision of the entire analytical procedure, a chromatographically pure, sequenced protein containing a known number of cysteine and methionine residues was analyzed. Three samples of ribonuclease A (Calbiochem

TABLE I

DETERMINATION OF CYSTEIC ACID AND METHIONINE SULFONE CONTENT OF RIBONUCLEASE A

R.S.D. = relative standard deviation.

Sample	*Actual cysteic acid content (g/100 g sample)*	*Adjusted cysteic acid content (g/100 g ribonuclease A)**	*Recovery (as % of theoretical)*	*Actual methionine sulfone content (g/100 g sample)*	*Adjusted methionine sulfone content (g/100 g ribonuclease A)**	*Recovery (as % of theoretical)*
1	8.61	10.06	101.8	4.79	5.60	105.7
2	8.22	9.60	97.2	4.93	5.76	108.7
3	8.08	9.44	95.4	4.72	5.51	104.0
		Mean	98.1		Mean	106.1
		R.S.D. (%)	3.3		R.S.D. (%)	2.4

* This value is calculated based on a Kjeldahl N analysis of 14.98% for the samples and a theoretical N content of 17.50% for ribonuclease A.

No. 55674) were oxidized with performic acid, acid hydrolyzed, and analyzed by HPLC. Table I shows the results of these analyses. Ribonuclease A was analyzed in duplicate for total nitrogen by the method described. The protein content of the samples was calculated based on the nitrogen analyses and the theoretical nitrogen content of ribonuclease A (17.5%, w/w). Since the ribonuclease A was chromatographically pure, it was assumed to be the only source of nitrogen in the samples. The percent theoretical calculations in Table I are based on ribonuclease A containing eight cysteine residues per mole of protein and four methionine residues per mole of protein. The recovery calculated from the cysteine and methionine found is in excellent agreement with the expected content based on nitrogen content and the sulfur amino acid composition of ribonuclease A.

Conclusion

This HPLC procedure to determine total sulfur amino acids in protein hydrolysates is more rapid, both in terms of chromatographic separation time and in sample preparation time, than the standard ion-exchange procedure. Furthermore, this procedure offers increased precision, compared to ion-exchange methods. The separation difficulties encountered with ion-exchange chromatography for cysteic acid and methionine sulfone are also eliminated with this HPLC procedure. Finally, this procedure uses standard HPLC instrumentation instead of expensive specialized instrumentation.

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QUANTITATIVE DETERMINATION OF NEUTRAL LIPIDS ON CHROMARODS*

E. R. FARNWORTH*
Animal Research Centre, Research Branch, Agriculture Canada, Ottawa, Ontario K1A 0C6 (Canada)
B. K. THOMPSON
Engineering and Statistical Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario K1A 0C6 (Canada)
and
J. K. G. KRAMER
Animal Research Centre, Research Branch, Agriculture Canada, Ottawa, Ontario K1A 0C6 (Canada)
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SUMMARY

Five lots of ten chromarods were spotted with 2, 4, 6, or 8 μg of cholesterol ester, cholesterol, triglyceride, methyl ester and free fatty acid and then analyzed using an Iatroscan. Rod-to-rod and lot-to-lot differences in the detector response were evident in the data. The standard deviation for the rod within lot response appeared to increase linearly as the amount of lipid applied was increased. The logarithms of the detector response data were analysed statistically to determine the relative magnitude of the rod-to-rod and lot-to-lot variances. When methyl ester was used as an internal standard or as a covariate, the variation from rod to rod and lot to lot was much smaller than in the original analysis.

INTRODUCTION

The Iatroscan has been welcomed by many workers using conventional thin-layer chromatography (TLC) as an instrument that can provide qualitative and quantitative analyses of lipids[1,2]. Careful examination of the chromatographic behaviour of lipid subclasses on the chromarods used with the Iatroscan has shown that TLC solvent systems may not be directly applicable to the chromarods. However, proper solvent selection can ensure good separation of a wide range of lipid classes[3]. This separation ability, combined with the small sample size and the speed of analysis, has led to the suggestion that the Iatroscan may be used routinely for clinical lipid analysis[4–6].

Studies of the quantitative capabilities of the Iatroscan have shown that there

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is a low precision in single estimations owing to the large coefficient of variation[6–10]. Most of this variation has been attributed to the inconsistent behaviour of individual rods[10]. Hammond[11] has recently suggested that the flame ionization detector design may be the source of quantitation problems. Initial work with the Iatroscan in this laboratory led to our questioning the uniformity of results obtained using individual rods within a single lot and also the uniformity of results obtained from different lots. This paper presents a detailed study of the quantitation of lipid subclasses using five different lots of ten chromarods. Methods of standardizing the data from the rods and lots are reported which attempt to minimize the rod-to-rod and lot-to-lot variation.

EXPERIMENTAL

The instrument and operating conditions used in this study have been reported previously[3]. Five lots of ten chromarods (type S, mean thickness of sintered coating of active absorbent, 75 μm) were received over a 7-month period. Standard solutions of 2, 4, 6, or 8 μg of cholesterol ester (CE), methyl ester (ME), triglyceride (TG), free fatty acid (FFA) and cholesterol (C) in heptane were spotted on the rods (standards purchased from Nu Check, Elysin, MN, U.S.A.). A hexane–diethyl ether–formic acid (85:15:0.04) mixture was used as the developing solvent. The differential and integral outputs from the Iatroscan were displayed on a two-pen recorder (Fisher Recordall, Model 5000). The step height of the integration signal was taken as the detector response.

A second experiment was carried out in which 6 μg of the lipid standards (CE, ME, TG, FFA and C) were spotted on the rods from three lots, then developed and analysed. The procedure was repeated five times for each rod.

STATISTICAL ANALYSIS

Analyses of variance (ANOVA) and covariance were applied to the results from the five lots of rods in order to study the variation among lots and among rods within lots. All tests of significance were carried out at the 5% level. Because the standard errors appeared to increase approximately linearly with increasing concentration, the original data (detector responses) were transformed prior to analysis using the logarithmic transformation[12]. The transformations also facilitated comparisons of the different analyses because an ANOVA of the logarithm of a ratio is analogous to an analysis of covariance of the logs of the components with a regression coefficient of 1. The multiple regression techniques used in introducing a series of independent variables and the concepts involved in the tests for parallel lines are discussed by Snedecor and Cochran[12].

The model associated with the analyses of variance in the first experiment is:

$$Y_{ijk} = \mu + l_i + a_j + (al)_{ij} + r_{ik} + \varepsilon_{ijk}$$

where Y_{ijk} is the $ijk$the observation, μ the overall mean, l_i represents the effect of the i^{th} lot, a_j the effect of the j^{th} amount, $(al)_{ij}$ the interaction between lot i and amount j, r_{ik} the effect of the k^{th} rod in the i^{th} lot and ε_{ijk} the random error as-

sociated with the individual rods. All effects were assumed random except a_{ij}. The structure was the same in the second experiment except that replicates replaced amounts. Variance components were estimated for all random effects. It may be noted that the component for interaction in the first experiment includes two constituents, one relating to the interaction *per se* and the other to the random variation among determinations for the lot as a whole. There was no appropriate error for testing lot differences in the first experiment because one choice —the rod within lot mean square— did not include the random variation among determinations for the lots, a component in the lot mean square, while the other choice —the interaction mean square— contained the interaction component which is not included in the lot mean square. However, as the interaction mean square was the appropriate error in the second experiment, it was used in the first as well. The choice of either error term would make little difference to the interpretation of the experiment.

RESULTS AND DISCUSSION

Table I contains raw data from two lots (hereafter referred to as lots 2 and 5) of ten chromarods obtained using the Iatroscan. The data are the detector responses to the five different lipid classes (CE, ME, TG, FFA and C) when 2, 4, 6 or 8 μg of each lipid were applied to the rod. As can be seen, the average response for the ten rods from the two lots is similar in some cases (CE, 2 μg and 8 μg; ME, 4 μg and 8 μg; TG, 2 μg, 4 μg and 8 μg; FFA, 2 μg, 4 μg and 8 μg; C, 2 μg and 8 μg), but very different in others (CE, 4 μg; ME, 2 μg; FFA, 6 μg; C, 6 μg). For both lots, the standard deviation increased as the amount of lipid applied was increased. The standard deviations for lot 5 were always greater than the standard deviations for lot 2. These smaller standard deviations could reflect either greater precision in lot 2 and/or greater systematic differences among the rods of lot 5. Subsequent analyses of variances within lots showed both factors contributed to the differences in standard deviations.

The data in Table I show that there were often large differences among rods in response to a given amount of an individual lipid. This is most evident for the rods in lot 5 (CE, 2 μg, rods 5 and 8; TG, 4 μg, rods 6 and 8; FFA, 8 μg, rods 6 and 8), a result already noted in the larger standard deviations. When the responses of individual rods are examined, it is evident that in lot 5, rod 8 had a low sensitivity, whereas rod 6 had a higher sensitivity; in lot 2, rods 6 and 7 gave low responses, whereas rods 5 and 10 usually gave relatively higher responses.

In order to determine if these differences in response by the individual rods in a lot were large enough to have a considerable impact on the variation from lot to lot, data (similar to that given in Table I) were obtained for the 5 lots of 10 rods. Table II is a summary of these data. As when only lots 2 and 5 were compared, it was evident that for the five lots the mean responses to a particular amount of an individual lipid were sometimes similar and in other cases very different. In all lots, the standard deviation increased when the amount of lipid applied was increased. But lots 1 and 5 had greater standard deviations than lots 2, 3 and 4 for all lipids at the four amounts of application.

Tables III–VI show the mean square values that were obtained from the ANOVA. In addition, the estimates of the relevant variance components are also

TABLE I

DETECTOR RESPONSES TO DIFFERENT AMOUNTS OF LIPID SUBCLASSES (LOTS 2 AND 5)

Rod	*Amount 2 μg*					*Amount 4 μg*				
	CE	*ME*	*TG*	*FFA*	*C*	*CE*	*ME*	*TG*	*FFA*	*C*
Lot 2										
1	1.09	0.94	0.72	0.88	1.13	2.57	2.41	1.92	2.14	2.97
2	1.09	0.94	0.76	0.89	1.39	2.15	1.93	1.51	1.68	2.42
3	1.18	0.99	0.82	0.88	1.26	2.50	2.20	1.77	2.01	2.70
4	1.33	1.11	0.87	0.93	1.21	2.28	2.00	1.63	1.78	2.81
5	1.38	1.18	0.90	0.98	1.32	2.90	2.62	2.14	2.36	3.13
6	1.00	0.87	0.67	0.76	1.62	2.43	2.09	1.64	1.69	2.96
7	1.00	0.82	0.60	0.67	0.89	2.81	2.45	2.00	2.12	2.92
8	1.22	1.05	0.89	0.90	1.25	2.82	2.57	2.16	2.25	2.99
9	1.28	1.10	0.86	0.90	1.33	2.68	2.31	1.81	1.90	2.79
10	1.33	1.19	1.04	1.05	1.40	2.70	2.46	2.07	2.04	2.96
Mean	1.19	1.02	0.81	0.88	1.28	2.58	2.30	1.87	2.00	2.87
S.D.*	±0.14	±0.13	±0.13	±0.11	±0.19	±0.25	±0.24	±0.23	±0.23	±0.20
Lot 5										
1	0.66	0.44	0.37	0.50	0.95	1.82	1.58	1.22	1.42	2.21
2	1.03	0.50	0.50	0.70	1.03	1.43	1.10	1.00	1.05	1.88
3	1.28	0.79	0.78	0.84	1.03	2.27	1.79	1.35	1.40	2.06
4	1.60	1.13	0.98	1.04	1.19	2.84	2.58	2.22	1.93	2.38
5	2.12	1.38	1.09	1.23	1.37	2.21	2.10	1.78	1.92	2.57
6	1.07	1.16	1.37	1.50	1.70	2.50	3.04	2.99	3.37	3.43
7	1.08	0.86	0.91	0.93	1.11	2.27	2.21	2.16	2.25	2.68
8	0.65	0.42	0.40	0.43	0.84	1.58	1.27	0.90	0.90	1.85
9	1.40	1.10	0.99	1.12	1.17	3.49	3.27	2.94	3.30	3.02
10	0.99	0.90	0.89	1.06	1.12	3.02	2.62	2.44	2.63	2.63
Mean	1.19	0.87	0.83	0.94	1.15	2.34	2.16	1.90	2.02	2.47
S.D.*	±0.44	±0.33	±0.14	±0.33	±0.24	±0.65	±0.73	±0.77	±0.87	±0.50

* Standard deviation.

presented. In analysis 1, the log transformed data from all five lots of rods were analysed. In analysis 2, the detector response for each compound was divided by the response to ME before transformation (thus essentially using ME as an internal standard). In analysis 3, the transformed detector response data were analysed using the transformed ME response as a covariate. In the analysis of each lipid it was assumed that the regression relation between ME and the compound being analysed was the same for all five lots.

When the transformed data alone were analysed (analysis 1, Tables III–VI, the F ratios for the lot-to-lot differences [L/(R*L)] and the rod-to-rod differences [(R/L)/(error)] both indicated significant differences. The estimates of the variance components from the different sources tended to be similar for each lipid, although the rod component was somewhat larger for TG and FFA. The random variation inherent in the determinations (represented by the error term) were very similar for the four lipids analysed. When the data for individual lots were analysed (not shown

Amount 6 μg					*Amount 8 μg*				
CE	*ME*	*TG*	*FFA*	*C*	*CE*	*ME*	*TG*	*FFA*	*C*
4.71	4.54	3.10	3.86	4.53	6.48	6.24	4.42	4.61	6.53
4.42	3.85	3.16	3.20	4.49	6.09	5.61	4.07	4.29	5.99
4.74	4.20	3.22	3.51	4.38	6.78	6.17	3.72	5.18	6.41
4.58	4.06	2.90	3.53	4.79	6.52	5.57	4.24	4.12	6.75
5.16	4.62	3.81	3.85	5.39	7.52	6.02	4.42	5.66	6.75
4.17	3.61	2.50	2.70	3.61	6.10	5.15	3.41	3.52	4.50
4.72	4.32	3.29	3.40	4.43	7.06	5.99	4.62	4.68	6.07
4.81	4.55	3.44	3.57	4.64	6.90	6.20	4.61	5.16	6.29
4.96	4.41	3.17	3.32	4.34	6.31	5.37	3.80	4.32	6.01
5.00	4.70	3.56	3.70	4.75	6.99	6.32	4.81	4.88	6.45
4.73	4.29	3.22	3.46	4.54	6.68	5.86	4.21	4.64	6.18
±0.29	±0.36	±0.36	±0.34	±0.45	±0.46	±0.41	±0.45	±0.62	±0.65
2.56	2.37	1.78	1.92	3.10	4.48	4.14	2.58	2.92	4.38
2.13	1.95	1.56	1.75	2.99	4.00	3.40	2.34	2.42	4.81
3.44	2.82	2.06	2.09	3.00	6.38	6.03	3.04	3.80	5.28
2.63	2.46	1.57	1.72	2.63	7.49	7.38	4.10	5.25	5.82
3.42	3.37	2.81	2.98	3.95	5.98	5.83	3.60	4.09	6.74
4.10	5.32	4.13	5.18	4.11	7.48	7.12	8.81	8.16	7.97
4.27	4.18	3.29	3.46	4.08	7.53	7.15	5.61	6.03	6.66
2.58	2.27	1.58	1.62	2.60	4.58	4.09	2.05	2.26	3.56
4.14	3.87	2.86	3.10	3.29	10.00	10.47	6.17	7.72	7.24
2.59	2.00	1.50	1.45	5.96	6.88	7.27	5.42	6.20	8.42
3.19	3.06	2.31	2.53	3.57	6.37	6.20	3.88	4.52	5.88
±0.79	±1.11	±0.91	±1.17	±1.11	±1.89	±2.19	±1.54	±1.89	±1.53

here), it was apparent that lots 1 and 5 had more rod-to-rod variation than lots 2, 3 and 4.

The use of an internal standard has been proposed as one way of overcoming the systematic differences in response[7,8]. To be useful in this application, an internal standard must (i) be soluble in organic solvents, (ii) have an R_F value on chromarods that does not overlap with other compounds of interest, (iii) be non-naturally occurring and (iv) have a response similar to the compound(s) being analysed. In this study, methyl ester was chosen as an internal standard in an attempt to eliminate the lot-to-lot and rod-to-rod differences. The results are represented in analysis 2.

Comparing the estimates of the variance components from analysis 2 with those from analysis 1 for each lipid (Tables III–VI), it is apparent that most components were reduced considerably by using ME as an internal standard. In spite of the large drops in variance components, the differences among rods within lots are still significant for all compounds. Analysis 2 shows that the amount mean square remains relatively large, other than perhaps for CE, suggesting a problem in accuracy

TABLE II

MEAN* DETECTOR RESPONSE TO VARIOUS AMOUNTS OF NEUTRAL LIPIDS DEVELOPED** ON CHROMARODS FROM DIFFERENT LOTS

*Subclass****	*Amount applied (μg)*	*Lot 1*	*Lot 2*	*Lot 3*	*Lot 4*	*Lot 5*
CE	2	0.83 ± 0.17	1.19 ± 0.14	1.16 ± 0.11	1.03 ± 0.09	1.19 ± 0.44
	4	1.91 ± 0.34	2.58 ± 0.25	2.52 ± 0.13	3.14 ± 0.36	2.34 ± 0.65
	6	3.37 ± 0.71	4.73 ± 0.29	4.24 ± 0.44	4.38 ± 0.28	3.19 ± 0.79
	8	5.54 ± 0.83	6.68 ± 0.46	5.56 ± 0.71	6.08 ± 0.60	6.38 ± 1.89
ME	2	0.67 ± 0.17	1.02 ± 0.13	1.21 ± 0.09	0.93 ± 0.09	0.87 ± 0.33
	4	1.74 ± 0.40	2.30 ± 0.24	2.30 ± 0.11	2.79 ± 0.31	2.16 ± 0.73
	6	3.11 ± 0.78	4.29 ± 0.36	3.79 ± 0.41	3.76 ± 0.24	3.06 ± 1.11
	8	5.51 ± 1.13	5.86 ± 0.41	5.00 ± 0.22	5.08 ± 0.41	6.29 ± 2.19
TG	2	0.68 ± 0.20	0.81 ± 0.13	1.13 ± 0.14	0.75 ± 0.08	0.83 ± 0.14
	4	1.54 ± 0.41	1.87 ± 0.23	2.18 ± 0.18	2.29 ± 0.27	1.90 ± 0.77
	6	2.32 ± 0.71	3.22 ± 0.36	3.16 ± 0.24	2.97 ± 0.25	2.31 ± 0.91
	8	3.35 ± 0.57	4.21 ± 0.45	3.93 ± 0.18	3.89 ± 0.34	3.99 ± 1.54
FFA	2	0.71 ± 0.22	0.88 ± 0.11	1.24 ± 0.15	0.82 ± 0.07	0.94 ± 0.33
	4	1.57 ± 0.45	1.99 ± 0.23	2.54 ± 0.24	2.31 ± 0.27	2.02 ± 0.87
	6	2.42 ± 0.78	3.46 ± 0.34	3.42 ± 0.28	3.04 ± 0.21	2.53 ± 1.17
	8	3.80 ± 0.85	4.64 ± 0.62	4.21 ± 0.17	3.88 ± 0.32	4.66 ± 1.89
C	2	0.93 ± 0.15	1.28 ± 0.19	1.58 ± 0.23	1.16 ± 0.11	1.15 ± 0.24
	4	2.18 ± 0.55	2.87 ± 0.20	3.21 ± 0.51	3.46 ± 0.08	2.47 ± 0.50
	6	3.12 ± 0.54	4.54 ± 0.45	5.06 ± 1.08	4.64 ± 1.14	3.57 ± 1.11
	8	5.04 ± 0.93	6.18 ± 0.65	6.11 ± 1.11	5.28 ± 1.05	5.94 ± 1.53

* Means represent average of ten rods from a lot run simultaneously ± standard deviation.
** Developing solvent hexane–diethyl ether–formic acid 85:15:0.04.
*** CE = cholesterol ester; ME = methyl ester; TG = triglyceride; FFA = free fatty acid; C = cholesterol.

of the internal standard method at the different concentration levels, perhaps because of a non-linear relationship between the lipids and ME, a point to be discussed below. Furthermore, the significant lot* amount interactions indicate that the response patterns differ somewhat from lot to lot. The differences among lots, however, are not significant, a result which can be attributed at least in part to the relatively large amount* lot interactions (the denominator in the F ratio) and to the small number of degrees of freedom involved in the F ratio (4 and 12 in the numerator and denominator respectively).

In an attempt to determine why the use of ME as an internal standard (analysis 2) left comparatively large differences among amounts and failed to explain some of the variation among lots and among rods within lots, regression analyses (within lots) were carried out on the untransformed data from the individual lipids (C, ME, TG, FFA, CE), using the amounts applied as the independent variable. The untransformed data were used here to retain the structure depicted in Fig. 1; the fact that the coefficient of variation remained constant over the range is unlikely to have affected

TABLE III

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES ON CHOLESTEROL ESTER DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.624	0.033	0.035
Amount (A)	45	27.397	0.041	0.006
L * A	3	0.121	0.075	0.051
Rods/L	12	0.086	0.020	0.011
Error	135**	0.013	0.004	0.003
		Variance components		
Lots (L)		0.013	−0.001	0.000
L * A		0.001	0.007	0.005
Rods/L (R/L)		0.018	0.004	0.002
Error		0.013	0.004	0.003

* 1 = ANOVA of log (detector response to CE); 2 = ANOVA of log (detector response to CE/detector response to ME); 3 = ANOVA of log (detector response to CE) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.84 ± 0.05.

** 134 d.f. for analysis 3.

TABLE IV

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF CHOLESTEROL DATA

Source variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.960	0.117	0.359
Amount (A)	45	22.184	0.604	0.344
L * A	3	0.098	0.058	0.042
Rods/L (R/L)	12	0.077	0.067	0.042
Error	135**	0.018	0.027	0.018
		Variance components		
Lots (L)		0.022	0.001	0.008
L * A		0.008	0.003	0.002
Rods/L (R/L)		0.015	0.010	0.006
Error		0.018	0.027	0.018

* 1 = ANOVA of log (detector response to C); 2 = ANOVA of log (detector response to C/detector response to ME); 3 = ANOVA of log (detector response to C) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.14 ± 0.10.

** 134 d.f. for analysis 3.

TABLE V

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF TRIGLYCERIDE DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.788	0.097	0.097
Amount (A)	45	22.107	0.626	0.084
L * A	3	0.114	0.071	0.049
Rods/L (R/L)	12	0.177	0.013	0.013
Error	135**	0.016	0.005	0.004
		Variance components		
Lots (L)		0.017	0.001	0.001
L * A		0.010	0.007	0.005
Rods/L (R/L)		0.040	0.002	0.002
Error		0.016	0.005	0.004

* 1 = ANOVA of log (detector response to TG); 2 = ANOVA of log (detector response to TG/detector response to ME); 3 = ANOVA of log (detector response to TG) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.88 $\pm$ 0.006.

** 134 d.f. for analysis 3.

TABLE VI

MEAN SQUARE VALUES AND VARIANCE COMPONENTS FROM ANALYSES OF FREE FATTY ACID DATA

Source of variation		*Analysis**		
		1	*2*	*3*
	d.f.		*Mean square*	
Lots (L)	4	0.904	0.171	0.168
Amount (A)	45	21.805	0.624	0.044
L * A	3	0.143	0.054	0.043
Rods/L (R/L)	12	0.187	0.020	0.019
Error	135**	0.016	0.004	0.004
		Variance components		
Lots (L)		0.019	0.003	0.003
L * A		0.013	0.005	0.004
Rods/L (R/L)		0.043	0.004	0.004
Errors		0.016	0.004	0.004

* 1 = ANOVA of log (detector response to FFA); 2 = ANOVA of log (detector response to FFA/detector response to ME); 3 = ANOVA of log (detector response to FFA) with log ME as covariate allowing for a single regression slope for all lots. Slope estimate 0.94 $\pm$ 0.05.

** 134 d.f. for analysis 3.

the results to any degree. The analyses indicated that for both CE and ME there was evidence of a curvilinear relationship between amount applied and detector response (see Fig. 1). However, *ca.* 80% of the total sums of squares for both compounds could be explained by including terms for the linear and quadratic effects, as well as lot differences, in the regression equation. The regression analysis of the ME data indicated that the regression lines were not parallel from lot to lot, a result that helps to explain why the lot* amount interaction could not be completely removed using a single slope in the analyses of covariance. It was also found that the regression equations generally did not pass through zero–zero, so that some change in the relationship can be expected as the detection limits are approached.

The regression coefficients presented in Table VII reflect the patterns illustrated in Fig. 1, that is, the responses of CE, C and ME are all similar and steeper than those of FFA and TG. The difference in slopes probably reflects a difference in detector response. When using the internal standard method, it is usually assumed that the ratio of the true concentration to detector response is the same for both the standard and the unknown. Hence, if ME is to be used as a standard for FFA and TG, an adjustment will be necessary to the standard formulation.

Another problem with the internal standard method is introduced by the non-linear standard curve for ME. The ratio of the detector responses for the lipids and ME did not remain constant over the range of amounts considered. In the present context, the impact of the non-linearity could be studied by using the ME value as a covariate rather than as the denominator in the ratio. On the log scale, the closer the regression coefficient β for the covariate is to unity, the more appropriate it is to use the ratio directly. Hence the analyses of covariance were carried out on the response values of the various lipids, with the ME detector response as a covariate; the results are given as analysis 3 in Tables III–VI. Generally, the estimates of the variance components for lots and rods within lots were similar or somewhat smaller than those of analysis 2. The most noteworthy result, however, was the substantial reduction in the amount mean square for all compounds, but especially for TG and FFA.

While the β estimates for CE, TG and FFA (0.84, 0.88 and 0.94, respectively) were all near unity, the accuracy was improved by taking account of the non-linearity, probably because the slight discrepancies were exaggerated by the relatively large range of concentrations. It is interesting to note that for C, the only compound with an estimate considerably different from unity (0.14), the error was actually increased by using the internal standard method (analysis 2), *i.e.*, by assuming $\beta = 1$.

One of the difficulties associated with the analyses of Tables III–VI was that it was impossible to determine the relative contributions of interaction and random error to the lot * amount and error mean squares. In order to present the error mean square as an estimate of the random variation from determination to determination within rod, it was necessary to assume that there was no rod * amount interaction. To examine this issue more closely, a second study was carried out, in which repeated measurements were taken at the 6-μg level, thereby eliminating interaction components involving amounts. The results (not presented here) were similar to those of the first study, with the corresponding entries for analysis 3 never differing by more than a factor of 2. The estimates were especially close for TG where, for example, the rod within lot and error mean squares of the second study were 0.017 and 0.004, respectively. With the exception of the lot mean squares, the results in the second

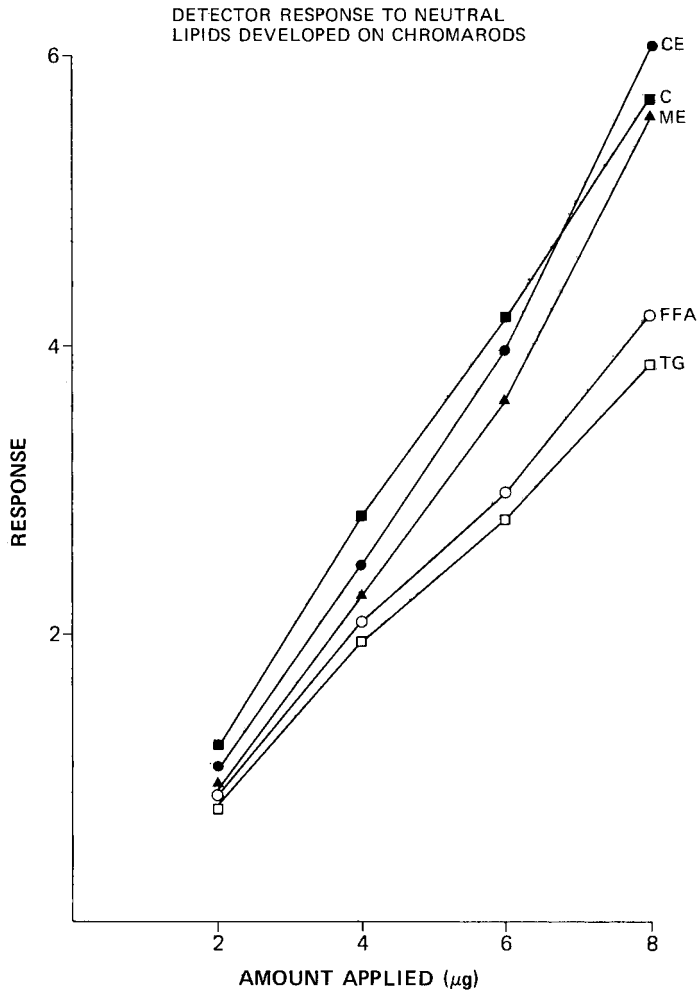

Fig. 1. Plot of detector response *vs.* the amount of lipid applied (2, 4, 6 or 8 μg) for cholesterol ester (CE), cholesterol (C), methyl ester (ME), free fatty acid (FFA) and triglyceride (TG). Each point is the mean of five lots.

study were usually somewhat smaller, suggesting that there was perhaps some interaction between amounts and lots in the original study. The fact that the replicate* lot mean squares in the second experiment were considerably larger than the corresponding error terms suggests that the random variation among replicates for a lot as a whole cannot be attributed solely to the precision of the individual rods. The estimates of β were generally somewhat smaller in this second study, the only exception being for C(0.24) but this pattern remained consistent with the estimate for C much smaller than for the other compounds.

TABLE VII

REGRESSION ANALYSIS* ON IATROSCAN LIPID DATA

Source of variation	*df.*	*CE*	*ME*	*TG*	*FFA*	*C*
			Mean squares			
Lots (L)	4	4.72	1.92	2.45	3.13	8.01
Amounts (A)	3	227.32	193.60	86.38	101.03	185.28
Linear**	1	675.63	575.03	258.26	301.63	555.22
Quadratic**	1	5.57	4.94	0.02	0.23	0.05
Cubic**	1	0.74	0.83	0.85	1.22	1.75
A * L	12	1.56	1.83	0.74	1.07	1.55
Lots * linear	4	0.97	1.85	0.66	1.04	0.99
Residual	8	1.85	1.82	0.79	1.09	1.84
Rods/L	45	0.87	1.08	0.99	1.21	1.20
Error	135	0.21	0.25	0.19	0.21	0.37
Linear*** regression coefficient		0.82 ± 0.02	0.75 ± 0.02	0.49 ± 0.02	0.54 ± 0.02	0.74 ± 0.02

* Using amount of lipid as independent variable.

** Based on orthogonal polyomials[12].

*** Based on an equation including only the linear, and not the quadratic and cubic, terms.

CONCLUSIONS

Several workers have investigated the use of an internal standard to improve on the quantitative capabilities of the Iatroscan technique[5,7,8]. However, statistical analyses to determine if the precision of the results from the rods had been increased by the use of internal standards were not included in their reports. Our results show that the variability in the measurements from the Iatroscan method can be improved by including an internal standard in the test solution. This approach does improve the precision of the method.

The use of an internal standard assumes a relationship between the concentration of the lipid and the concentration of the standard of the form:

$$\text{true concentration of lipid} = \frac{\text{detector response to lipid}}{\text{detector response to standard}} \times \text{true concentration of standard}$$

In some applications, it will be necessary to adjust this formula to allow for differences in detector response[5]. The results of the present study indicate that use of the internal standard method with Iatroscan measurements will improve precision considerably. However, it was found that the standard curves were not always linear. The effect of the slight departure from linearity was notable only in the amounts mean square, probably because of the magnitude of this term relative to the other mean squares. Hence, care should be taken to ascertain whether or not standard curves relevant in a particular application are linear. While precision is unlikely to be affected unduly by a slight lack of linearity, it may be necessary to make adjustments to

the internal standard method in order to enhance the accuracy of the method, especially if a wide range of concentrations are expected.

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USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO QUANTITATE THYMINE-CONTAINING PYRIMIDINE DIMERS IN DNA

JACK D. LOVE and ERROL C. FRIEDBERG*
Laboratory of Experimental Oncology, Department of Pathology, Stanford University, Stanford, CA 94305 (U.S.A.)
(Received December 24th, 1981)

SUMMARY

We have developed two high-performance liquid chromatographic systems for the measurement of pyrimidine dimers in hydrolysates of DNA. Normal-phase chromatography on an NH_2 column in methanol–ethyl acetate (3:97) at an elution rate of 2.0 ml/min allowed quantitation of thymine-containing (thymine–thymine plus thymine–uracil) pyrimidine dimers at levels as low as 0.1 % of the total radioactivity as thymine in DNA. This system was unaffected by the presence of up to 1 mg of contaminating protein (bovine serum albumin) or 40 μg of DNA in hydrolysates prepared for chromatography. Reversed-phase chromatography on a μBondapak C_{18} column allowed measurement of thymine–thymine dimers at concentrations as low as 0.02 % of the total radioactivity. With 0.1 % tetrahydrofuran in water as the solvent at a flow-rate of up to 0.6 ml/min, thymine–thymine, thymine–uracil, and uracil–uracil dimers were completely resolved. We were not able to quantitate the latter two dimeric forms, however, owing to the presence of other radioactive components of undefined origin that eluted concomitantly with the uracil-containing dimers.

INTRODUCTION

The accurate measurement of thymine-containing pyrimidine dimers is an important and widely-used technique, since these photoproducts serve as the lesion in one of the most extensively studied models for the repair of damaged DNA. Various procedures have been employed for the quantitative separation of thymine-containing dimers from thymine monomer in hydrolysates of radiolabeled ultraviolet (UV)-irradiated DNA. These include thin-layer chromatography (TLC)[1,2], paper chromatography[3,4], ion-exchange chromatography,[5,6] high-performance liquid chromatography (HPLC)[7] and others[8,9]. Most published procedures are somewhat cumbersome and time-consuming, and, except for the use of HPLC, are limited because of the extreme sensitivity required for accurate measurement of very low levels of thymine dimers (*i.e.*, in the range 0.01–0.02 % of total thymine in DNA).

Breter *et al.*[7] reported the measurement of thymine-containing pyrimidine

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dimers down to 0.01% of the total thymine content of DNA by cation-exchange HPLC. However, their technique requires the use of a column (300 × 0.18 cm) that is not commercially available. In addition, the back pressure generated by the use of such a long and narrow column requires heating the column to 75°C to reduce the viscosity of the solvent to a level that facilitates elution at less than the 6000 p.s.i. that most available pumps can generate. A recent report by Cadet *et al.*[10] demonstrated that the four isomers of cyclobutyl thymine-containing pyrimidine dimers could be separated by reversed-phase HPLC. However, these investigators did not report the measurement of thymine-containing pyrimidine dimers in hydrolysates of UV-irradiated DNA.

We have previously reported the use of TLC techniques that reliably measure thymine-dimer contents of *ca.* 0.2% or higher[2]. We have recently sought to develop methods that are both faster and more sensitive than TLC. In this paper, we describe two HPLC procedures for the measurement of thymine-containing pyrimidine dimers in hydrolysates of radiolabeled DNA. Both procedures use commercially available equipment and each has its own merit for particular applications.

MATERIALS AND METHODS

Instrumentation and supplies

We used a Waters Associates liquid chromatograph (Model 6000 A pump and U6K injector) attached to a variable-wavelength UV-detector (Model 450) and the following HPLC columns from Waters Associates: μBondapak C_{18} (30 cm × 3.9 mm), and μStyragel (100 Å column, 30 cm × 7.8 mm). We also used an NH_2 column (25 cm × 4.6 mm) and a Nucleosil C_{18} column (25 cm × 2.1 mm) from Alltech Associates. All solvents were Burdick and Jackson UV-grade. Thymine, thymidine and uracil were from Sigma (St. Louis, MO, U.S.A.). [2-^{14}C]Uracil and [methyl-^{3}H]-thymine were from Amersham. [Methyl-^{3}H]thymidine was from ICN.

The solvent output was connected to a Gilson Mini-Escargot or Micro-frac (for high flow-rates) fraction collector. Aqueous samples were collected and transferred to liquid scintillation vials containing 1.0 ml of water and 10.0 ml of aqueous counting fluor [1 part of Triton X-100 plus 2 parts of toluene-based Omnifluor (New England Nuclear)] for radioactivity determinations. Organic solvents were added directly to 5.0 ml of toluene-based Omnifluor for liquid scintillation counting. The quenching by 2.0 ml of ethyl acetate–methanol was approximately 25%; as it was uniform in all fractions, no quench corrections were made.

Preparation of pyrimidine dimer standards

Authentic pyrimidine dimers were prepared for use as chromatographic standard markers by UV-irradiating the free base (thymine, uracil or both) in the frozen state with 12 kJ/m^2 at 254 nm, essentially as described by Beukers and Berends[11]. Dimers were separated from monomer bases by TLC as described by Cook and Friedberg[1] or by Reynolds *et al.*[2], and were harvested by elution from the silica layer matrix by soaking the relevant regions of the chromatograms overnight in distilled water. Each water fraction was dried down, and the dimer residue was redissolved in the appropriate solvent.

During the hydrolysis of DNA before chromatographic resolution of dimers

from monomeric bases, cytosine is deaminated to form uracil[12]. Therefore, we prepared markers representing cytosine–cytosine or thymine–cytosine dimers by UV-irradiating uracil or a thymine–uracil mixture, respectively. As regards the latter, we irradiated [2-^{14}C]uracil in the presence of a 100-fold excess of thymine to ensure that the majority of radioactively labeled dimers were of the mixed (U < >T) form.

Preparation of hydrolysates of radiolabeled DNA from Escherichia coli

E. coli B was labeled with [methyl-^{3}H]thymidine or thymine in L-broth (5 g/l of yeast extract, 10 g/l of tryptone, 5 g/l of NaCl) for at least two generations. Cells were harvested by centrifugation, washed free of medium, resuspended in 50 m*M* Tris–HCl (pH 7.6), 10 m*M* EDTA, 100 μg/ml of lysozyme and 200 μg/ml of RNase A, and incubated at 37°C for 60 min or until the suspension became very viscous. Cell debris was removed by centrifugation at 6000 *g* for 10 min. DNA in the aqueous phase was extracted extensively with buffer-saturated phenol, chloroform–isoamyl alcohol (20:1), and diethyl ether, successively, until the ratio of absorbance at 260 nm to that at 280 nm was >1.85. The final specific radioactivity was between 14,000 and 28,000 cpm/μg of DNA.

DNA was irradiated under UV light with constant stirring. The UV-light source was a standard 15-W germicidal bulb (General Electric G8T5); the UV fluence was determined with a calibrated photometer (International Light Model IL254), and detector (Model PT100), and was corrected for absorbance at 254 nm by the method of Morowitz[13].

Irradiated DNA (not more than 100 μg of DNA per tube) was dried in a vacuum centrifuge (Speed Vac, Savant, Inc.) and solubilized in 0.2 ml of 97% formic acid. Solutions were placed in 75 × 10 mm ignition tubes, and the ends were sealed as described[2]. Hydrolysis was typically for 1 h at 175°C, but was for up to 4 h at 220°C in certain experiments. Following heating, the tubes were submerged in liquid nitrogen until frozen. Frozen tubes were opened by breaking the tips with a hemostat clamp. Hydrolysates were then dried in a vacuum centrifuge and redissolved in the appropriate solvent.

Calculations and measurements by HPLC

Since the advent of the widespread use of HPLC technology, a number of specifically defined terms have become accepted parameters that allow for quantitative comparisons between different chromatographic systems; the interested reader is referred to references 14–16 for detailed explanations of these terms.

The retention volume (V) of a given component during HPLC was measured as the distance (mm) from the point of injection to the eluted peak of the component. The width (W) of eluted peaks was determined by drawing tangents to the linear portion of the sides of the peaks to the baseline and measuring the distance (mm) between the intercepts. The total volume of each column was calculated from the known dimensions. Void volumes (V_0) were determined by injection of water into the column and observing the position of the peak caused by the Schlieren effect. The capacity factor (k') was calculated for each component as the ratio $(V - V_0)/V_0$. The selectivity factor (α) was calculated as the ratio k'_2/k'_1, where k'_1 is for thymine–thymine dimers and k'_2 is for thymine. Resolution $(R_s) = 2(V_2 - V_1)/(W_1 + W_2)$, where V_1 is the retention volume and W_1 is the peak width for thymine–

thymine dimers, and V_2 is the retention volume and W_2 is the peak width for thymine. Column efficiency (N) was calculated by the formula $N = 16(V/W)^2$.

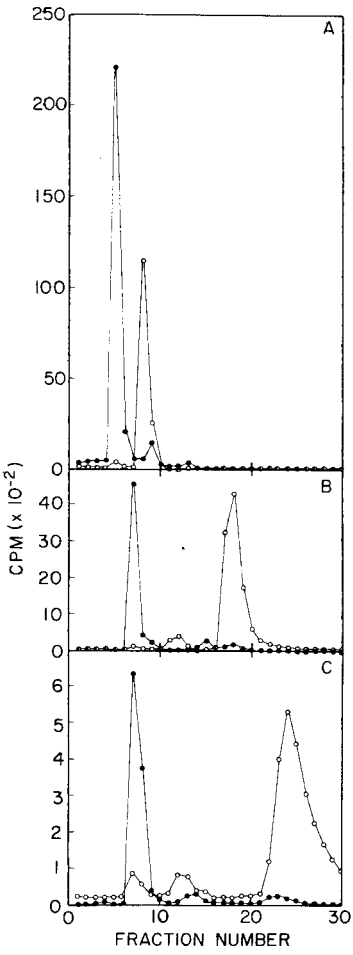
RESULTS

Normal-phase chromatography (NH_2 column)

Both thymine and thymine-containing pyrimidine dimers contain several polar groups. In particular, the N-1 and N-3 groups of pyrimidines carry lone-pair electrons that have potential for hydrogen-bonding. In addition, the covalent hydrogens of these secondary amines can form hydrogen-bonds. The double-bonded oxygens at C-2 and C-4 are negatively polarized and thus should be able to form bonds with amine hydrogens. Consequently, the choice of a column containing bound amine groups (*i.e.*, NH_2) for the chromatographic resolution of thymine from thymine dimers seemed logical. When either 100% methanol or pure water was used as the exclusive solvent, both thymine and dimers eluted at $k' = 0$ (*i.e.*, at the solvent front). Fig. 1 shows the elution profiles of thymine and thymine–thymine dimer standards at several solvent strengths. In every instance, thymine eluted ahead of the dimer. We settled on a compromise between reduced separation time and increased separation distance by establishing our routine elution solvent as 3.0% of methanol in ethyl acetate, and this solvent was used for the remainder of the studies described. Pure ethyl acetate is a solvent with an intermediate polarity index[17], in which neither thymine nor dimers have much solubility[18]. Both components were somewhat soluble in 100% methanol, 100% *n*-propanol or 100% tetrahydrofuran, and all of these solvents are miscible with ethyl acetate. We noted that 3% of methanol in ethyl acetate absorbed air very readily and therefore we degassed the solvent by filtration through a sintered-glass filtration apparatus (Millipore) fitted with a PTFE filter (pore size 0.2 μm) at least once a day. We also noted that different lots of ethyl acetate had different properties. Therefore, when changing lots, it was often necessary to readjust the exact percentage of methanol required for reproducible elution profiles.

By using the system just described, we carried out a series of experiments with hydrolysates of UV-irradiated *E. coli* DNA labeled as described in Materials and methods. For these experiments, we prepared markers of pyrimidine dimers containing thymine only, thymine and uracil, or uracil only, as described in the Materials and methods section. The uracil-containing dimers are representative of cytosine-containing dimers that occur naturally in DNA exposed to UV radiation, because, as previously indicated, cytosine is deaminated to uracil during hydrolysis at 175°C in formic acid[12]. The uracil-containing dimers (U<>U and U<>T) eluted just behind the thymine–thymine dimers at about 34 to 40 min (Fig. 2), and the U<>T dimers were included in the calculation of total thymine-containing pyrimidine dimers. In practice, one ordinarily labels only thymine in DNA, and hence would detect only thymine-containing dimers (*i.e.*, not U<>U dimers).

Table I shows a comparison of the results obtained by HPLC and by TLC. Replicate samples at a given dimer content gave reasonably comparable results by either method, but the accuracy of the measurement decreased progressively as the dimer content was lowered. The measurement of thymine-containing pyrimidine dimers by normal-phase HPLC was relatively unaffected by the presence of protein during hydrolysis and elution (Table II). Table III shows that the total DNA content



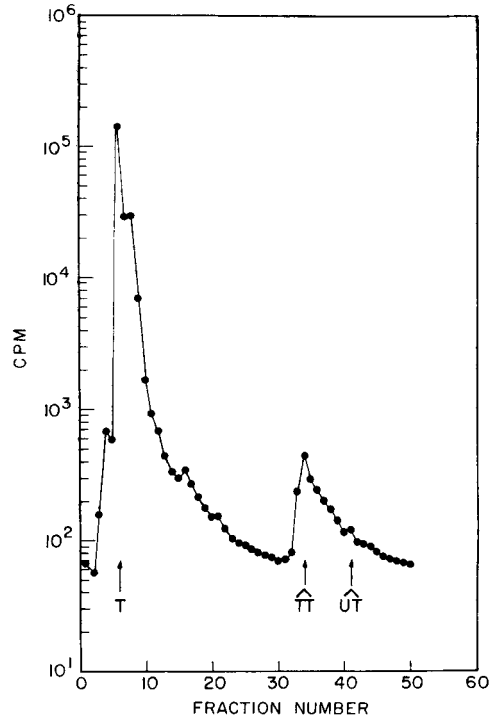


Fig. 1. Separation of thymine and thymine–thymine dimers by normal-phase chromatography. The ^{14}C-labeled thymine–thymine dimers (○) and ^{3}H-labeled thymine (●) were prepared and purified as described in Materials and methods. Chromatography on an NH_2 column (0.46 × 25 cm) was at 2.0 ml/min, and 2.0-ml fractions were collected and measured for radioactivity as described in the text. The elution solvents contained the following amounts (%) of methanol in ethyl acetate: A, 10; B, 8; C, 3.

Fig. 2. Chromatography of a hydrolysate of *E. coli* DNA that had been UV-irradiated as described in the text and contained 0.58% of thymine-containing pyrimidine dimers as total radioactivity. Hydrolysis of the DNA was at 170 C for 1 h. Chromatography and measurement of radioactivity were under the conditions described in Fig. 1, with methanol–ethyl acetate (3:97) as elution solvent. The arrows indicate the peak positions of the standard markers shown.

can be varied over at least a 150-fold range with only a small effect on the measured dimer content.

The single most significant factor that limited the accurate quantitation of very low levels of thymine-containing pyrimidine dimers by normal-phase chromatography was the consistent tailing of the thymine peak into the dimer area (Fig. 2). This problem was not satisfactorily eliminated, even when the flow-rate was reduced to as little as 0.2 ml per min. When we collected the peak thymine fractions and con-

TABLE I

MEASUREMENT OF THYMINE-CONTAINING PYRIMIDINE DIMERS BY HPLC AND BY TLC

E. coli DNA was UV-irradiated as described in the text to give varying dimer contents. Thymine-containing pyrimidine dimers in each sample were then quantitated both by TLC and by HPLC. Samples were eluted isocratically on an NH_2 column with methanol–ethyl acetate (3:97) at 2.0 ml per min, and 2.0-ml fractions were collected. The TLC was performed as described by Reynolds *et al.*[2]. The figures to the right of each quoted dimer value are the standard deviations and the percentages of the mean encompassed by the standard deviations.

Experiment No.	*Thymine dimers (% of total radioactivity)*	
	HPLC	*TLC*
1	6.36 (10)*–0.44 (6.9%)	6.89 (4)*–0.53 (7.8%)
2	3.29 (7)–0.27 (8.3%)	4.15 (4)–0.51 (12.1%)
3	1.29 (4)–0.15 (11.7%)	1.76 (3)–0.21 (11.8%)
4	0.55 (4)–0.10 (18.2%)	–
5	0.076 (4)–0.023 (30.3%)	–

* Number of replicates.

centrated and re-chromatographed them, the tail reappeared, suggesting poor column efficiency as a factor contributing to the tailing (data not shown). In addition, when we collected the tail region in separate fractions and concentrated and re-chromatographed it, we recovered two peaks, one at the original position of the tail and one at the position of thymine (data not shown). This indicated that the tailing also represented the presence of some non-thymine radiolabeled material, perhaps arising from sample hydrolysis or from breakdown of multiply-labeled thymine by tritium decay. We also observed that [^{3}H]thymidine and thymine stocks delivered by

TABLE II

EFFECT OF ADDED PROTEIN ON THYMINE-DIMER MEASUREMENTS

The effect of adding protein (bovine serum albumin) to DNA samples before hydrolysis is shown. The samples were chromatographed on an NH_2 column as described in Table I. After hydrolysis, samples were dried in a vacuum centrifuge, and 100 µl of 100% methanol were added to each. Samples were vigorously mixed (vortex-type mixer) and precipitates were removed by centrifuging for 2 min in an Eppendorf Microfuge before HPLC. Each value represents the average of two analyses.

Added bovine serum albumin (µg)	*Thymine dimers (% of total radioactivity)*
25	6.8
50	6.2
100	6.3
250	5.5
500	5.9
1000	6.0

TABLE III

EFFECT OF TOTAL RADIOACTIVITY AND MASS OF DNA ON THYMINE-DIMER MEASUREMENTS

The effect of total radioactivity and mass of DNA on the measured dimer content is shown. Samples, after treatment as described in Table II, were chromatographed on an NH_2 column as described in Table I. The volume of the samples varied from 3 to 20 μl. Each value represents the average of two analyses.

Total mass of DNA dimers (μg)	*Total radioactivity as DNA (cpm)*	*Thymine (% of total radioactivity)*
0.26	3600	6.6
0.98	13,500	6.4
2.72	37,500	6.2
5.43	75,000	6.7
41.67	575,000	6.0

the manufacturer eluted with a significant tail before hydrolysis, the severity of which increased with age over a few months.

With samples containing relatively high levels of thymine-containing pyrimidine dimers, as shown in Table III, the tailing problem had little effect on quantitation, even when >500,000 cpm were present as thymine. However, at thymine-dimer levels as low as 0.1%, the presence of more than 200,000 cpm as thymine precluded accurate quantitation of the dimer species (data not shown).

Gel-permeation chromatography

In light of the limitations of normal-phase chromatography using the system described above, we sought a means by which we could reverse the elution order of the two peaks, since, if the dimers could be eluted first, tailing from the thymine peak would not present a problem in their accurate quantitation. We initially experimented with gel-permeation chromatography on a μStyragel column. This matrix is quoted to have a resolving capacity in the molecular mass range of >100 to <700 daltons. Thymine is 125 daltons in mass, whereas the thymine–thymine dimer is twice that value. In gel-permeation chromatography, larger molecules elute first, so that we anticipated the elution of dimers ahead of thymine. Although the expected result was achieved, the actual resolution was poor ($R_s = 0.18$, $\alpha = 1.05$) when only 600 cpm as radioactivity in dimers were chromatographed (data not shown). Resolution was further decreased as the amount of radioactivity in the dimer peak was increased to 6000 cpm ($R_s = 0.13$), due to increased peak width (data not shown). Slower flow-rates somewhat reduced the band width, but not enough to achieve the required resolution.

Reversed-phase chromatography

We successfully reversed the elution order and achieved satisfactory resolution of thymine from thymine-containing pyrimidine dimers by reversed-phase chromatography on a C_{18} column, with a mixture of water and tetrahydrofuran as the elution solvent. We compared the resolving capacity of two C_{18} columns for the standard markers referred to above, over a range of solvent strengths (Fig. 3 and Table IV).

Surprisingly, the Nucleosil C_{18} column, with a particle size averaging 5 μm, had a lower efficiency than the μBondapak C_{18} column, with a particle size of about 10 μm, at the same solvent strength. Essentially, the same elution profile was observed at tenfold lower solvent strength with the former column, but, due to the difference in column dimensions and respective void volumes, the k' values were approximately the same at similar solvent strengths. When we eluted from the Nucleosil C_{18} column using pure water as the solvent, the maximum k' for thymine was 8.2. The same conditions generated a k' of 6.8 for thymine with the μBondapak C_{18} column.

We then used reversed-phase chromatography for the resolution of thymine and thymine-containing dimers present in hydrolysates of UV-irradiated radiolabeled

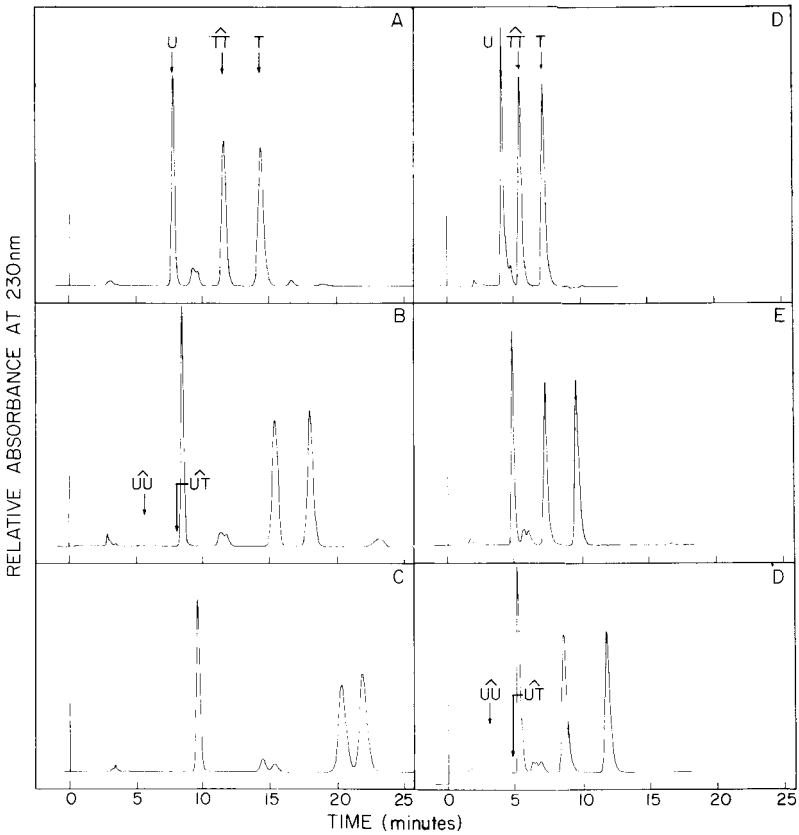


Fig. 3. Reversed-phase chromatography showing the separation of pyrimidine monomers from various pyrimidine-dimer standards. Thymine, thymine–thymine dimers, uracil–thymine dimers and uracil–uracil dimers were prepared as described in Materials and methods. Chromatography on μBondapak C_{18} (panels A–C) and on Nucleosil C_{18} (panels D–F) columns was at 0.3 ml/min, and the effluents were monitored at 230 nm (0.4 a.u.f.s.). The total sample volume injected was 0.05 ml in each instance. In panels B and F, the eluting positions of uracil–uracil (U < > U) and uracil–thymine (U < > T) dimers are indicated with arrows. These positions were determined by collecting 4-drop fractions (*ca.* 0.1 ml) and counting radioactivity. The solvents used contained the following amounts (%) of tetrahydrofuran in water: Panels A and D, 0.4; panels B and E, 0.1; panels C and F, 0.

TABLE IV

QUANTITATIVE PARAMETERS REFLECTING EFFICIENCY OF HPLC BY REVERSED-PHASE CHROMATOGRAPHY

Some useful descriptive values for reversed-phase chromatography at various solvent strengths are shown. Markers were prepared as described in the text. See "*Calculations and measurements by HPLC*" for identification of symbols and for calculation procedures. Values for all markers were obtained from strip-chart recordings of the absorbance at 230 nm. Samples were eluted in the solvent indicated at 0.3 ml per min. Retention volume (V) and peak width (W) are presented in mm as described in Materials and methods; 1 mm = 0.06 ml.

Component	*For μBondapak C_{18} column*						*For Nucleosil C_{18} column*					
	V	k'	W	α	N	R_s	V	k'	W	α	N	R_s
With pure water as mobile phase												
T	223	6.8	11	1.1	6600	1.5	138	7.4	9	1.5	3800	4.5
T< >T	207	6.3	10		6900		100	5.1	8		2500	
Thymidine	>600*						319	18.3	25		2600	
U	94	2.3	6		3900		52	2.2	5		1700	
With aqueous 0.1% tetrahydrofuran as mobile phase												
T	178	5.2	9	1.2	6300	3.0	95	4.8	8	1.4	2300	3.1
T< >T	152	4.3	8.5		5100		72	3.4	7.0		1700	
Thymidine	554	18.5	31		5100		240	13.5	17		3200	
U	84	1.9	6		3100		48	1.9	4.5		1400	
*With aqueous 0.4%** tetrahydrofuran as mobile phase*												
T	141	4.0	9	1.3	3900	3.4	70	3.2	7	1.5	1600	2.6
T< >T	114	3.0	7		4200		53	2.2	6		1200	
Thymidine	344	11.0	19		5200		146	7.8	10		3400	
U	77	1.7	5		3800		40	1.4	4.5		1200	
With aqueous 1.0% tetrahydrofuran as mobile phase												
T	100	2.5	9	1.7	2000	3.8						
T< >T	72	1.5	6		2300							
Thymidine	174	5.1	10		4900							
U	61	1.1	5		2400							

* Thymidine never came off this column in pure water.

** Aqueous 0.5% tetrahydrofuran was used with the Nucleosil column.

DNA (Fig. 3 and Table V). We obtained the most satisfactory results using the μBondapak C_{18} column with 0.1% of tetrahydrofuran in water as the solvent at a flow-rate of 0.3 ml/min. We have also performed these analyses at 0.6 ml per min (the upper limit for collecting 0.1-ml fractions by the Gilson Mini-Escargot) with no discernible effects on peak width or resolution. When we used hydrolysates of DNA, we could still resolve uracil–uracil, uracil–thymine, and thymine–thymine dimers. However, quantitation of the former two species was consistently precluded by the presence of unidentified contaminating peaks of radioactivity, the source of which

TABLE V

MEASUREMENT OF THYMINE–THYMINE DIMERS IN DNA BY REVERSED-PHASE CHROMATOGRAPHY

Precision and lower limit of detection of thymine–thymine dimers by reversed-phase chromatography. The samples were chromatographed as described in the text. Samples A–E were prepared by irradiating *E. coli* DNA with the following UV fluences (J m^{-2}) corrected for the absorbance at 254 nm of the sample as described in Materials and methods: A, 30; B, 15; C, 6; D, 3; E, 1. Samples F and G were prepared by mixing ^{3}H-labeled thymine–thymine dimers, prepared by irradiating frozen thymine solutions and purified by TLC (see Materials and methods), with unirradiated hydrolyzed [^{3}H]thymine-labeled *E. coli* DNA.

Sample	*T< >T (%)*	*Number of replicates*	*Standard deviation*
A	1.02	3	0.06
B	0.46	3	0.01
C	0.31	3	0.03
D	0.21	3	0.01
E	0.13	3	0.02
F	0.033	4	0.003
G	0.022	4	0.005

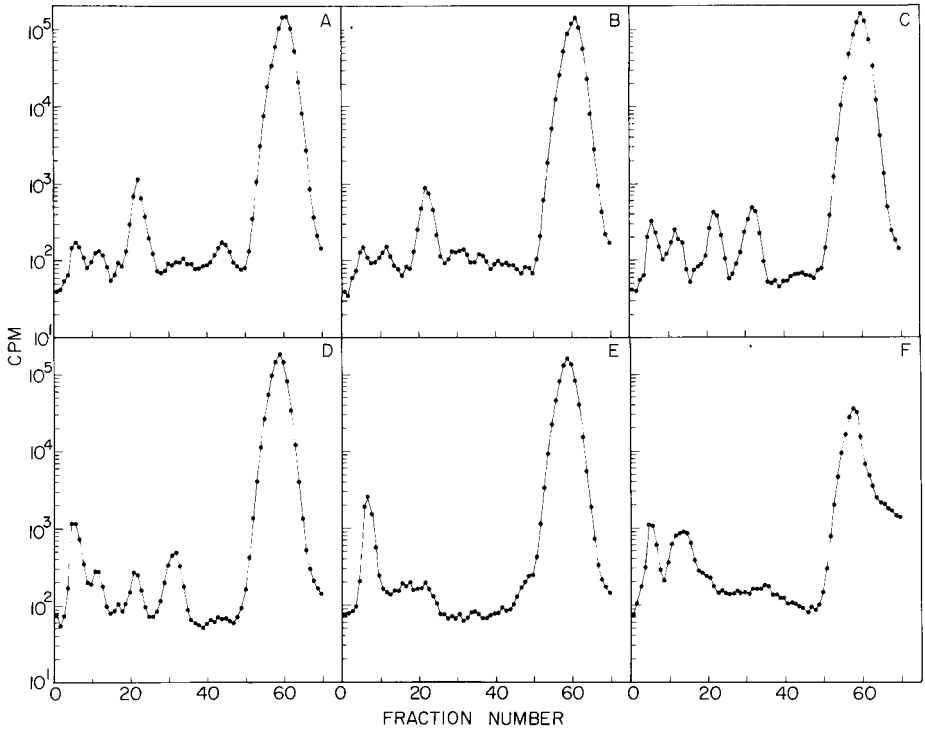


Fig. 4. Reversed-phase chromatography on hydrosylates of unirradiated *E. coli* DNA. The [^{3}H]thymine-labeled unirradiated *E. coli* DNA was prepared and hydrolyzed as described in the text. Hydrolysis was at 170°C (panels A–C) or 220°C (panels D–F) for the following times: A, 30 min; B, 1 h; C, 4 h; D, 1 h; E, 2 h; F, 20 h. Chromatography on μBondapak C_{18} was at 0.3 ml/min, and 5-drop (0.12-ml) fractions were collected; only fractions 11–80 were counted. The elution solvent was 0.1% of tetrahydrofuran in water.

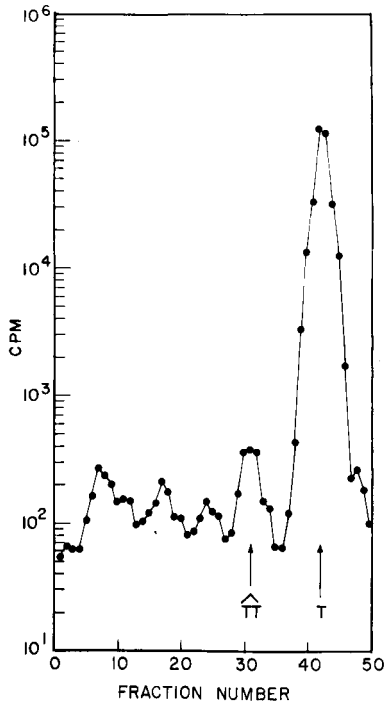


Fig. 5. Reversed-phase chromatography of a hydrolysate of UV-irradiated *E. coli* DNA. The [^{3}H]thymine-labeled *E. coli* DNA was prepared as described in the text and UV-irradiated to yield a thymine–thymine dimer content of 0.34 % of the total radioactivity. Hydrolysis was at 220°C for 2 h. Chromatography was as described in Fig. 4. The first 20 fractions eluted were not measured for radioactivity, since none was detected in this region of the chromatogram in numerous previous experiments carried out under identical conditions. The positions of thymine (T) and thymine–thymine dimers (T< >T) are indicated. The exact elution positions of U< >T and U< >U dimers are not shown, but are to the left of the T< >T dimers (see Fig. 3).

was independent of irradiation of the DNA (Figs. 4 and 5). The measurement of thymine–thymine dimers was unaffected by this problem, and this species could be readily quantitated in hydrolysates of UV-irradiated DNA (Table V and Fig. 5).

In order to test the lower limits of the sensitivity for measuring thymine–thymine dimers by reversed-phase chromatography, we mixed ^{3}H-labeled dimers prepared by irradiating frozen thymine and purified by TLC, with hydrolysates of unirradiated labeled *E. coli* DNA. We used this approach to avoid the difficulty of accurately measuring the extremely small UV fluences (< 1 J m^{-2}) required for direct irradiation of the DNA. The results shown in Table V (samples F and G) indicate that this technique can be used to measure thymine–thymine dimers down to at least 0.02 % of the total radioactivity. This level of quantitation makes the technique useful for studying thymine–thymine dimer excision at levels of UV radiation well within the biological dose range for both prokaryote and eukaryote cells.

For the measurement of thymine–thymine dimers at levels below 0.1 % by reversed-phase chromatography, we found it necessary to adhere to the strictest technical guidelines. We observed that radiolabeled DNA generated thymine degradation products after only a few weeks during storage at 4°C. One of these products had a k'

value of 4.8 that placed it between the dimers and thymine and hence interfered with accurate measurements. We have avoided this potential problem by using freshly prepared radiolabeled DNA. In addition, certain conditions of hydrolysis preclude the formation of some interfering species. We compared the results of hydrolysis of radiolabeled unirradiated DNA in 97% formic acid at 170°C or at 220°C for various times (Fig. 4). Hydrolysis at 220°C consistently generated a lower background with greater separation between thymine and interfering peaks than did hydrolysis at 170°C. Therefore, we routinely hydrolyze DNA samples at 220°C for 2 to 4 h. Under these conditions, sealed ignition tubes build up a great deal of pressure, probably due to liberation of gases. Thus, these tubes must be handled with extreme caution because they are highly explosive. We allow them to stand in liquid nitrogen for at least 15 min, until the gases have liquified, before opening them. Finally, it is noteworthy that we have observed that as much as 20% of the radioactivity in stocks of [methyl-^{3}H]thymine, as shipped to us by the manufacturer, did not elute at the position of authentic thymine (unpublished results). We recommend purifying radiolabeled DNA precursors before labeling cells when thymine-dimer detection is desired at levels lower than 0.1%.

DISCUSSION

We have presented two methods for the separation and quantation of thymine-containing pyrimidine dimers using standard commercially available HPLC equipment. With normal-phase chromatography on a NH_2 column and with 3.0% of methanol in ethyl acetate as eluting solvent, we can clearly separate radiolabeled thymine from uracil–thymine and thymine–thymine pyrimidine dimers present in hydrolysates of UV-irradiated *E. coli* DNA. The two dimer species elute as a single peak of radioactivity and collectively constitute a measure of thymine-containing pyrimidine dimers in DNA. This technique has the significant advantage of being relatively unaffected by the presence of considerable amounts of protein or DNA in the hydrolysates and is recommended for the measurement of thymine-containing pyrimidine dimers at levels of 0.1% or higher. However, since thymine elutes ahead of the dimers during normal-phase chromatography, even slight tailing of the vast excess of radioactivity that constitutes the thymine peak into the relative paucity of radioactivity present in the dimer peak limits accurate measurement of the latter peak at levels below 0.1% of total radioactivity.

The order of elution of thymine and thymine dimers can be altered by reversed-phase chromatography. For such work, we have found the μBondapak C_{18} column to be the most satisfactory. For the most sensitive measurements we recommend the use of 0.1–0.4% of tetrahydrofuran in pure water as the eluting solvent. This system separates thymine from thymine–thymine, thymine–uracil, and uracil–uracil dimers and in theory could allow the quantitation of total pyrimidine–dimer contents of UV-irradiated DNA. However, despite all manner of experimental precautions, we have thus far consistently observed that the regions of the chromatogram containing thymine–uracil and uracil–uracil dimers are contaminated by very small amounts of unidentified radiolabeled products, even in unirradiated DNA (Fig. 4). Such products could result from radiation damage associated with the use of DNA of high specific radioactivity, as well as from the use of multiply-labeled thymine or thymidine as a source of radiolabel. With respect to the latter, we have carried out experiments using singly labeled (ring-labeled) thymine for preparing radioactive DNA. However, this problem has not been significantly alleviated. Reversed-phase chromatography does,

however, allow the measurement of thymine–thymine dimers at levels at least as low as 0.02% of the total radioactivity.

The level of sensitivity achieved by reversed-phase chromatography represents a significant improvement over other chromatographic techniques for separating very small amounts of thymine dimers from thymine; thus, we would recommend this particular procedure for biological studies on living cells. In addition, with a total chromatographic time of 20 min, this procedure has the distinct advantage of being much more rapid than most other published techniques. In applying a procedure such as this, in which one is attempting to resolve *ca.* 99.98% of the total radioactivity from the remaining 0.02%, it is mandatory that scrupulous experimental technique be used. We thus recommend pre-purification of thymine or thymidine used for labeling the DNA of living cells, and do not recommend storing radioactively labeled DNA for more than about 2 weeks. Finally, DNA should be extensively purified from biological sources before hydrolysis, since we have observed that protein and large amounts of hydrolyzed nucleic acids had adverse effects on the resolution of peaks in reversed-phase chromatography.

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Note

Compensation of baseline drift in temperature-programmed capillary gas chromatography with electron-capture detection

G. BENGTSSON*
Laboratory of Ecological Chemistry, Ecology Building, Helgonavägen 5, S-223 62 Lund (Sweden)
and
C. CAVALLIN
Varian AB, Ribersborgsvägen 17, S-217 53 Malmö (Sweden)
(Received December 15th, 1981)

Reduction in the standing current of an electron-capture detector due to bleeding from the stationary phase is one of the major problems in temperature programmed gas chromatography–electron-capture detection (GC–ECD)[1]. The reduction may arise from trace contaminant vapours in the carrier gas or pyrolysis products of the stationary phase. Bleeding increases exponentially with temperature[2], producing a dramatically rising baseline at higher oven temperatures, which can make quantitation of peaks difficult. Use of non-polar phases reduces bleeding effects, while high-molecular-weight compounds with large electron affinities may cause severe baseline drift with polar phases.

The conventional approach to compensate for baseline drift, dual column analysis, is usually not practised in capillary GC–ECD owing to the high cost of duplicating the detector and column. Recently, a single column compensation method, which stores the column bleed profile during a blank run, was presented[3].

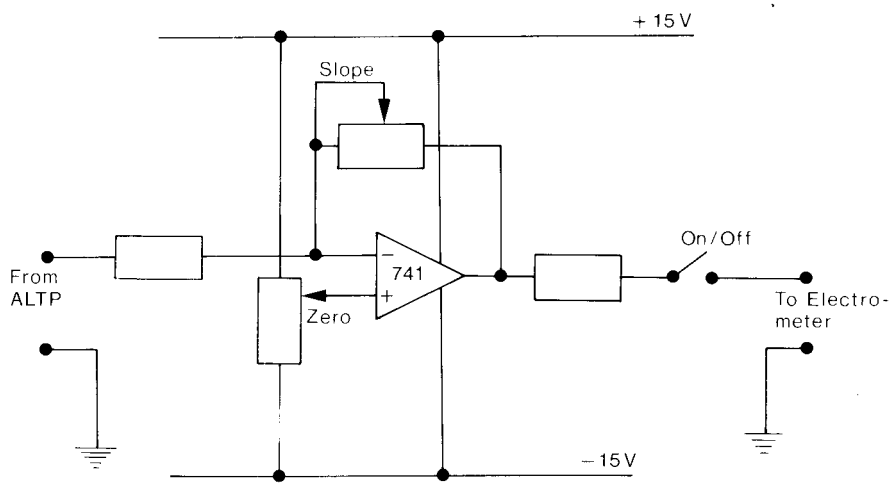

Fig. 1. Wiring diagram for a linear compensation of baseline drift.

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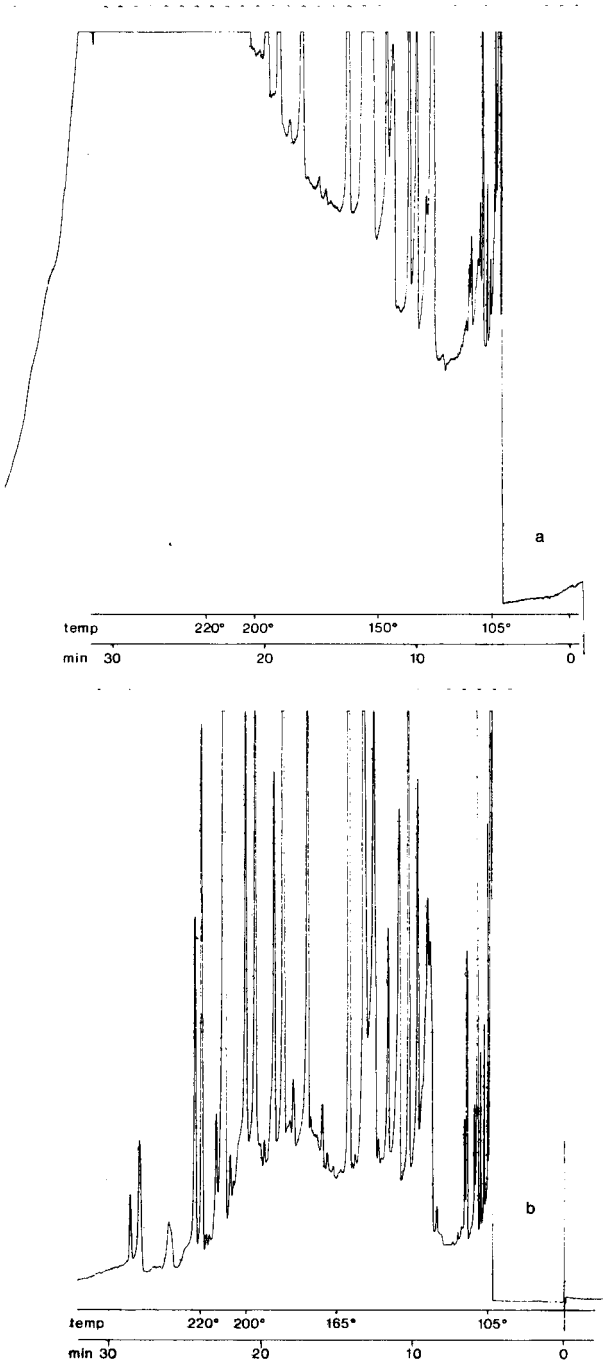

Fig. 2. Gas chromatograms after injection of a 0.5-μl sample of amino acids on a 50-m SCOT column with temperature program and a ^{63}Ni detector; without (a) and with (b) baseline correction.

We here report on a simple displacement of the electrometer voltage to compensate for baseline drift in capillary GC–ECD.

The experiments were performed with a Varian 3700 gas chromatograph equipped with a ^{63}Ni detector. Baseline compensation was achieved by utilizing a voltage in the automatic linear temperature programmer (ALTP) (Fig. 1). The voltage, which decreases proportionally with increasing temperature, was inverted and amplified so that it cancelled out the drift caused by temperature programming when fed into the electrometer. The slope control sets the gain for the correct amount of compensation and the zero control is for zeroing at the initial temperature. With the On/Off switch the circuit can be switched out when no compensation is required.

The performance of the compensation mechanism was demonstrated with a sample of amino acids, a class of compounds with a wide range of boiling points and which consequently require temperature-programmed separation. Owing to the low volatility of amino acids, suitable derivatives have to be prepared for gas chromatography. After purification by ion-exchange, the amino acids were converted into their corresponding heptafluoroisobutyl derivatives[4]. These are highly electron absorbing and volatile and have the potential of analysing amino acids at the trace levels found in biological microenvironments[5].

Derivatives were separated on an SE30/OV17 SCOT column (50 m × 0.5 mm I.D.). The oven was maintained at 105°C for 5 min, then programmed at 6°C/min to 220°C and held there for 10 min. Injections were made with no splitting at 250°C. Nitrogen was used as a carrier gas at 4.5 ml/min and the ECD was maintained at 300°C. The volume injected was 0.5 μl, representing *ca.* 0.76 pmole for the large peaks.

At the attenuation and range used ($16 \cdot 10^{-10}$ A/mV) the uncompensated analysis shows a heavy bleed and the baseline runs off the scale (Fig. 2a), while the compensation keeps the baseline in its starting position (Fig. 2b), thus facilitating integration of the peaks.

The compensation method was successfully applied to samples with less than 10 fmole of substance, but at even lower levels a baseline hump between 110 and 150°C could not be avoided if the last eluted peaks were not to be overcompensated for and thus appear below the baseline. This effect was certainly due to the exponential relationship between bleeding and temperature. For these cases an exponentially compensating voltage may be applied to the electrometer.

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Note

Increased signal-to-noise ratio through grounding and the proper selection of a pump for high-performance liquid chromatography with electrochemical detection

J. A. HELPERN*, J. R. EWING and K. M. A. WELCH
Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202 (U.S.A.)
(Received January 21st, 1982)

In recent years, high-performance liquid chromatography with electrochemical detection (HPLC–ElCD) has made a significant impact on trace analysis in various research applications. By far, however, its most elegant and extensive use has been in the detection and quantitation of tissue biogenic amines and their metabolites[1–3].

As with any analytical method, there has been a continuing search for a "limit of detection". Of prime importance in the determination of this sensitivity is the signal-to-noise (S/N) ratio. In the case of HPLC–ElCD, noise can result from power line surges, electrical interference from the use of other equipment in proximity, poorly packed electrochemical cells, inadequate grounding, mobile-phase flow-fluctuation (pump noise), incompatible mobile phase selection, and many more. Most of these sources of noise can be easily minimized or eliminated.

In our laboratory, we have found that elimination of ground loops, using a scheme of serial grounding of equipment with braided grounding cable (No. 12 AWG), has increased our S/N ratio by a factor of two. Fig. 1 shows a schematic of the grounding configuration for dual-system HPLC–ElCD.

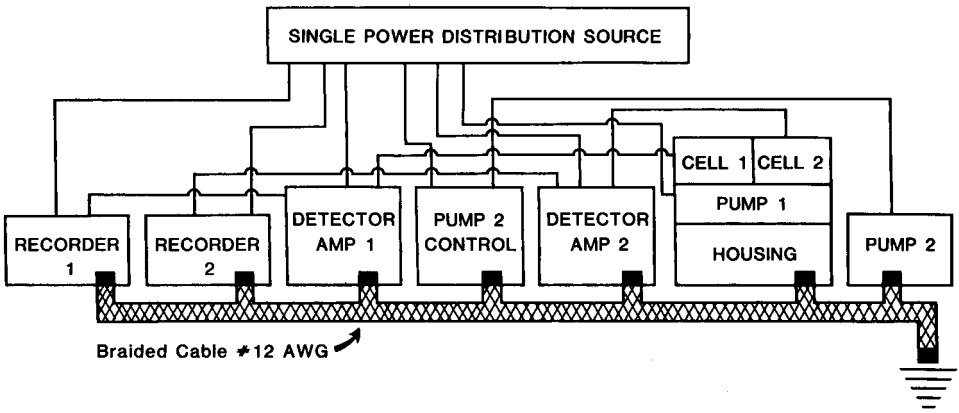

Fig. 1. Schematic of serial grounding with braided cable for dual-system HPLC–ElCD (Bioanalytical Systems, West Lafayette, IN, U.S.A.).

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The connection of braided cable to each component as well as the connection to a ground source must be bonded[4] contact. In our system the ground source is a copper pipe. Special care should be taken to make sure that the ground source chosen is truly grounded. For instance, copper water pipes in many newly constructed buildings are sometimes interrupted with plastic piping and may not be a suitable ground source. We advise that you contact the respective department in your building, such as physical facilities, and specify that you require a ground source with an impedance-to-ground as low as possible. All other ground connections are eliminated, including the ground connection normally supplied to the electrochemical cell from its corresponding detector–amplifier. In addition, the HPLC columns are grounded to the housing, and power for the entire system is supplied by a single source. Short of building a Faraday cage, this configuration affords an inexpensive and easy method for the elimination of most extraneous electrical noise.

Another major source of noise in HPLC–ElCD originates from the pump. A simplified equation for current detection in HPLC–ElCD is shown below:

$$i_d = \frac{-n \cdot F \cdot A \cdot J}{\delta}$$

where i_d = detected current; n = number of electrons involved in the electrolysis per mole of material; F = Faraday's constant; A = surface area of the working electrode; J = diffusion flux; δ = thickness of diffusion layer.

The product of the expression $n \cdot F \cdot A$ is a constant for a given chemical. The diffusion layer is the region where molecules diffuse to the electrode surface and is flow dependent. Flow surges result in changes of the thickness of the diffusion layer (δ), which then indirectly cause noise in the detected current (i_d). The need for a totally pulsationless constant-flow pump is apparent.

In our examination of various expensive and elaborate flow-feedback dual-piston reciprocating pumps, we found that at high sensitivity (0.1 nA/V, 1 V full scale) we could still see flow-surge contributions to baseline noise. We chose, therefore, to utilize a relatively inexpensive syringe pump manufactured by Instrumentation Specialties, Lincoln, NE, U.S.A.). This pump supplies truly constant flow to our HPLC-ElCD and has virtually eliminated pump noise. As a result, we have again increased our S/N ratio by a factor of two, thereby affording higher sensitivity.

HPLC–ElCD is by far the state of the art in biogenic amine trace analysis. It is unsurpassed in sensitivity and selectivity. With our HPLC-ElCD system (Bioanalytical Systems) and the modifications mentioned in this paper, we have been able routinely to measure < 50 fmole quantities of monoamine neurotransmitters in biological samples with an S/N ratio $\geqslant 4$.

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Note

Multi-parallel detection in high-performance liquid chromatography

HISANOBU YOSHIDA*, SHOZO KITO, MOTOHISA AKIMOTO and TERUMI NAKAJIMA
Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734 (Japan)
(Received January 8th, 1982)

High-performance liquid chromatography (HPLC) is now widely used in many fields, including routine work such as clinical tests, but the selectivity of the detection method is sometimes poor. Also, in the analysis of a series of metabolites with different chemical characteristics, it is desirable to have a detection method involving several appropriate procedures in a single HPLC run.

In this paper we describe a multi-parallel detection method, based on the separation of the flow from the HPLC column by a simple flow separator with pressure coils.

EXPERIMENTAL

Chemicals

Catecholamines were obtained from Sigma (St. Louis, MO, U.S.A.) and other chemicals from Yoneyama Pharmaceutical (Osaka, Japan). All were of guaranteed grade and were used without further purifications.

Flow separator

The flow separator was made of a stainless-steel multi-way joint (Kyowa Seimitsu, Tokyo, Japan) and pressure coils (1 m × 0.1 mm or 2.5 m × 0.25 mm PTFE or stainless-steel tubing).

HPLC system

The HPLC system used was a TSK 805 (Toyo Soda, Tokyo, Japan), Catecholamines were separated on a 7.5 × 7.5 mm I.D. column of TSK LS 410 (ODS-type resin, 5 μm; Toyo Soda) using 0.1 *M* potassium dihydrogen orthophosphate (pH 3.1) at 25°C at a flow-rate of 1.0 ml/min. All of the catecholamines were eluted within 10 min and were then subjected to different detection methods.

Sample preparation

Sample of catecholamines from biological materials were prepared by the alumina method with dihydroxybenzylamine (DHBA) as the internal standard. For example, 70 mg of neutral alumina were used to adsorb catecholamines at pH 8.6 from 3 ml of human urine; they were subsequently eluted with 0.2 ml of 1 *M* acetic acid by the column method, followed by washing with 5 ml of 0.1 *M* potassium

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dihydrogen orthophosphate (pH 8.6) and with 0.5 ml of water. The catecholamine fraction was analysed directly by HPLC.

Detection methods

In the *o*-phthalaldehyde (OPA) method, the reagent (according to Benson and Hare[1]) was mixed with an equal volume of the column eluate. The reaction time was 20 sec for 70°C. The fluorometer used was an FLD-1 (Shimadzu, Kyoto, Japan) with a back-pressure of 1 kg/cm^2 to prevent air-bubble formation. In the trihydroxyindole (THI) method[2], the flow reaction system with air segmentation was a PRR-2[3] (Shimadzu) with an RF 500 fluorometer (Shimadzu, Japan). In the electrochemical method[4], an EC-8 electrochemical detector (Toyo Soda) was used. The working electrode was glassy carbon and the applied voltage was 0.8 V *vs.* silver-silver chloride (0.1 *N* potassium chloride solution).

RESULTS AND DISCUSSION

Flow separator

It was important to have a good flow separator, and this was very easy to achieve. The only requirements were a low dead volume and reasonable pressure coils. Supposing that the separated flow was an aqueous solution at 0.3 ml/min, a pressure coil of 1 m × 0.1 mm of PTFE tubing correspond to a pressure barrier of about 10 kg/cm^2, and in practice this was good enough in the experiments under consideration. The volume of the pressure coil was 7.8 μl, which was negligible with respect to diffusion. A schematic diagram of the flow separator is shown in Fig. 1.

With the pressure coils with a pressure barrier of 5–10 kg/cm^2 was important to maintain the flow separation constant, but even so there were some pressure differences in each flow line (for example, ± 1 kg/cm^2) after the flow separation owing to the different detection reactions involved. Also, as shown in Fig. 1, if there was no diffusion at the flow separator then the concentration of the solute was the same

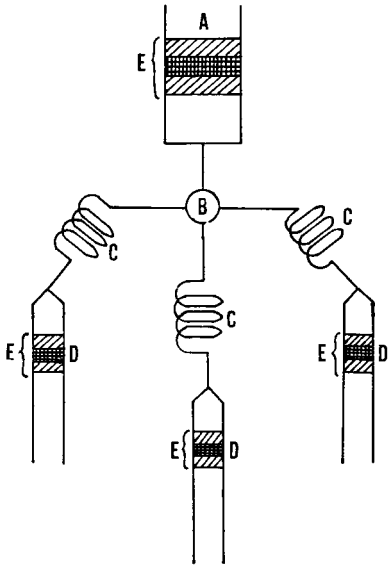

Fig. 1. Schematic diagram of the flow separator. A = HPLC separation column; B = flow separator (multi-way joint); C = pressure coils; D = pressure coils (expanded view to illustrate the concentration of the solute in the pressure coils); E = concentration of the solute in the column and in the pressure coils.

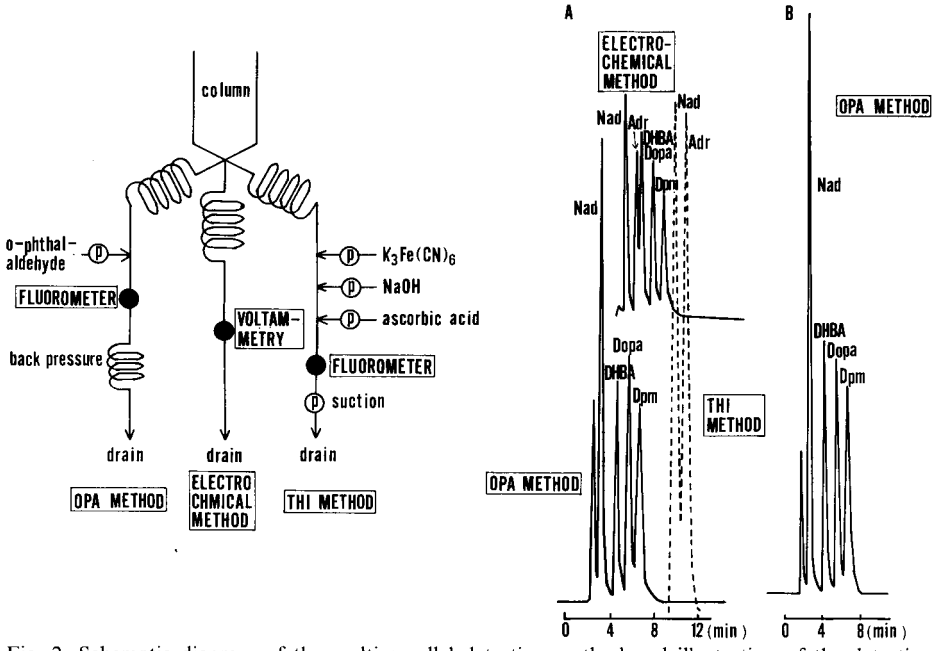

Fig. 2. Schematic diagram of the multi-parallel detection method and illustration of the detection of standard catecholamines, (A) by the multi-parallel detection method and (B) by the OPA method only (without the flow separator). The retention time of Dopa by the OPA method was 6 min. Nad = Noradrenaline; Adr = adrenaline; DHBA = dihydroxybenzylamine; Dopa = dihydroxyphenylalanine; Dpm = dopamine.

before and after the flow separator. This effect ensured that there was no loss of sensitivity in the multi-parallel detection method, as demonstrated in Fig. 2.

Standard analysis of catecholamines using the three detection methods

Catecholamines separated on the ODS-type column (TSK LS 410) were detected by the THI, OPA and electrochemical methods with the multi-parallel detection procedure as shown in Fig. 2A using a three-pen recorder. When analysing 50 pmol of each amine, the reproducibility of the peak height by the three detection methods was excellent (the coefficient of variation was less than 3%; $n = 10$). In the THI method the peaks came out later than other method because of the longer reaction time involved (*ca.* 7 min).

When the flow separator was not used, all of the eluate from the column was subjected to the OPA detection method, keeping the ratio of the eluate to the reagent constant, and the sensitivity was almost identical with that obtained in the multi-parallel detection method (Fig. 2B). The slight difference in the peak heights in Fig. 2A and B obtained with the OPA method might be due to slight differences in the reaction conditions (possibly due to a shorter reaction time). In practice, the multi-parallel detection method did not show a lower detection sensitivity, although the absolute amount to be analysed was reduced.

Analysis of catecholamine fractions from biological samples

Catecholamine fractions of rat whole brain and human obtained by the alumina method, were separated on the ODS-type column (TSK LS 410) and were detected as the above three methods (Fig. 3). Although the sample enrichment method was the same, the reliabilities of the peaks obtained by the three methods were different.

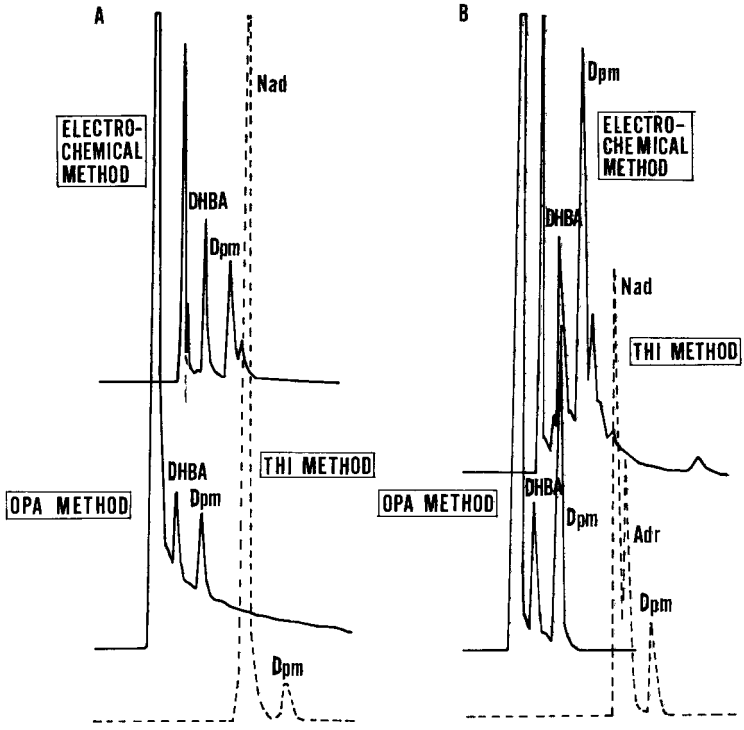

Fig. 3. Chromatogram of catecholamine fraction from (A) rat whole brain and (B) human urine by the multi-parallel detection method. The retention time of Dopa by the OPA method was 6 min. Abbreviations: see Fig. 2.

Comparison of the three methods indicates that the results obtained with the THI method were the most reliable, although another internal standard was necessary. The results obtained with the electrochemical method for rat whole brain were also reliable, but not those for human urine. The OPA method was not suitable for the analysis of either sample. The different reliabilities of the three detection methods for these samples might be due to different impurities in the catecholamine fractions.

In conclusion, the multi-parallel detection method is a useful adjunct to HPLC, and might be useful for detecting a series of metabolites that require different detection methods owing to their different chemical characteristics.

ACKNOWLEDGEMENT

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Note

Adaptable system for microdialysis

SIDNEY W. WHITEHEART, ROBIN D. CAMPBELL, and SAMUEL H. LOVE*
Department of Microbiology and Immunology, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103 (U.S.A.)
(Received January 20th, 1982)

Dialysis of microliter quantities of a sample can be a valuable technique in many laboratory situations. Several methods which are applicable to this problem have been proposed[1–4]. This paper describes a microdialysis method which overcomes some of the limitations encountered in other methods and utilizes common laboratory equipment. Extensive studies in our laboratory have shown that this system combines quantitative recovery of protein retentates and ease of sample manipulation into a relatively inexpensive microdialysis technique.

EXPERIMENTAL

Microdialysis chambers (3 cm long) were prepared from standard bore gel electrophoresis tubing (7 mm O.D. × 5 mm I.D., Bio-Rad) and were fitted with a Vacutainer tube top as illustrated in Fig. 1. Wet dialysis membranes (Spectrapor Nos. 2 and 3, Fisher Scientific Co.) were arranged as double thicknesses on one end of the tube and were held in place by an O-ring (No. 2-0.007 NC 74-7, Southern Rubber Co., Greensboro, NC, U.S.A.) which was positioned with an O-ring applicator supplied with a YSI Model 53 oxygen analyzer (Yellow Springs, OH, U.S.A.). After the dialysis chambers were assembled, they were placed into the top chamber of a Bio-Rad Model 150A electrophoresis cell, and the lower chamber, placed over a magnetic

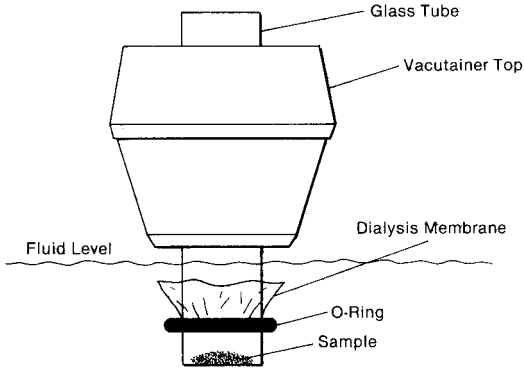

Fig. 1. Schematic diagram of microdialysis chamber.

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stirrer, was filled with distilled water. A Lauda K-2/R cooling circulator (Brinkmann Instruments) controlled the temperature of the electrophoresis unit.

The dialysis chambers were filled with 100 μl of [1-^{14}C]glycine or [^{3}H]acetic acid in distilled water, and samples were removed from the dialysis chambers at various times to measure the effectiveness of dialysis. To measure recovery of protein in the dialysate, 50 μl of [1-^{14}C]glycine and 50 μl of bovine serum albumin (BSA) (10 mg/ml) were added to duplicate chambers. At the various times, samples were removed and tested for protein concentration and remaining radiolabel.

The effect of sample volume on dialysis efficiency was determined by using [^{3}H]acetic acid. Different volumes of dilute radiolabel were dialyzed for 1 h, samples were removed, and the remaining radiolabel was determined.

All samples were counted in a Beckman LS-133 liquid scintillation system while using Bray's Solution as a fluor. Bovine serum albumin concentrations were determined by ultraviolet absorbance[5]. The radiolabels were chosen because of their small molecular weight and availability to our laboratory.

RESULTS

Figs. 2 and 3 illustrate the efficiency of dialysis with and without BSA at 5 and 25°C. Temperature and pore size had the greatest effects on the speed of dialysis, the apparent enhancement of dialysis by BSA in one case (Fig. 2) was not evident at 3 h. In all cases, the Spectrapor No. 2 (mol.wt. cutoff, 12,000–14,000) gave a more efficient dialysis than did Spectrapor No. 3 (mol.wt. cutoff, 3500). The experiments performed at 5°C indicated slower dialysis rates than did experiments at 25°C, regardless of the membrane used.

For samples with and without BSA, 3.5 and 5.0% of the radiolabel remained in the samples dialyzed with Spectrapor No. 2 at 25°C for 3 h. At 5°C, 12.5 and 11.5% of radiolabel remained in samples dialyzed with Spectrapor No. 2 (Fig. 2). For samples with and without BSA, 24.5 and 17.7% of radiolabel remained in the samples dialyzed at 25°C with Spectrapor No. 3, while 40.0 and 35.0% of radiolabel remained in samples dialyzed at 5°C (Fig. 3).

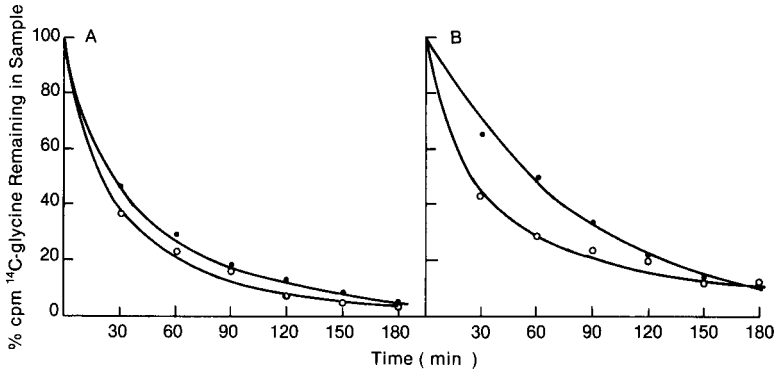

Fig. 2. Effect of protein on dialysis of [1-^{14}C]glycine at 25°C (A) and 5°C (B) utilizing Spectrapor No. 2 membranes. Samples contain 100 μl of [1-^{14}C]glycine (●) or 50 μl of [1-^{14}C]glycine and 50 μl BSA (10 mg/ml) (○).

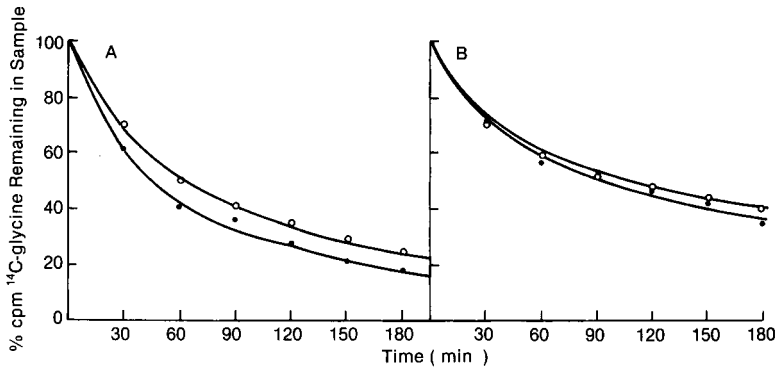

Fig. 3. Effect of protein on dialysis of [1-^{14}C]glycine at 25°C (A) and 5°C (B) utilizing Spectrapor No. 3 membranes. Samples contain 100 μl of [1-^{14}C]glycine (●) or 50 μl of [1-^{14}C]glycine and 50 μl BSA (10 mg/ml) (○).

When Spectrapor No. 2 was used for dialysis, the extent of dialysis at 3 h was not altered significantly when protein was present in the dialysand. However, the initial rates of dialysis were affected. Fig. 2 indicates that the presence of BSA increased the rate at which initial dialysis occurred, especially at 5°C. The presence of BSA in chambers in which Spectrapor No. 3 was used (Fig. 3) appeared to slow the rate of dialysis at 25°C but did not significantly alter the rate of dialysis at 5°C. In all studies, the concentration of BSA was measured after dialysis (data not shown), and the results clearly indicated 100% recovery.

Volumetric studies indicated similar results for both Spectrapor No. 2 and 3. The efficiency of dialysis was greatest for the smaller volumes (25 to 75 μl) dialyzed for 1 h (Fig. 4).

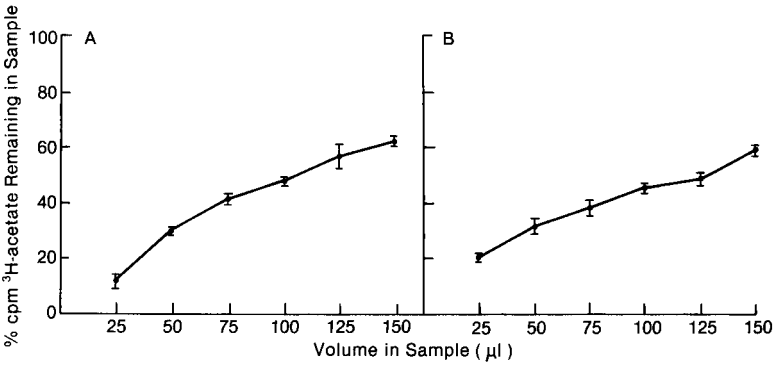

Fig. 4. Effect of sample volume on efficiency of dialysis at 25°C for 1 h. Dialysis was performed with [^{3}H]acetic acid and Spectrapor No. 2 membranes (A) and Spectrapor No. 3 membranes (B).

DISCUSSION

Studies using this microdialysis method have shown it to be a useful and predictable technique for small samples. The difference in rate of dialysis seen in the temperature change experiments was in keeping with the role that temperature plays in most equilibrium reactions. Colder temperatures tend to slow the progression to equilibrium, and this is illustrated by the experiments at 5 and 25°C. Pore size of the membrane also affected the rate of dialysis by limiting the flow of molecules through the dialysis chamber. Our experiments with Spectrapor No. 2 and 3 demonstrated this relationship of pore size to dialysis rate.

The experiments in which BSA was included presented interesting and unexpected results. The presence of protein in the dialysand should have caused an increase in osmotic pressure resulting in an increased dialysis rate. This increase occurred with the Spectrapor No. 2 membrane but not with the Spectrapor No. 3 membrane. We postulate that the pore size of the Spectrapor No. 3 compensated for the effect caused by the increased osmotic pressure. This suggestion is supported by the similarity of dialysis rates of the test and controls in the Spectrapor No. 3 experiments (Fig. 3).

Fig. 4 demonstrates the extent to which different volumes were dialyzed in 1 h. A direct relationship between sample size and extent of dialysis was expected. As the sample volume increased, the proportion of sample actually in contact with the membrane decreased, thus requiring longer dialysis times for large samples.

Certain precautions must be observed when using this microdialysis system. The seal made by the O-ring must be tight and should be examined before any sample is added to the chamber. One method of insuring a proper seal is to equilibrate the chambers in the dialyzing fluid before adding any sample. Leakage of fluid into the chamber will be evident before the sample is added. The use of double-thickness membranes greatly reduced leakage problems, while the use of single-thickness membranes led to more difficulty in obtaining a tight seal. Care also should be taken to avoid puncturing the membrane while introducing samples into the chamber.

The advantages of this microdialysis system include its simplicity, rapidity and reproducibility. The basic unit is easily constructed from readily available, inexpensive laboratory equipment. The microdialysis chambers, once placed in the dialyzing fluid, provide ready access to the samples during the dialysis process. A buffer change is not required during dialysis, and buffer temperature is easily regulated. Except for the dialysis membranes, the components of the system are reusable. An important advantage is the ability to recover samples. As was stated the recovery of BSA was virtually 100% in all cases, thus allowing confident dialysis of very small volumes. The microdialysis chamber (glass tube, membrane and O-ring) can be adapted to many situations. However, the O-ring applicator is essential for correctly positioning the membrane and O-ring. We used the electrophoresis cooling unit because it was available to our laboratory, but any other similar apparatus could be used. An ordinary test tube rack can be used to support the chambers in a refrigerator tray filled with desired buffer.

Application of this system is as varied as the techniques that require small volume samples. Our laboratory has used this system for dialysis of samples prior to electrophoretic and immunological assays. Testing enzyme activity during purifi-

cation studies is another indication for the use of this method. This microdialysis system should prove to be adaptable to suit many needs.

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Note

Effect of methanol in the mobile phase on the ion chromatographic determination of some monovalent cations

R. C. BUECHELE* and D. J. REUTTER
Research Unit, Forensic Science Research and Training Center, FBI Academy, Quantico, VA 22135 (U.S.A.)
(Received January 19th, 1982)

The determination of several amines, ammonia and alkali metals by ion chromatography has been reported previously[1,2]. The moderate efficiencies still exhibited by ion-exchange columns make the determination of many of these ions impossible in the presence of other ions with similar retention times unless sample pre-treatment or other independent methods are used.

Similar difficulties in the separation of some amino acids by ion-exchange chromatography have been resolved by changing column selectivities with the addition of an organic solvent to the mobile phase[3–5]. These selectivity changes have been attributed to a combination of several retention mechanisms which can be important in chromatographic separations using ion-exchange resins. The addition of an organic solvent to the mobile phase has a significant effect on several of these mechanisms.

The application of this phenomenon to ion chromatography has received little attention. This is due, in part, to the fact that organic solvents suppress ionization and consequently decrease sensitivity with conductivity detection. Furthermore, organic solvents swell the sulfonated polystyrene–divinyl benzene resins commonly used in ion chromatography thus increasing back pressure[6,7]. We have investigated these problems and found that the advantages gained by large changes in column selectivities can outweigh the disadvantages for some analyses.

EXPERIMENTAL

Apparatus

The ion chromatograph used was a Dionex Model 16 (Dionex, Sunnyvale, CA, U.S.A.) which was equipped with a 6-μl flow-through conductivity detector. Samples were introduced by an air actuated valve injector with a 100-μl sample loop. The analog output from the conductivity detector (which is proportional to the square of the conductivity in the cell) was recorded on a Shimadzu C-RIA integrating recorder.

Columns

The analytical column was a 6 × 250 mm "cation separator" column

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(Dionex). A 9 × 250 mm anion exchange suppressor column (Dionex) preceded the conductivity cell to strip the highly conductive HCl from the mobile phase.

Chemicals

Grade one deionized water (Hydro Services), Baker analyzed HPLC grade methanol and ACS certified 1 *N* hydrochloric acid (Fisher) were used to prepare the ion chromatography eluents. Sodium nitrate, ammonium nitrate, potassium nitrate, lithium carbonate and rubidium chloride (certified ACS grade, Fisher) were used to prepare the inorganic cation standards. Cesium chloride and the amines were supplied by Sigma (St. Louis, MO, U.S.A.). Each reagent was dissolved in deionized water to yield standard solutions of approximately 10 ppm (w/w) of the cation.

Mobile phases

The mobile phases consisted of 0.01 *N* hydrochloric acid in water–methanol solutions. The quantity of methanol was varied in each of the five mobile phases as follows: (a) 40 ml 1 *N* hydrochloric acid diluted to 4 l with deionized water (0% v/v, methanol); (b) 40 ml 1 *N* hydrochloric acid, 400 ml methanol, diluted to 4 l with deionized (10% v/v, methanol); (c) 40 ml 1 *N* hydrochloric acid, 800 ml methanol, diluted to 4 l with deionized water (20% v/v, methanol); (d) 40 ml 1 *N* hydrochloric acid, 1200 ml methanol, diluted to 4 l with deionized water (30% v/v, methanol); (e) 40 ml 1 *N* hydrochloric acid, 1600 ml methanol, diluted to 4 l with deionized water (40% v/v, methanol).

Procedure

Retention times for the cations shown in Table I are reported as mean values from at least three injections with a relative standard deviation of less than 2%. In all

TABLE I

RETENTION TIMES FOR MONOVALENT CATIONS USING 5 DIFFERENT MOBILE PHASES

Each mobile phase contained 0.01 *N* HCl and varied only in methanol content. Flow-rate was held constant at 1.53 ml/min.

Ion	*Retention time (min)*				
	Methanol (%)				
	0	*10*	*20*	*30*	*40*
Li^+	9.69	9.83	9.99	10.15	10.66
Na^+	11.36	11.52	11.89	12.27	13.67
NH_4^+	14.69	14.65	15.00	15.27	16.70
K^+	16.16	16.61	18.23	19.74	23.33
Rb^+	19.12	17.87	19.96	22.97	27.27
Cs^+	23.26	21.14	23.64	27.30	32.57
Ethanolamine	15.26	15.21	15.61	15.87	17.49
Diethanolamine	16.50	16.11	16.51	16.45	17.55
Triethanolamine	19.29	18.18	17.93	17.69	19.10
Ethylamine	18.66	17.92	17.91	18.10	19.89
Diethylamine	26.88	24.07	23.19	22.58	24.08
Triethylamine	39.66	31.68	28.15	25.95	26.92
Methylamine	16.84	16.53	16.86	17.34	19.34
Trimethylamine	23.13	21.61	21.55	21.50	23.60

cases, the flow of the mobile phase was maintained at 1.53 ml/min. The selectivity was determined by the equation:

$$\alpha = \frac{K_2}{K_1} \tag{1}$$

where K_2 is the retention time of the sample ion and K_1 is the retention time of the least retained species, (Li^+). At 40% methanol concentration the linearity was studied for selected cations to determine if addition of methanol had an adverse effect. The signal and signal-to-noise (S/N) ratios were compared at zero and 40% methanol. The signal was measured as peak height of sodium at 10 ppm, and the noise was determined as the peak-to-peak voltage of the sinusoidal variation in the baseline over a 2-min period.

RESULTS

The addition of methanol to the mobile phase of the cation ion chromatography system altered the selectivity of the separator column. For many pairs of ions the retention orders were reversed when going from 0 to 40% methanol. Table I lists retention times for all the ions studied as a function of methanol concentration in the mobile phase. Fig. 1 plots selectivity *versus* percentage methanol in the mobile phase for inorganic monovalent cations, and Fig. 2 shows the same functions for the monovalent amines which were considered.

Fig. 3 shows a graph of integrated signal as a function of the concentrations of Na^+, NH_4^+, K^+ and methylamine, over the range 1 to 50 ppm for the system containing 40% methanol. Others have reported response *vs.* concentration for these ions using eluents without organic modifiers[2]. Comparison of the peak heights of representative ions when they are run on the 0 and 40% methanol systems, indicates that at an ion concentration of 10 ppm, the 40% methanol system gives only 1/3 of the response measured in the 0% methanol system. However, when the S/N ratio is measured for Na^+ at 10 ppm as is described in the procedure, the ratio drops from

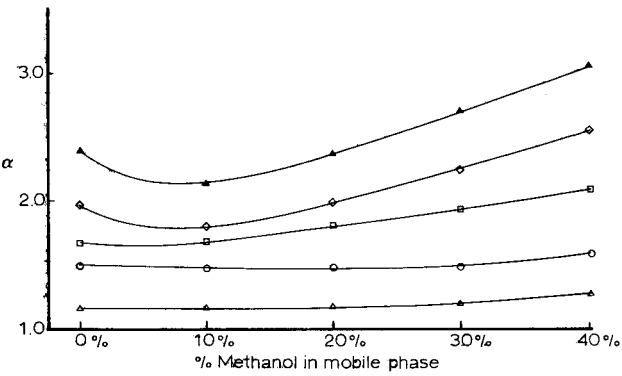

Fig. 1. Relationship between selectivity (α) and percent methanol in the mobile phase for inorganic monovalent cations. △ = Na^+, ○ = NH_4^+, □ = K^+, ◇ = Rb^+, ▲ = Cs^+.

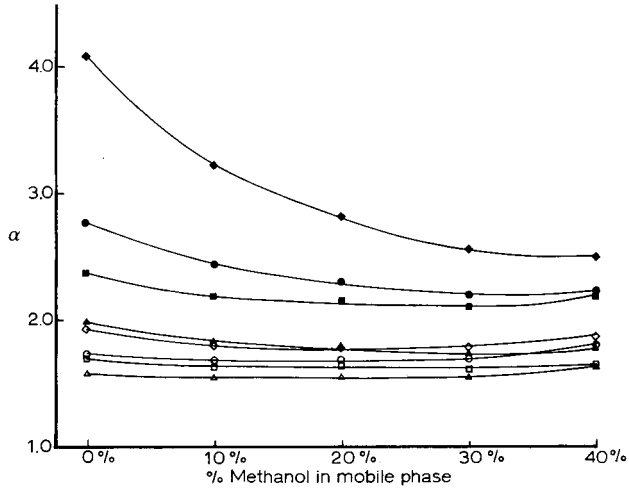

Fig. 2. Relationship between selectivity (α) and percent methanol in the mobile phase for monovalent amines. △ = Ethanolamine, □ = diethanolamine, ○ = methylamine, ▲ = triethanolamine, ◇ = ethylamine, ◆ = triethylamine, ● = diethylamine, ■ = trimethylamine.

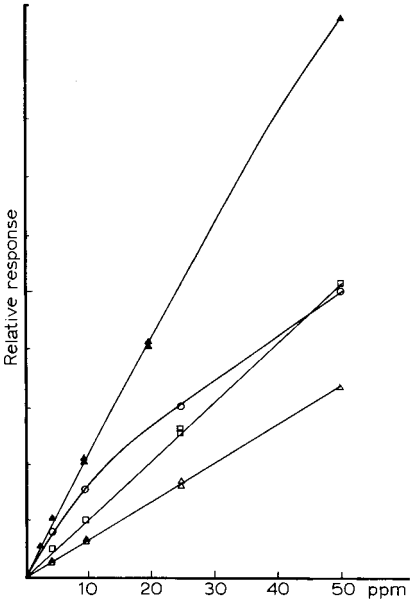

Fig. 3. Relationship between cation concentration and relative detector response for the mobile phase containing 40% methanol. △ = K^+, □ = Na^+, ○ = NH_4^+, ▲ = methylamine.

20,500 to 18,000. This is due to a significant drop in the background noise which accompanies the suppression of the signal.

DISCUSSION

The addition of methanol to the mobile phase of a cation ion chromatography

system can be used to separate ions which would not be separated by aqueous eluents. The large drop in detector response which is due to suppression of ionization by the organic solvent is not a significant detriment to the use of such solvents in ion chromatography. Even at 40% methanol concentration, the S/N ratio is decreased by approximately 10%; therefore, the detector output can be electronically amplified with little overall loss in performance. Moreover, an eluent containing 40% methanol produces similar results for linearity of signal *vs.* ionic concentration when compared to the previous study[2].

The influence of organic solvents in the mobile phase on retention mechanisms for ion-exchange chromatography has been previously discussed[5,8]. For simplicity, we can characterize the phenomenon as one involving two separate processes, both of which are affected by the addition of the organic solvent. First, there is the interaction of the methanol with the ion-exchange resin swelling the resin and increasing the capacity by better exposing the available ion-exchange sites and increasing the area for adsorption. Second, the addition of methanol to the aqueous eluent changes the free energy of solvation for each cation. These effects are apparent upon examining Figs. 1 and 2. The alkali earths are generally more retained with the addition of methanol while the amines are generally less retained.

One application which takes advantage of these selectivity changes is the separation of potassium and methylamine. The eluent recommended by the manufacturer of the ion chromatograph (0.005–0.0075 *N* hydrochloric acid) will not resolve these two ions. With the addition of 40% methanol to the mobile phase, baseline resolution is obtained. These separations are shown in Figs. 4 and 5, respectively. Other successful applications of this elution method depend upon the needs of the particular separation. Table I offers a basis for selecting an eluent which may provide workable separations for monovalent cations. Other organic solvents which are compatible with the ion-exchange resins could be equally applicable. Our studies have included only acetonitrile which yields results similar to those reported here with methanol.

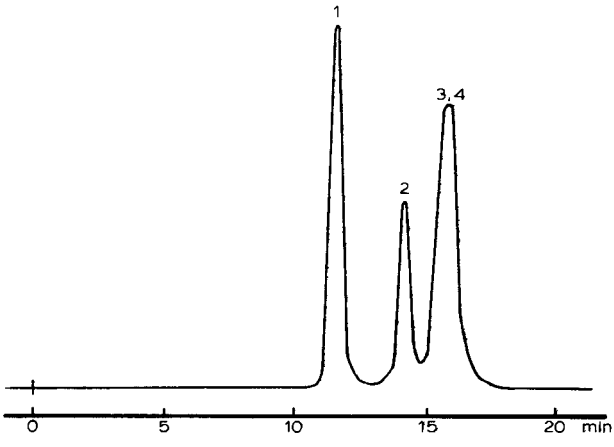

Fig. 4. Ion chromatogram of a solution containing 10 ppm each of Na^+, NH_4^+, K^+ and methylamine. Mobile phase is 0.01 *N* HCl in deionized water with 0% methanol. Peaks: 1 = Na^+, 2 = NH_4^+, 3 = K^+ and 4 = methylamine.

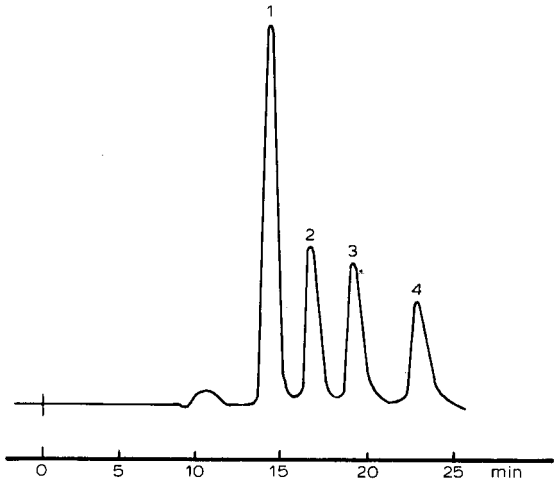

Fig. 5. Ion chromatogram of a solution containing 10 ppm each of Na^+, NH_4^+, K^+ and methylamine. Mobile phase is 0.01 *N* HCl in deionized water with 40% methanol added. Peaks: 1 = Na^+, 2 = NH_4^+, 3 = methylamine and 4 = K^+.

This research has applied to a modern ion chromatography system the proven practice of changing ion-exchange column selectivities by the addition of an organic solvent to the mobile phase. This has proven useful for some of our applications and creates potential for further research in ion chromatography.

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Note

Determination of water content in toluene using a novel method of calibration

CONSTANTINE DUMAS and C. C. HSU*
Chemical Engineering Department, Queen's University, Kingston, Ontario K7L 3N6 (Canada)
(First received November 9th, 1981; revised manuscript received December 29th, 1981)

Toluene is used as a solvent for many polymerization processes and the water content can affect the rate of polymerization, microstructure and molecular weight distribution[1].

Among the published methods of water content analysis[2–7], the calibration procedure is always the limiting factor for the accuracy of water determinations. Approximate measurements have been made by relating the water peak area to the solvent peak area[3,5]. Internal standardization requires an internal standard with a known moisture content[6]. Another method involves analysing mixtures in which water is the major component[4]. External standard methods[2,7] involve blending various amounts of water with toluene, previously dried using molecular sieves. However, the calibration curves show significant responses at a water content supposedly of zero, indicating that the molecular sieves did not dry the toluene completely[2,7].

To overcome these problems a more accurate calibration method for the determination of water content in toluene using gas chromatography was investigated. Various volumes of water vapour with known quantities were used as calibration standards. To recover moisture adsorbed on the syringe walls, each sample of standard required re-injections with carrier gas.

EXPERIMENTAL

Apparatus and method

A Hewlett-Packard 5710A gas chromatograph with a thermal conductivity detector was used. A nickel column (0.5 m × 3 mm I.D.) packed with Porapak R (100–120 mesh) was used to separate air, water and toluene. The column temperature was 90°C and the helium flow-rate was 40 cc/min. Porapak R, with an upper temperature limit of 250°C, allowed rapid column clearing of higher-boiling-point components by temperature programming to 240°C. The retention time for air was 5 sec, for water 56 sec and for toluene 236 sec. The complete temperature-programme cycle ran for 12 min.

For the water calibration standards, saturated water vapour at a known temperature was used. Various volumes of saturated water vapour were drawn from a flask with 100- and 250-μl gas-tight syringes. Before sampling, the syringe was dried by flushing several times with carrier gas. Complete dryness was ensured by re-

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injecting this carrier gas into the gas chromatograph, and observing the response. Sampling involved drawing up the syringe plunger to the exact level required and then injecting this sample into the gas chromatograph. In order to recover material adsorbed on the walls of the barrel, a volume of dry carrier gas equal to the sample volume was immediately redrawn into the syringe. The syringe was left in this state, in the injection port, until the water peak of the initial injection had appeared. Then, the syringe contents were injected into the gas chromatograph and dry carrier gas was again redrawn into the syringe. This redrawing procedure was repeated several times until all the moisture in the sample had been recovered. For the analysis of the moisture content in toluene, samples were injected with a 10-μl liquid syringe.

RESULTS AND DISCUSSION

Toluene samples in the 44.6–601.4 ppm water-content range were analysed. The accuracy of these determinations ranged from ± 2.4 to $\pm 16.8\%$, as shown in Table I.

TABLE I

CONFIDENCE INTERVALS FOR TOLUENE ANALYSES

Toluene batch	*Sample number*	*Sample volume (μl)*	*Water content (ppm)*	*Mean water content (ppm)*	*Calibration standard error (ppm)*	*Sampling standard error (ppm)*	*Overall standard error*	*Error (%)*
A	1	7.2	47.4	47.4	4.6	–	–	
	2	7.2	42.4	44.9	3.4	3.5	4.9	
	3	7.2	41.9	43.9	3.0	3.0	4.2	
	4	7.2	44.6	44.1	2.7	2.5	3.7	16.8
B	1	8.0	122.6	122.6	4.0	–	–	
	2	8.0	119.8	120.9	3.0	2.4	3.8	
	3	8.0	119.2	120.5	2.5	1.8	3.1	
	4	8.0	117.4	119.8	2.3	2.2	3.2	5.4
C	1	7.0	207.6	207.6	4.5	–	–	
	2	7.0	210.9	209.3	3.3	2.4	4.1	
	3	7.0	211.3	209.9	2.8	2.0	3.4	
	4	7.0	213.7	210.9	2.5	2.5	3.5	3.4
D	1	8.2	353.3	353.3	3.8	–	–	
	2	8.2	355.0	354.2	2.8	1.3	3.1	
	3	8.2	357.7	355.3	2.3	2.2	3.2	
	4	8.2	349.1	353.8	2.1	3.6	4.2	2.4
E	1	8.3	521.3	521.3	4.0	–	–	
	2	8.3	539.2	530.3	3.0	12.7	13.0	
	3	8.3	530.8	530.4	2.6	9.0	9.4	
	4	8.3	530.5	530.5	2.4	7.3	7.7	3.0
F	1	6.1	588.8	588.8	5.2	–	–	
	2	6.1	612.2	600.5	3.9	16.5	17.0	
	3	6.1	603.3	601.4	3.3	11.8	12.3	4.0

Results obtained by calibrating with water vapour are shown in Table II. A 250-μl syringe was used for samples larger than 100 μl. For the other samples, a 100-μl syringe was used. The relative standard deviation on the repeats ranged from ± 0.2 to $\pm$ 3.2%. Approximately 85% of the water vapour per sample was recovered with the first injection. Water adsorbed on the walls of the syringe was recovered in subsequent re-injections. Fig. 1 shows water peaks in a series of re-injections. Air peaks were also present on re-injections. The air must be retained on the syringe walls by adsorption because it does not condense at room temperature.

By calculating the water vapour moisture content per unit volume, the data of Table II were used to construct a calibration curve (Fig. 2). Inspection of the calibration curve indicates a straight line passing very near the origin. A slope of 1559 and intercept of -147 were calculated by linear regression. Confidence intervals for the calibration curve were computed using eqn. 1, an approximation of Fieller's Theorem[8], assuming negligible error in the water vapour standards:

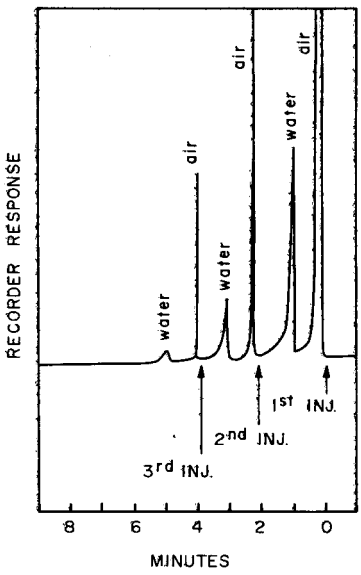
$$\text{C.S.E. } (X_0) = \frac{\sigma}{b}\sqrt{\left\{\frac{1}{m} + \frac{1}{n} + \frac{(y_0 - \bar{y})^2}{b^2 S_{xx}}\right\}} \quad (1)$$

where C.S.E. (X_0) is the standard error for X_0, n is the number of samples used for the calibration, σ is the standard deviation based on $n - 2$ degrees of freedom, b is the slope of the standard curve, m is the number of samples used for the analysis, $\bar{y}$ is the

TABLE II

WATER CALIBRATION DATA

Nominal sample volume (μl)	*Corrected sample volume (μl)*	*H_2O (μg)*	*Injection number*				*Total*	*Mean*	*σ (%)*
			1	*2*	*3*	*4*			
40	40	0.6481	762	86	–	–	848		
			742	70	–	–	812		
			777	89	–	–	866	842	3.2
60	60	0.9721	1237	109	–	–	1346		
			1238	138	–	–	1376		
			1265	110	–	–	1375	1366	1.2
80	80	1.2961	1762	160	–	–	1922		
			1722	133	–	–	1855		
			1738	152	–	–	1890	1889	1.8
100	100	1.6202	2193	192	–	–	2385		
			2184	197	–	–	2381		
			2238	195	–	–	2433	2400	1.2
150	149.9	2.4286	3077	390	82	42	3591		
			3117	432	98	62	3709		
			3128	407	90	40	3665	3665	1.7
200	199.1	3.2257	3923	762	143	69	4897		
			4040	601	131	80	4852		
			3930	690	137	51	4808	4852	0.9
250	248.6	4.0277	5113	751	185	79	6128		
			5258	649	161	79	6147		
			5269	683	124	78	6154	6143	0.2



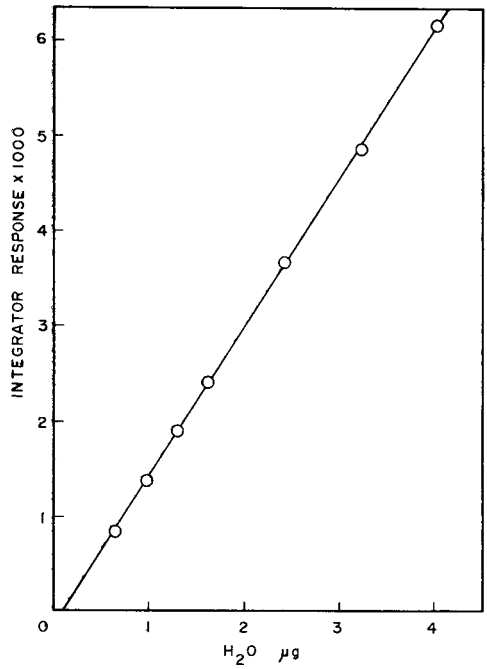


Fig. 1. Recovery of total water vapour sample by re-injection using Porapak R column packing.

Fig. 2. Calibration curve constructed using water vapour standards.

mean value of the calibration response, y_0 is the mean value of the analyses response, and S_{xx} equals $\Sigma(x - \bar{x})^2$.

The calibration curve data were obtained using relatively large gas-tight syringes whereas toluene samples were analysed with a 10-μl liquid syringe. Lesser readability and thus reproducibility with the liquid syringe led to uncertainties in the toluene analysis which could not be estimated by eqn. 1 alone. The overall standard error was estimated using eqn. 2,

$$\text{O.S.E.}\ (X_0) = \sqrt{\text{C.S.E.}\ (X_0)^2 + \sigma(X_0)^2} \tag{2}$$

where O.S.E. (X_0) is the overall standard error, C.S.E. (X_0) is the standard calibration error and $\sigma(X_0)$ is the standard deviation of the toluene analysis.

The number of re-injections required to completely recover adsorbed water depended on a number of factors and varied from 1 to 5. Cleaning and silanizing the syringes reduced the number of re-injections required; however, more than one re-injection was always necessary. The results are shown in Table III.

The gas-tight syringes were calibrated by weighing, using pure mercury of known density. When taking water vapour samples, the plunger was brought exactly to the required level on the first draw. Drawing a sample larger than required and expelling the excess sample prior to injection resulted in a higher water response. By

TABLE III

EFFECTS OF VARIOUS TREATMENTS ON SYRINGE WATER ADSORPTION

Syringe A was new; syringe B was previously silanized.

syringe A (100 µl sample)					*Syringe B (100 µl sample)*				
Injection number					*Injection number*				
1	*2*	*3*	*4*	*Total*	*1*	*2*	*3*	*4*	*Total*
1373	521	106	65	2065	1437	583	77	–	2097
1027	738	142	42	1945	1197	659	91	38	1985
1454	502	113	80	2149	1228	630	99	–	1957
1372	508	106	74	2060	1047	772	102	49	1970
5% NaOH treatment									
1335	473	137	68	2013	2041	216	–	–	2257
1387	790	203	76	2456	1993	283	–	–	2276
1455	481	139	64	2139	2071	236	–	–	2307
1191	664	184	61	2100	2075	216	–	–	2291
Organosilane treatment									
1586	454	117	66	2223	1785	385	52	–	2222
1723	390	97	28	2238	1762	338	56	–	2156
1774	358	67	30	2260	1898	351	39	–	2288
1919	322	78	49	2368	1839	386	54	–	2279
5% NaOH treatment									
2091	301	66	–	2458	2193	192	–	–	2385
2040	304	80	–	2424	2184	197	–	–	2381
2201	243	67	–	2511	2238	195	–	–	2433

expelling the excess sample, the walls of the syringe came into contact with and adsorbed more water than required. This same effect made the use of a sampling valve unacceptable. Water response was much higher than that obtained with a syringe of the same volume and peak tailing was observed.

Calibration using various volumes of water-saturated toluene was unsuccessful. Results were not reproducible because it was difficult to prevent the formation of a suspension of small water droplets in the toluene. This may explain the scattered literature values for water content in saturated toluene. Also, toluene rapidly absorbs moisture from the atmosphere.

CONCLUSIONS

Accurate gas chromatograph calibration, for low levels of water, is performed with water vapour standards. This can be applied to toluene water-content analysis or other organic solvents in the ppm range.

Sorption of water and air on the syringe walls, with water vapour standards, is significant. This material is recovered by re-drawing dry carrier gas into the syringe and re-injecting into the chromatograph. This method of recovering sample adsorbed on the syringe walls finds application in all types of gas analyses.

APPENDIX

Sample calculations

Calibration parameters for eqn. 1 using data from Table II.

$n = 21$

$$\bar{y} = \frac{848 + 812 + \ldots. 6154}{21} = 3021$$

$$\bar{x} = \frac{0.6481 + 0.9721 + \ldots.. 4.0277}{7} = 2.031$$

$b = 1559.57$ (by linear regression)

$\sigma = 41.482$

$S_{xx} = 27.94$

For toluene batch D of Table I

$m = 4$

$$y_0 = \frac{3786 + 3804 + 3835 + 3739}{4} = 3791$$

$x_0 = 2.5239\ \mu g\ H_2O$

$$\text{C.S.E.}(2.5239) = \frac{41.482}{1559.57}\left[\frac{1}{4} + \frac{1}{21} + \frac{(3791 - 3021)^2}{6.796 \cdot 10^7}\right]^{1/2} = 0.0147\ \mu g\ H_2O \quad (1)$$

$$\text{Now, } \frac{2.5239 \pm 0.0147\ \mu g\ H_2O}{8.2\ \mu l \text{ toluene}} = 353.8 \pm 2.1 \text{ ppm } H_2O \text{ (C.S.E.)}$$

Overall standard error calculation:

C.S.E. (353.8) = 2.1 ppm

$\sigma = 3.6$ ppm

$$\text{O.S.E. } (353.8) = [(2.1)^2 + (3.6)^2]^{1/2} = 8.4 \text{ ppm} \quad (2)$$

Therefore 353.8 ± 4.2 ppm.

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Note

Spray reagent for steroids and triterpenoids on thin-layer plates

P. GHOSH and S. THAKUR*
Chemistry Department, University of Burdwan, Burdwan 713104 (India)
(First received August 25th; revised manuscript received December 15th, 1981)

A number of spray reagents for the selective and non-selective detection of triterpenoids and steroids[1] on silica gel G thin-layer plates are known. The Lieberman–Burchard reaction with acetic anhydride–sulphuric acid is widely used and other reagents claimed to be effective in the detection of both classes include chlorosulphonic acid–acetic acid[2], antimony trichloride–acetic acid[3,4] and arsenic trichloride–acetic acid[5]. The use of molybdophosphoric acid[6,7] as a very sensitive reagent, although non-specific, is well established.

In this paper we describe another very sensitive spray reagent, carbazole–sulphuric acid, which is capable of detecting triterpenoids in amounts as low as 0.8 μg and steroids at levels of 0.2–6 μg.

EXPERIMENTAL

The reagent is prepared by adding 2 ml of concentrated sulphuric acid per 10 ml of a 1% solution of highly purified carbazole (BDH, Poole, Great Britain; recrystallised six times from benzene) in ethanol. Steroids and triterpenoids, after development on silica gel G thin-layer plates (0.1 mm), are sprayed with the reagent and heated at 120°C for 5 min.

RESULTS AND DISCUSSION

The observed colours and the limits of detection are presented in Table I, which shows that only two out of thirty steroidal samples fail to respond even at very much higher concentrations. The colour reaction with phytosterols develops within 3 min, whereas almost all other steroids show no coloration, thereby providing a convenient method for the identification of phytosterols in sample mixtures. The range of colours indicates the diagnostic value of this reagent in differentiating different classes of steroids. The chemistry behind the colour reaction is not clear and it is obvious that neither the stereochemistry of the ring junctions nor the nature of the side-chains and functional characteristics in the molecule can be responsible for the observed coloration.

Amongst the triterpenoids no coloration is observed for samples with the friedelane skeleton, but seven other skeletons *viz.*, lupane, oleanane, ursane, taraxerane, glutinane, taraxasterane and bauerane, responded well. Possibly this is the

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TABLE I

COLOUR REACTIONS OF STEROIDS AND TRITERPENOIDS WITH CARBAZOLE–SULPHURIC ACID REAGENT ON SILICA GEL G THIN-LAYER PLATES

Type	*Compound*	*Colour on heating for 5 min at 120°C*	*Limit of detection (μg)*
Steroids	β-Sitosterol	Pinkish violet	0.2
	β-Sitosterol acetate	Pinkish violet	0.2
	Stigmasterol	Pinkish violet	0.2
	Stigmasterol acetate	Pinkish violet	0.2
	α-Spinasterol	Pinkish violet	0.2
	Cholesterol	Pinkish violet	0.2
	Cholesterol acetate	Pinkish violet	0.2
	Cholesterol palmitate	Pinkish violet	0.2
	Ergosterol	Green	0.5
	Progesterone	No coloration	–
	11α-Hydroxyprogesterone	Light yellow	6.0
	17α-Hydroxyprogesterone	Light brown	6.0
	Pregnenolone	Pinkish violet	0.2
	Testosterone	Brown	6.0
	Cortisone	Light blue	1.2
	Hydrocortisone	Light blue	1.2
	Estrone	Light brown	4.0
	Estrone 3-methyl ether	Orange	0.8
	Estradiol	Deep orange	0.4
	17α-Ethinylestradiol	Pinkish violet	1.2
	17α-Ethinylestradiol 3-methyl ether	Pinkish violet	1.6
	cis-Androsterone	Light brown	0.4
	Dehydro-epi-androsterone	Pinkish violet	1.6
	Androsta-4-ene-17β-ol-3-one	Light green	1.6
	5α-Androsta-17β-ol-3-one	Light green	1.6
	5α-Androsta-3β-ol-17-one	Light green	4.0
	Androsta-1,4-diene-3,17-dione	Light brown	4.0
	17-Ketal of androsta-1,4-diene-3,17-dione	Light brown	4.0
	Norethisterone	Light brown	1.6
	Androsta-4-ene-3,17-dione	No coloration	–
Triterpenes	Lupeol	Reddish brown	0.8
	Methyl betulate	Reddish brown	0.8
	β-Amyrin	Reddish brown	0.8
	β-Amyrin acetate	Reddish brown	0.8
	Taraxerol	Reddish brown	0.8
	Taraxerone	Reddish brown	0.8
	Taraxerol acetate	Reddish brown	0.8
	Multiflorinyl acetate	Reddish brown	0.8
	Methyl oleanolate	Reddish brown	0.8
	Taraxasterol	Reddish brown	0.8
	Taraxasteryl acetate	Reddish brown	0.8
	Glutinol	Reddish brown	0.8
	Bauernyl acetate	Reddish brown	0.8
	Methyl crategolate	Reddish brown	0.8
	Methyl ursolate	Reddish brown	0.8
	Friedelin	No coloration	–
	Epi-friedelinol	No coloration	–
	Putranjivadione	No coloration	–

only report of the differentiation of friedelane derivatives from other triterpenoids through a colour reaction. The sterochemistry of A/B ring junction and the staggered arrangement of the 4- and 5-methyl groups compared with the usual 4,4-dimethyl arrangement in other classes of triterpenoids are probably responsible for the non-participation of friedelane derivatives in colour reactions with this reagent. It is expected that samples with the filicane skeleton, the only system bearing a resemblance to friedelane skeleton, will also exhibit a negative reaction but a lack of samples prevented us from testing this suggestion. It appears that triterpenoids having a *gem*-dimethyl arrangement at C-4 with proper ring A/B stereochemistry and a free hydroxyl or carbonyl group or groups capable of producing a hydroxyl function under the hydrolytic action of sulphuric acid are important in determining the sensitivity of the reaction, as all other triterpenoids examined respond equally and produce the same coloration in spite of the varying stereochemistry of the ring junctions.

It is worth mentioning that the same reagent is capable of producing colorations with other terpenoids also. A few representative samples have already been examined and more are awaiting confirmation. It is expected that this reagent will be versatile for detecting terpenoids and steroids, in addition to its accepted use in the carbohydrate field[8].

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Note

Zur Farbreaktion der Anisaldehyd-Schwefelsäure als Reagenz in der Dünnschicht-Chromatographie

EGON STAHL* und A. GLATZ
Pharmakognosie und Analytische Phytochemie, Universität des Saarlandes, D-6000 Saarbrücken (B.R.D.)
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Aldehyd-Säurereagenzien, insbesondere Anisaldehyd-Schwefelsäure, werden bei dünnschicht-chromatographischen (DC) Untersuchungen häufig verwendet. Bei der Detektion von Alkoholen, Zuckern, Phenolen und Terpenoiden erhält man damit farblich gut differenzierte Zonen[1]. In der Regel liegen die erreichten Erfassungsgrenzen knapp unterhalb 1 μg pro Zone. Allerdings sind die Detektionsfarben nur schlecht reproduzierbar; sie hängen von zahlreichen experimentellen Faktoren, insbesondere von der Detektionszeit und Detektionstemperatur, ab[3]. Quantitative Bestimmungen mit diesen Reagenzien liefern meist streuende Werte. Auf die Ursachen hierfür soll im folgenden am Beispiel von Steroiddetektionen eingegangen werden[2].

EXPERIMENTELLES

Schichtmaterial: Selbstbeschichtete Glasplatten (Streichverfahren 1), des Formats 20 × 20 cm, TLC-Kieselgel 60 HF_{254} mit einer mittleren Korngrösse von 15 μm (E. Merck, Darmstadt, B.R.D.) unter Zusatz von Acronal 250 D (BASF). Zusammensetzung der Suspension: 30 g Kieselgel + 98 ml Wasser + 1.8 ml Acronal-Dispersion; Mengenangabe für 5 DC-Platten. Nass-Schichtdicke 250 μm.

Aldehyd-Säurereagenzien: Zu 100 ml eines Gemisches aus Methanol–Eisessig–Schwefelsäure (85:10:5) wurden jeweils zugesetzt: 0.5 ml Anisaldehyd; 0.5 g 2,4-Dimethoxybenzaldehyd; 0.5 g Vanillin; 0.3 g Benzaldehyd. Davon wurden 20 ml pro Schichtfläche von 20 × 20 cm aufgesprüht. Die Erwärmung der Schichten erfolgte bei 100°C auf einer Heizplatte vom Typ Thermoplate (Desaga, Heidelberg, B.R.D.).

Spektralphotometrische Messungen: Chromatogramm-Spektralphotometer PMQ II mit Kreuztisch (Zeiss, Oberkochen, B.R.D.); Monochromator-Spaltlänge 6 mm, Spaltbreite 0.7 mm.

ERGEBNISSE UND DISKUSSION

Der primäre Schritt der Nachweisreaktion ist die Umsetzung der Steroide mit einer starken Mineralsäure. Dabei entstehen zahlreiche Produkte, die zum Teil farbig sind[4–6]. Einige davon kondensieren dann mit dem vorhandenen Aldehyd zu farbintensiven Verbindungen. Die dabei ablaufenden Vorgänge sind mehrfach untersucht worden[7–10]; dennoch ist der Reaktionsablauf ungeklärt. Je nach Reaktionsbedingun-

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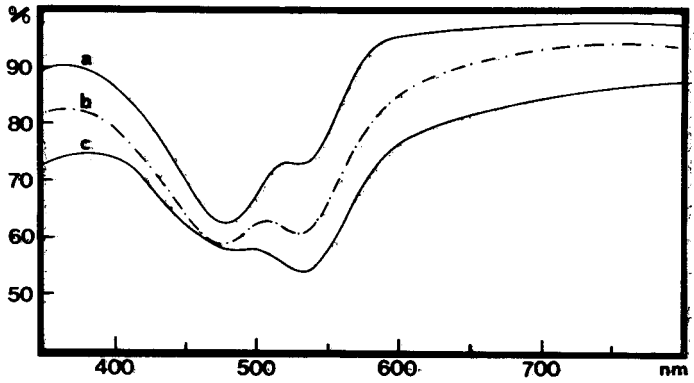

Fig. 1. Remissionsspektren von Östradiol nach Umsetzung mit einem Gemisch aus Methanol–Eisessig–Schwefelsäure (85:10:5), ohne Aldehydzusatz, 100°C, nach (a) 2 min, (b) 15 min, (c) 30 min.

gen wird eine Vielzahl von Produkten beobachtet. Allerdings scheint dem Auftreten von Cyclopentenylkationen zentrale Bedeutung zuzukommen[11,12].

Im Gegensatz zu Versuchen mit einem Überschuss an Lösungsmitteln ändern sich die Reaktionsbedingungen auf der DC-Schicht während der Detektion ständig. Unter dem Temperatureinfluss verdunstet das Methanol —und zum Teil auch der Eisessig— der Detektionslösung. Dadurch erhöht sich die Schwefelsäurekonzentration kontinuierlich. Sie erreicht bei 100°C nach 10 min etwa 50–70 %. Parallel dazu werden die Steroide zu farbigen Produkten umgelagert. Remissionsmessungen zeigen, dass die Ausprägung der Spektren von der Detektionsdauer abhängt (Fig. 1).

In der Regel verschiebt sich das Absorptionsmaximum bei längerer Temperatureinwirkung hin zu grösseren Wellenlängen. Es bleibt jedoch meist unter 500 nm, seltener werden auch 550 nm erreicht. Die visuell wahrgenommenen Zonenfärbungen sind somit gelb–orange, eventuell auch rot oder rot–violett.

Wird dieser Mischung ein aromatischer Aldehyd zugesetzt (Fig. 2), so tritt im

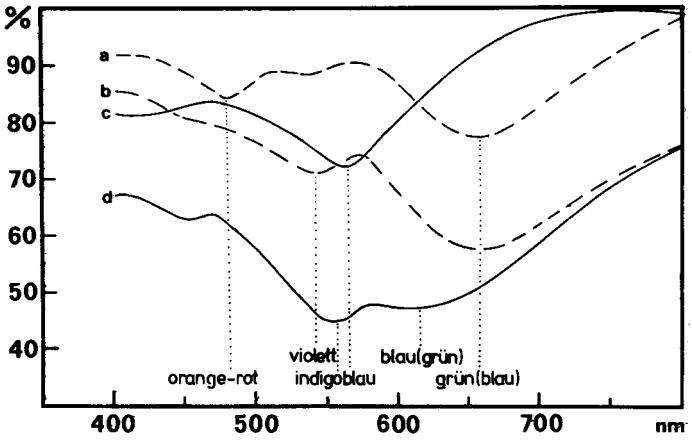

Fig. 2. Remissionsspektren von Östradiol nach Umsetzung mit Anisaldehyd-Schwefelsäure bei 100°C, nach (a) 2 min, (b) 5–10 min, (c) Schichtfärbung nach 15 min, (d) Gesamtabsorption nach 15 min. a und b gegen benachbarte Schichtfläche gemessen, c und d gegen unveränderte Schichtfläche gemessen.

Spektrum eine zusätzliche Absorptionsbande auf. Diese zeigt hohe Intensität und wird stets oberhalb von 550 nm beobachtet. Gleichzeitig verschieben sich die relativen Intensitäten der säurebedingten Absorptionsmaxima. Sie bleiben jedoch auch nach längeren Detektionszeiten nachweisbar. Das zeigt, dass die Umsetzung mit der Säure tatsächlich die Produkte schafft, mit denen der Aldehyd reagiert. Es zeigt aber auch, dass die Umsetzung mit dem Aldehyd zumindest auf der DC-Schicht nur eine von mehreren Reaktionen ist und keinesfalls quantitativ verläuft.

Mit zunehmender Säurestärke tritt allmählich eine Untergrundverfärbung auf. Diese ist, wegen des normalerweise im Überschuss vorhandenen Aldehydes (0.25–0.5 mg/cm^2) auch in der Zone selbst nachweisbar. Ursache hierfür ist eine Autokondensationsreaktion der aromatischen Aldehyde zu uneinheitlich polymeren Farbstoffen des Di- oder Triphenylmethantypes.

In ihrer Überlagerung bestimmen die drei beschriebenen Reaktionsabläufe den Farbton der detektierten Zonen. Alle drei laufen als Funktionen der Säurestärke, jedoch mit unterschiedlichen Geschwindigkeiten ab. Deshalb verändert sich die beobachtete Färbung während der Detektion ständig. Im vorliegenden Beispiel von grau–braun über grün–blau hin zu violett. Dazu kommt noch, dass die Mengenverhältnisse der vorliegenden Reaktionspartner die verschiedenen Reaktionen in unterschiedlichem Masse begünstigen. Das wiederum verändert die Farbausprägung. In der Tabelle I sind einige Einflussgrössen und deren Auswirkungen aufgelistet.

TABELLE I

EINFLUSSGRÖSSEN UND DEREN AUSWIRKUNGEN AUF DIE DETEKTIONSFARBEN BEI VERWENDUNG VON ALDEHYD-SÄUREREAGENZIEN

Einflussgrösse	*Auswirkung*
Hohe Schwefelsäurekonzentration im Reagenz Grosse zu detektierende Substanzmenge Wenig Reagenz pro Schichtfläche Kurze Detektionszeiten Niedrige Detektionstemperatur	Hervortreten säurebedingter Färbungen
Geringe Schwefelsäurekonzentration im Reagenz Grosse Reagenzmenge pro Schichtfläche Niedrige Detektionstemperatur	Hervortreten der aldehydbedingten Färbungen
Hohe Schwefelsäurekonzentration im Reagenz Hohe Aldehydkonzentration im Reagenz Grosse Reagenzmenge pro Schichtfläche Hohe Detektionstemperatur Lange Detektionszeiten	Untergrund- und Zonenverfärbungen

Die Vielzahl der Einflussgrössen bedingt auch die geringe Eignung dieser Reagenzien für direkte quantitative, photometrische DC-Bestimmungen. Bei der photometrischen Auswertung wird stets nur ein Teil der Substanz erfasst. Standardabweichungen unter 10 oder 15% lassen sich nur unter strengster Kontrolle aller Reaktionsbedingungen erreichen.

In der Praxis haben sich als Aldehydkomponenten neben Anisaldehyd insbesondere Vanillin und 2,4-Dimethoxybenzaldehyd bewährt[13,14]. Bei etwa gleichen Er-

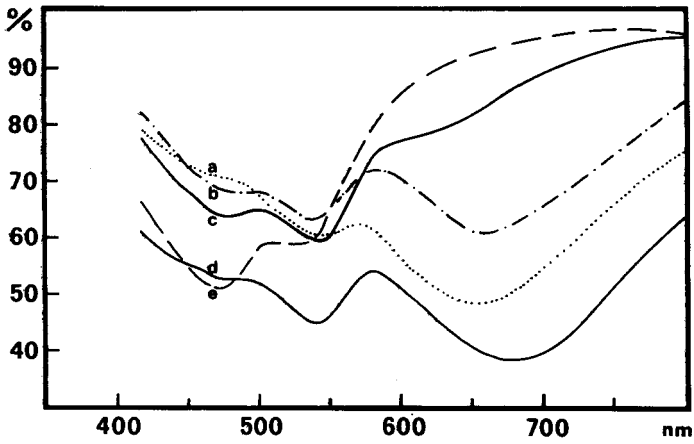

Fig. 3. Remissionsspektren von Östradiol nach Umsetzung mit verschiedenen Aldehyd-Säurereagenzien bei 100°C, nach 10 min. (a) Anisaldehyd-Schwefelsäure, (b) Vanillin-Schwefelsäure, (c) Benzaldehyd-Schwefelsäure, (d) 2,4-Dimethoxybenzaldehyd-Schwefelsäure, (e) aldehydfreies Reagenz. Messung gegen benachbarte Schichtfläche.

fassungsgrenzen werden zum Teil jedoch völlig verschiedene Färbungen beschrieben[3]. Fig. 3 zeigt, dass dies nicht auf einer Verschiebung der Absorptionsbanden, bedingt durch Art, Zahl und Anordnung der Chromophore beruht. Massgeblich sind zum geringeren Teil unterschiedliche Carbonylaktivitäten und Extinktionskoeffizienten der verschiedenen Aldehyde. Stärker ist der Einfluss, den veränderte Konzentrationsverhältnisse im Reagenz oder geänderte Detektionstemperaturen oder Detektionszeiten ausüben. Unter konstanten Reaktionsbedingungen jedenfalls werden mit den drei Reagenzien, zeitlich etwas versetzt, sehr ähnlich Färbungen erhalten.

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Note

Gas chromatographic determination of some methyl α-D-glucopyranoside pentyloxymethyl ethers

JACEK STANIEWSKI and JAN SZYMANOWSKI*
Poznań Polytechnic, Institute of Chemical Technology, Sklodowskiej-Curie 2, 60-965 Poznań (Poland)
(Received December 9th, 1981)

In previous work[1–6] we discussed the synthesis and properties of non-ionic surfactants having sucrose or glucose as the hydrophilic part of the surfactant molecule and an alkyloxymethyl group as the hydrophobic part. Thin-layer chromatography[7,8] was used to determine the relative proportions of the mono-, di- and trisubstituted derivatives. However, the proportions of the individual positional isomers were not determined.

The aim of this work was to determine the chromatographic conditions for separating the positional isomers of methyl mono-O-pentyloxymethyl-α-D-glucopyranoside and methyl di-O-pentyloxymethyl-α-D-glucopyranoside, the main products in the reaction of methyl α-D-glucopyranoside with pentyloxymethyl chloride.

EXPERIMENTAL

Materials

The products from the reaction of methyl α-D-glucopyranoside with pentyloxymethyl chloride, the standard isomers of methyl mono-O-pentyloxymethyl-α-D-glucopyranoside having a pentyloxymethyl group at the 2, 3, 4 or 6 positions and a mixture of the positional isomers of methyl di-O-pentyloxymethyl-α-D-glucopyranoside, were obtained in our laboratory. Methyl α-D-glucopyranoside (Fluka, Buchs, Switzerland) was used as substrate.

The products were analysed as their trimethylsilyl derivatives. A mixture of anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane (9:3:1) was used as solvent.

Chromatographic separation

A Jeol 1100 TFP gas chromatograph equipped with a flame-ionization detector and coupled with a Takeda Riken TR-2215A electronic integrator was used. For the separation, glass columns (2 m × 3 mm I.D.) were used with SE-30, OV-1, OV-17 and OV-25 (3%) as the liquid phases. Gas-Chrom Q (80–100 mesh) was used as the support. Helium was used as the carrier gas with a flow-rate of 1 cm^3/sec. The detector and injector temperatures were 555 ± 2 and 565 ± 5°K, respectively. The column temperature was raised from 453 to 533°K at a rate of 2°K/min.

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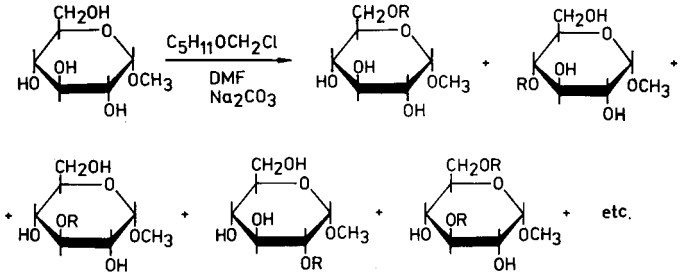

Fig. 1. Reaction scheme.

RESULTS AND DISCUSSION

In the reaction of methyl α-D-glucopyranoside with pentyloxymethyl chloride the products may contain four different positional monoether isomers, six diether isomers, four triether isomers and one tetraether (Fig. 1).

When columns having OV-17 or OV-25 semi-polar phases were used we did not separate all the monoisomers: the isomers having a pentyloxymethyl group at

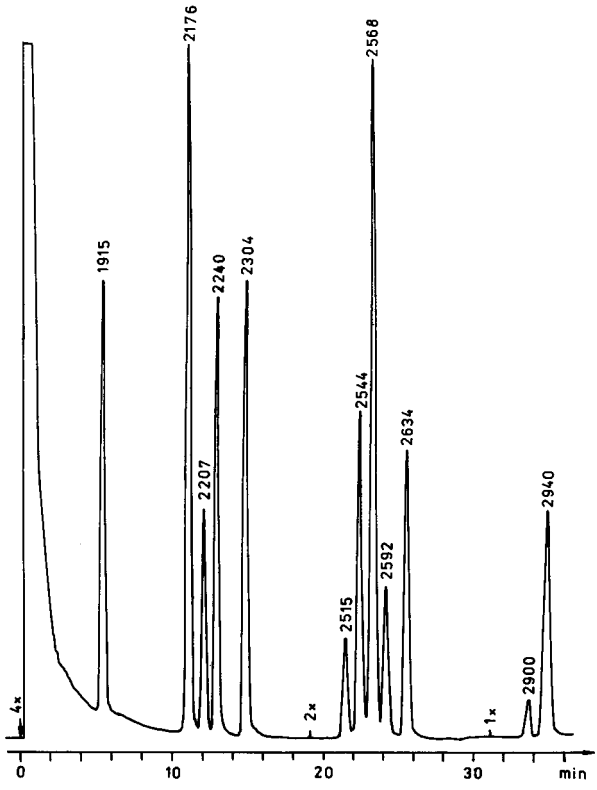

Fig. 2. Chromatogram of methyl α-D-glucopyranoside pentyloxymethyl ethers.

TABLE I

RETENTION INDICES OF METHYL α-D-GLUCOPYRANOSIDE PENTYLOXYMETHYL ETHERS MEASURED FOR TRIMETHYLSILYL ETHERS

Compound	*Isomer*	*Retention index*
Methyl α-D-glucopyranose		1915
Monoether	3-O-	2176
	4-O-	2207
	2-O-	2249
	6-O-	2304
Diether	3,4-O-	2515
	2,3-O- + 2,4-O-	2544
	3,6-O-	2568
	4,6-O-	2592
	2,6-O-	2634
Tri- and tetraethers	Tri- and tetraethers	2900
		2942

positions 2 and 4 eluted together. Similarly, we obtained only four partly separated peaks for the diether.

When columns having SE-30 or OV-1 phases were used we separated all four isomers of the monoether and all four isomers of the diether. Only methyl 2,3-di-O-pentyloxymethyl-α-D-glucopyranoside and methyl 2,4-di-O-pentyloxymethyl-α-D-glucopyranoside eluted together as one single peak. A somewhat better separation was obtained for the SE-30 phase (Fig. 2).

TABLE II

COMPOSITION OF THE REACTION PRODUCTS

Temperature, 320°K; N,N-dimethylformamide used as solvent. Product A = methyl α-D-glucopyranoside–pentyloxymethyl chloride (2:1, mole/mole); product B = methyl α-D-glucopyranoside–pentyloxymethyl chloride = (1:2, mole/mole).

Compound	*Isomer*	*Content (%)*	
		Product A	*Product B*
Monoether	3-O-	33.5	22.9
	4-O-	9.8	4.8
	2-O-	17.8	8.8
	6-O-	22.3	11.2
Diether	3,4-O-	0.9	2.1
	2,3-O- + 2,4-O-	3.2	8.0
	3,6-O-	6.6	18.8
	4,6-O-	1.5	4.7
	2,6-O-	4.4	11.8
Tri- and tetraethers		–	6.9

The first five peaks were identified by comparison with standard samples of methyl α-D-glucopyranoside and methyl mono-O-pentyloxymethyl-α-D-glucopyranosides. Successive isomers of the diether were identified by comparison of the chromatograms (*i.e.* of the retention indices of the separated components) of the products obtained separately from four different positional isomers of the monoether. The isomers of the tri- and tetraethers were not considered.

The values of the retention indices for the separated isomers of the mono- and diethers are given in Table I. The values of the retention indices depend upon the position of the pentyloxymethyl group, especially when positions 3 and 6 are considered. The range of the retention indices, both for the mono- and diethers, is *ca.* 120–130, the smallest differences being observed for isomers substituted at the 3-, 4- or 2-positions (25–30 units).

For the separated mono- and diethers, the relative molar response is *ca.* 1 (this was checked and the error of such an assumption found to be less than 5%). The proportions of the different isomers of the mono- and diethers in two exemplary products are given in Table II.

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Note

Simplified method for determining acephate and methamidophos residues in several substrates

S. Y. SZETO
Research Station, Agriculture Canada, Vancouver, B.C., V6T 1X2 (Canada)
J. YEE
Department of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6 (Canada)
M. J. BROWN
Research Station, Agriculture Canada, Vancouver, B.C. V6T 1X2 (Canada)
and
P. C. OLOFFS
Department of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6 (Canada)
(Received January 18th, 1982)

Acephate (O,S-dimethyl acetylphosphoramidothioate) is a broad-spectrum insecticide, introduced by the Chevron Chemical Company under the trade name Orthene® in 1971 (ref. 1). The uses of this chemical for pest control in agriculture, forestry, and for domestic purposes have increased rapidly in the past because of its effectiveness against a wide range of pest insects, such as aphids, thrips, leaf miners, sawflies and lepidopterous larvae and because of its low toxicity to higher animals, especially fish. However, acephate is partially metabolized in some plants and animals to yield the highly toxic methamidophos (O,S-dimethyl phosphoramidothioate), an excellent insecticide in its own right, and marketed under the trade names Monitor® and Tamaron® in North America and Europe, respectively.

In order to monitor the fate of acephate and methamidophos following their application for purposes of insect pest control, sensitive, reliable, and relatively simple analytical methods for these compounds are essential. A gas chromatographic (GC) method for the analysis of residues of these compounds in crops was described by Leary[2] and modified for citrus foliage by Nigg *et al.*[3]. According to these authors, plant tissues are extracted with ethyl acetate or acetonitrile by blending. The crude extracts are cleaned by eluting acephate and methamidophos from silica gel columns with 10% (v/v) methanol–ether prior to analysis by gas chromatography–alkali flame-ionization detection (GC–AFID). This fraction still contains considerable amounts of extraneous substances, such as plant pigments and other co-extractants, which are deleterious to the GC columns and reduce sensitivity progressively[4].

An activated coconut-charcoal column was described for the extraction and clean-up of acephate and methamidophos from natural waters[5], a method sensitive to sub-ppb concentrations. However, the preparation and activation of the coconut charcoal, as well as the elution of the samples from the charcoal columns are exceedingly tedious and cricital for consistent results.

This paper describes simpler and less time-consuming methods for the analyses

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of acephate and methamidophos residues in natural waters, sediments, and tissues from plants (asparagus ferns and spears) and animals (trout).

MATERIALS AND METHODS

Analytical grades of acephate (99.3%) and methamidophos (99.6%) were obtained from the Chevron Chemical Company. Standard solutions for sample fortifications and gas–liquid chromatographic (GLC) analyses were prepared by appropriate dilutions with acetone or ethyl acetate. Charcoal (Nuchar C, Fisher Scientific) was acid-washed prior to use[6]. Pyrex glass wool and Whatman CF-11 cellulose were used in clean-up columns. Glass-distilled, pesticide-grade solvents were used throughout.

Asparagus ferns and spears were obtained from the Research Station, Agriculture Canada, Summerland, B.C., Canada. Rainbouw trout (*Salmo gairdneri*), mean weight 5 g, were purchased from a trout farm in the Lower Fraser Valley, east of the city of Vancouver, B.C. Water and sediment samples were collected from a pond, located in the Coastal Mountains, approximately 50 km north-east of Vancouver.

Prior to fortification with acephate and methamidophos to give concentrations of either 1.0 or 0.01 ppm, plant tissues were macerated; sediments, placed in Büchner funnels, were stripped of excess water by aspiration; whole fish, one per sample, were weighed and cut into small pieces. Water was treated as received.

Extraction and clean-up

Tissue and sediment samples were extracted, three times each, according to Table I (solvents: EA = ethyl acetate; An = acetonitrile. Na_2SO_4 = anhydrous sodium sulphate.

TABLE I

EXTRACTION PROCEDURES

Sample	Asparagus	Fish	Sediment
Sample size (g)	10	5	50
Solvent (ml per extraction)	EA, 100	An, 100	An, 100
Time (min per extraction)	5	1	5
Na_2SO_4 (g)	20	10	50
Apparatus	Omni-Mixer	Polytron PT20	Omni-Mixer

The Na_2SO_4 was added before the first extraction; the Sorvall Omni-Mixer was equipped with threaded 400-ml jars.

The asparagus and fish extracts were filtered through glass-fibre disks in Büchner funnels, the sediment extracts through Pyrex glass wool plugs in filter funnels. The three fractions from each sample were combined in measuring cylinders and the volumes recorded. Aliquots of crude fish extracts, equivalent to 2 g of body weight, were transferred to 250-ml separatory funnels and extracted three times with 20 ml of hexane to remove hexane-soluble lipids. The combined hexane phases were back-extracted with 20 ml of acetonitrile before being discarded.

Crude extracts, equivalent to 2 g of asparagus or fish or 10 g of sediment, were

used for clean-up. In 100-ml round-bottom flasks, they were evaporated just to dryness by flash evaporation at 38°C. The residues were picked up in 1 ml of ethyl acetate, followed by the addition of 4 ml of hexane. Glass columns (30 × 1.1 cm I.D.) with PTFE stop-cocks were packed, from the bottom up, with a glass wool plug, 2 cm of Na_2SO_4, 4 cm of a 2:5 (w/w) mixture of Nuchar C and Whatman CF-11, 2 cm of Na_2SO_4 and a glass wool plug. The packed columns were pre-washed with 10 ml of ethyl acetate, followed by 10 ml of hexane. Then the redissolved extracts were transferred quantitatively from the round-bottom flasks to the clean-up columns. The resulting eluates were discarded. Finally, acephate and methamidophos were eluted from the columns with 40 ml of ethyl acetate. These eluates were suitably concentrated for GLC analysis.

Water samples (5 ml) were mixed with approximately 75 ml of acetonitrile, to form an azeotropic mixture, and then evaporated just to dryness in a flash evaporator at 38°C. The residues were picked up in appropriate volumes of acetone for GLC analysis, no further clean-up being required.

GLC analysis

Three Pyrex glass columns were used: (1) 36 cm × 4 mm I.D., Ultra-Bond II, 100–120 mesh; (2) 75 cm × 2 mm I.D., 1% OV-225 on Ultra-Bond 20M, 100–120 mesh; (3) 36 cm × 3 mm I.D., 1% Carbowax 20M TPA on Chromosorb W HP, 100–120 mesh. Columns 1 and 2 were operated isothermally at 150 and 140°C, respectively, with the carrier gas flow-rate at 40 ml/min; column 3 was programmed from 145 to 190°C at 30°C/min, carrier gas flow-rate at 60 ml/min.

The operating parameters were as follows: air 120 ml/min, hydrogen 3.5 ml/min and helium as carrier 40 ml/min with columns 1 and 2 and an alkali flame-ionization detector (Tracor 702-NP) at 240°C; hydrogen 200 ml/min, air 150 ml/min and nitrogen as carrier 60 ml/min with column 3 and a flame photometric detector (phosphorus mode) at 170°C; inlet and outlet temperature 210°C.

Detector response was calibrated daily before and after sample analysis with authentic reference-grade standards. Quantification of acephate and methamidophos was based on average peak heights of these external standards, injected before and after sample analysis.

RESULTS AND DISCUSSION

Performance of the GC columns

All three columns gave good separation of acephate and methamidophos; absolute retention times are given in Table II. Columns 1 (Ultra-bond II) and 2 (1% OV-225 on Ultra-Bond 20M) are recommended because good separation of acephate and methamidophos can be obtained isothermally at relatively low temperature, and because of the inertness of these packings[7]. It was observed, however, that moisture has deleterious effects on these columns, as well as on column 3, and that, in order to maintain their high performance, it is extremely important to analyse on these columns moisture-free extracts only. Fig. 1 shows typical chromatograms obtained with these columns.

Recoveries of acephate and methamidophos

Recoveries from the four substrates (plant tissue, fish tissue, sediments and

TABLE II

ABSOLUTE RETENTION TIMES OF ACEPHATE AND METHAMIDOPHOS

GC column	*Temperature*	*Retention times (min)*	
		Acephate	*Methamidophos*
(1) Ultra-Bond II	150°C	1.25	0.75
(2) 1% OV-225 on Ultra-Bond 20M	140°C	1.76	0.88
(3) 1% Carbowax 20M TPA on Chromosorb W HP	145–190°C (30°C/min)	1.05	0.47

natural water) were 85%, or higher, following fortification with both acephate and methamidophos to give either 1.0 or 0.01 ppm of both (Table III). The exceedingly high recovery of methamidophos from water treated to contain 0.01 ppm, namely 116 ± 7.2%, is difficult to explain. De-acetylation of acephate was not indicated since recovery of acephate from these samples was not correspondingly lower (Table III). Methamidophos-resembling responses in control samples, discussed below, evidently were not responsible either.

The clean-up achieved with the charcoal–cellulose (Nuchar C–Whatman CF-11) column was excellent. Following elution from this column, extracts equivalent to 10 g of asparagus spear, 2 g of asparagus fern, 2 g of fish or 10 g of sediment were pigment-free and contained no other extraneous substances interfering with subsequent analysis by GLC of acephate and methamidophos using either of the columns, detectors or conditions described.

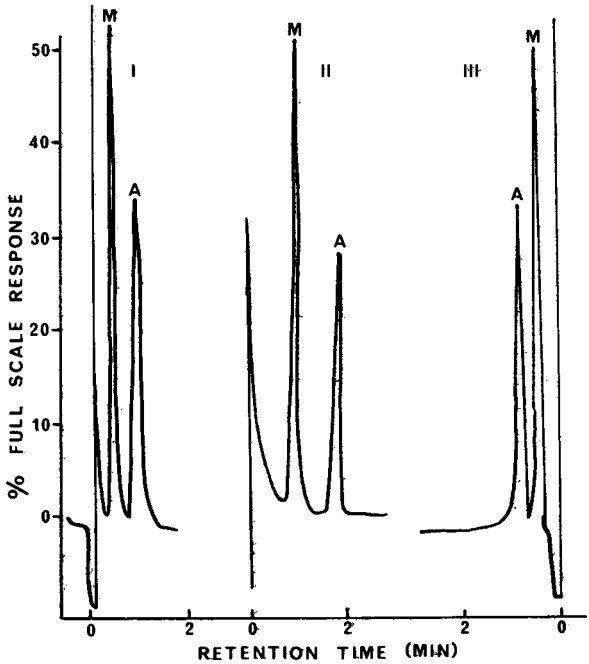

Fig. 1. Chromatograms of acephate (A) and methamidophos (M), 0.4 ng each, on (I) Ultra-Bond II, (II) 1% OV-225 on Ultra-Bond 20M and (III) 1% Carbowax 20M TPA on Chromosorb W HP.

TABLE III

RECOVERY OF ACEPHATE AND METHAMIDOPHOS FROM ASPARAGUS, FISH, POND WATER AND SEDIMENT

(Mean ± S.D., in %, $n = 4$)

Origin	*Fortification*			
	1.0 ppm		*0.01 ppm*	
	Acephate	*Methamidophos*	*Acephate*	*Methamidophos*
Asparagus	95.1 ± 2.3	93.1 ± 2.3	91.5 ± 2.3	86.8 ± 3.2
Fish	99.0 ± 7.9	84.8 ± 4.3	101 ± 7.5	84.6 ± 5.9
Sediment	105 ± 1.5	103 ± 1.7	96.7 ± 2.9	102 ± 6.9
Water	97.9 ± 1.9	95.5 ± 4.1	102 ± 8.2	116 ± 7.2

Figs. 2 and 3 show chromatograms of acephate and methamidophos, extracted from the four substrates following fortification to 0.01 ppm. Extracts of asparagus and sediment samples contained substances interfering with methamidophos analysis. These were removed, however, by discarding the first eluate following the transfer of the residue in ethyl acetate–hexane (1:4) to the Nuchar–cellulose clean-up column. Extracts without these interfering substances could be transferred to the clean-up columns in 5 ml of ethyl acetate (no hexane) and without discarding the resulting eluate.

In preparing the azeotropic mixtures, an excess of acetonitrile was added to the water samples to ensure complete removal of water during flash evaporation as trace

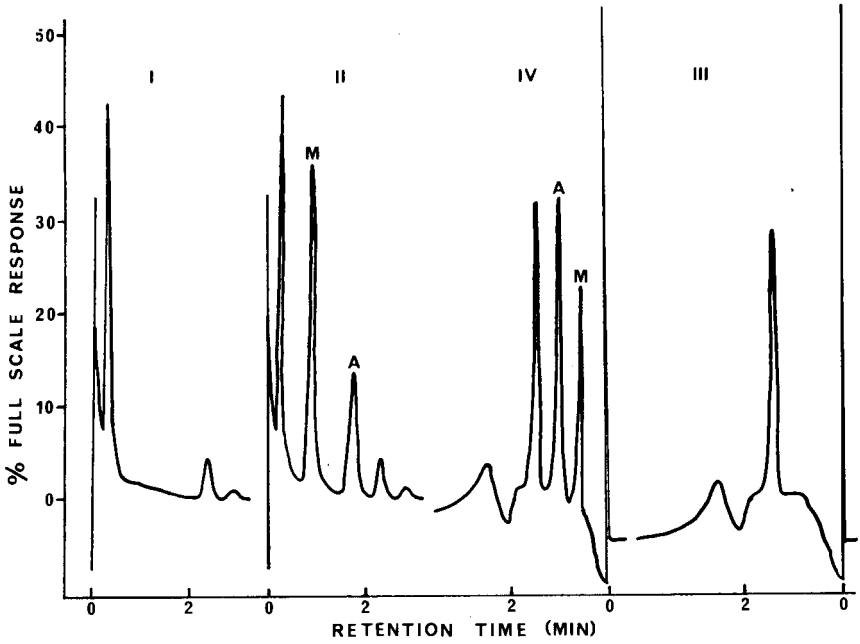

Fig. 2. Chromatograms of (I) asparagus blank, (II) asparagus with acephate (A) and methamidophos (M) at 0.01 ppm each, (III) fish blank and (IV) fish with A and M at 0.01 ppm each.

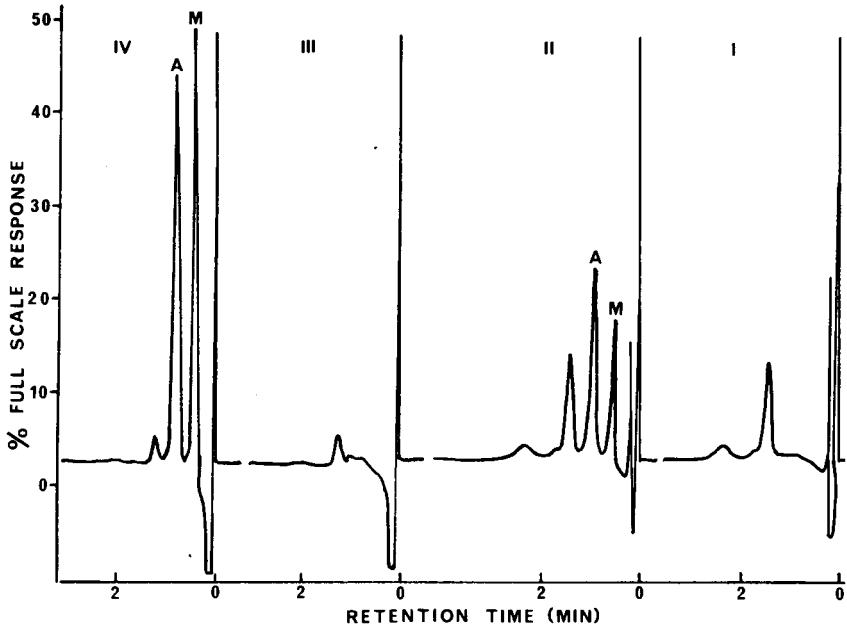

Fig. 3. Chromatograms of (I) sediment blank, (II) sediment with acephate (A) and methamidophos (M) at 0.01 ppm each, (III) water blank and (IV) water with (A) and (M) at 0.01 ppm each.

quantities of water present in the extracts drastically and rapidly shortened column life. This method for analysing acephate and methamidophos in water is rapid, simple, and reliable for sensitivities as low as 1–5 ppb. To improve the sensitivity levels, larger water samples and correspondingly larger volumes of acetonitrile would be required. Further clean-up, following evaporation of the azeotropic mixture, may also become necessary, rendering this method impractical and/or uneconomical if sub-ppb sensitivities are imperative. In that case, the coconut-charcoal method[5] might be more desirable.

ACKNOWLEDGEMENTS

We thank Mr. F. Huen for his valuable technical contribution. We gratefully acknowledge financial support by the Ministry of Forestry, The Province of British Columbia, and by the National Research Council of Canada, Ottawa. We also thank the Chevron Chemical Company for providing analytical standards of acephate and methamidophos.

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Note

High-performance liquid chromatography of phosfolan, mephosfolan and related compounds

SHERIF A. ABOU-DONIA* and MOHAMED B. ABOU-DONIA*
Department of Pharmacology, Duke University Medical Center, Durham, NC 27710 (U.S.A.)
(First received November 11th, 1981; revised manuscript received January 18th, 1982)

The organophosphorus compounds phosfolan (O,O-diethyl 1,3-dithiolan-2-ylidene phosphoramidate) and mephosfolan (O,O-diethyl 4-methyl 1,3-dithiolan-2-ylidene phosphoramidate) are systemic insecticides that have been used extensively for pest control on cotton, corn, rice, sorghum and sugar cane, especially in the Middle East and Asia[1]. There are several reports concerning the use of phosfolan and mephosfolan against the Egyptian cotton leaf worm (*Spodoptera littoralis*)[2], the pink bollworm (*Pectinophora spp.*) and the spiney bollworm (*Earious insulana*)[3,4].

Numerous methods have been developed for the determination of phosfolan and mephosfolan. These insecticides have been analyzed frequently by spectrophotometry[5,6], gas–liquid chromatography[6] and thin-layer chromatography[6–11]. All these methods have been used mainly for determination of the intact compounds, although in some cases a few metabolites were identified. For example, a sequential thin-layer chromatographic method has been used to analyze phosfolan and mephosfolan and some of their possible metabolites in animals given the ^{14}C-labeled insecticide[12].

The present study describes a high-performance liquid chromatographic (HPLC) method that was developed for quantitative and qualitative analysis of phosfolan, mephosfolan and some of their degradation products. This method offers rapid and accurate analysis of these compounds in metabolic studies and in the analysis of their residues in the environment. In this study, to demonstrate its effectiveness in biological and non-biological systems, the method was used to analyze the residue of these chemicals in human plasma and tomato leaves.

EXPERIMENTAL

Materials

Phosfolan (Cyolane), mephosfolan (Cytrolane), and their related compounds were provided by American Cyanamid (Princeton, NJ, U.S.A.). The following analytical-grade compounds were studied: phosfolan (O,O-diethyl 1,3-dithiolan-2-ylidene phosphoramidate), mephosfolan (O,O-diethyl 4-methyl 1,3-dithiolan-2-ylidene phosphoramidate), hydroxy mephosfolan (O,O-diethyl 4-hydroxymethyl-1,3-dithiolan-2-

* Permanent address: Department of Plant Protection, Al Azhar University, Cairo, Egypt.

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ylidenephosphoramidate), PDIC (propylene dithioimidocarbonate hydrochloride), PDC (propylene dithiocarbonate), EDIC (ethylene dithioimidocarbonate hydrochloride) and EDC (ethylene dithiocarbonate). Stock solutions of these compounds were prepared in methanol (1 mg/ml) and appropriate dilutions were made to produce working standards. Stock and standard solutions were stored at −20°C in amber glass vials with no detectable decomposition over a period of 2 months.

High-performance liquid chromatography

Reversed-phase HPLC was performed using a Waters Assoc. liquid chromatograph (Milford, MA, U.S.A.) consisting of two M6000-A pumps, an M660 solvent programmer, a M440 ultraviolet (UV) detector, a U6-K universal injection system and a stainless steel precolumn (10 cm × 2 mm I.D.) containing a reversed-phase μBondapak C_{18} followed by a μBondapak C_{18} column (30 cm × 3.9 mm I.D.). The HPLC-grade solvents were filtered through Millipore membrane filters, type HA or FH, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.) and degassed under vacuum prior to use. Test compounds were injected in 2–25 μl of methanol solutions and separated by gradient elution at room temperature. The initial solvent composition was 5% methanol, 1% glacial acetic acid and 94% water, changing to a final solvent composition of 100% methanol in 60 min. The gradient shape employed was Waters No. 6 gradient shape. The solvent flow-rate was 2.0 ml/min, and the column inlet pressure was 2000 p.s.i. All compounds were detected and quantitated by monitoring the UV absorbance of the column eluates at 254 nm using a 10-mV chart recorder with a chart speed of 1.0 cm/min. Peak areas were measured with a Shimadzu Chromatopak ElA (Shimadzu, Kyoto, Japan) reporting integrator.

Application and extraction of phosfolan, mephosfolan and their related compounds from tomato leaves

Aliquots (5 μl) of methanol solutions (1 mg/ml) of phosfolan, mephosfolan and their related metabolites were spotted on the upper surface of ten tomato leaves. The leaves were placed in a 150-ml Erlenmeyer flask, stoppered tightly and kept for 10 min at room temperature. Then, the leaves were homogenized in 30 ml of hexane–acetone (4:1) for 3 min using a Polytron Ultrasonic homogenizer (Brinkmann, Westbury, NY, U.S.A.). To clean up the solution, 0.1 g of charcoal (Norite A) and 1 g of anhydrous sodium sulfate were added. The flask was swirled gently for 2 min and then stirred by a magnetic stirrer for 5 min. The homogenate was filtered through Whatman No. 1 filter paper which was washed with 5 ml of the solvent mixture, and the filtrate and the wash were combined in the same flask. The solvent was evaporated to dryness using a rotary evaporator at 40°C under reduced pressure and then diluted to 50 μl by methanol. Aliquots of 5 μl were injected into the HPLC columns, eluted and detected as described above.

Application and extraction of phosfolan, mephosfolan and their related compounds from human plasma

Aliquots (5 μl) of methanol solutions (1 mg/ml) of phosfolan, mephosfolan and their related compounds were added to 5 ml of human plasma in 13 × 100 mm screw-capped culture tubes and mixed for 1 min using a vortex mixture. Ethyl acetate (10 ml) was added to the tube, mixed for 1 min, and then centrifuged at 3000 rpm for

5 min. The ethyl acetate layer was transferred to a small flask by Pasteur pipette, and evaporated to dryness using a rotary evaporator at 40°C under reduced pressure. The residue was dissolved to 50 μl of methanol. Aliquots of 5 μl were analyzed by the HPLC procedure described above.

RESULTS AND DISCUSSION

Most organophosphorus insecticides are composed of highly lipophilic molecules; however, biotransformation of these insecticides within the affected organism generally results in the molecule becoming more polar and more water-soluble. This biological modification of organophosphorus insecticides usually results in great changes in the toxicity of the compounds. Therefore, the prime objective of the present study is to develop a rapid and sensitive HPLC method which could be used specifically and quantitatively for the analysis of phosfolan, mephosfolan and some of their metabolites (Fig. 1) in both biological and non-biological systems. To determine the applicability of this method for both systems, it was used to analyze the test compounds in tomato leaves and human plasma.

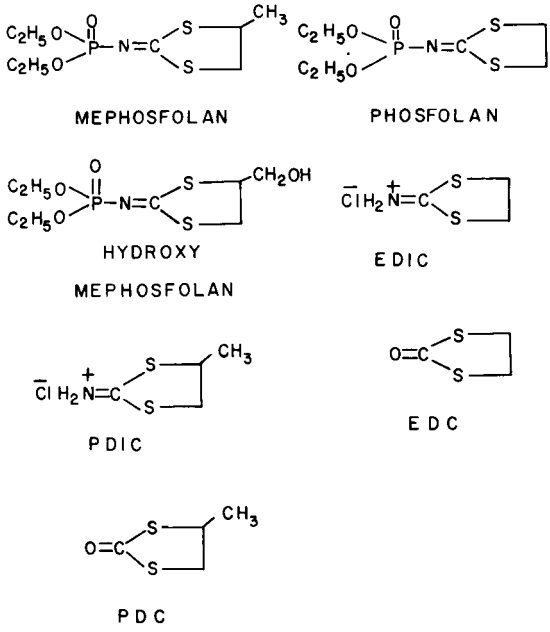

Fig. 1. Chemical structures of phosfolan, mephosfolan and their possible degradation products. Abbreviations are listed under Materials.

These compounds could be classified into two groups according to their separation on HPLC: non-polar compounds (mephosfolan and phosfolan) and polar compounds (PDIC, PDC, EDC and EDIC). Hydroxy mephosfolan exhibited behaviour intermediate between non-polar and polar. Generally, these two groups can be resolved from each other; however, the individual compounds within the polar group may not be readily separated. Because all the compounds have a wide range of

polarity, several HPLC solvents were evaluated to develop a method to distinguish quickly the parent compounds from each other and from their possible degradation products. The best solvent system for the separation of non-polar compounds from each other, as well as from the polar compounds on the reversed-phase μBondapak C_{18} column, was the solvent gradient of 5–10% methanol in water. In this system, non-polar substances moved in good separable distances from each other. Mephosfolan was the slowest followed by phosfolan and then the intermediate hydroxy mephosfolan. By contrast, although the polar compounds eluted faster and separated well from the non-polar substances, they eluted as two peaks with shoulders. EDC appeared as a shoulder on the EDIC peak and PDC appeared as a shoulder on the PDIC peak.

The problem of resolution of EDC from EDIC and PDC from PDIC was solved by adding 1% HPLC-grade glacial acetic acid to the initial eluting water. When this method was used all the pairs of compounds with overlapping peaks were resolved, *i.e.*, PDIC–PDC and EDIC–EDC. Good resolution of the parent phosphates and their degradation products was obtained by reversed-phase HPLC using μBondapak C_{18} columns (Fig. 2). At a flow-rate of 2.0 ml/min, all compounds eluted as sharp symmetrical peaks with baseline resolution. A sharply concave gradient was employed to decrease the retention of the highly non-polar compounds.

The retention times and UV absorbance of the phosfolan and mephosfolan are listed in Table I. These data were highly reproducible with the elution solvents and

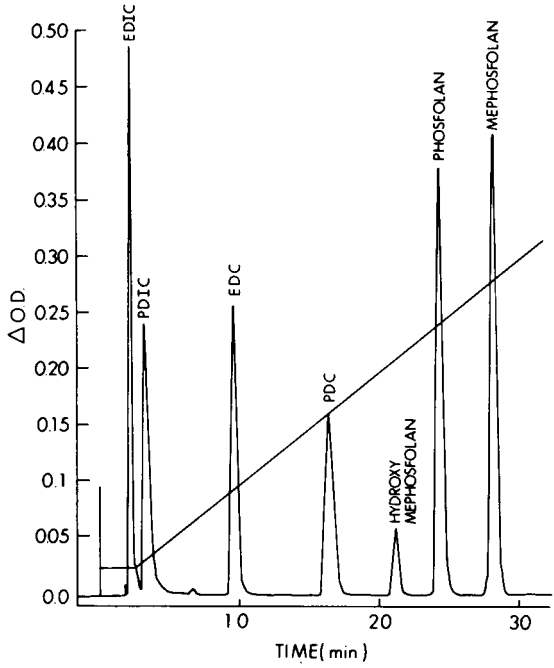

Fig. 2. Separation of phosfolan, mephosfolan and their related compounds by reversed-phase HPLC. Elution from μBondapak C_{18} column using a gradient of 5–100% methanol–water (initial solvent also contained 1% glacial acetic acid) in 60 min. Flow-rate 2 ml/min at 25°C. The shape of gradient used is described by the solid tracing.

TABLE I

RETENTION TIMES AND RELATIVE UV ABSORBANCE OF PHOSFOLAN, MEPHOSFOLAN AND RELATED COMPOUNDS USING HPLC

Experimental conditions are listed under Experimental.

Compound	*Retention time* (min)*	*Relative UV absorbance***
Mephosfolan	29.05	1.00
Hydroxymephosfolan	21.79	0.13
PDIC	3.93	0.59
PDC	17.14	0.52
Phosfolan	25.01	0.96
EDIC	2.56	0.63
EDC	10.36	0.55

* Mean of four successive injections of 5 μg of each compound.

** Values are expressed relative to the UV absorbance at 254 nm of 5 μg of mephosfolan with detector sensitivity at 0.5 a.u.f.s.

column used. The UV detection limit at 254 nm was 10 ng for phosfolan, mephosfolan, EDIC, EDC, PDIC and PDC. Hydroxy mephosfolan had a detection limit of 100 ng.

The relationship between peak area and amount injected remained linear over a 1000-fold range (10 ng–10 μg) (Figure not shown).

This method was successful in analyzing these compounds in tomato leaves and human plasma (Figs. 3 and 4). All test chemicals were detected in both leaves and

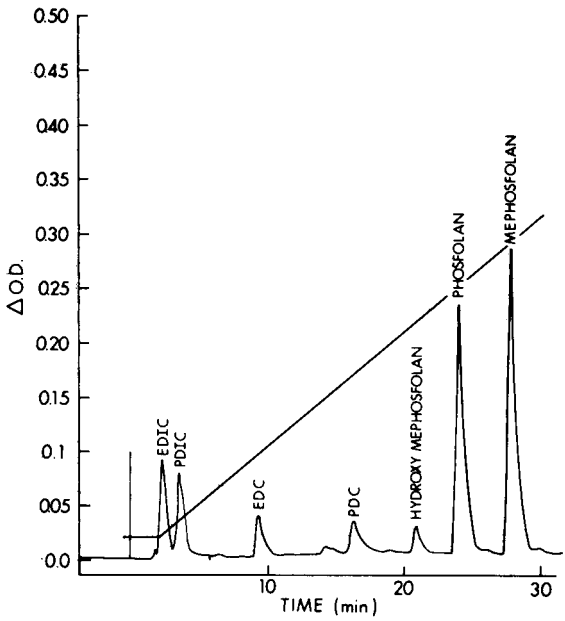

Fig. 3. HPLC profile of phosfolan and mephosfolan and related compounds extracted from tomato leaves. HPLC conditions as in Fig. 2.

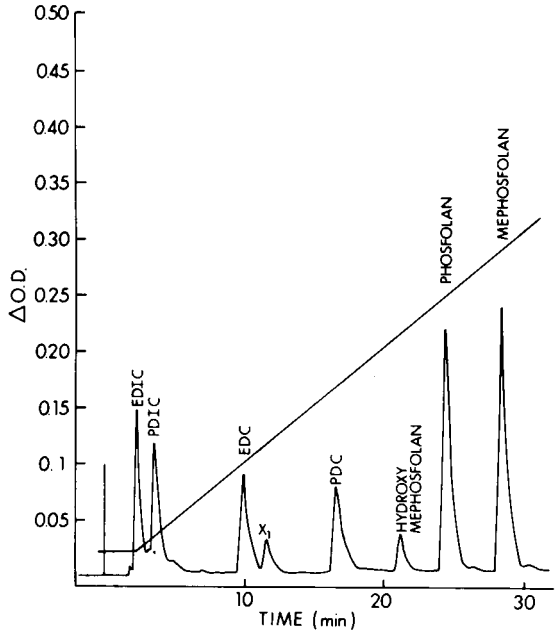

Fig. 4. HPLC profile of phosfolan and mephosfolan and related compounds extracted from human plasma. HPLC conditions as in Fig. 2.

plasma extracts. The percentages of recovery of phosfolan and mephosfolan from tomato leaves were 73 and 83%, respectively. Hydroxy mephosfolan recovery was 62%. All other polar metabolites had much lower recoveries: EDIC 23%, EDC 17%, PDIC 31% and PDC 21%. The low recoveries of the polar compounds may be attributed to the use of charcoal to clean up the tomato extracts from interfering materials. Highly polar compounds also tend to bind to biological tissues. Thus, although the percentages of recovery of the parent compounds from human plasma were similar to those recovered from leaves, the percentages of recovery of the polar compounds from human plasma were higher than from tomato leaves ranging between 33 and 52%. Also the chromatogram obtained from the human plasma extract contained an unidentified peak X_1 (Fig. 4). Since the retention time of this peak was 11.5, it did not interfere with analysis of these compounds.

In conclusion, the method developed is a rapid and sensitive analytical method which resolved phosfolan, mephosfolan and five of their metabolites in a single chromatogram. The present study demonstrates that HPLC fulfills the requirements for quantitative and qualitative analysis of phosfolan and mephosfolan and some of their metabolites.

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The supply of phosfolan and mephosfolan by American Cyanamid (Princeton, NJ, U.S.A.) is acknowledged.

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Note

High-performance liquid chromatography of 6-caprolactam and its cyclic oligomers present in polyamide 6

VÁCLAV KRAJNÍK* and PETER BOŽEK
State Hospital, F. Kraľa 14, 821 24 Bratislava (Czechoslovakia)
and
JAROSLAVA KONDELÍKOVÁ and JAROSLAV KRÁLÍČEK
Department of Polymers, Institute of Chemical Technology, 166 28 Prague 6 (Czechoslovakia)
(Received January 11th, 1982)

Cyclic oligomers of 6-caprolactam, together with the monomer, are equilibrium products of its polymerization and form the greater part of oligomers in water extracts, *i.e.*, in the waste product from the production of fibres from polyamide 6. Our need to determine the individual cyclic oligomers in this waste product led us to seek a simple and rapid experimental method. Previously published methods are unsatisfactory because they are rather time consuming.

Ongemach and Moody[1] determined 6-caprolactam in water extracts of polyamide 6 by gas chromatography, and the content of cyclic oligomers together with 6-caprolactam by differential refractometry and IR spectrophotometry. Their method did not allow the determination of the individual cyclic oligomers, as was the case with the method of Anton[2], who determined the cyclic oligomers directly in water extracts. Reinisch *et al.*[3] used Craig's method of separation in the system *n*-heptane–methanol for direct determination of oligomers. Even when the long times and experimental requirements of this method are compensated by its absolute reliability, the method is not suitable for routine analyses. Heikens[4] determined the cyclic dimer, trimer and tetramer by a fractional sublimation of a methanolic extract, which was previously freed from monomer and linear oligomers. Rothe[5] separated cyclic oligomers by paper chromatography and determined the individual components by colorimetry. Both methods, fractional sublimation and paper chromatography, are complicated and time consuming.

Gas–liquid chromatography (GLC) was utilized in the determination of cyclic oligomers of 6-caprolactam for the first time by Mori *et al.*[6]. The monomer and oligomers were reduced to the corresponding amines which were then determined chromatographically. The method has again high experimental and time requirements. However, Mori and Takeuchi[7] published at the same time a method based on gel permeation chromatography (GPC). A good separation of 6-caprolactam and its cyclic oligomers up to the hexamer was attained on the gels Sephadex G-15, G-25 and Bio-Gel P-4 using 0.1 *N* HCl as the eluent. The method was experimentally simple and the analysis of one sample was relatively fast —it took 6 h. At the same time, Mulder and Buytenhuys[8] described the separation of cyclic oligomers of 6-capro-

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lactam on a column of Sephadex 20 with methanol as eluent, while Andrews *et al.*[9] used Sephadex G-25 and 50% acetic acid as eluent.

In spite of the good separation achieved, all methods based on gel permeation chromatography remain, due to their relatively high demand on time, just at the border of possible utilization for laboratory practice, and the more so for technological purposes.

In comparison with the above methods, high-performance liquid chromatography (HPLC) appears to be the method with lowest demand on time, and its application to the separation of cyclic oligomers of 6-caprolactam is described in this paper.

EXPERIMENTAL

Reagents and chemicals

6-Caprolactam (K.p. Spolana, Neratovice, Czechoslovakia) was recrystallized twice from acetone and three times from benzene, dried for 50 h at 50°C and 2 kPa and then for 50 h at 20°C and 0.2 kPa.

The cyclic dimer of 6-caprolactam (1,8-diaza-2,9-dioxocyclotetradecane) was obtained from concentrated extracts in the production of polyamide 6 (Chemlon n.p., Humenné, Czechoslovakia) by the procedure according to Fritzche and Körösi[10] followed by the recrystallization five times from methanol. It was dried similarly to 6-caprolactam; m.p. 339–342°C.

Polyamide 6 was prepared by polymerization of 6-caprolactam initiated with 2 mol.% of 6-aminocaproic acid and carried out at 260, 270 or 280°C for 24 h in a glass ampoule which was sealed under vacuum (0.2 kPa). Turnings of polyamide 6 of thickness *ca.* 0.1 mm were extracted in methanol (125:1, w/w) under reflux for 1 h. The extract was directly injected into a chromatographic column. The quantitative completeness of extraction was confirmed by repeated extractions.

Equipment

The mixture of oligomers was separated in a column from E. Merck packed with the carrier LiChrosorb having the anchored non-polar phase RP-18. Aqueous acetic acid (5 mmol/l)–methanol (70:30, v/v) was used as the eluent; flow-rate 0.64 ml/min. A 7-μl volume of the extracts was injected.

Separations were carried out with a Spectra-Physics SP 8000 liquid chromatograph and monitored with a SP 8400 UV–VIS detector at 210 nm.

RESULTS AND DISCUSSION

The HPLC method was used to solve this problem by Brodilová *et al.*[11], however they failed completely to separate the cyclic dimer and monomer. The ternary eluents tetrahydrofuran–heptane–water and 1-butanol–acetic acid–water and the less sensitive refractometric detection were used in that case. Consequently, separation was possible only with the concentrated mixture of cyclic oligomers of 6-caprolactam.

We have used the much more sensitive UV spectrophotometric detection which enables the direct quantitative analysis of extracts of 6-caprolactam polymers. Anoth-

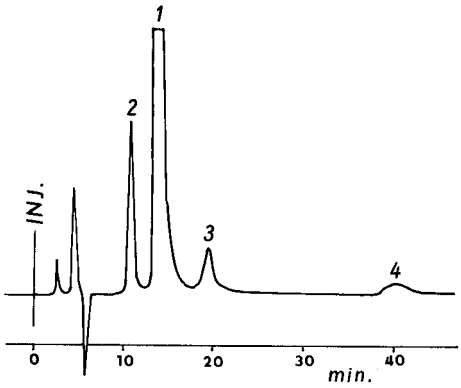

Fig. 1. HPLC separation of 6-caprolactam and its cyclic oligomers. Peaks: 1 = 6-caprolactam; 2 = cyclic dimer of caprolactam; 3 = cyclic trimer; 4 = cyclic tetramer.

er advantage of the procedure described is the application of methanol as the extraction agent and also as a component of the elution mixture. As shown in Fig. 1, a very good separation of 6-caprolactam and its cyclic oligomers was achieved (retention times of pentamer, hexamer and heptamer were 27, 37 and 45 min, respectively).

The peaks were identified by means of individual oligomers obtained by the GPC separation according to Mori and Takeuchi[7], where the cyclic oligomers were eluted in order of decreasing molecular weight. The method was tested with 6-caprolactam and the cyclic dimer as standards. The need for the complicated equipment is outweighed by the simplicity, rapidity and accuracy of the method.

It is known that the content of oligomers and monomer in polyamide 6 at equilibrium is constant and depends only on the polymerization temperature. Results

TABLE I

CONTENT OF 6-CAPROLACTAM (M_1) AND ITS CYCLIC OLIGOMERS (M_i) IN POLYAMIDE 6

A, B and C = equilibrium polyamide 6 prepared at 260, 270 and 280°C, respectively; D = industrial extract from production of fibres (Chemlon n.p.); a = content in the product of polymerization (%, w/w); b = content in extract (%, w/w).

Method	*Sample*	M_1	M_2	M_3	M_4	M_5	M_6	M_7 *and higher*
HPLC	A–a	7.1	1.3	0.6	0.3	0.1		
	A–b	75.5	13.8	6.4	3.2	1.1		
	B–a	7.4	1.3	0.6	0.4	0.1		
	B–b	77.1	12.5	6.3	3.1	1.0		
	C–a	8.3	1.3	0.6	0.3	0.1		
	C–b	78.3	12.3	5.7	2.8	0.9		
	D–b	67.1	6.5	11.0	8.2	4.5	1.3	0.6
GPC[7]	a	8.70	0.75	0.47	0.30	0.17	0.16	
GLC[6]	a	8.50	0.71	0.43	0.22	0.13	0.10	
Sublimation[12]	b	76.0	12.5		4.9	3.6	3.0	
Craig[13]	b*	–	23.3	27.9	23.3	25.6		

* After removal of the monomer.

of the quantitative HPLC analyses of equilibrium polymers of 6-caprolactam, prepared at 260, 270 and 280°C, are presented in Table I. However, exact comparison with the results of other methods is impossible, because the quoted authors mostly used unspecified industrial samples for analysis.

Table I shows that the equilibrium content of cyclic oligomers changes little with temperature, while that of the monomer changes substantially. The difference in the content of individual oligomers in the concentrated industrial water extracts from the production of polyamide 6 fibres and in the extract of equilibrium polyamide 6 prepared in the laboratory is obviously caused by the loss of the monomer and cyclic dimer which readily sublime during concentration of industrial water extracts.

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Note

High-performance liquid chromatography of chromenes and benzofurans from the genus *Encelia (Asteraceae)*

PETER PROKSCH and ELOY RODRIGUEZ
Phytochemical Laboratory, Department of Ecology and Evolutionary Biology, University of California, Irvine, Irvine, CA 92717 (U.S.A.)
(Received January 27th, 1982)

Chromenes (benzopyrans) and benzofurans are common natural products of the family *Asteraceae*[1]. These naturally occurring phytochemicals are known to exhibit a variety of biological activities ranging from cytotoxicity[2] to poisoning insects and livestock[2,3]. They have also been used for chemotaxonomic studies in the *Asteraceae*[1,4–7].

Analyses of these compounds has so far been done mainly by thin-layer chromatography (TLC) or column chromatography. This present study describes the application of reversed-phase high-performance liquid chromatography (HPLC) to the separation of chromenes, benzofurans and coumarins in crude plant extracts from species of the genus *Encelia*.

EXPERIMENTAL

Encelia ventorum, *E. palmeri*, *E. laciniata* and *E. halimifolia* were collected in Baja California, Mexico. Voucher specimens are on file in the UCI herbarium. The stems were dried, ground and extracted for 48 h with chloroform. The crude extracts were taken to dryness, redissolved in methanol, filtered and chromatographed on a Sephadex LH-20 column, eluent methanol. Further purification of analyzed compounds was achieved by repeated preparative TLC on silica gel, solvent system light petroleum (b.p. 30–60°C)–chloroform (50:50 or 70:30). ^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Varian 390 EM NMR spectrometer.

For HPLC analysis a Waters liquid chromatograph was used, equipped with a solvent pump Model 6000 A, a solvent pump Model M 45, a solvent programmer Model 660, a universal injector Model U6K and a UV detector Model 440. Detection was achieved at 254 nm. The HPLC column was LiChrosorb RP-8 (250 × 4 mm), pore size 5 μm (Alltech, Los Altos, CA, U.S.A.). The crude chloroform extract of *Encelia palmeri* was taken to dryness, redissolved in methanol, filtered, injected and separated using a linear gradient of A (water) and B (acetonitrile–acetic acid, 98:2), starting from 40% B in A to 100% B in 30 min, flow-rate 1.5 ml/min. Identification of the peaks was done by simultanous injection of the isolated and identified compounds. Ammonium acetate (NH_4OAc) was added to solvent B to prevent intramolecular hydrogen bonding, B (methanol, $9.0 \cdot 10^{-2}$ *M* ammonium

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acetate, 2% acetic acid). Methanol was chosen because of the better solubility of ammonium acetate in this solvent.

RESULTS AND DISCUSSION

The genus *Encelia* (tribe *Heliantheae*, family *Asteraceae*) consists of at least 15 species with their principal geographical distribution in the arid regions of the south western United States and Mexico. *Encelia californica* and *E. farinosa* have previously shown to contain chromenes and benzofurans[8,9]. During our chemical screening of the genus we isolated one coumarin, four chromenes and two benzofurans (Fig. 1) from *Encelia ventorum*, *E. palmeri*, *E. laciniata* and *E. halimifolia* and identified them by spectroscopical means (Table I).

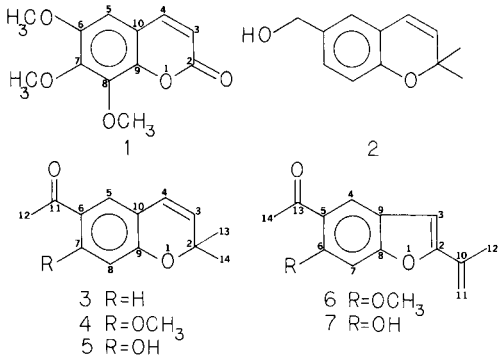

Fig. 1. Structures of coumarin (1), chromenes (2–5) and benzofurans (6, 7) isolated from *Encelia ventorum*, *E. palmeri*, *E. laciniata* and *E. halimifolia*.

TABLE I

^{1}H NMR SIGNALS OF COUMARIN (1), CHROMENES (2–5) AND BENZOFURANS (6,7)

Numbering follows Fig. 1. Chemical shifts are given in δ. All spectra were recorded at 90 MHz in C^2HCl_3 with Tetramethylsilane (TMS) as internal standard.

	1	*2*	*3*	*4*	*5*	*6*	*7*
H-3	d 6.30*	d 5.62*	d 5.60*	d 5.32*	d 5.52*	s 6.98	s 6.90
H-4	d 7.55*	d 6.30*	d 6.30*	d 6.25*	d 6.25*	s 7.88	s 7.86
H-5	s 6.60	d 7.52**	d 7.57**	s 7.46	s 7.25		
H-7		dd 7.65***	dd 7.69***			s 6.53	s 6.48
H-8		d 6.76§	d 6.72§	s 6.29	s 6.28		
H-11		s 4.72				br s 5.68, 5.10	br s 5.70, 5.10
H-12			s 2.50	s 2.50	s 2.50	br s 2.10	br s 2.10
H-13		s 1.47	s 1.47	s 1.47	s 1.45		
H-14		s 1.47	s 1.47	s 1.47	s 1.45	s 2.65	s 2.76
OCH_3	s 3.88, 3.97, 4.05			s 3.88		s 3.92	
OH		s 3.65			s 12.59		s 12.38

* J = 10 Hz.
** J = 3 Hz.
*** J = 9 Hz, 3 Hz.
§ J = 9 Hz.

These seven structurally related compounds were separated by reversed-phase HPLC within 17 min, using an RP-8 column and a linear gradient of water and acetonitrile with 2% acetic acid (Fig. 2A). Addition of acetic acid to the acetonitrile resulted in remarkable improvement of the peak symmetry of compounds 2, 5 and 7, exhibiting hydroxyl groups. The elution sequence of compounds 5 and 7 is affected by intramolecular hydrogen bonding of the hydroxyl groups to the ketone moieties. Addition of ammonium acetate, as previously described with the HPLC separation of phenolic acids[10], partially prevented the hydrogen bonding and caused compounds 5 and 7 to coelute with 4 and 6, respectively. Increasing the amount of ammonium acetate above $9.0 \cdot 10^{-2}$ *M* showed no further effect on the separation.

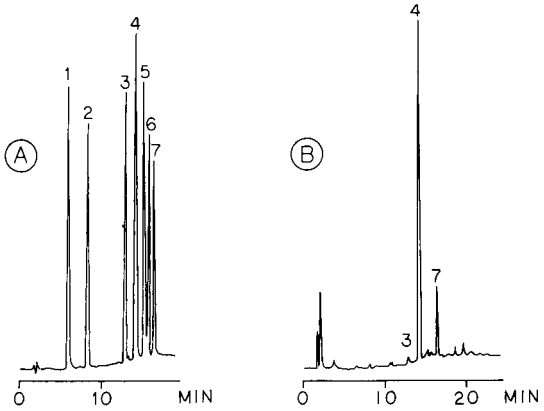

Fig. 2. (a) Separation of compounds 1–7 by reversed-phase HPLC.

Compound No.	t_R *(min)*
1	6.35
2	8.40
3	12.98
4	14.20
5	15.20
6	15.84
7	16.50

(b) HPLC separation of a crude stem extract of *Encelia palmeri*. Only main components are marked. Attenuation 1.0 a.u.f.s., chart speed 0.5 cm/min. For chromatographic conditions see Experimental.

The application of the described method is shown in Fig. 2B. Compounds 3, 4 and 7, the main components of *Encelia palmeri*, were determined in the crude stem extract of the species without any previous purification.

The use of HPLC, as described in this study, offers a quick and sensitive method to screen crude plant extracts for the presence of chromenes, benzofurans and structurally related compounds.

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Note

High-performance liquid chromatographic separation of the diastereoisomers of propoxyphene

Determination of microquantities of β-*dl*-propoxyphene in commercial preparations of α-*d*-propoxyphene

SUSAN BARKAN* and I. W. WAINER
Division of Drug Chemistry, Food and Drug Administration, Washington, DC 20204 (U.S.A.)
(Received January 27th, 1982)

Propoxyphene, 1,2-diphenyl-2-propionoxy-3-methyl-4-dimethylaminobutane (Fig. 1), exists in two diastereoisomeric forms, α-*dl* and β-*dl*. The α-isomers are pharmacologically active; the α-*d*-isomer is an analgesic, whereas the α-*l*-isomer is a clinically useful antitussive[1]. The β-*d*- and β-*l*-isomers, however, are therapeutically almost inactive[2] and are unwanted contaminants in pharmaceutical preparations containing the α-diastereoisomers.

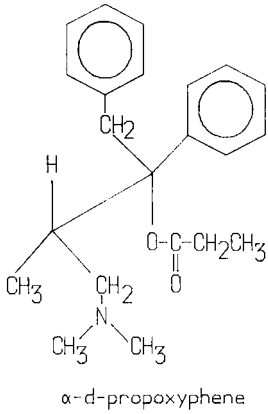

Fig. 1. Structure of propoxyphene.

Thin-layer chromatography (TLC), infrared spectroscopy and nuclear magnetic resonance spectroscopy have been used to identify the two diastereoisomeric forms of propoxyphene[3]. Although these methods are useful identification tests, they cannot be used to accurately quantify small amounts of one diastereoisomer in the presence of the other.

Soni and Van Gelder[4] have recently reported a reversed-phase high-performance liquid chromatographic (HPLC) method for the separation and characterization of the propoxyphene diastereoisomers. However, in their method the α-isomers elute

before the β-isomers, and the assay does not appear to be suitable for determination of microquantities of the β-diastereoisomers in the presence of the α-diastereoisomers.

As part of the ongoing program in our laboratory for the development of assays for the isomeric purity of therapeutic agents, we have also developed an HPLC assay for the α- and β-diastereoisomers of propoxyphene. In this assay, the β-diastereoisomers elute before the α-isomers, and as little as 0.1 % of the β-*dl*-isomer can be detected in the presence of the α-*d*-isomer. The analytical procedure is a rapid and accurate method for determining the content and isomeric purity of commercial propoxyphene preparations as well as those of standards and the bulk drug substance; it can be used for single dose analysis.

EXPERIMENTAL

Apparatus

The HPLC determinations were performed with a Spectra-Physics Model 3500 high-performance liquid chromatograph equipped with a 4000 S-P data system (Spectra-Physics, Santa Clara, CA, U.S.A.); a Spectra-Physics Model 770 UV-visible detector set at 220 nm; and a temperature-controlled column compartment. A stainless-steel DuPont-packed Zorbax Sil column, 5–6 μm particle size (25 cm × 4.6 mm I.D.) was used for all determinations. The injector (Valco Instruments, Houston, TX, U.S.A.) was equipped with a 10-μl sampling loop.

Materials

All solvents, including water, were of suitable grade for HPLC. All solutions were filtered through micropore Millipore LS filters (Millipore, Bedford, MA, U.S.A.), or equivalent and then degassed before use, α-*d*-Propoxyphene hydrochloride was a proposed United States Pharmacopeia Reference Standard, Lot H, β-*dl*-Propoxyphene hydrochloride was supplied by the Mid-Atlantic Regional Laboratory of the Drug Enforcement Administration. This sample, which was analyzed by TLC[3] and by the proposed HPLC method, was found to contain impurities. The β-*dl*-propoxyphene was isolated by preparative TLC on precoated plates of silica gel GF, 2.0 mm thick (Analtech, Newark, DE, U.S.A.). Each plate was viewed under short-wavelength UV light; the upper, isolated band, which contained the β-*dl*-propyxphene, was scraped from the plate, vigorously mixed with methanol and centrifuged. The supernatant liquid was removed, filtered through a micropore filter, and analyzed by the proposed HPLC method.

General procedures

Standard solutions of α-*d*-propoxyphene hydrochloride in the mobile phase and in methanol were prepared in concentrations ranging from 0.001 to 1 mg/ml. The standard solutions were chromatographed and a calibration curve was obtained by using the peak area measurements generated by the data system.

A mixture containing 1 % β-*dl*-propoxyphene hydrochloride in α-*d*-propoxyphene hydrochloride was prepared both in the mobile phase and in methanol.

Single dose units (tablets or capsules) were dissolved individually in the mobile phase and in methanol in volumetric flasks with the aid of an ultrasonic bath. If

necessary, the samples were crushed with a glass rod. After dissolution, the samples were diluted to volume with either the mobile phase or methanol, and the resulting solutions were filtered through micropore filters. This procedure produced solutions with α-*d*-propoxyphene concentrations of approximately 1 mg/ml. The sample solutions were then chromatographed and the peak areas were used to determine the actual concentrations of the samples.

Chromatographic conditions

The mobile phase was isopropanol–hexane (80:20) containing 1% water. When the mobile phase was being prepared, vigorous shaking was required to ensure the miscibility of the water. A flow-rate of 0.25 ml/min and a column temperature of 25°C were maintained throughout the experiment. When the column was not in use, the mobile phase was continuously circulated over it.

RESULTS AND DISCUSSION

Under the experimental conditions, baseline separations of β-*dl*-propoxyphene and α-*d*-propoxyphene were obtained with an average resolution factor (R_s) of 5.60 (Fig. 2). The retention time of the β-isomer ranged from 20.9 to 21.7 min; the retention time of the α-isomer ranged from 27.8 to 28.7 min. The separation between the two peaks and their characteristics permitted determination of as little as 0.1% of β-*dl*-propoxyphene in the presence of α-*d*-propoxyphene (Fig. 2).

Methanol was used as the diluent in initial work; however, spurious peaks were observed in the chromatograms. These peaks were shown to be associated with the

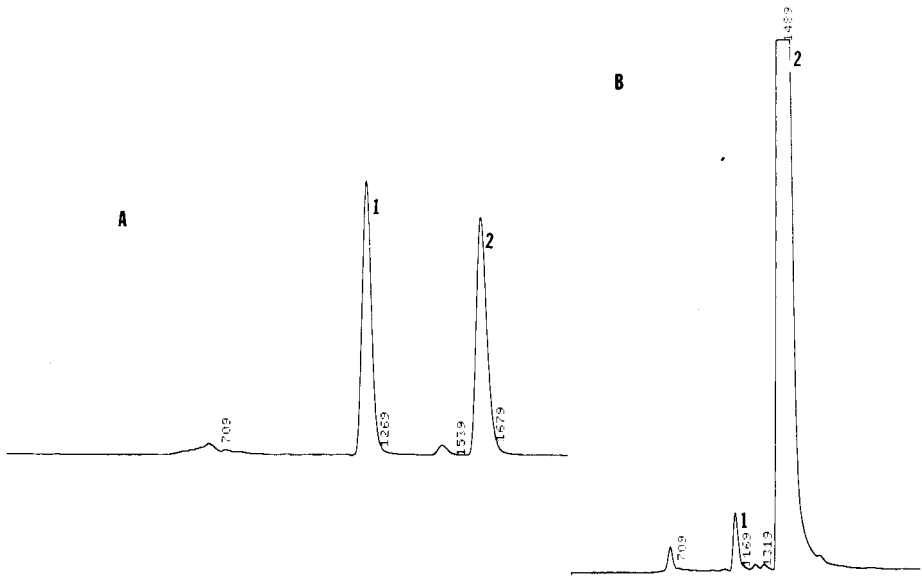

Fig. 2. Chromatograms of mixtures of β-*dl*-propoxyphene (1) and α-*d*-propoxyphene (2): (A) Equimolar solutions of β-*dl*-propoxyphene and α-*d*-propoxyphene, [1] = [2] = 100 μg/ml; (B) 0.5% β-*dl*-propoxyphene in α-*d*-propoxyphene, [1] = 10 μg/ml. [2] = 2000 μg/ml.

methanol. The problem was avoided by preparing the drug standards and dosage forms in the mobile phase. The relationship of the area under the UV response curve to the concentration of α-*d*-propoxyphene was linear over a 1000-fold range (0.001 to 1 mg/ml) (Fig. 3). The response curves with the mobile phase and with methanol as diluents were identical.

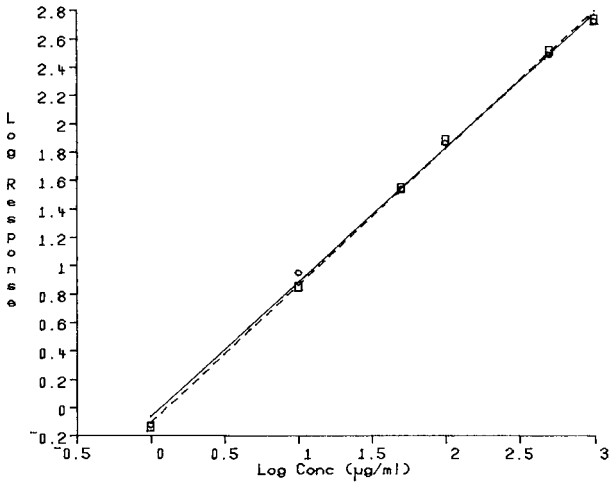

Fig. 3. UV response curve for α-*d*-propoxyphene concentrations of 0.001 to 1 mg/ml. O——O, methanol; □---□, isopropanol–hexane (80:20).

Six dosage forms of commercially available α-*d*-propoxyphene hydrochloride were analyzed for β-*dl*-propoxyphene and for content (Table I). Propoxyphene is often formulated in combination with such drugs as aspirin, acetaminophen and naloxone. Aspirin, acetaminophen and naloxone hydrochloride elute very slowly from the column and do not interfere with the analysis. However, after a series of samples, the column must be flushed with mobile phase to prevent interference with the assay.

Solubility problems were encountered with two of the preparations —sample 4 (propoxyphene HCl, sustained action capsule) and sample 5 (propoxyphene N/acetaminophen, tablet). These problems were overcome by placing each sample in a 100-ml volumetric flask, adding 20 ml of isopropanol and 1 ml of water, and sonicating until dissolution. After the solutions cooled to room temperature, hexane was added to volume. The samples were then treated as described.

Single tablet assays for α-*d*-propoxyphene content varied from 97.8 to 113.7% of label claim (Table I). After the determination of α-*d*-propoxyphene content, the samples were rerun at maximum detector sensitivity to assay for the β-isomer. Of six samples analyzed, four samples contained the β-isomer in concentrations ranging from 0.16 to 0.45%; samples 2 and 3 contained no β-isomer (Table I).

This method is a rapid and sensitive assay for the content and isomeric purity of α-*d*-propoxyphene preparations and can be used for commercial samples as well as for the bulk drug.

TABLE I

ANALYSES OF INDIVIDUAL PROPOXYPHENE TABLETS AND CAPSULES FROM COMMERCIAL SOURCES FOR *β-dl*-PROPOXYPHENE AND CONTENT

Sample	*Dosage form*	*Label claim*	*α-Propoxyphene found (mg/tablet or mg/capsule)*	*Percent of label claim*	*Percent of β-isomer*
1	Capsule	Propoxyphene HCl, 65 mg/capsule	73.3	112.7	0.35
2	Tablet	Propoxyphene napsylate (N), 100 mg/tablet	97.8	97.8	0.00
3	Tablet	Propoxyphene N, 100 mg/tablet Aspirin, 325 mg/tablet	113.7	113.7	0.00
4	Sustained-action capsule	Propoxyphene HCl, 130 mg/capsule	127.9	98.4	0.24
5	Tablet	Propoxyphene N, 50 mg/tablet Acetaminophen, 325 mg/tablet	53.4	106.7	0.16
6	Capsule	Propoxyphene HCl, 65 mg/capsule Naloxone HCl, 0.5 mg/capsule	72.5	111.5	0.45

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Note

Rapid procedure for isolation of earthworm bacteriostatic factor isoforms using chromatofocusing

PHILIPPE G. ROCH* and NICOLE DAVANT
Centre de Morphologie Expérimentale du CNRS and Département de Biologie du Développement de l'Université de Bordeaux 1, Avenue des Facultés, 33405 Talence Cédex (France)
(Received January 21st, 1982)

The coelomic fluid of a Lumbricidae *Eisenia fetida andrei* (*E.f. andrei*) contains a molecular product called *E.f. andrei* factor (EFAF), able to hemolyze various vertebrate erythrocytes[1] and to inhibit the growth of some telluric bacteria isolated from manure containing earthworms[2]. As demonstrated by injections, only the EFAF-sensitive bacteria were pathogeneous for the earthworms and rapidly killed the animals[3]. In analytical polyacrylamide gel electrophoresis (PAGE), which separates *E.f. andrei* coelomic fluid into 18 proteic components, EFAF appears as 2 different lipoproteins, with apparent molecular weights of 40,000 and 45,000 (ref. 4). In analytical isoelectric focusing (IEF), EFAF migrates as four different molecules referred to as isoforms characterized by their isoelectric points (p*I*), ranging from 5.9 to 6.3. All the animals possessed either two or three isoforms and among all the populations tested, only six different patterns have been found. Each pattern is genetically defined[5].

According to our present knowledge, although no invertebrates have developed true immunoglobulin, most of them possess humoral defense mechanisms. To understand the invertebrate defense system(s), as well as the phylogenetic evolution of the vertebrate immune system, it is of importance to investigate the biochemical characteristics of invertebrate humoral defense molecules. For that purpose, it is first necessary to isolate pure molecules in quantities compatible with biochemical studies. The present report is devoted to chromatofocusing[6], a preparative technique separating *E.f. andrei* coelomic fluid proteins in a chromatographic column according to their p*I* values.

EXPERIMENTAL

The coelomic fluid of at least 50 earthworms (Annelida, Lumbricidae, *Eisenia fetida andrei*) was harvested by electric stimulation of the worms[5]. After 10 min, 11,000 *g* centrifugation to remove cells, 10 ml of pooled coelomic fluid was filtrated on Ultrogel AcA-44 (LKB)[4]. The two fractions containing EFAF activity as assayed by hemolysis were pooled, then equilibrated with start buffer (0.025 *M* imidazole–HCl, pH 7.4) and concentrated to a volume of 10 ml by ultrafiltration on Amicon PM 10. Chromatofocusing was performed in a K 9-30 column (Pharmacia) filled with 19 ml of PBE 94 gel (Pharmacia) previously equilibrated with starting buffer and degassed. After 1 h packing with starting buffer (60 ml/h), 1 ml of Sephadex G 50 (Pharmacia)

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was layered onto the top of the PBE 94 gel in order to prevent disturbance during sample application. The column was then equilibrated with starting buffer (60 ml/h, 5 h). The sample (10 ml in start buffer) was applied by first running 5 ml (25 ml/h) of eluent PB 74 (Pharmacia) diluted 1:8 with water and adjusted at pH 5.0 with 1 *M* hydrochloric acid, followed by applying the sample and then switching back to the eluent (25 ml/h, 8 h). In this way, the sample proteins were kept close to the physiological pH of the worm coelomic fluid.

RESULTS AND DISCUSSION

The elution profile obtained in chromatofocusing with a sample constituted by the two EFAF fractions of AcA-44 gel filtration of pooled *E.f. andrei* coelomic fluid is indicated in Fig. 1. The pH gradient of the eluate gradually decreased from 7.9 to 5.2. The slight shifting observed when compared to the selected pH gradient (7.4–5.0) was unexplainable but highly reproducible.

The protein diagram was determined by absorption at 280 nm. The first peak obtained corresponded to the elution of sample proteins with p*I* values higher than the gel pH (7.9). Since the eluent pH was not high enough to neutralize the charges of these proteins, they remained positively charged and did not bind to the ion exchanger which was also positively charged. These proteins were carried along in the eluent buffer and simply filtrated, leaving the column in the first 1–3 bed volumes of eluate.

The last peak, obtained when the column was regenerated with 2 *M* NaCl, corresponded to strongly bound proteins with p*I* values lower than the final pH of elution (5.2). Under the present experimental conditions, these proteins were negatively charged and bound to the gel matrix. As a pH corresponding to their p*I* was not used, they still remained bound. These proteins were removed instead by increasing

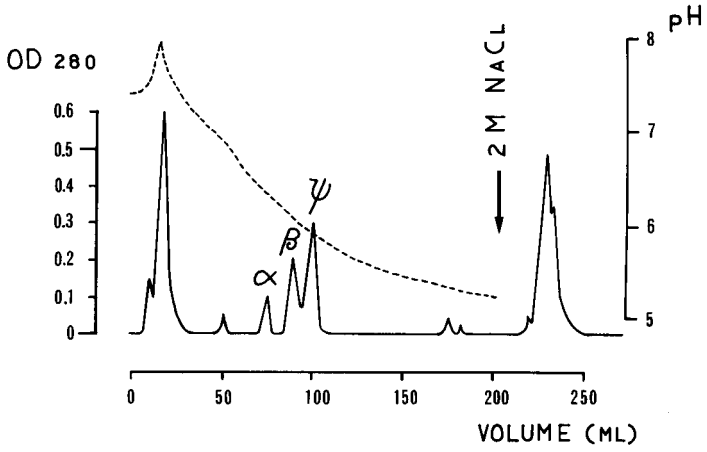

Fig. 1. Separation of the EFAF isoforms from the coelomic fluid by chromatofocusing. Column: K 9-30. Gel: 19 ml of PBE 94. Sample: 10 ml of hemolytic fractions obtained by gel filtration of 10 ml crude coelomic fluid. Elution conditions: Start buffer: 0.025 *M* imidazole–HCl, pH 7.4; Elution buffer: PB 74 adjusted to pH 5.0; Flow-rate: 25 ml/h. Elution profile measured by absorption at 280 nm (solid line) and pH gradient measured in fractions leaving the column (dotted line).

the ionic strength of the eluent with NaCl. Immediately after regeneration, the column was re-equilibrated with start buffer (60 ml/h, 5 h).

The peaks α, β and ψ were eluted respectively by pH values of 5.90, 6.15 and 6.30. The fractions corresponding to each peak were pooled, giving a volume of 12–15 ml that corresponded to 1.2–1.5 sample dilution. For each peak, the polybuffers contained in the eluate were eliminated by ultrafiltration on Amicon PM 10 and the peak volumes adjusted to the start sample volume. The purity of each peak was assayed by analytical IEF in flat-bed polyacrylamide gel containing 6 *M* urea according to the method previously described[5]. In a pH gradient of 5–8, the protein content of peak α focused as a single band of pH 6.30 (Fig. 2). This band corresponded to the isoform of p*I* 6.30 previously described in the hemolytic patterns A, C and E[5]. The 2 preparative steps considered in this paper, gel filtration and chromatofocusing, were sufficient to isolate this particular isoform in a pure form.

Peak β was constituted by two molecules of p*I* 6.00 and 6.30. As they were eluted as a single peak of p*I* 6.15, these two different isoforms must be associated in the pooled coelomic fluid used as sample. The presence of 6 *M* urea in the analytical IEF gel split the molecule into two fractions: one with a p*I* identical to the protein of peak α (6.30), the other with a p*I* of 6.00 (Fig. 2) which represented the EFAF isoform common to all the *E.f. andrei*[5].

Finally, peak ψ was also constituted by two different molecules corresponding to the EFAF isoforms of p*I* 5.90 and 5.95. With the protocol described above, these two isoforms have p*I* values too close to be separated. But according to the hemolytic patterns, some animals did not possess the p*I* 5.95 EFAF band and others did not possess the 5.90 band; thus, their coelomic fluid can be used as a sample to isolate the other band. Although they were characterized by close p*I*, the proteins of peaks β and

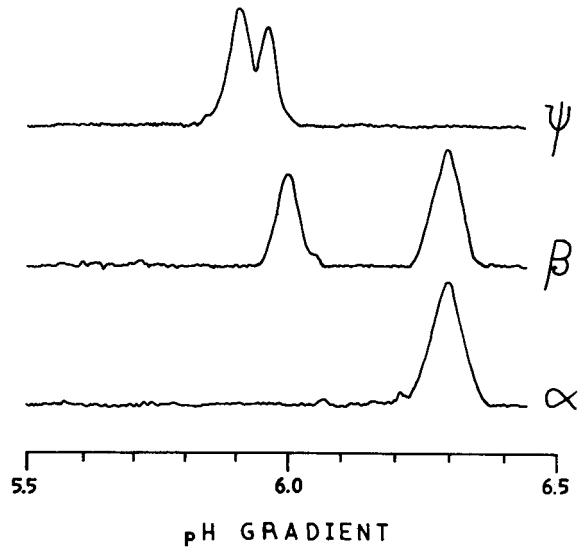

Fig. 2. Analytical flat-bed IEF of peaks α, β and ψ isolated in chromatofocusing. Densitometric scan of Coomassie Blue stained gel. Experimental conditions: pH gradient 5–8; Samples: 20 μl, salt free; Focusing: 3 h, 4°C, 10 W constant power.

ψ were different as evidenced by running a mixture of β and ψ fractions in analytical IEF.

Chromatofocusing performed with a sample containing all the hemolytic patterns led to the isolation of one isoform. Repeating the same protocol with a sample containing only one hemolytic pattern will (1) elucidate the *in vivo* relationships between the isoforms and (2) isolate the three other isoforms in a preparative way for further biochemical studies of one component of the invertebrate humoral defense system.

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Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,735

Book Review

Chemical derivatization in gas chromatography (*Journal of Chromatography Library*, Vol. 19), by J. Drozd, Elsevier, Amsterdam, Oxford, New York, 1981, XIII + 232 pp., price Dfl. 120.00, US$ 58.50, ISBN 0-444-41917-9.

The use of stable, volatile, low-polarity derivatives for the analysis of a large number of polar compounds of relatively low molecular weight which are impossible to elute from a gas chromatographic (GC) column is almost as old as GC itself. A huge amount of literature is available on this problem and only one book so far (King and Blau, *Handbook of derivatives*, Heyden, London, 1978) has attempted to review this literature and to condense it into material easy to use by analysts. Although that book gave a wealth of data on reaction procedures and mechanisms, it was difficult to find in it which derivative to select for a given type of analysis. Accordingly, there was certainly a need for a specialized book on this topic. Almost at the same time as the present book was published, however, a new series has been initiated by Plenum Press, *Chemical derivatization in analytical chemistry*, edited by R. Frei and J. Lawrence.

The present book has five chapters of very different lengths. Chapter 1 deals very briefly (7 pp.) with the reasons for using derivatives in GC. It should have been combined with the Introduction or expanded. As it is, it is too shallow and does not tell the average analyst anything new. Chapter 2 gives a brief description of sample treatment and derivatization procedures. It is sound and fairly complete, but why are the 9 pages dealing with purification of chemicals and solvents given at the end as Appendix I? Chapter 3 is a very shallow discussion of identification and quantitation. The first part has almost nothing to do with the topic of the book, while the latter part is to quantitative analysis what integration of first-order equations is to kinetics.

Fortunately, the last two chapters are better. Chapter 4 discusses the most frequently used derivatives and methods for their preparation. It is complete and presents a thorough discussion of the preparation procedures and their problems; different methods of preparation are well described and sufficient details are given to reproduce the reactions. The reviewer has the feeling that as much attention has been given to reaction yields as the data available in the literature permitted.

The last chapter deals with the derivatization of individual types of compounds. It comprises half the book (115 out of 232 pages). Each type of compound, from alcohols to inorganic anions and cations through amino acids, sugars and many others, are discussed, and for each type the different derivatives used are described. A thorough literature survey is given (654 references for this chapter alone) with examples of applications, tables of retention data, recovery, electron-capture detection sensitivity and typical chromatograms. Unfortunately, this is a collection of many results with no or very little critical review. The analyst faced with a new problem will have no serious indication of which type of derivative is best to try first in order to solve his problem.

In spite of this flaw the book will be a valuable addition to the libraries of

analytical laboratories, especially those which have a variety of new samples to handle. It will be helpful to the seasoned analyst who needs a quick literature survey and has developed a good feeling for what works and what does not. It will save others time, as in most instances they will need to check only a very small fraction of the existing literature in their field.

Palaiseau (France) G. GUIOCHON

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CHROM. 14,711

Book Review

Applications of glass capillary gas chromatography (*Chromatographic Science Series*, Vol. 15), edited by W. G. Jennings, Marcel Dekker, New York, Basel, 1981, VIII + 629 pp., price SFr. 186.00, ISBN 0-8247-1223-4.

This book is a collection of reviews written by experts in their respective fields and all are concerned with applications of glass capillary gas chromatography.

The first chapter, on the evolution of the technique (by L. S. Ettre), deals with the historical development. It also advances the idea that "open tubular columns" would have been a better term than capillary columns. The reviewer doubts this. First of all, "open" is the opposite of "closed" and not of "packed". Second, in early work on paper chromatography it had already been pointed out that flat-bed techniques were an "open column", in the sense that the column should be considered as being slit open longitudinally and exposed to the atmosphere. It is therefore not certain that the term "open tubular column" would cause less confusion than "capillary columns".

The second chapter, on "System requirements", is not really exhaustive enough for the beginner and offers little of interest for the experienced chromatographer.

The other thirteen chapters cover most analytical applications of glass capillary gas chromatography; they are mainly of good quality and will be useful to many analysts. There is some overlapping, such as between the chapter on clinical medicine and that on steroid hormones, but this is difficult to avoid in a book of this type.

Some figures are poorly reproduced, *e.g.*, Figs. 11 and 12 on p. 527, and some references are wrong *e.g.*, p. 508–509, refs. 61 and 86.

Lausanne (Switzerland) M. GUDIENS

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CHROM. 14,651

Book Review

Advances in chromatography, Vol. 19, edited by J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown, Marcel Dekker, New York, Basel, 1981, XVIII + 312 pp., price SFr. 98.00, ISBN 0-8247-1246-3.

Three of the six chapters of this volume on progress in chromatographic separation techniques are of more general character (Chapters 2, 4 and 5), whereas the others deal with aspects of practical interest (Chapters 1, 3 and 6).

Among the former chapters hydrophobic interaction chromatography (Chapter 2) is treated briefly, too briefly I feel in view of the growing importance of this technique in the separation of bioactive compounds and bioparticles.

Chapter 2, Calibration of the separation systems in gel permeation chromatography for polymer characterization, gives an exellent review of interest to all those working in the field of gel permeation chromatography of synthetic and naturally occurring polymers. After discussion of the different procedures employed for the construction of calibration plots, consideration is given to universal calibration parameters proposed by different authors. A table surveys applications of these universal calibration parameters in synthetic polymer analysis. Possibilities for the application of universal calibration parameters in the study of polymer structure are discussed, together with the limitations of its application. The reviewer regrets the limitation of this chapter to problems of the analysis of synthetic polymers because of the necessity to treat biopolymers in the same manner.

In Chapter 5, Liquid chromatography with programmed composition of the mobile phase, the author gives a critical review of the theory of elution with programmed composition of the mobile phase and provides hints for those working in chromatographic practice. The very comprehensive treatment of the theoretical basis of the different separation principles is directed to the theoretical scientist rather than to those working in analytical practice, although it is of equal importance to both. The section on practical aspects of chromatography with programmed composition of the mobile phase is therefore of special interest for the latter.

Chapter 1, Roles of high performance liquid chromatography in nuclear medicine, is the first of the more practical chapters. High-performance liquid chromatography (HPLC) in nuclear medicine is discussed in terms of preparation, purification and quality control of radiopharmaceuticals. Among the short-lived radionuclides ^{99m}Tc plays a predominant role. Preparation and purification using HPLC are reviewed for nucleosides and bases, steroids, alkaloids, amino acids, carboxylic and fatty acids, sugars and macromolecules. Because of the application of ^{99m}Tc-labelled radiopharmaceuticals in most nuclear medicine diagnostic procedures, problems of the analysis of these compounds are included in a special section. The review is completed by three tables listing recoveries of radioactivity after HPLC analysis, columns and conditions used for the HPLC of labelled nucleosides and the chromatographic analysis of labelled amino acids of interest in nuclear medicine.

Chapter 3, Isomer specific assay of 2,4-D herbicide products by HPLC: Regulatory methodology, discusses the development of the use of microparticulate column technology for a routinely usable and inter-laboratory precise isomer-specific assay method. The separation of impurities by ion-exchange chromatography, reversed-phase chromatography, column stability, precision and linearity of the method are discussed. The efficiency of the method, including separation times, is illustrated by several examples.

Chapter 6, Chromatographic separation of aldosterone and its metabolites, deals with problems in the separation of aldosterone and its metabolites from aqueous extracts of tissues, plasma and urine. Extraction with dichloromethane, chromatography on Sephadex LH-20 and DEAP-Sephadex LH-20 and HPLC are used to separate reduced and polar aldosterone metabolites. Improved systems are presented using aqueous methanol or aqueous acetonitrile. HPLC is the method preferred in this field also.

All chapters are supported by a large number of references up to 1979.

The book continues the tradition of the *Advances in Chromatography* series by including chapters of more general content together with chapters of more practical interest. Therefore, it can be recommended not only to chemists and biochemists working in chromatography but also to those using chromatography as a practical tool.

Leipzig (G.D.R.) J. WAGNER

Author Index

PUBLICATION SCHEDULE FOR 1982

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

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