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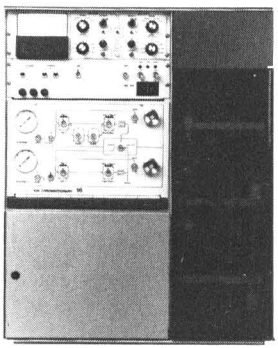

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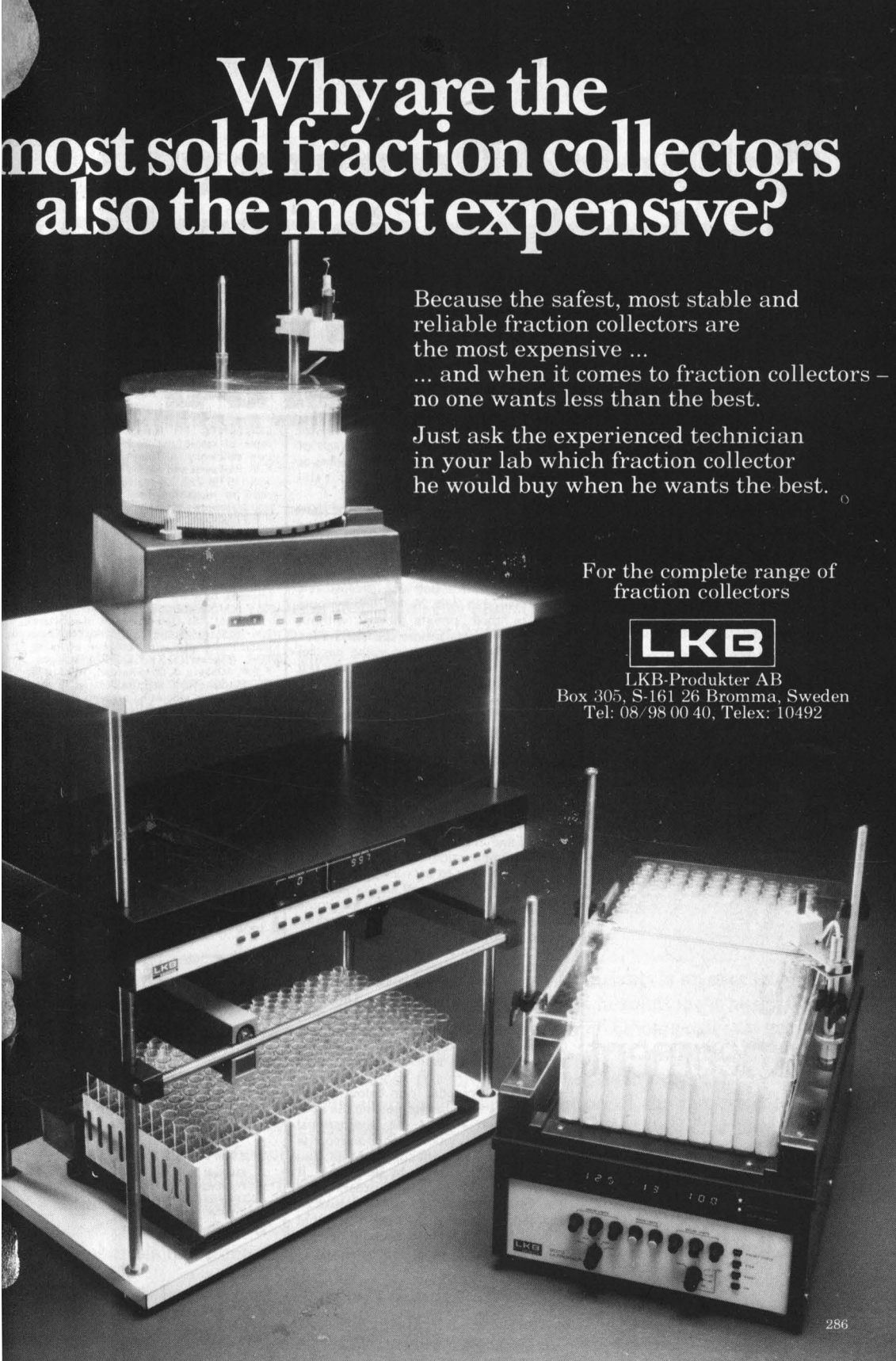
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These considerations do not however change the fact that nobody can afford to take the journal home and read it after dinner, and the editor thinks that this is one of the functions of a good journal.

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CONTINUOUS-FLOW, SUPPORT-FREE, ELECTROPHORETIC SEPARATION IN THIN LAYERS: TOWARDS LARGE-SCALE OPERATION

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(Received March 26th, 1980)

SUMMARY

The operation of continuous-flow, support-free electrophoretic separators of the Philpot thin-layer configuration (*i.e.* thin in the direction of electrophoretic migration) is modelled with the simplifying assumptions of: (i) solution properties uniform (except for electrophoretic mobility) and not affected by temperature; (ii) zero thermal resistance of the separator walls; (iii) plug flow; and (iv) diffusion as the only zone-broadening influence. It is shown for both adiabatically operated and cooled separators that the four main operational variables, *viz.* maximum temperature, resolving power, processing rate and separator size, are in each case inseparably united in a single relationship. These relationships indicate on the one hand that certain separations would be impossible in apparatus of this sort because of the high temperatures that would necessarily be involved, while on the other that the processing capacity with permitted separations would be increased indefinitely by narrowing of the separator in the direction of electrophoretic migration. The significance of these findings for real, large-scale electrophoretic separators is discussed.

INTRODUCTION

On a micro scale, zone electrophoresis in its many variants is an excellent and widely used technique for the separation of ionic materials in solution: it has proved especially useful for proteins, nucleic acids and other polymeric biologicals that are not readily separable without damage by the classical analytical methods. However, most attempts to scale-up this process for preparative use have been disappointing, largely because of the problems involved in the dissipation of the increased ohmic heat that is produced. Whereas most workers have approached this shortcoming by concentrating on efficiency of cooling, few have considered the alternative, *viz.* minimising heat production, in spite of the elegant exposition of the implications of this approach in a short paper by Philpot[1] as early as 1940.

Philpot considered the continuous, support-free configuration for zone electrophoresis represented in Fig. 1, a configuration which has been the basis for many

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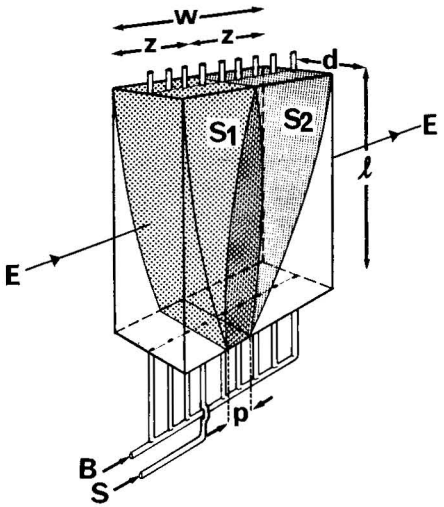

Fig. 1. Diagram of model separator; see text for explanation.

actual working models[1–11]. In this variant the sample solution for analysis, S, is pumped into the rectangular separator sandwiched between zones of electrolyte, B, which are pumped in on either side. Under the influence of the electric field, E, the sample components (two, S_1 and S_2, shown in the diagram) are deflected from the straight course through the separator to an extent dependent on their individual electrophoretic mobilities. Providing that these deflections are not equal, the component zones diverge, emerging more or less separately from the top of the apparatus.

To understand how ohmic heating might be minimised in this separator, it is instructive to consider what would happen if it were narrowed in the particular sense of w being reduced. To simplify the following argument it is assumed that throughout the separator (i) the intrinsic solution properties (thermal and electrical conductivity, specific heat, etc.) are uniform and (ii) the liquid flow is uniform in magnitude and direction (plug flow). With the proviso of linearity of zone-broadening discussed below, it is evident that if w were reduced while all other factors were kept constant (*i.e.* separator length and depth, potential gradient of the electric field, pumping rates of B and S, and the number of outlets), then the S_1 and S_2 zones would be narrowed proportionately and the resolution of these components not altered. It is to be noticed particularly that the sample throughput, proportional to the pumping rate of S, would not be reduced. However, the ohmic heat production per unit time (or, what amounts to the same thing, per unit of sample throughput) would be reduced in proportion to the narrowing. Thus, as far as heat production goes, it would seem to be advantageous to work with very narrow separators.

Narrowing would have two other important advantages. Firstly, passage-time for the solutions through the separator would be proportionately less so that convective and possibly other disturbances to uniform flow would have less time to develop. And secondly, if the separator were narrow enough, cooling could be applied to its right- and left-hand sides rather than, as is more usually done, to the front and back. In this way the depth of the separator, d, would be freed from the

need for it to be small for the sake of cooling and this dimension could be increased indefinitely with a proportionate increase in processing capacity, an increase which could, without undue enlargement of the apparatus, be of the order of hundreds of times relative to the capacity of existing apparatus with the conventional (cooled-front-and-back, Hannig-type[7]) configuration. Still further narrowing of such (cooled-at-the-sides) apparatus would, according to the argument developed above, lead to even lower rates of heat production as well as to an increased efficiency of cooling, tendencies that would seem to provide an infinite possibility for reducing the problem of ohmic heat production and therefore for scaling up.

However, implicit in the above argument is the assumption that the widths of the S_1 and S_2 zones at the output end of the separator would be reduced in proportion to the narrowing. One component of zone-broadening that would not conform to this requirement is diffusion. The width of a narrow diffusing zone increases approximately with the square-root of time and is almost independent of the initial width of the zone. Therefore, halving, for example, the width of a narrow separator would halve the passage time but reduce the width of the sample zone at the output end by a factor of only *ca.* $1/\sqrt{2} = 0.707$, so that narrowing would lead to this zone occupying a greater proportion of the width of the separator. Thus, while narrowing at constant throughput and voltage gradient would lead to less heat production, in the face of diffusion it would also eventually lead to an unacceptable loss of resolution, and because diffusion is irreducible in principle this effect would constitute a fundamental limitation in separator design.

Using essentially this approach, Philpot[1] considered whether by narrowing the model separator shown in Fig. 1 it might be possible to reduce ohmic heat production to such an extent that all of the heat could be taken up by the flowing solutions themselves to result in a harmless temperature rise, obviating altogether the need for cooling. In formulating rate of heat production in a separator operating to produce, in the face of diffusion, a 95% separation of the two hypothetical components, he derived the expression

$$\Delta T = 7.38kD/\delta m^2$$

relating ΔT, the increase in temperature of the solutions that occurs as they traverse the (adiabatically operated) separator, to k, the specific electrical conductance of the solutions (taken to be the same for B and S), D, the diffusion constant of the two sample components (taken to be the same for both), and δm, the difference between the electrophoretic mobilities of the two sample components. Philpot's conclusion was that a given degree of separation of the sample components would involve a certain expenditure of electrical energy per unit throughput of sample (proportional to adiabatic temperature rise) which would be determined entirely by solution parameters of the system and independent of the dimensions of the apparatus. Or, in other words, that there would be a one-to-one relationship, independent of separator dimensions, between heat production and resolution. Philpot calculated that as a consequence of this certain separations would be impossible in adiabatic apparatus because of the extent of the heating that would necessarily be involved, but at the same time that many useful separations would not be precluded by this effect.

However, Philpot's published analysis was based on a conveniently simple but approximate expression for diffusion, which would apply strictly only to the

limiting situation in which the initial sample zone width and therefore the processing rate were both vanishingly small, his treatment of finite sample zones and finite processing rates, as well as of the effects of cooling, being rather cursory. Therefore the present study was undertaken to extend Philpot's analysis to cover explicitly the case of finite sample zones. It will be shown that, for both cooled-at-the-sides separators conforming to the above model, and for those operated adiabatically, the four critical factors maximum temperature, resolving power, processing rate and separator size are in each case inseparably united in a single relationship, which involves diffusion rate and other solution indices as parameters. These relationships indicate on the one hand that certain separations would be impossible in apparatus of this sort because of the high temperatures that would necessarily be involved, while on the other, that the processing capacity with permitted separations would be increased indefinitely by narrowing of the separator in the sense considered above.

ANALYSIS

In the following it is taken that non-turbulent plug flow and uniform solution properties (except of course for the electrophoretic mobilities of the two sample components), unaffected by temperature, prevail within the separator.

Consider the separation of two sample components, S_1 and S_2, in the apparatus outlined above (Fig. 1). In general, during passage through the separator the two sample components will, owing to various influences, each spread out to occupy a broader zone at the outlet end. For the present purpose it is convenient to consider these broadening tendencies to be the same for each sample and therefore the width of each of the zones at the outlet end is put equal to z. Since the width of the separator, w, has only to be large enough for the two components to emerge side by side, it is convenient to put

$$w = 2z \tag{1}$$

The operating conditions of the separator must be consistent with the S_1 and S_2 zones just separating at the outlet end of the apparatus. The condition that this is so — *i.e.* that particles of the two components starting from the same point at the input end migrate apart a distance z in the passage time t — is:

$$E_g \delta m t = z \tag{2}$$

where E_g is the electrical potential gradient and δm is the difference between the electrophoretic mobility coefficients of S_1 and S_2. Substituting for z from eqn. 1, one obtains the basic operational equation for the model

$$E_g \delta m t = w/2 \tag{3}$$

The processing rate, U, defined as the pumping rate of the sample solution, is:

$$U = pdl/t \tag{4}$$

where p is the width of the sample zone at the inlet end of the separator (defined by the relative pumping rates of B and S) and d and l are the separator depth and length respectively.

Zone broadening could in practice have many causes as discussed below but in the present analysis only diffusional broadening will be considered. Such broadening is of special significance because it is unavoidable in principle, and because, as outlined above, it is especially marked in narrow separators. Dispersion of a zone of material under the combined influence of electrophoresis and diffusion acting along the same (x) axis is described by the differential equation

$$D(\partial^2 c/\partial x^2) - E_g m\,(\partial c/\partial x) - \partial c/\partial t = 0$$

where c is the concentration and D the diffusion constant of the material, and t is time. Putting $x = x' + E_g mt$ leads to the equation

$$D\,(\partial^2 c/\partial x^2) - \partial c/\partial t = 0$$

which is Fick's equation for purely diffusional dispersion. This transformation shows that combining the electrophoretic and diffusional influences merely leads to translation of the diffusional dispersion along the x axis at a rate equal to that of the electrophoretic migration. In other words the resultant dispersion is equal to the sum of the electrophoretic and diffusional dispersions calculated separately.

According to diffusion theory the distribution of material diffusing in one dimension away from an initial slab-shaped zone of width, p, and uniform concentration, c_0, is given by the equation:

$$c = \frac{c_0}{2}\left(\operatorname{erf}\frac{p/2 + x}{2\sqrt{Dt}} + \operatorname{erf}\frac{p/2 - x}{2\sqrt{Dt}}\right) \tag{5}$$

where x is the distance from the middle of the slab along the diffusion axis, and erf is the error function defined by:

$$\operatorname{erf} u = (2/\sqrt{\pi})\int_0^u \exp(-v^2)\,\mathrm{d}v \tag{6}$$

Because zones of diffusing material are strictly speaking infinitely broad, it is convenient to define zone width as containing a given large proportion, say 0.95, of the original material. With r equal to the proportion of the diffusing material to be found within a zone of width z disposed symmetrically about the initial zone, and $c_0 p$ equal to the total quantity of diffusing material per unit depth of the slab,

$$rc_0 p = \int_{-z/2}^{z/2} c\,\mathrm{d}x$$

whence, from eqn. 5,

$$rc_0 p = \frac{c_0}{2}\left[\int_{-z/2}^{z/2} \operatorname{erf}\frac{p/2 + x}{2\sqrt{Dt}}\,\mathrm{d}x + \int_{-z/2}^{z/2} \operatorname{erf}\frac{p/2 - x}{2\sqrt{Dt}}\,\mathrm{d}x\right]$$

Cancelling c_0 and putting $-y = x$ in the second integration, one has

$$\begin{aligned} rp &= \frac{1}{2}\left[\int_{-z/2}^{z/2} \operatorname{erf}\frac{p/2 + x}{2\sqrt{Dt}}\,\mathrm{d}x + \int_{-z/2}^{z/2} \operatorname{erf}\frac{p/2 + y}{2\sqrt{Dt}}\,\mathrm{d}y\right] \\ &= \int_{-z/2}^{z/2} \operatorname{erf}\frac{p/2 + x}{2\sqrt{Dt}}\,\mathrm{d}x \end{aligned} \tag{7}$$

Putting $(p/2 + x)/2\sqrt{Dt} = u$, $dx = 2\sqrt{Dt}\cdot du$, and when $x = \pm z/2$, $U = (p \pm z)/4\sqrt{Dt}$. Substituting in eqn. 7

$$rp = 2\sqrt{Dt}\int_{(p-z)/4\sqrt{Dt}}^{(p+z)/4\sqrt{Dt}} \operatorname{erf} u\, du \tag{8}$$

At the outlet end of the separator, $z = w/2$ (eqn. 1) and t is the passage time. Substituting for z in eqn. 8 and rearranging, one has

$$\int_{(p-w/2)/4\sqrt{Dt}}^{(p+w/2)/4\sqrt{Dt}} \operatorname{erf} u\, du = rp/2\sqrt{Dt} \tag{9}$$

$$\text{Putting } (p - w/2)/4\sqrt{Dt} = A \tag{10}$$

$$\text{and} \quad (p + w/2)/4\sqrt{Dt} = B \tag{11}$$

in eqn. 9 one has

$$\int_A^B \operatorname{erf} u\, du = rp/2\sqrt{Dt} = r(B + A),$$

and, using the identity

$$\int_A^B \operatorname{erf} u\, du = (1/\sqrt{\pi})\,[\exp(-B^2) - \exp(-A^2)] + B \operatorname{erf} B - A \operatorname{erf} A,$$

one has

$$(1/\sqrt{\pi})\,[\exp(-B^2) - \exp(-A^2)] + B \operatorname{erf} B - A \operatorname{erf} A = r(B + A) \tag{12}$$

Eqns. 10–12 relate the width, w, of the electrophoretic separator to the width of the initial sample zone, p, the passage time, t, and the diffusion constant, D, for a given degree of resolution, r. Elimination of A and B, which are dimensionless auxiliary variables of no obvious physical significance, would result in a single equation relating, p, w and t in terms of the parameters D and r. We have not been able to solve these equations analytically, but Fig. 2 shows graphs of A *vs.* B for values of r of 0.90, 0.95 and 0.99 which were plotted with the help of a computer, while Fig. 3 represents graphically in two dimensions the normalised relationships between w, p, t and D.

For the limiting situation in which the effect of diffusion is vanishingly small (*i.e.* A, B, $w/\sqrt{Dt}$ and $p/\sqrt{Dt}$ infinite), $w = 2rp$, whence $w = 1.80p$, $1.90p$ and $1.98p$ for the three values of r, respectively. These lines are shown dashed in Fig. 3. In the same situation, from eqns. 10 and 11, $B = 19A$, $39A$ and $199A$ respectively, as shown (dashed) in Fig. 2.

Fig. 2 shows that $A = -B$ when $A = -1.165$, -1.389 and -1.825, respectively. These points correspond to the situation where all of the sample is initially concentrated in an infinitesimally narrow zone ($p = 0$, the situation studied by Philpot). From physical considerations A and B must always be greater than these values respectively.

The special cases where $A = 0$ (Fig. 2) correspond to the physical situation where $w/2 = p$, *i.e.* where just 90%, 95% and 99% respectively of either sample remains within a zone of width p at the outlet end of the separator.

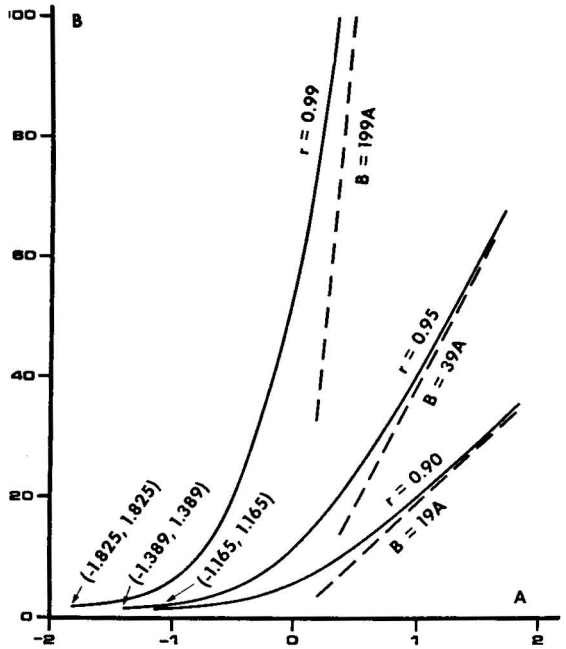

Fig. 2. Relationships between A and B (of the diffusion equation) and the resolution index (r).

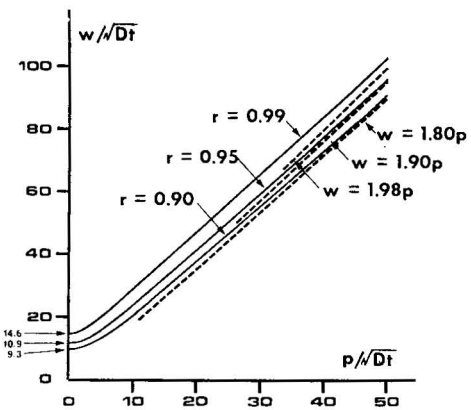

Fig. 3. Relationships between separator width (w), initial width of sample zone (p), passage time (t) and resolution index (r).

Implications of adiabatic heating

The temperature increase in the solutions flowing through the model separator operated adiabatically would be given by

$$\Delta T = kE_g^2 t/\varrho s$$

where ϱ is the density of the solutions, s the specific heat, and k the specific electrical

conductance. Assuming for the sake of simplicity that the solutions enter at zero temperature (°C), then the maximum temperature would be

$$T_{a,max} = kE_g^2 t/\varrho s$$

Substituting for E_g from eqn. 3 one has

$$T_{a,max} = kw^2/4\varrho s\delta m^2 t \tag{13}$$

and for w^2/t from eqns. 10 and 11

$$T_{a,max} = 4kD(B - A)^2/\varrho s\delta m^2 \tag{14}$$

It is of special interest in the present context to examine the relationship between $T_{a,max}$ and analytical rate. From eqns. 10, 11 and 4,

$$Uw/dlD = 8(B^2 - A^2) \tag{15}$$

By the elimination (numerically) of A and B between eqns. 15, 14 and 12, one obtains the relationship between U and $T_{a,max}$ in terms of the solution parameters k, D, ϱ, s and δm, and the separator dimensions l, d and w, for resolutions determined by r = 0.90, 0.95 and 0.99 as shown in Fig. 4.

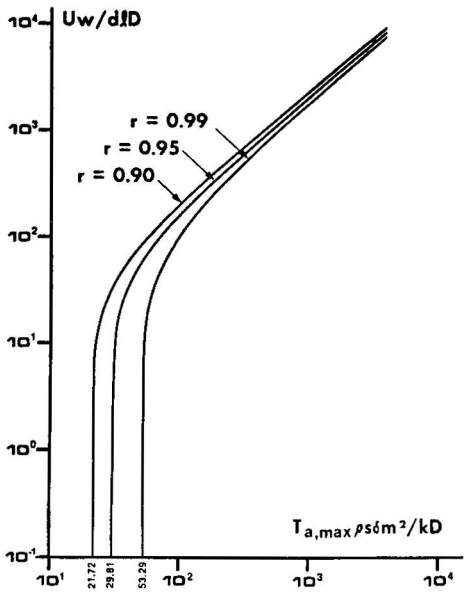

Fig. 4. Relationships between processing rate (U), adiabatic temperature rise ($T_{a,max}$) and resolution index (r).

Effects of cooling

If cooling of the pair of sides of the model separator normal to the electric field were introduced ("isothermal" operation), there would be developed across the separator at any point along its length a temperature profile with highest value half way between the cooled sides. This mid-plane temperature would be lowest at

the input end, rising towards a maximum, steady-state, value with increasing distance up the separator. With the assumption of zero temperature (°C) for the solutions at the input end of the separator, and also for the sidewalls, the mid-plane temperature at the output end would be given by the equation

$$T_{i,max} = \frac{kw^4}{32K\delta m^2 t^2}\left\{1 - \frac{32}{\pi^3}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)^3}\exp\left[-\frac{K}{\varrho s}(2n+1)^2\pi^2 t/w^2\right]\right\}$$

where t is the passage-time for solutions through the separator and K is the thermal conductivity[12]. A normalised plot of $T_{i,max}$ *vs.* t is shown in Fig. 5. Substituting in this equation for w from eqns. 10 and 11, and rearranging, one obtains

$$T_{i,max}\frac{K\delta m^2}{kD^2} = 8(B-A)^4\left\{1 - \frac{32}{\pi^3}\sum_{n=0}^{\infty}\frac{(-1)^n}{(2n+1)^3}\times\right.$$

$$\left.\times\exp\left[-\frac{K}{\varrho sD}(2n+1)^2\pi^2/16(B-A)^2\right]\right\} \qquad (16)$$

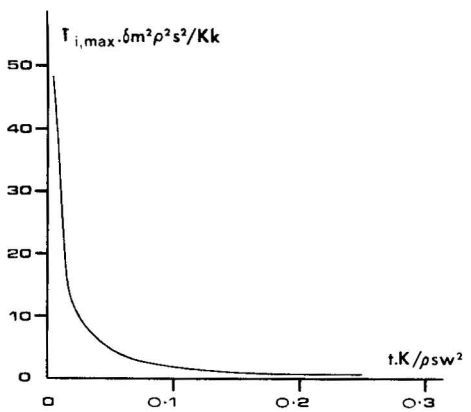

Fig. 5. "Isothermal" temperature rise ($T_{i,max}$) *vs.* passage time (t).

As for the adiabatic case, corresponding values of $T_{i,max}$ $K\delta m^2/kD^2$ and Uw/dlD may be obtained by elimination of A and B between eqns. 16, 15 and 12, but in the present instance, because $T_{i,max}$ cannot be expressed explicitly in terms of A and B, it is necessary to give particular values to the term $K/\varrho sD$ in order to do this. In all systems of practical interest, the values of K, ϱ and s would be near those for pure water, *viz.* $K = 6.03\cdot10^{-3}$ J cm^{-1} sec^{-1}°C^{-1}, $\varrho = 1.0$ g cm^{-3} and $s = 4.186$ J g^{-1}°C^{-1} so that $K/\varrho s = ca.\ 1.4\cdot10^{-3}$ cm^2 sec^{-1}. Therefore, for the sake of illustration, the relationships between analytical rate and temperature have been calculated for three values of $K/\varrho sD$ (dimensionless), *viz.* 10^2, 10^4 and ∞, corresponding to values of D of *ca.* $1.4\cdot10^{-5}$ cm^2 sec^{-1} (as, for example, for sodium chloride), $1.4\cdot10^{-7}$ cm^2 sec^{-1} (largest viruses), and zero respectively. These relationships calculated for the three values of r are shown in Fig. 6. It is to be noted that the $K/\varrho sD = 10^4$ plots begin to diverge markedly above the $K/\varrho sD = \infty$ plots only beyond the extreme right-hand side of this figure.

TABLE I

REPRESENTATIVE HYPOTHETICAL SEPARATIONS

Limiting T_{max} values, processing rates and passage times for hypothetical separations made with the Fig. 1 model separator. The following values were assumed for calculation of the processing rates and passage times: $l = 30$ cm; $d = 10$ cm; $w = 0.1$ cm; sample concentration, 1% except for Cl^- (last example) for which 0.05% is assumed; and pure water values for K, ϱ and s; and a I_{max} of 10°C. In the last example the processing rates are with respect to Cl^-, those with respect to glucose being twenty times greater.

	k (A/V cm)	*D (cm²/sec)*	*δm (cm²/sec V)*	*Philpot's separation temperature (°C)*	*r*	*Adiabatic operation*			*Isothermal operation*		
						Limiting $T_{a,max}$ (°C)	*Processing rate (g/h)*	*Passage time (sec)*	*Limiting $T_{I,max}$ (°C)*	*Processing rate (g/h)*	*Passage time (sec)*
Phycocyan	$7.7 \cdot 10^{-4}$	$2.2 \cdot 10^{-7}$	$1.0 \cdot 10^{-4}$	0.13	0.90	0.09	131	4.6	$1.5 \cdot 10^{-4}$	304	2.0
from					0.95	0.12	121	4.6	$2.7 \cdot 10^{-4}$	286	2.0
phycoerythrin					0.99	0.22	111	4.6	$8.6 \cdot 10^{-4}$	274	2.0
Yeast flavo-	$1.19 \cdot 10^{-3}$	$6.28 \cdot 10^{-7}$	$3.8 \cdot 10^{-5}$	3.8	0.90	2.7	0.148	49	$1.3 \cdot 10^{-2}$	88	6.5
protein from					0.95	3.7	0.113	49	$2.4 \cdot 10^{-2}$	81	6.5
impurities					0.99	6.6	0.068	49	$7.5 \cdot 10^{-2}$	72	6.5
Pepsin	$8.0 \cdot 10^{-4}$	$5 \cdot 10^{-7}$	*ca.* $2 \cdot 10^{-5}$?	7.4	0.90	5.2	3.4	12	$2.0 \cdot 10^{-2}$	57	10.2
from					0.95	7.1	2.0	12	$3.7 \cdot 10^{-2}$	52	10.2
impurities					0.99	12.9	0.0	∞	$11.6 \cdot 10^{-2}$	46	10.2
Horse anti-	$9.0 \cdot 10^{-3}$	$2.2 \cdot 10^{-7}$	$9.0 \cdot 10^{-6}$	181	0.90	127	0.0	∞	0.21	100	76
pneumococcus I					0.95	174	0.0	∞	0.40	94	76
from serum					0.99	311	0.0	∞	1.25	84	76
Albumin	$1.0 \cdot 10^{-3}$	$5.9 \cdot 10^{-7}$	$3.1 \cdot 10^{-5}$	4.5	0.90	3.2	7.6	62	$1.4 \cdot 10^{-2}$	80	7.3
from					0.95	4.4	5.7	62	$2.7 \cdot 10^{-2}$	74	7.3
prealbumin					0.99	7.8	2.9	62	$8.4 \cdot 10^{-2}$	66	7.3
Glucose	$1.0 \cdot 10^{-3}$	*ca.* $1.0 \cdot 10^{-5}$	$6.8 \cdot 10^{-4}$	0.16	0.90	0.11	229	0.13	$0.85 \cdot 10^{-2}$	232	0.13
from					0.95	0.15	213	0.13	$1.59 \cdot 10^{-2}$	216	0.13
Cl^-					0.99	0.28	197	0.13	$5.02 \cdot 10^{-2}$	197	0.13

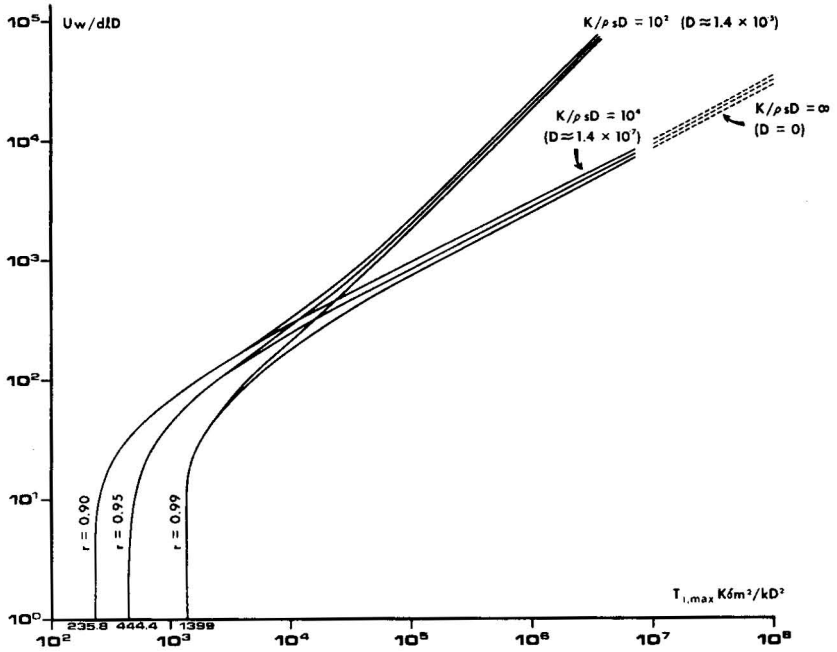

Fig. 6. Relationships between processing rate (U), "isothermal" temperature rise ($T_{i,max}$) and resolution index (r) for sample components with diffusion constants of *ca.* $1.4 \cdot 10^{-5}$, *ca.* $1.4 \cdot 10^{-7}$ and ∞.

HYPOTHETICAL SEPARATIONS

As shown in Figs. 4 and 6, with both the isothermal and adiabatic modes of operation there are limiting maximum temperatures (defined by solution parameters) below which separation to the given degree of resolution (defined by r) is not possible. It is instructive to calculate these for given hypothetical separations, a variety of which are shown in the accompanying table. The examples chosen and the values for k, δm and D (except for the separations of glucose from chloride and albumin from prealbumin) are those given by Philpot[1]. (The Philpot separation temperatures for the last two examples were calculated using his formula, $\Delta T = 7.38kD/\delta m^2$.)

Table I also shows processing rates and passage times for these hypothetical separations derived on the basis presented above using a separator of length 30 cm, depth 10 cm and width 0.1 cm; pure-water values for K, ϱ and s (quoted above) and a T_{max} of 10°C were assumed. The samples were taken to contain 1% of each of the components, except for Cl^- in the last example for which a 0.05% concentration was assumed so that the specific electrical conductance would have a value comparable with those of the other examples —in this example the analytical rates quoted in the table are for the Cl^-, those for glucose being twenty times greater.

DISCUSSION

Figs. 4 and 6 show that for the model separator shown in Fig. 1,

$T_{a,max} \geqslant XkD/\varrho s\delta m^2\,^\circ C$, and

$T_{i,max} \geqslant YkD^2/K\delta m^2\,^\circ C$,

where X and Y are functions of r. It is to be noted that these limiting separation temperatures are independent of separator parameters, and also that processing rate vanishes at these values, all finite rates involving higher temperatures. Figs. 4 and 6 also show that for given values of r, T_{max} (adiabatic or isothermal) and solution parameters, the processing rate, U, is proportional to separator depth, d, and length, l, and inversely proportional to separator width, w. And finally these figures show that higher values of r are associated with higher temperatures for a given processing rate, or alternatively lower processing rates for a given temperature.

In other words, the above analysis shows that, with the model separator shown in Fig. 1 operated either adiabatically or with cooling applied to the pair of sides normal to the electric field (the so-called isothermal mode), then: (i) with neither thermal mode of operation could the separation be achieved with less than a certain rise in temperature of the solutions as they traverse the separator; (ii) this limiting separation temperature would be entirely independent of separator dimensions; (iii) the processing rate would be vanishingly small at the limiting separation temperature; (iv) operation at finite rates would necessarily involve temperatures higher than the limiting value, the temperature increasing with processing rate; (v) for a given separation temperature processing rate would be increased indefinitely by increasing the separator depth, d, by increasing the length, l, or by decreasing the width, w; and (vi) improved resolution of the sample components could only be achieved at the expense of higher operating temperatures or lower processing rates or both. It follows that if for a particular separation the limiting adiabatic or isothermal separation temperature were too high (*e.g.* higher than the sample denaturation temperature), then there would be no way in which it could be carried out in apparatus of this sort operated in that particular thermal mode. If, on the other hand, the limiting separation temperature were favourable, changing the dimensions of the separator would allow an increase in throughput which in principle would seem to be without limit. Since enlargement of the separator by increasing length or depth would clearly be limited by physical constraints, and since narrowing (decreasing w) would confer additional advantages, this latter is the effect of special interest here.

In arriving at the above conclusions the following simplifying assumptions and approximations were made: (i) solution parameters were taken to be uniform (except for electrophoretic mobility) and not affected by temperature; (ii) in the case of the isothermal separator, the walls between the coolant and the solutions were taken to have zero thermal resistance; (iii) flow in the separator was taken to be uniform in magnitude and direction throughout ("plug flow"); and (iv) the only zone-broadening considered was that due to diffusion. It is instructive to consider how these assumptions might affect the conclusions.

The assumption of uniformity of solution parameters, as well as of their not being affected by temperature, would both seem to be sufficiently reasonable

approximations to reality for the purpose of the present analysis —in practice in electrophoretic analysis, especially of biological polymers such as proteins, the conductivity contributed by the sample itself is relatively small, while the effects of temperature on electrical conductivity and therefore on electric field-strength, electrophoretic mobilities and heating, are modest. Hinckley[13] has shown that, while neglecting the positive temperature coefficient of electrolytic conductivity (*ca.* 2%/°C) can lead to gross (up to *ca.* 50%) underestimation of the temperature in the centre of a column under some conditions, the effect is very small for narrow, flat separators such as those being considered here. The temperature coefficient of viscosity (*ca.* −2.4%/°C at 0°C) would affect the validity of the plug-flow assumption as discussed below, while that of diffusion (*ca.* 2%/°C) would affect the zone spreading calculations. However, on the face of it neither approximation would seem to grossly affect either the values or the characters of the relationships shown in Figs. 4 and 6.

The effect of assuming zero thermal resistance at the walls of the separator would be to underestimate temperature rises occurring during "isothermal" operation: the model considered here would represent the best possible situation, but how nearly it could be approximated to by the use of thin highly conducting materials for the walls of the separator is beyond the scope of the present enquiry.

The assumption of non-turbulent plug-flow, a major departure from the reality of laminar flow increasing in velocity from zero at the walls of the separator to a maximum at the mid plane, was made because without it a convincing analytical description of transport phenomena in the separator seemed unattainable. The consequences of this simplification would be generally to distort patterns of sample component migration as well as of heat production and flow, but the details would depend greatly on the relative magnitudes of d and w (Fig. 1). In the case of $d \gg w$, the model of especial interest here, axial movement of a sample component, instead of being uniform, would fall off as the component migrated under the influences of the electric field towards one or other of the side-walls of the separator, with the consequence that the trajectory, instead of being straight, would be curved outwards towards the side-wall. This effect would certainly increase with narrowing of the model separator (*i.e.* with w decreasing), but it is hard to see how it could affect separability or invalidate the existance of separation temperatures and the qualitative predictions of the effects of narrowing on analytical rate. On the other hand, in the alternative case (Hannig-type separator, $d \ll w$), this effect would be the major cause of zone-broadening under some conditions[14,15,44]: the markedly non-uniform flow profile across the narrow dimension of the separator would combine with the uniform electrophoretic migration at right angles to it to cause an initially straight sample component zone to become progressively more and more curved between the front and back faces of the apparatus during passage through it, giving rise to the special sort of dispersion described by Taylor for an initially discrete zone of material flowing down a tube[16]. Taylor-type dispersion would be counteracted by simple diffusion operating across the curved zone of the sample component, this corrective effect being greater the thinner the conduit and the longer the passage time. In narrow, rectangular electrophoretic separators of the present sort ($d \gg w$), Taylor-type dispersion would operate only at the very edges of the conduit so that its effects would be small, while in annular separators with a radial field such as the Philpot-

Harwell separator[36], conforming essentially to the same model, it would be completely absent.

Clearly, temperature distribution within both adiabatic and cooled separators would also be implicated in the plug-flow simplification in that: (i) laminar flow would involve the outside layers of electrolyte spending longer in the electric field than the inner ones and therefore generating more heat; (ii) it would also involve physical translation of the layers of heated electrolyte relative to each other in the direction of flow; and (iii) the temperature distribution would affect the flow pattern itself via its effect on viscosity. However, considering the decreasing heat production and increasing cooling efficiency with narrowing of separators of the $d \gg w$ sort, we see no reason why the properties predicted for the plug-flow models should not remain at least qualitatively true in practice, especially the existence of limiting separation temperatures and the increasing processing capacity with narrowing for given operating temperatures.

Of the variety of factors that could conceivably disturb the flow pattern or otherwise affect zone sharpness in thin-layer separators of the $d \gg w$ type, diffusion has been highlighted here because it was felt to be the most fundamental in that it is unavoidable in principle, imposing absolute restrictions on separator performance. Other factors that could affect zone sharpness in such separators under some conditions include; (i) convection due to temperature and concentration gradients; (ii) electro-osmosis; (iii) simple turbulence; (iv) electro-turbulence; (v) Kohlrausch-type phenomena, and (vi) chemical interactions between sample components.

Convection would be reduced by separator narrowing (*i.e.* decreasing w) since this would result in smaller temperature differentials and reduced passage-times. Furthermore, convection can be counteracted by the addition of thickening agents[4,17]. Electro-osmotic circulation within a separator can be eliminated by special treatments of the separator walls[18,19], but in any case, it would occur in the thin-layer separator only at the side-walls —*i.e.* the pair of walls parallel to the electric field— and would therefore be small because of their relatively small area. On the other hand, electro-osmotic flow through the pair of walls conducting the electric field into and out of the separator could be quite a different matter —clearly, because of their influence on both thermal and material exchanges, the nature of these latter walls would be a major technical concern in separator construction. Simple turbulence would not be a problem special to narrow separators because the Reynolds number, which describes this tendency, is proportional to mean flow velocity $\times$ w, which, other things being equal, would not be affected by narrowing.

The effects on zone sharpness of Kohlrausch-type phenomena and sample component interactions would depend largely on the particular separation being carried out. The former depends on the progressive redistribution of material within zones during electrophoresis as formulated by Kohlrausch in 1897[20]; the effects increase with sample concentration, and they can lead to sharpening of zones as well as to broadening depending on the composition of both the sample and the background electrolyte. Mikkers *et al.*[21] have described this effect as it applies to support-free electrophoresis, and also shown practically (on a very small scale) how it may be applied to improve resolution[22]. Interaction between different sample components (*e.g.* reversible association), as well as the existence of a single component in two or more states with different electrophoretic mobilities, could clearly affect zone

sharpness. These situations have been dealt with extensively by Cann and co-workers[23–26] and by Boyack and Giddings[27], but are beyond the scope of the present investigation.

Electro-turbulence is a phenomenon that has apparently received little attention, especially in connection with electrophoresis. Early on in his development of the thin-layer separator, Philpot found that, at least at higher intensities (100–200 V/cm), the electric field caused a peculiar mixing of the solutions, distinct from ordinary convection, which he referred to as dielectric instability[28]. Similar phenomena have been reported for a range of different liquids placed in a d.c. electric field[29–31,47]. It has been reported that the activity of the turbulence increases with dielectric constant of the liquid, that it is absent in a.c. fields, and that it appears only when the field-strength is increased beyond a threshold value. It would seem to be caused by a force gradient, caused somehow by the electric field acting on the liquid in much the same way as gravity acts on a horizontal liquid film heated from below in the system studied by Rayleigh[32] —tendency to convection is damped out at low intensities of the force-field, but active, localised convection cells appear at field strengths above a certain value which depends on the nature of the liquid[48]. Clearly, an understanding of this phenomenon will be fundamental to future developments in support-free electrophoretic separators, but the observation that it occurs only at higher field-strengths suggests that it might be avoidable.

The influence of viscosity on separator operation was considered to be too complex for it to be included in the above analysis, but clearly viscosity would be involved in the rate of diffusion of the sample components and in their electrophoretic mobilities, as well as in convection and flow of the liquid within the separator. Since diffusion and electrophoretic migration are each affected by viscosity in the same way, it would not seem to be possible to reduce the diffusional component of zone overlapping by manipulation of viscosity. On the other hand, as discussed by Dobry and Finn[4] and Finn[17], it is possible by the judicious use of certain viscous additives to stabilize laminar flow while not appreciably affecting motion at a molecular level —electrically neutral, long-chain polymeric additives such as methyl cellulose, dextran and polyvinyl alcohol reduce convective and turbulent tendencies without significantly affecting either diffusional or electrophoretic migration rates. Furthermore, while Finn considered only relatively high levels of additive (final viscosity 10 cP or greater), it has also been found that minute levels of such long-chain additives[33,34] can bring about spectacular reductions in turbulent friction and therefore presumably in tendency to turbulence. Even suspensions of microscopic fibres such as asbestos have this effect[35]. Obviously viscosity is an important factor in the operation of support-free electrophoretic separators —the possibility of stabilizing laminar flow with microscopic fibre additives, which would be easily removed from the product by filtration, is at first sight a particularly attractive idea.

In addition to the increased analytical capacity that a narrow (w small) separator would have because of low rate of heat production, certain other advantages would accrue. For one thing, the need for d (Fig. 1) to be small for cooling (as exists in most continuous electrophoretic separators, which are of the Hannig-type[7]) would be obviated so that analytical capacity could be increased still further by increasing d (Figs. 4 and 6): since in the Hannig-type of apparatus the value of d is typically of the order of 0.5 mm, an increased capacity of several hundred times

could be attained in a separator of very modest dimensions. Furthermore, a small w/d ratio would virtually eliminate Taylor-type dispersion, as discussed above. Additionally, the short passage times of narrow separators would be conducive to preservation of heat-labile sample materials and, as discussed above, to avoidance of convection and electro-osmosis.

In order to bring out the principles of separator operation, the argument developed above was based on the separation of only two sample components, while in practice, of course, one would expect to be able to collect many more fractions. To cope theoretically with multiple fractions, the model separator would need to be correspondingly increased in width (w, Fig. 1), which would give rise to corresponding increases in the separation temperatures, but obviously the same principles would apply, in particular the existence of limiting separation temperatures, and the form of the relationships between analytical rate, resolution, separator dimensions and T_{max}.

Collecting multiple fractions from a very narrow separator would also introduce some technical problems, but this has been overcome neatly in the more recent Philpot separator[3,28,36] (outlined below), by withdrawing the fractions from relatively large slots or circular holes at the side of the separation chamber near the outlet end, instead of across the end itself as done by Dobry and Finn[5].

The present analysis confirms Philpot's values[1] for the limiting separation temperatures in certain hypothetical separations carried out adiabatically (see table). (Philpot used 95.4% separation —*i.e.* $\pm$ 2 standard deviations of the normal distribution equation describing diffusion from an infinitesimally narrow starting zone— to our 95.0%, which probably explains the small discrepancies.) The table also shows, as one would expect, that cooling increases the scope of the method in that: (i) in every case the limiting values of $T_{i,max}$ are considerably lower than those of $T_{a,max}$ and (ii) the "isothermal" processing rates (for a separator of length 30 cm, depth 10 cm and width 0.1 cm, with pure-water values for K, ϱ and s, and a T_{max} of 10°C) are higher than the corresponding adiabatic rates (with a single exception). The improvement in processing capacity with the introduction of cooling for a given degree of resolution of the sample components is considerable for the separations of larger molecules, but insignificant for the separation of Cl^- from glucose, a finding confirmed by Philpot's own calculations[37]. The table also shows the relatively short passage times ranging from a fraction of a second for the glucose-from-Cl^- separation to *ca.* 1 min for the most difficult ones. These are in contrast to the passage times in Hannig-type apparatus, which are of the order of minutes or hours. Finally, it is clear from the examples given that only the most difficult of separations by the thin-layer method would be precluded by temperature rise, even without cooling of the apparatus.

Philpot's published analysis[1,28] depended on the diffusional relationship

$$z = C\sqrt{t}$$

where z is the width of a zone of diffusing material containing a given proportion of the original, t is the duration of the diffusion, and C is a constant depending on both the diffusion constant and the proportion of material to be found within z. This relationship is a valid approximation to reality in situations in which the original zone width is sufficiently small relative to the duration of diffusion. Thus Philpot's

analysis gave the correct limiting adiabatic separation temperatures for the several hypothetical systems considered (see table) since these correspond to operation of the separator at zero analytical rate as would occur if the sample input zone were infinitely thin. However, we found this simplified approach to the influence of diffusion to be inappropriate when applied to the calculation of processing rate, which necessarily involves finite width sample zones, and therefore used the exact equation as a basis for the analysis. Philpot did consider processing rate, but his earlier treatment of it[1] was brief and rather obscure while in his only other theoretical publication[28] he merely stated without derivation that

$$F = 4.186STm^2/\eta k$$

where F is the flow-rate of carrier electrolyte (ml/min) to accommodate migration of fastest component; S is the scale factor which stands for migration distance in cm/area in cm^2 normal to migration; T is the adiabatic temperature rise; m is the mobility of fastest component at 0°C; η is the average relative viscosity of carrier electrolyte over the actual temperature range; and k is the conductivity of carrier electrolyte at 0°C. Allowing for what seems to be a misprint in this paper (inversion of the scale factor, S), this equation predicts proportionality between processing rate and $ldT_{a,max}/w$ (our notation) much the same as do the combined eqns. 14 and 15 of the present communication —eqn. 15 shows directly that processing rate is proportional to l and d and inversely proportional to w, while Fig. 4, derived from these two equations, shows that processing rate and $T_{a,max}$ increase together, though not proportionately. From its simplicity, it would seem that Philpot derived this equation using the same approximation as he used for his earlier analysis.

However, in spite of the lack of analytical documentation in Philpot's published work, it is clear from exchanges of letters with him that he fully appreciates the nature of the relationships between resolving power, processing rate and temperature in the Fig. 1 model, and furthermore that his unpublished calculations fully support the potential of the thin-layer principle as a basis for scaling-up.

In spite of this promise, the thin-layer approach seems to have attracted little attention by others, either theoretical or practical.

On the theoretical side, a few studies have been published on limited aspects of this approach. Reis *et al.*[14] analysed the operation of continuous electrophoretic separators at vanishingly small current densities and sample concentrations. They concluded that, while Philpot's concept of the limiting effects of diffusion ("separation temperature") was correct for model separators with plug-flow, with the admission of non-uniform flow, Taylor-type dispersion would be a far greater cause of zone-broadening, except in cases in which the depth of the separator (d in Fig. 1) was extremely small. However, these workers considered only the Hannig-type configuration of separator ($d \ll w$) while Philpot was preoccupied by the alternative configuration ($d \gg w$), because of its much greater potential for scaling-up, in which, as discussed above, Taylor-type dispersion would seem to be far less important. A number of other groups also have considered theoretically this and other aspects of operation of Hannig-type separators[10,15,38,44–46].

In a recent paper, Strickler[39], largely concerned with the possibility of testing such apparatus in a weightless environment to avoid the effects of unwanted convection, presented an analysis of the functioning of continuous separators, examin-

ing the effects of non-uniform flow profiles on sample component trajectory, electro-osmosis (considered negligible in the thin-layer separator) and turbulence, as well as the problem of electrode design and of fraction collection. He, and also Hinckley in his paper on temperature patterns[13], have pointed out the advantages of the cooled, thin-layer ($d \gg w$) configuration over the alternative Hannig-type apparatus, for large-scale working, but these workers based their preference simply on the absence of restrictions on depth implicit in this configuration and were apparently unaware of the existence of limiting separation temperatures and of the additional scale-up that would be made possible by reducing width.

On the constructional side, Philpot himself described a very simple rectangular separator in his 1940 paper[1] ("classed with those aeroplanes at South Kensington which never left the ground"). (This apparatus may have the distinction of being the first electrophoretic separator ever built in "Perspex", so widely used for this purpose today.) He subsequently developed a much refined annular version of this apparatus, which was described in a British patent in 1969[2]. In a second patent in 1970[3], Philpot introduced "angular velocity gradient stabilization" (rotation of the outer cylinder of the separator relative to the inner cylinder) to stabilize the fluid sandwich within the separator against mixing tendencies, especially that of electro-turbulence[28,36]. This latter development was pursued under the auspicies of the National Research Development Corporation, more recently in collaboration with the U.K. Atomic Energy Authority at Harwell, to the stage where a commercial separator was offered[36]. The specifications of this commercial separator include adiabatic operation; carrier electrolyte input rate 0.5–1.5 l/min; sample input rate 15–25 ml/min; sample concentration, up to 6% protein; outlets, 30; resolution (peak widths of single components), 3 outlets; good resolution at processing rates up to 0.2 g/min for bovine serum albumin; dilution 2–10× depending on mobilities; passage time, 30–60 sec; temperature rise, up to 20°C depending on mobilities; outer cylinder rotation at 100–200 rpm; radial thickness of the separation chamber, 5 mm. Satisfactory fractionations of blood proteins, muscle extracts, microbiological culture filtrates and other organic compounds including dyes and antibiotics, as well as of particulate preparations, are claimed. Both larger and smaller versions of this basic separator have been successfully built[40].

Both continuous[41] and batch-wise[42] separators based on the electroconvection (electrodecantation) principle are essentially very like the thin-layer method under study here in spite of their being restricted to producing only two fractions. Of special significance in the present context is the high-capacity capability of these separators.

Tippetts *et al.*[6] experimented extensively with a separator very similar to Philpot's original one[1] in that it was horizontal with flow-stability maintained by a vertical gradient in density within the separator; a special fraction collection system was required. Both adiabatic and cooled versions were tried. However, the thickness of the Mel separator (corresponding to w in Fig. 1) was quite large (of the order of 1 cm), and its operation so dominated by gravitational considerations that its relevance to the present discussion is small. High analytical rates were never claimed.

Dobry and Finn[4,5] and Finn[17] have described the fractionation of dye mixtures in a vertical apparatus designed to explore the use of the thin-layer principle for large-scale operation. Although they considered such factors as non-uni-

form flow, electro-osmosis, resolving power, heating and convection, and obviously believed in the thin-layer principle for scaling-up, their approach was a largely practical one and their brief theoretical analysis did not demonstrate the essential relationships between separator dimensions, processing rate, resolution, and temperature.

To sum up, the rather limited analysis of the operation of the continuous electrophoretic separator presented here confirms the contention of Philpot and others concerning the promise of the thin-layer principle for scaling-up. This contention is endorsed on a practical level by the high performance of electroconvectors, and especially by that of the Philpot–Harwell separator itself[36]. The great value that such support-free, high capacity separators would have for both industry and research, especially for the purification of delicate biological materials such as enzymes, polypeptide hormones and antibodies, goes without saying.

However the fundamental relationships between processing rate, resolving power and temperature in terms of solution and separator parameters do not seem to be at all widely appreciated. Although in the present analysis we have found it necessary to make many simplifying assumptions, the most significant probably being the assumption of plug instead of laminar flow, nevertheless we feel that the relationships derived for the model have important implications for real separators, in particular in indicating the existence of limiting separation temperatures imposed by diffusion, and the tendency for electrical heating to be reduced indefinitely on narrowing. This being so, if the purely technical problems of: (i) collecting multiple fractions from very narrow separators; (ii) maintaining well-behaved laminar flow; and (iii) providing satisfactory materials for the pair of separator walls through which the electric field enters and leaves, could be solved, then, with the proviso of favourable limiting separation temperatures, there would seem to be no limit in principle to the extent of scale-up possible with such an apparatus, simply by narrowing.

With regard to the future, there would seem to be three especially important areas of thin-layer electrophoresis development into which effort could usefully be directed. Firstly, exploration of operation, both adiabatic and cooled, at thicknesses less than the 5 mm of the Philpot-Harwell apparatus. Secondly, a thorough study of the factors affecting flow-stability, so as to put on a firm basis the requirements for stabilization. Philpot in a recent letter remarked that he was impressed by how much the flow stability in his latest separator was improved by the use of a rapidly circulated, cooled, electrode solution of the same concentration as in the flow channel instead of the relatively concentrated solution used previously; so much so that he was led to suggest that special means of stabilize flow might be done away with altogether. If this proved to be so, the annular configuration could be replaced by the original rectangular configuration, which would be cheaper and easier to produce and which would be more flexible in that the thickness (w in Fig. 1), the most critical of the dimensions, could be made easily adjustable. Furthermore it would facilitate the putting together of large, multicellular, units for really large-scale or complex analytical programmes. And finally, the development of a relatively small version of the Philpot-Harwell, or similar rational, flexible and efficient support-free preparative separator, would be most welcome in biochemical laboratories throughout the world.

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EINIGE MATHEMATISCHE ASPEKTE DER ROHRSCHNEIDERKONZEPTION

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SUMMARY

Mathematical aspects of the Rohrschneider concept

In this paper the relationships between the retention indices used in the calculation system were examined, relying on Rohrschneider's concept and equation. Our statements were also extended to the McReynolds-system. By means of the revealed relationships, the possibility is given, in addition to the estimations of the individual interactions, for the examinations of the resultant interactions.

EINLEITUNG

In seinem in 1966 erschienenen Artikel zeigte Rohrschneider[1] den Weg zur Vorausberechnung von Retentionsindizes, ausgehend von entsprechenden Daten von Standardsubstanzen. Hierbei wurde zwischen den Indexdifferenzen, ΔI, der Substanzen (polare stationäre Phase–Squalan) ein linearer Zusammenhang angenommen. In einer früheren Arbeit[2] setzten wir, Rohrschneider entsprechend, einen linearen Zusammenhang zwischen den Indexquotienten voraus[3]. Die in der Praxis angewandte Methode ist dass man für die unbekannten Koeffizienten ein lineares Gleichungssystem mit n Variablen aufstellt und das mit den Lösungen dieses Systems weitergerechnet wird. Deshalb war der erste Schritt unserer Untersuchungen, einen Zusammenhang zwischen den Lösungen der linearen Gleichungssysteme vom Rohrschneidertyp und vom Quotiententyp zu bestimmen, wobei einfachheitshalber auf die ausführlichen Beweise verzichtet wurde.

BESCHREIBUNG DES MATHEMATISCHEN MODELLS

Sei $A = \{a_{ij}\}$ eine quadratische reelle Matrix der Ordnung n, und $\overline{Y}$ ein reeller Vektor mit $n + 1$ Elementen, dann gilt für das Gleichungssystem von Rohrschneider:

$$\sum_{i=1}^{n} (a_{ji} - y_i)\, x_i = b_j - y_{n+1} \quad 1 \leqslant j \leqslant n \tag{1}$$

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und für das Gleichungssystem des Quotientenausdruck

$$\sum_{i=1}^{n} \frac{a_{ji}}{y_i} z_i = \frac{b_j}{y_{n+1}} \tag{2}$$

wobei

i, j und n = Ordinalzahl
a = Retentionsindex von Standardsubstanzen auf der polaren stationären Phase
b = Retentionsindex von untersuchten Substanzen auf der polaren stationären Phase
x = Wurzel des Rohrschneider-Gleichungssystem
y = Retentionsindex auf Squalan
z = Wurzel des Gleichungssystems des Quotientenausdruck

Dabei sind die Elemente der Matrix $\{a_{ij}\}$ die Retentionsindizes gemessen an den verschiedenen stationären Phasen, und die Elemente des Vektors $\overline{Y}$, die Indizes der Standardsubstanzen, gemessen an Squalan. Auf den Faktor 100 im Nenner wurde dabei verzichtet, da er nur die Grössenordnung des Ergebnisses beeinflusst. Das folgende Gleichungssystem

$$\sum_{i=1}^{n} a_{ji} x_i' = b_j \qquad 1 \leqslant j \leqslant n \tag{3}$$

werden wir im folgenden als Basissystem bezeichnen. Um den zwischen dem Gleichungssystem von Rohrschneider und dem Quotienten-Gleichungssystem bestehenden Zusammenhang herauszufinden, suchten wir zunächst nach dem Zusammenhang von die Lösungen mit denen des Basissystems.

Sind x_i' die Lösungen des Basissystems, dan gilt:

$$x_i' = x_i - c_i \qquad 1 \leqslant i \leqslant n \tag{4}$$

wobei c_i die Lösungen des folgenden, sogenannten Ergänzungssystems, sind

$$\sum_{i=1}^{n} a_{ji} c_i = -\varrho \tag{4}$$

und

$$-\varrho = y_{n+1} + \sum_{i=1}^{n} x_i y_i \tag{6}$$

wobei

ϱ = Rohrschneiderische Systemkonstante

Der Zusammenhang zwischen den Lösungen des Quotientensystems und des Basissystems ist trivial:

Sind z_i die Lösungen des Quotientensystems und x_i' die des Basissystems, dann gilt:

$$z_i = \frac{x_i' y_i}{y_{n+1}} \qquad 1 \leqslant i \leqslant n \tag{7}$$

Wenn wir den obigen Ergebnisse zusammenfassen erhalten wir für den Zusammenhang zwischen den Lösungen des Rohrschneidersystems und des Quotientensystems:

$$z_i = \frac{y_i(x_i - c_i)}{y_{n+1}} \qquad 1 \leqslant i \leqslant n \tag{8}$$

Auf Grund früherer Untersuchungen[3], wo wir das von McReynolds publizierte Material analysierten, stellte sich heraus, dass die Summe der Lösungen des Quotienten-Gleichungssystems einen Wert ergibt, der um 1 schwankt.

Betrachten wir also, welchen Einfluss die Voraussetzung $\sum_1^5 z_i = 1$ auf den Lösungen der verschiedenen Gleichungssysteme ausübt. Sei $\sum_1^5 z_i = 1$, dann stimmen die Lösungen des Basissystems überein mit die der Gleichung von Rohrschneider, das heisst $x_i' = x_i$. Deshalb sind die Gleichung von Rohrschneider und das Basissystem äquivalent. Die Herleitungen wurden, im Hinblick auf die Allgemeinheit, für beliebig viele Variablen n hergestellt.

Rohrschneider[1] formulierte das Problem für fünf Standardsubstanzen, deshalb verwendeten wir bei der Untersuchung der Arbeit von McReynolds auch fünf Standardsubstanzen. Die Gleichung von Rohrschneider bestimmt im 6-dimensionalen Raum eine solche 5-dimensionale Hyperfläche, die den Punkt (0,0,0,0,0,0) schneidet. Es ist daher offensichtlich, dass die ursprünglichen Messdaten auch auf einer 5-dimensionalen Hyperfläche liegen. Die Rohrschneider-Hyperfläche erhält man aus der Hyperfläche der ursprünglichen Messdaten, indem man auf der Hyperfläche der Messdaten beliebig ein fester Punkt auswählt, und die Koordinaten dieses Punktes von den entsprechenden Koordinaten der anderen Punkte subtrahiert. Das so erhaltene Punkt ist der durch die am Squalan gemessenen Daten bestimmte Punkt. Durch die obige Koordinatentransformation führen wir eine Parallelverschiebung der Hyperfläche der ursprünglichen Messdaten derart durch, dass sie durch den Punkt (0,0,0,0,0,0) geht. Wie gesagt, haben wir bei der Untersuchung des Systems von McReynolds in unseren früheren Arbeiten bemerkt, dass die Summe der Lösungen des Quotientensystems ≈ 1 beträgt. Wenn diese Näherung gut ist, bedeutet das:

$$\sum_{i=1}^{5} x_i' y_i = y_{n+1} \tag{9}$$

Das heisst, der Punkt $(y_1,y_2,y_3,y_4,y_5,y_6)$ liegt auf der von Rohrschneider bestimmten Hyperfläche. Genau dasselbe wird durch der im Verlaufe der algebraischen Untersuchungen gefundene Äquivalenz von Rohrschneider- und Basissystem ausgedrückt.

Der Ausdruck $\Sigma z_i = 1$ deutet also darauf hin, dass die von Rohrschneider benutzte und die aus den ursprünglichen Messdaten gewonnene Hyperfläche zusammenfallen. Deshalb verschiebten wir mit der obigen Koordinatentransformation die Hyperfläche an sich selbst. Wenn die beiden Hyperflächen nicht zusammenfallen, hat die Hyperfläche der ursprünglichen Messdaten die Form

$$\sum_{i=1}^{n} a_{ji} x_i + \varrho = b_j \tag{10}$$

wo der j. Punkt der Hyperfläche $(a_{j1},a_{j2},a_{j3},a_{j4},a_{j5},a_{j6})$ ist und das Absolutglied, ϱ, übereinstimmt mit dem bei algebraischen Untersuchung benutzten ϱ, dann

$$\sum_{i=1}^{n} x_i y_i + \varrho = y_{n+1} \tag{11}$$

und

$$\varrho = y_{n+1} - \sum_{i=1}^{n} x_i y_i \tag{12}$$

Den Wert ϱ können wir auch als Lösung eines linearen Gleichungssystems bestimmen

$$a_{11}x_1 + \ldots . + a_{15}x_5 + \varrho = b_1 \tag{13}$$

bis

$$a_{61}x_1 + \ldots . + a_{65}x_5 + \varrho = b_6 \tag{14}$$

Mit diesem Gleichungssystem sind sämtliche Parameter der Hyperfläche der ursprünglichen Messdaten zu berechenen. Selbstverständlich sind die erhaltenen Resultate nur dann weiter anzuwenden, wenn die Berechnungen auf der Grundlage verhältnismässig genauer Messdaten durchgeführt wurden. In vielen Fällen ist die Anwendung der Methode der kleinsten Quadrate, zur Bestimmung der Parameter der Hyperfläche zweckmässig. Unter der Bedingung konstanter Kolonnentemperatur ist, bei der Arbeit mit denselben Standardsubstanzen, der Wert ϱ charakteristisch für die untersuchte Substanz und ausserdem ist er konstant, und unabhängig von der verwendeten stationären Phase. Im Hinblick auf die, von Rohrschneider auf diesem Fachgebiet geleistete Pionierarbeit und deren hervorragende Ergebnisse, benannten wir den Wert ϱ mit "Rohrschneiderische Systemkonstante".

UNTERSUCHUNGEN

Bei unseren Berechnungen verwendeten wir Daten des Rohrschneider[1] bzw. McReynoldssystems[4]. Aus dem letzten System entnahmen wir als Standardsubstanzen:

Benzol, 2-Pentanon, Nitropropan, Pyridin und 2-Methyl-2-Pentanol und als zu untersuchen Substanz betrachteten wir 1-Butanol. Die, mit der Methode der kleinsten Quadrate, zuerst bestimmten Koeffizienten der Hyperfläche waren die folgenden: $x_1 = 0.0245$; $x_2 = -0.5823$; $x_3 = 0.2245$; $x_4 = 0.0999$; $x_5 = 1.3603$ und $\varrho = -201.1035$. Unter Benutzung der Angaben[4] des McReynoldssystems untersuchten wir an 217 stationären Phasen den Zusammenhang von Predictingdaten und gemessenen Daten. Die gefundenen Ergebnisse sind in Tabelle I zusammengefasst.

TABELLE I

VERGLEICH DER BEI 120°C AN 217 STATIONÄREN PHASEN GEMESSENEN[4] UND BERECHNETEN WERTE DES 1-BUTANOLS

Mass der Abweichung	*Anzahl der Fälle*	*Anzahl der Fälle in % zur Gesamtanzahl*
0	17*	7.83
0–5	72	33.18
5–10	36	16.59
10–20	11	5.07
20–50	81	37.33

* Fünf stationäre Phasen im Gleichungssystem.

In Tabelle I ist zu sehen, dass es in vielen Fällen bedeutende Abweichungen gibt, dessen Ursache in einer gewissen Ungenauigkeit einiger verwendeten Retentionsangaben zu finden ist. Korrelationsmatrix:

1	1.00					
2	0.97	1.00				
3	0.99	0.99	1.00			
4	0.97	0.97	0.97	1.00		
5	0.96	0.97	0.97	0.97	1.00	
6	0.95	0.95	0.96	0.96	0.99	1.00
	1	2	3	4	5	6

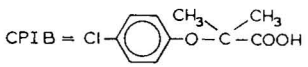
Darin bedeuten die Zahlen 1, ..., 6 die Standardsubstanzen in der oben gegebenen Reihenfolge.

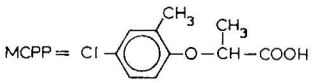
Auf Grund der Differenz zwischen den gemessenen und berechneten Werten stellten wir fest, dass es relativ wenige Punkte gab, die eine kleine Abweichung lieferten. Das wird verständlich, weil schon öfter darauf hingewiesen wurde, dass unter den Daten des McReynoldssystems falsche (d.h. mit grosser Abweichung) Angaben vorhanden sind, die das Verfahren auf Kosten der guten Messdaten bei Bestimmung des kleinsten quadratischen Fehlers in Betracht zieht. Auf Grund von den Ergebnisse früherer Untersuchungen könnten viele solche Punkte aus dem System selektiert worden, wobei die Summe der Lösungen des aus diesen Punkten aufgestellten Quotienten-Gleichungssystems praktisch gleich 1 ist. Nach der Lösung zahlreicher solcher Gleichungssysteme fanden wir die nachfolgenden stationären Phasen als die besten: Nujol[4], Octoil-S[4], Span-60[4], EGA[4] und OS-138[4]. Die auf 5 Dezimalen gerundeten Lösungen waren die folgenden: $x_1 = -0.50700$; $x_2 = -0.42836$; $x_3 = 0.45072$; $x_4 = 0.41121$; $x_5 = 0.88378$ und $\varrho = 0$. Die praktische Anwendbarkeit der Basisgleichung stellen wir im folgenden am Hand einiger Beispiele, dar. Beim ersten Beispiel berechneten wir, gegründet auf eine Arbeit von Gröbler und Bálizs[5], den Retentionsindex für eine gemischte stationäre Phase (Squalan–PEG-1500, 26:74) bei 100°C. In diesen Fälle benutzten wir die Rohrschneiderkonzeption mit den drei Standardsubstanzen: Benzol, Äthanol und Methyläthylketon. Die Modellsubstanz war 2-Heptanon. In dem gegebenen System waren die Werte des Retentionsindexes der Standardsubstanz die folgenden: I(Benzol) = 772.3 Indexeinheit (i.u.); I(Äthanol) = 765.8 i.u.; I(Methyläthylketon) = 760.2 i.u. Die substanzspezifischen Faktoren des 2-Heptanon waren: $s_1 = 1.265$; $s_2 = 0.039$; $s_3 = -0.232$. Die Rohrschneiderkonstante des Systems war 139.058. Der im gegebenen System von Gröbler und Bálizs[5] bestimmte Wert war I(2-Heptanon) = 969.2 i.u. Der durch uns berechnete Wert war

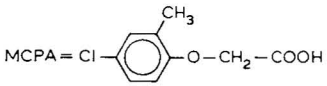
$$772.3s_1 + 765.8s_2 + 760.2s_3 + \varrho = 969.9 \text{ i.u.} \tag{15}$$

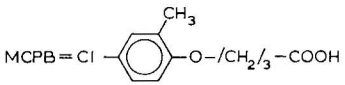
Die Übereinstimmung ist als sehr gut zu bezeichnen.

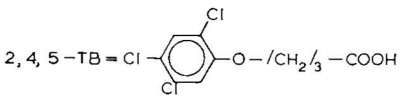
Im folgenden Beispiel stellten wir unter Benutzung einiger Retentionszeitangaben aus der Arbeit von Beer *et al.*[6], und unter Verwendung der 5 Standardsubstanzen: CPIB-pentafluorobenzyl, MCPP-pentafluorobenzyl, MCPA-pentafluorobenzyl, MCPB-pentafluorobenzyl und 2,4,5-TB-pentafluorobenzyl, wobei



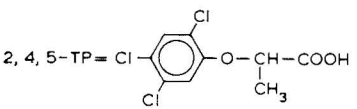








eine Predictingrechnung für die stationäre Phase OV-225 bei 210°C vor. Die Modellsubstanz war in diesem Falle 2,4,5-TP-Pentafluorobenzyl wobei



Die Substanzspezifischen Faktoren des 2,4,5-TP-pentafluorobenzyl waren die folgenden
$s_1 = 2.949$; $s_2 = 5.883$; $s_3 = -0.104$; $s_4 = -1.171$ und $s_5 = 0.327$. Die Rohrschneidersche Systemkonstante, ϱ, war -21.169. Die unter den gegebenen Bedingungen[6] gemessene Retentionszeit des 2,4,5-TP-pentafluorobenzyl war 9 Minuten und 39 Sekunden. Die Berechnung ergab

$$3'31''s_1 + 4'26''s_2 + 6'19''s_3 + 13'28''s_4 + 32'24''s_5 - 21.169 = 9'32'' \qquad (16)$$

Auch in diesem Falle ist das Übereinkommen gut, der Unterschied beträgt 7 Sekunden.

Schliesslich möchten wir ein Beispiel darstellen, wobei die Basisgleichung, weil sie keine an der apolaren stationären Phase gemessenen Werte des Retentionsindexes enthält, auch für polare Substanzen zuverlässigere Angaben liefert. Als Beispiel stellen wir die Predictingrechnung des Retentionsindexes von 1-Butanol bei 120°C für die stationäre Phase OV-22 vor.

$$I_1\text{-Butanol (120°C) OV-22} = 813s_1 + 818s_2 + 935s_3 + 952s_4 + 823s_5 + \\ + \varrho = 777.7 \text{ i.u.} \qquad (17)$$

Der gemessene Wert[4] war 778.0 i.u. d.h. die Abweichung ist minimal. Die substanzspezifischen Faktoren und die Systemkonstante ϱ werden in Tabelle II angegeben.

TABELLE II

1-BUTANOL SUBSTANZSPEZIFISCHE FAKTOREN BEI 120°C UND $\varrho = 0$

Substanzspezifische Faktor	
s_1	−0.50700
s_2	−0.42836
s_3	0.45072
s_4	0.41121
s_5	0.88378

Im folgenden stellen wir die Vorherberechnung des Ansteigs der *n*-Alkankurve mit Hilfe der Basisgleichung an der stationären Phase Apolane-87 bei 100°C vor. Die Ausgangsdaten sind in Tabelle III zusammengefasst.

TABELLE III

AUSGANGSDATEN

Temperature (°K)	*SE-30*	*APL*	*SE-52*	*DC-550*	*OV-17*	*Apolane-87*
323.16	0.380	0.418	0.367	0.376	0.368	0.389
358.16	0.310	0.347	0.303	0.311	0.305	0.329
383.16	0.267	0.304	0.264	0.272	0.267	0.293
398.16	0.244	0.280	0.243	0.250	0.246	0.274
433.16	0.197	0.232	0.200	0.207	0.204	0.233
473.16	0.152	0.186	0.158	0.165	0.163	0.195

Die substanzspezifischen Faktoren und die Systemkonstante werden in Tabelle IV angegeben.

TABELLE IV

SUBSTANZSPEZIFISCHE FAKTOREN (SYSTENKONSTANTE, ϱ, IST 0.0370)

Substanzspezifische Faktor	
s_1	−0.0004
s_2	1.2186
s_3	−0.0287
s_4	−0.3903
s_5	−0.0001

Der gemessene Wert war 0.307 bei 100°C (Apolane-87 stationäre Phase).

Die Predictingrechnung ergab $b_{100°C}^{\text{APOLANE-87}} = 0.284s_1 + 0.320s_2 + 0.279s_3 + + 0.287s_4 + 0.281s_5 + 0.037 = 0.307$ (18)

Mit Hilfe der berechneten Daten stellen wir abschliessend die Zusammenhänge zwischen den Lösungen im Falle der Basis- und Quotientengleichungen vor. Da wir im allgemeinen schreiben können:

$$z_i = \frac{I_M(i)\, x_i}{I_M(s)} \tag{19}$$

wobei:

i = die Nummer der Standardsubstanz im System;

s = die sich auf die untersuchte Substanz beziehende Bezeichung;

I_M = der molekulare Indexzusatz, i.u. (ref. 3), sind die Werte der einzelnen

Lösungen z_i, bzw. die Summe der Lösungen z_i, unter Benutzung der angaben aus Tabelle II leicht au berechnen:

$$z_1 = \frac{162.25\,(-0.507)}{146.60} = -0.56112 \tag{20}$$

$$z_2 = \frac{155.79\,(-0.42836)}{146.60} = -0.45521 \tag{21}$$

$$z_3 = \frac{162.00\,(0.45072)}{146.60} = 0.49807 \tag{22}$$

$$z_4 = \frac{173.68\,(0.41121)}{146.60} = 0.48717 \tag{23}$$

$$z_5 = \frac{171.44\,(0.88378)}{146.60} = 1.03353 \tag{24}$$

$$\sum_{1}^{5} z_i = 1.002 \tag{25}$$

Aus diesem Ergebnis findet man heraus, dass die Summe der Lösungen in der Tat nahe zu 1 liegt. Das Basisgleichungssystem, dem die Rohrschneiderkonzeption zur Grunde liegt, eröffnet also die Möglichkeit zur Untersuchung der zwischen der untersuchten Substanz und der benutzten stationären Phase bestehenden resultierenden Wechselwirkungen —die ursprüngliche Rohrschneidergleichung ermöglichte die Berechnung der individuellen Wechselwirkungen— womit das Fachgebiet um eine neue Art des Predicting bereichert werden konnte.

DANK

Die Autoren entsprechen Herr Hannes Henkel ihren Dank für die fachgerechte Übersetzung aus.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit untersuchten wir, ausgehend von der Rohrschneiderkonzeption und -gleichung, die auf Grund dieses Rechnungssystems zwischen den Retentionsindizes bestehenden Zusammenhänge. Die erhalten Ergebnisse wurden auf das McReynoldssche System übertragen. Mit Hilfe der gefundenen Zusammenhänge ist es möglich, ausser der Schätzung der individuellen Wechselwirkungen auch die resultierenden Wechselwirkungen zu untersuchen.

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CHROM. 13,178

LINEAR RELATIONSHIP BETWEEN THE LOGARITHM OF THE EQUILIBRIUM CONSTANTS AND THE LOGARITHM OF THE LIQUID CHROMATOGRAPHIC SEPARATION FACTORS FOR TAUTOMERS OBTAINED IN DIFFERENT SOLVENTS

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SUMMARY

The linear relationship between the logarithm of the separation factor, α, in liquid–solid chromatography (silica gel) using a less-polar solvent, such as a *n*-hexane–ethyl acetate, and the logarithm of the equilibrium constant, K, for a pair of tautomers in a different solvent such as ethanol was confirmed experimentally using seventeen pairs of tautomers of steroid ketoximes and their corresponding O-methylketoximes. Theoretical considerations using a static model of the adsorption–desorption equilibrium in chromatography afforded the equation

$$\log \alpha = (\Delta\Delta G^0/\Delta G^0) \log K + (\Delta\Delta G^0/\Delta G^0) \log m$$

where m is a constant relating the equilibrium constants of a pair of tautomers in two different solvent systems, namely, the solvent used in the chromatographic separation and that used in the equilibrium constant determination. The correlation coefficient obtained with 40 mol/mol % ethyl acetate in *n*-hexane as the chromatographic solvent system was 0.9804. The two constants of the above equation were determined as $\Delta\Delta G^0/\Delta G^0 = 0.3898$ and $\log m = 0.3999$ with the same solvent system.

INTRODUCTION

As is well known, the free energy change between two tautomers P and Q is proportional to the logarithm of their equilibrium constants in solution. In chromatography, one can obtain the capacity ratios, k'_P and k'_Q, from the difference in distribution constants on the stationary and mobile phases, yielding the separation factor, α, of the two solutes, which is defined as k'_P/k'_Q. The logarithm of the separation factor and the difference in the free energy changes between P and Q in distribution equilibrium between two phases are known to be related. Consequently, if the same solvent system could be applied to obtain the equilibrium constant of P and Q in solution and the separation factor in liquid chromatography, the logarithm of these parameters would be directly proportional to the coefficient of $\Delta\Delta G^0/\Delta G^0$. From this relationship it would be possible to calculate either of the parameters K or α, if its

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counterpart is available. These considerations induced us to confirm this hypothetical equation experimentally.

THEORETICAL

For a pair of tautomers P and Q in equilibrium in an appropriate protic solvent A, the following thermodynamic equation holds:

$$\Delta G^0 = -RT \ln K^A \tag{1}$$

To resolve these tautomers by liquid chromatography, it is necessary to use an aprotic solvent B in which the rate of attainment of equilibrium is minimized. In such a system, the separation factor may be expressed by:

$$\alpha^B = k'_P/k'_Q \tag{2}$$

Since the separation factor represents the ratio of distribution constants, K_P and K_Q, of the tautomers P and Q, respectively, between the stationary and mobile phases, the corresponding difference in free energy changes can be expressed by:

$$\Delta\Delta G^0 = -RT \ln \alpha^B \tag{3}$$

A static model of chromatographic separation and equilibrium is illustrated in Fig. 1. The thermodynamic expression of the equilibrium between two tautomers P and Q in the mobile phases B is:

$$\Delta G^0 = -RT \ln K^B \tag{1a}$$

From eqns. 1a and 3 the following relation can be obtained:

$$\ln \alpha^B = (\Delta\Delta G^0/\Delta G^0) \ln K^B \tag{4}$$

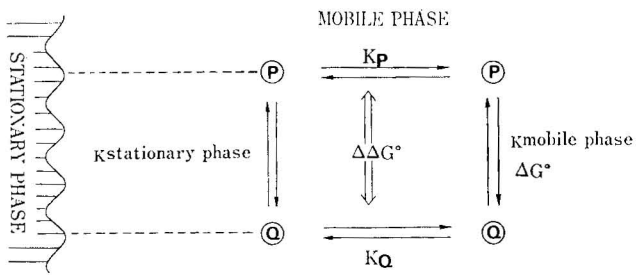

Fig. 1. Static model of the chromatographic separation of the isomers P and Q. P and Q are the solutes in the mobile phase, and - - - P and - - - Q are those on the stationary phase. K_P and K_Q represent the adsorption–desorption equilibrium constants of the solutes; $K^{\text{mobile phase}}$ represents the equilibrium constant between P and Q in the mobile phase. Of course, these isomers do not undergo equilibration during the chromatographic operation. $\Delta\Delta G°$ is the difference between the adsorption–desorption free-energy changes of both solutes P and Q; $\Delta G°$ is the free energy change of the isomers in the mobile phase.

This equation represents the linear relationship between the equilibrium constant and separation factor in a chromatographic system. However, because it is difficult to obtain the value of K^B experimentally in less-polar solvents owing to the high stabilities and low solubilities of the tautomers, we applied an approximation by means of the coefficient m, which assumes that the equilibrium constants are dependent upon the dielectric constants of the solvents:

$$K^B = mK^A \tag{5}$$

Then, we obtain from the eqns. 4 and 5 the following relation:

$$\ln \alpha^B = (\Delta\Delta G^0/\Delta G^0) \ln K^A + (\Delta\Delta G^0/\Delta G^0) \ln m \tag{4a}$$

This equation expresses the linear relationship between the two parameters α^B and K^A. To confirm this experimentally, we need to obtain the capacity ratios using a large number of tautomeric pairs under common chromatographic conditions, and to obtain the equilibrium constants using a common solvent system for all pairs of tautomers.

EXPERIMENTAL

Chromatography

A KP-9H reciprocating pump (Kusano Scientific, Tokyo, Japan) and a glass column were connected to a R 401 differential refractometer (Waters Assoc., Milford, MA, U.S.A.). The glass columns (CIG, Kusano), 30 cm × 4 mm I.D. for analytical work and 30 cm × 8 mm I.D. for preparative work, were packed with a silica gel slurry prepared in a mixture of chloroform, carbon tetrachloride and dioxan (2:1:2, v/v) using irregularly shaped silica (10 μm, pore size 70 Å) (Wakogel LC-10H; Wako, Osaka, Japan). 4500 Theoretical plates per 30 cm were obtained using diethyl phthalate as sample and *n*-hexane–ethyl acetate (9:1 v/v) as mobile phase.

Various mixtures of *n*-hexane and ethyl acetate were used as the solvent system. Solutions of 300 mg of the equilibrated tautomers in 1 or 2 ml of chloroform were injected into preparative columns by the on-column technique so that the K values could be obtained by weight. Capacity ratios, $k' = (t_s - t_m)/t_m$, for each pair of isomers were obtained using an analytical column system at a constant temperature of 20°C and three different solvent compositions. The hold-up volume, t_m, was obtained using cyclohexane, and flow-rates were 0.3 ml/min for analytical columns and 3 ml/min for preparative columns.

NMR spectroscopy

Structural assignments of pure, isolated tautomers were performed with a JEOL PS-100 spectrometer in deuterochloroform solution at 22°C. Equilibrium constants of mixtures which could not be separated by chromatography were determined by NMR analysis.

Samples

3-Oxosteroid oximes were prepared in 95% ethanol with $NH_2OH \cdot HCl$ and

sodium acetate at 20°C overnight. The crude products were allowed to stand in anhydrous ethanol at 20°C for 24 h. O-Methyloximes were prepared in pyridine with $NH_2OCH_3 \cdot HCl$ at 20°C overnight. The crude products were allowed to stand in anhydrous ethanol containing catalytic amounts of *p*-toluenesulphonic acid at 20°C for 2–20 days to obtain the equilibrium mixtures. Evaporation of the solvent at 20°C *in vacuo* gave the analytical samples.

RESULTS AND DISCUSSION

The proposed scheme requires the chromatographic separation of a pair of tautomers without isomerization. For this purpose, we have chosen seventeen pairs of geometrical isomers consisting of steroidal ketoximes and O-methylketoximes, some of which had been previously resolved by silica gel liquid–solid chromatography[1–4]. To obtain equilibrated mixtures of these oximes, the crude products, prepared in the usual manner, were allowed to stand in ethanol at constant temperature of 20°C. In the case of O-methylketoximes, equilibration was very slow, requiring 2–20 days, and *p*-toluenesulphonic acid was added as a catalyst. Although the constitutions of equilibrium mixtures could be determined by NMR spectroscopy[5] for the compounds which involve olefinic protons, accurate values required preparative separation on silica gel (*n*-hexane–ethyl acetate) and weighing of each isolated isomer. Thus the equilibrium constants, $K_{Z/E}^{EtOH}$, for all pairs of isomers were obtained with minimal experimental errors.

The configurations of the geometrical isomers were assigned according to chemical shifts in their NMR spectra; however, in order for these assignments to correspond with results of chromatographic separations, further considerations are required. If one draws a plane bisecting the angle O–N–lone pair (see Fig. 2), whose lone pair of electrons is the driving force in retention, the larger part of the hydrocarbon moiety is on the same side of the plane as the lone pair of electrons in the *Z*-form, while polar substituents in addition to the oxime group are located on the same side as the

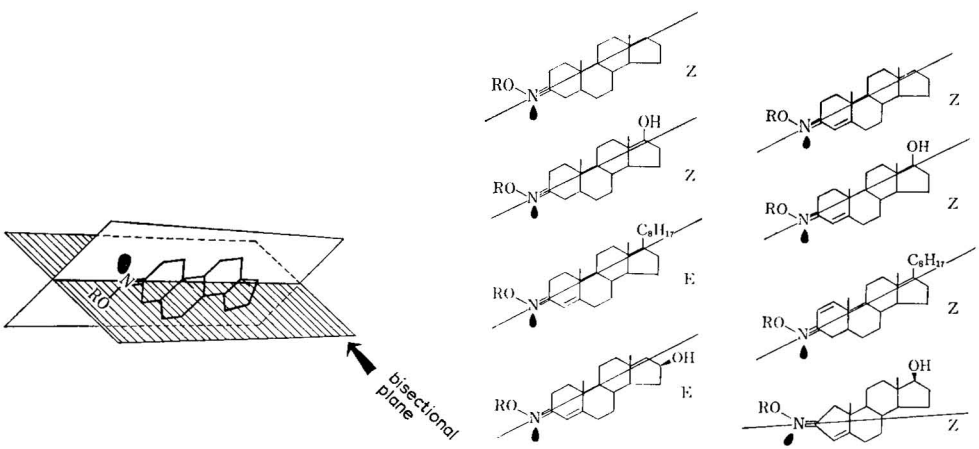

Fig. 2. Symbolism for *Z*- and *E*-isomers of steroid oximes, showing the bisectional plane (the lone pair of electrons and alkoxyl group are exchangeable in accordance with the geometrical isomerism) for 5α-androstan-3-one *E*-oxime.

lone pair of electrons in the *E*-form. For such compounds the hydroxyl group shows a distinct contribution to adsorption, the olefinic group shows a lesser contribution and bulky hydrocarbon groups show negative effects. These principles are not completely consistent with the stereochemical symbolism of the Cahn–Ingold–Prelog system; however, they may be more flexible and reliable for the retention sequence in liquid–solid adsorption chromatography. Thus we assigned the geometrical isomerism of the solutes as illustrated in Fig. 2.

A chromatographic system to obtain the separation factor, α^{B}, was designed, using silica gel as the stationary phase and a binary solvent involving *n*-hexane and ethyl acetate as the mobile phase. Since the steroid molecules contained various D-ring substituents such as hydrocarbon, hydroxyl and acetoxyl groups, along with the oxime and methyloxime groups on the A-ring, it is very difficult to obtain the capacity ratios or separation factors using binary solvents of the same concentration. To overcome this difficulty we applied the interpolation and/or extrapolation using experimentally obtained data for binary solvent mixtures of three different compositions.

In adsorption–desorption chromatography using binary solvent systems, the

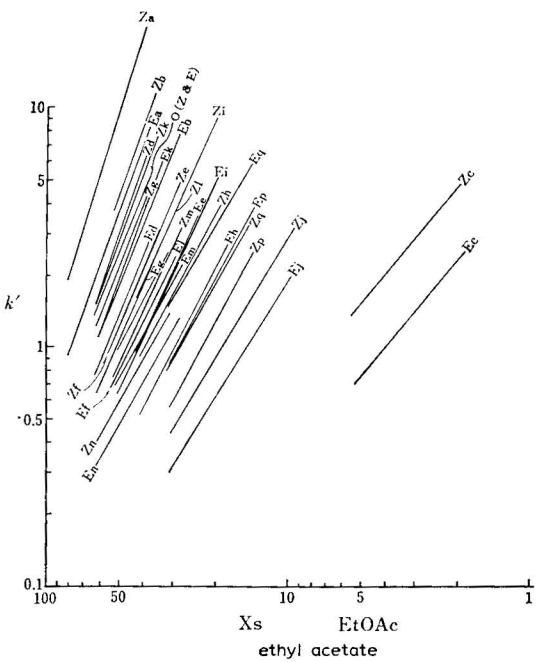

Fig. 3. Graph of the logarithm of the capacity ratio *versus* the logarithm of the mole fraction of ethyl acetate in *n*-hexane. Compounds: a = A-nortestosterone oxime; b = testosterone oxime; c = androst-4-en-3-one O-methyloxime; d = 17β-hydroxy-17α-methyl-4-androsten-3-one oxime; e = testosterone acetate oxime; f = testosterone O-methyloxime; g = 19-nor-17β-hydroxy-17α-ethyl-4-androsten-3-one oxime; h = androst-4-en-3-one oxime; i = testosterone benzoate oxime; j = 17β-acetoxy-5α-androstan-3-one O-methyloxime; k = 17β-hydroxy-5α-androstan-3-one oxime; l = 17β-acetoxy-5α-androstan-3-one oxime; m = 17β-hydroxy-5α-androstan-3-one O-methyloxime; n = 5α-androstan-3-one oxime; o = 16β-hydroxy-4-androsten-3-one oxime; p = 5α-cholest-1-en-3-one oxime; q = cholestenone oxime.

relation between the retention of the solute and the composition of the solvent is given by[6–11]

$$\log k' = c - n \log X_s \tag{6}$$

where c and n are constants and X_s is the mole fraction of the stronger solvent component. Thus we obtained the capacity ratios of 34 compounds using appropriate solvent constitutions at a constant temperature of 20°C. The plot of the logarithms of the capacity ratios *versus* the logarithms of the mole fractions of ethyl acetate was linear for each compound (Fig. 3).

Interpolation and/or extrapolation of the straight lines in Fig. 3 gave the ratio of the capacity ratios of each *E*- and *Z*-isomer ($\alpha^B = k'_E/k'_Z$) at a desired solvent composition for all pairs of isomers. Solvent compositions selected for these experiments were 1, 10, 40, 80 and 100 mole per mole % of ethyl acetate. Plots of the logarithms of the separation factors thus obtained *versus* the logarithms of the equilibrium constants in ethanol solutions are shown in Fig. 4a–e. The correlation coeffi-

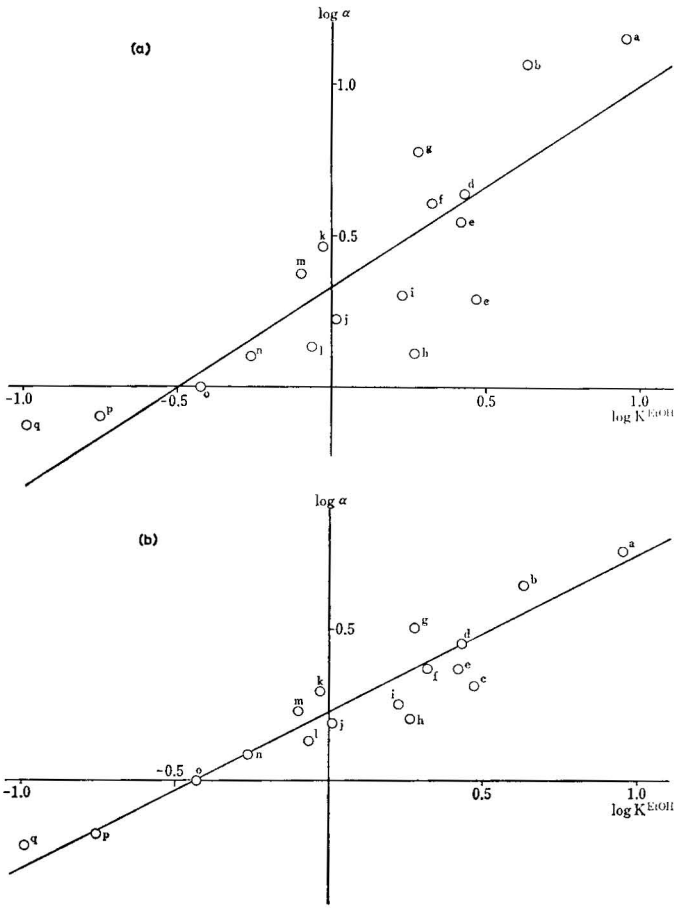

Fig. 4.

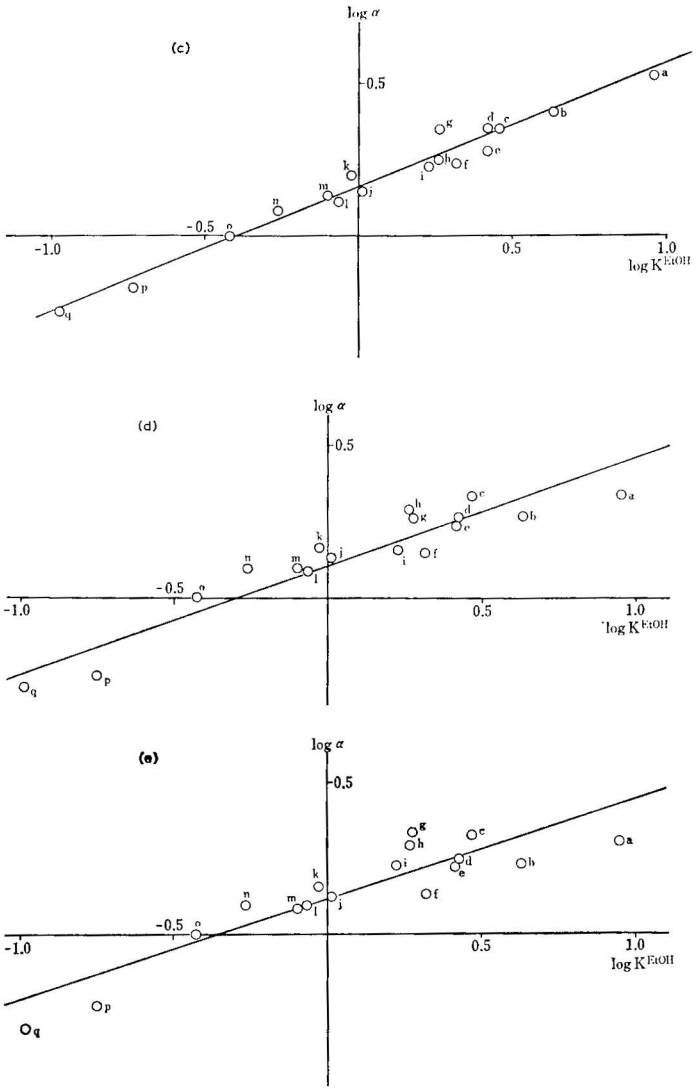

Fig. 4. Linear relationship between logarithms of separation factors and equilibrium constants. The logarithms of separation factors were obtained using a silica gel column and a mixture of *n*-hexane and ethyl acetate while the logarithms of equilibrium constants were determined in ethanol at 20°C. (a) $\log \alpha = 0.6698 \log K^{EtOH} + 0.3310$, 1% EtOAc in *n*-hexane, $R = 0.8511$; (b) $\log \alpha = 0.4905 \log K^{EtOH} + 0.2232$, 10% EtOAc in *n*-hexane, $R = 0.9401$; (c) $\log \alpha = 0.3898 \log K^{EtOH} + 0.1559$, 40% EtOAc in *n*-hexane, $R = 0.9804$; (d) $\log \alpha = 0.3549 \log K^{EtOH} + 0.1040$, 80% EtOAc in *n*-hexane, $R = 0.9346$; (e) $\log \alpha = 0.3441 \log K^{EtOH} + 0.1082$, 100% EtOAc, $R = 0.9060$.

cients, R, based on the least-squares method varied from 0.8511 for 1% to 0.9060 for 100% ethyl acetate taking the maximized R value of 0.9804 for 40% ethyl acetate. The two constants in eqn. 4a at 40% ethyl acetate were $\Delta\Delta G^0/\Delta G^0 = 0.3898$ and $\log m = 0.3999$.

The R values in Fig. 4a–e suggest that the linear relations given by eqn. 6 are reliable for medium-range solvent concentrations, less trustworthy for high concentrations of the stronger component and unreliable for low concentrations of the stronger component.

From the above results, a highly reliable estimation of $\Delta\Delta G^0/\Delta G^0$ and m values can be achieved by the use of capacity ratios at medium concentrations of the binary solvent and by extrapolations and interpolations based on eqn. 6. These results also demonstrate the linear relationship between the equilibrium constants, in appropriate solvents, and the separation factors in different solvent systems as in eqn. 4a. This physicochemical relationship may also exist for many isomer pairs besides the present steroid oximes. If the equilibrium constant of a pair of isomers is known, a chromatographic separation can be performed and if their equilibrium constants and/or free-energy changes are not known, they can be calculated by simple chromatographic techniques. Thus the relationship expressed by eqn. 4a may provide a useful connection between chromatographic separations and the properties of solutes and provide wide application to both analytical and physical chemistry.

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CHROM. 13,240

CRITICAL STUDY OF THE ALDITOL ACETATE METHOD FOR QUANTITATING SMALL QUANTITIES OF HEXOSES AND HEXOSAMINES IN GANGLIOSIDES

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SUMMARY

An extensive investigation of and improvement in the method for quantitating the carbohydrates of glycolipids by the gas–liquid chromatography of their alditol acetate derivatives is described. The effects of duration and temperature of hydrolysis, neutralization after hydrolysis, and acetylation time and temperature on the relative detector responses of mixtures of free as well as ganglioside hexoses and hexosamines were extensively studied. It is concluded that optimum results are obtained with the following conditions: hydrolysis at 100°C for 8 to 12 h, a 40–60 min reduction, acetylation at 100°C for 30 min. When quantitating the carbohydrate components of gangliosides, the most reliable results will be obtained using as an external standard a glycolipid whose chemical structure is similar to that of the sample. Using this procedure, reliable results can be obtained with the initial glycolipid sample containing as little as 1 μg of each sugar.

INTRODUCTION

Glycosphingolipids comprise a class of cell membrane constituents which are the source of much speculation and investigation. They have been implicated as being cell surface receptors[1], blood group antigens[2], and recognition molecules involved in intercellular communication and growth control[3]. The major differences among these molecules are the number and types of monosaccharide units within their carbohydrate chains. Therefore, investigations of their functions must allow for identification and quantification of their carbohydrate units.

Methods for analyzing the carbohydrate moieties in glycosphingolipids include colorimetry[4–6], gas–liquid chromatography (GLC)[7–10] and mass spectroscopy[11,12]. GLC has the advantages of being more sensitive and specific than colorimetry while being less costly and simpler to interpret than mass spectroscopy. GLC methods for

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glycosphingolipids require chemical derivatization of the monosaccharides obtained from hydrolysis of the parent compound. Commonly used carbohydrate derivatives include trifluoroacetates (TFA)[10], alditol acetates[8,9] and trimethylsilyl (TMS) derivatives[7]. While TMS has been widely employed, inconsistencies during quantitation of hexosamines and problems with the $AgCO_3$ neutralization step resulting in loss of the internal standard[13–16] have been reported. Furthermore, these problems are enhanced when working in the microgram range. We report here an extensive examination of the alditol acetate derivatization procedure and present improvements in the method which allows reproducible quantitation of as little as one microgram (approx. 5 μmol) of glucose, galactose and hexosamine derived from glycosphingolipids.

EXPERIMENTAL

Materials

Diethyl ether was obtained from Chemical Samples, Columbus, OH, U.S.A. Benzene and acetic anhydride were purchased from Drake Bros., Menomonee Falls, WI, U.S.A. Hexane and sodium borohydride were procured from Fisher Scientific, Fairlawn, NJ, U.S.A. Phosphorus pentoxide was purchased from Matheson, Coleman and Bell, Norwood, OH, U.S.A. Sigma, St. Louis, MO, U.S.A., was the source of D-xylose. D-Galactose, D-glucose and N-acetyl-D-glucosamine were obtained from Calbiochem, San Diego, CA, U.S.A. N-Acetyl-D-galactosamine and GM_2* were purchased from Supelco, Bellefonte, PA, U.S.A. All other gangliosides were prepared from normal human cerebral cortex[18]. Chloroform and methanol were distilled prior to use and glacial acetic acid, hydrochloric acid and ammonium hydroxide were of reagent grade. All glassware was acid washed prior to use. Reacti-vials, total capacity 3 ml, were purchased from Pierce, Rockford, IL, U.S.A. and Tufbond PTFE–silicone septums for the reacti-vials' screw caps were obtained from Supelco.

Gas chromatography

GLC analyses were carried out on a Hewlett-Packard No. 5710A gas chromatograph with a flame-ionization detector and a dual differential electrometer (range 1, attenuation 32). The temperature program was set for 32 min at 190°C, followed by 16 min at 230°C (rate, 4°C/min). Glass columns, 6 ft. $\times$ $^1/_4$ in. O.D., were obtained prepacked with 1% OV-225 (ref. 19) on 100–120 mesh Gas-Chrom Q from Supelco. Peak areas were either determined by weighing, or electronically, using a Hewlett-Packard No. 3385 integrator. Injection port temperature was maintained at 200°C and the detector temperature was set at 300°C. The carrier gas was nitrogen which flowed at a rate of 15 ml/min.

Optimized derivatization procedure

Standard sugars. D-Galactose (Gal), D-glucose (Glc), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) (4 sugars predominant in glycosphingolipids) and xylose (Xyl) (internal standard) were desiccated overnight before weighing and being brought to a concentration of 100 μg/ml in methanol. Equal amounts of each sugar, except xylose, (3 μg usually, but also 1, 5, 10, 25 and 50 μg) were placed in

* Nomenclature of gangliosides is according to Svennerholm[17].

a methanolysis tube and taken to dryness under a stream of nitrogen. A 0.5-ml volume of 1 *M* aqueous hydrochloride was added to each tube[20]. The volumes of reagents used were as mentioned when the amount of each monosaccharide varied between 1 and 5 μg. When derivatizing 10 to 50 μg of a monosaccharide, each reagent volume was doubled. The tubes were capped tightly with PTFE-lined screw caps and placed in a heating block at 100°C for 8 h[20,21]. The tubes were then removed from the heating block, cooled and an equivalent amount of xylose was added. The samples were then dried under nitrogen at 60–80°C. A 0.5-ml volume of freshly made $NaBH_4$ in 1 *M* NH_4OH (2 mg/ml) was added and each tube capped, vortexed and allowed to stand at room temperature for 40 min[22]. The excess reducing reagent was then destroyed by dropwise addition of glacial acetic acid[9] until all effervescence ceased (6–7 drops from a Pasteur pipet). The samples were then taken to near dryness under nitrogen at 60–80°C. The viscous boric acid remaining in each tube was removed by adding 1 ml of methanol–benzene (5:1), capping tightly, vortexing vigorously and then heating 5 min at 90°C. The samples were allowed to cool slightly, then evaporated to near dryness again. This procedure was repeated five more times using pure methanol[23]. By the end of the third repetition, the samples evaporated to complete dryness and could then be loosely capped and desiccated over P_2O_5 (no vacuum) overnight if the rest of the procedure could not be completed in the same day. Acetic anhydride, 0.75 ml, was added to each tube which was capped tightly, vortexed and placed in a heating block at 100°C for 30 min[22,23]. Following acetylation, the samples were cooled to room temperature and dried under a light stream of nitrogen at 38°C. Each sample was reconstituted with 0.5 ml chloroform and the salts removed by partitioning against 0.5 ml distilled water 5 times[21]. The desalted samples were taken to dryness under nitrogen at 38°C and transferred to reacti-vials with 3 additions of 0.5 ml chloroform. The contents of the reacti-vials were taken to dryness and the samples tightly capped and stored in the P_2O_5 desiccator until analyzed (never longer than 4 days). When analyzed, the samples were reconstituted to a volume of 10–50 μl, depending upon the amount of starting sugar; the volume equivalent to 1 μg of each sugar was injected (except for 1 μg samples where 0.5 μg was injected). Triplicate runs were performed on each sample (except 1 μg samples which had duplicate runs).

Gangliosides. Sets of gangliosides were prepared to correspond to 1, 3 and 5 μg of their glucose content. This was done by assigning their identity via thin-layer chromatographic mobilities and quantitating their sialic acid content colorimetrically[5,24]. Gangliosides were hydrolyzed in 0.5 ml of 1 *M* aqueous hydrochloride for 8 h at 100°C. Xylose, the internal standard, was added after hydrolysis, and the fatty acids extracted by partitioning against 0.5 ml of hexane 3 times. The pH was then adjusted to between 10 and 12 with 2 drops of 7.4 *M* NH_4OH and the sphingosines were extracted with 3 × 0.5 ml of diethyl ether[25]. (When sphingosines and fatty acids were not to be saved, pH adjustment was the initial step followed by the hexane extraction. No diethyl ether was used.) The samples were then subjected to the same steps as described above for the standard sugars. Standard sugars and GM_1 standard were always derivatized at the same time as the ganglioside samples.

Changes in derivatization procedure

Various conditions of the method such as hydrolysis time, reduction time, acetylation time and temperature, were examined using sugar standards and ganglio-

sides. Details denoting how these experimental protocols differed from the optimized derivatization procedures already described are noted under the appropriate heading within the Results section.

Statistics

Analyses included Student's *t*-test, one way analysis of variance, two way analyses of variance and Duncan's multiple range test[26].

RESULTS

Chromatogram

Fig. 1 shows a typical chromatogram of alditol acetates of xylose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. Xylose, the internal standard, is well separated from galactose, and there is little overlap of either the glucose and galactose peaks or of the N-acetylglucosamine and N-acetylgalactosamine peaks.

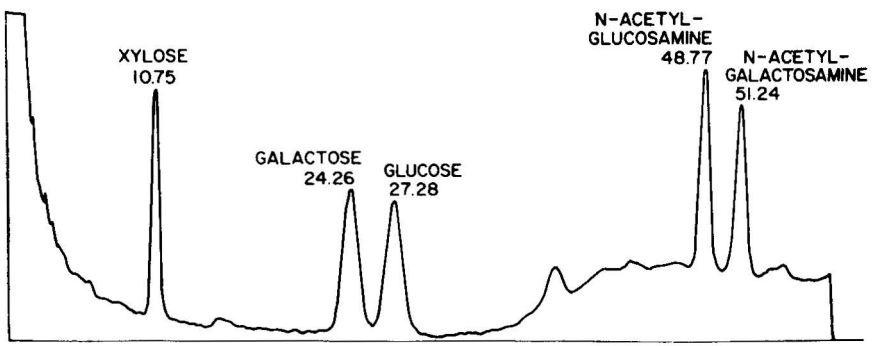

Fig. 1. Chromatogram of alditol acetates prepared from 3 μg each of xylose (internal standard), galactose, glucose, N-acetylglucosamine and N-acetylgalactosamine, chromatographed on a Hewlett-Packard No. 5710A GLC with a flame-ionization detector, dual differential electrometer (range 1, attenuation 32), and Hewlett-Packard No. 3385 integrator. Columns (1% OV-225 on 100–120 mesh Gas-Chrom Q) were 6 ft. $\times$ 1/4 in. O.D. Temperature program was 32 min at 190°C, then 16 min at 230°C (rate, 4°C/min). Injection port temperature was 200°C and detector temperature was 300°C. Carrier gas, nitrogen, flowed at 15 ml/min. A slight upward drift of the baseline occurred during temperature programming but did not affect electronic peak integration. The numbers represent the retention time of each sugar in minutes. Sample amount injected corresponds to 1 μg of each sugar. The ordinate is detector response; the abscissa is time in minutes.

Effects of varying the amounts of starting sugars upon the sugar ratios

All sugars relative to xylose decreased significantly (Table I) as the amount of starting sugar decreased from 50 to 25 ($P < 0.05$) and from 25 to 10 μg ($P < 0.01$), but only GalNAc decreased significantly from 3 to 1 μg ($P < 0.01$). The only sugar to change significantly relative to glucose (Table I) was GalNAc which decreased from 50 to 25 μg ($P < 0.05$).

Changes from the optimized derivatization procedure included a 16-h hydrolysis wich xylose added before hydrolysis, a 60-min $NaBH_4$ reduction and a 3-h acetylation.

Set to set variation in sugar ratios

All sugar ratios (Table I), except Glc:Xyl at 50, 25, 10 μg levels, varied significantly ($P < 0.01$) when prepared on different days (set number). The coefficient of variation (standard deviation/mean $\times$ 100) of data from multiple sets for Gal:Xyl averaged $\pm 12\%$; for Glc:Xyl, $\pm 9\%$; for GlcNAc:Xyl, $\pm 22\%$; for GalNAc:Xyl, $\pm 19\%$; for Gal:Glc, $\pm 11\%$; for GlcNAc:Glc, $\pm 22\%$; and for GalNAc:Glc, $\pm 18\%$.

Addition of the internal standard before or after hydrolysis

Fig. 2 shows the advantage of adding xylose after the hydrolysis step. Gal:Xyl is 290% greater ($P < 0.025$); Glc:Xyl, 200% greater ($P < 0.01$); GlcNAc:Xyl, 150% greater ($P < 0.05$); and GalNAc:Xyl, 130% greater ($P < 0.005$) when xylose is added before hydrolysis. Derivatization changes were as described under *Effects of varying the amounts of starting sugars upon the sugar ratios.*

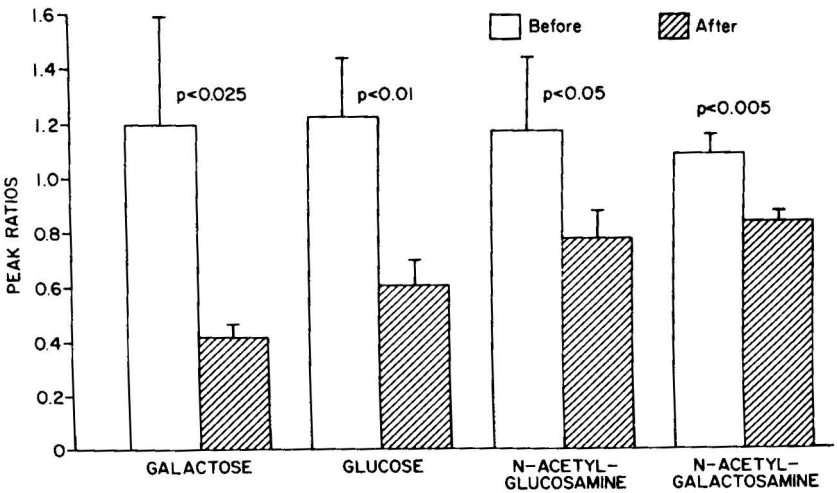

Fig. 2. Effects of addition of the internal standard, xylose, before and after 16 h of acid hydrolysis. A 3-μg amount of each sugar was used to prepare alditol acetate derivatives. Each sugar relative to xylose is represented as a bar graph with the mean of 3 separate derivatizations $\pm$ standard deviation shown. Notice how much greater the ratios are when xylose is also hydrolyzed indicating xylose degradation. Significance was determined by Student's *t*-test.

Effects of length of hydrolysis on sugar standards

Fig. 3 graphs the decrease in each sugar (relative to xylose) with increasing length of hydrolysis. Gal:Xyl decreased 26% ($P < 0.001$), Gly:Xyl decreased 20% ($P < 0.005$), GlcNAc:Xyl decreased 14% ($P < 0.005$) and GalNAc:Xyl decreased 4% ($P < 0.01$) between 0 and 4 h. Glc:Xyl also decreased between 8 and 12 h ($P < 0.01$). No further changes of significance were noted. Xylose was added following a 16-h hydrolysis. Other changes included a 60-min $NaBH_4$ reduction and a 3-h acetylation. Each time point (0, 4, 8, 12 and 16 h) was run in triplicate.

TABLE I

VARIATION IN ALDITOL ACETATE SUGAR RATIOS AS A FUNCTION OF AMOUNTS OF STARTING MATERIAL AND OF PREPARATION ON DIFFERENT DAYS (SET NO.)

Derivatives were prepared from standard sugar as described in the optimized derivatization procedure except that xylose was added before hydrolysis, hydrolysis time was 16 h, $NaBH_4$ reduction time was 60 min and acetylation time was 3 h. Statistical analyses included a two-way analysis of variance and a Duncan's multiple range test. Each number represents the mean of three samples ± standard deviation.

Set No.	*Sugar (μg)*	*Gal:Xyl*	*Glc:Xyl*	*GlcNAc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GlcNAc:Glc*	*GalNAc:Glc*
1	50	1.077 ± 0.026	1.592 ± 0.118	0.738 ± 0.112	1.051 ± 0.167	0.678 ± 0.032	0.464 ± 0.049	0.659 ± 0.057
1	25	1.024 ± 0.042	1.564 ± 0.084	0.726 ± 0.026	0.882 ± 0.106	0.657 ± 0.057	0.465 ± 0.037	0.566 ± 0.091
1	10	0.906 ± 0.076	1.346 ± 0.121	0.635 ± 0.111	0.776 ± 0.077	0.675 ± 0.057	0.470 ± 0.044	0.576 ± 0.016
2	50	1.014 ± 0.143	1.663 ± 0.102	1.077 ± 0.163	1.051 ± 0.144	0.586 ± 0.068	0.631 ± 0.082	0.614 ± 0.049
2	25	0.839 ± 0.047	1.428 ± 0.027	1.018 ± 0.070	0.824 ± 0.110	0.583 ± 0.033	0.709 ± 0.056	0.570 ± 0.068
2	10	0.760 ± 0.038	1.456 ± 0.046	1.094 ± 0.072	0.836 ± 0.055	0.521 ± 0.011	0.743 ± 0.048	0.573 ± 0.022
3	50	1.153 ± 0.111	1.705 ± 0.097	1.295 ± 0.105	1.493 ± 0.092	0.676 ± 0.112	0.771 ± 0.029	0.853 ± 0.083
3	25	0.960 ± 0.031	1.567 ± 0.091	0.866 ± 0.259	1.078 ± 0.227	0.616 ± 0.053	0.548 ± 0.140	0.685 ± 0.113
3	10	0.827 ± 0.028	1.385 ± 0.147	1.022 ± 0.124	0.987 ± 0.080	0.613 ± 0.099	0.739 ± 0.044	0.743 ± 0.080
4	50	0.980 ± 0.029	1.652 ± 0.086	1.039 ± 0.133	1.590 ± 0.172	0.595 ± 0.016	0.625 ± 0.034	0.961 ± 0.056
4	25	1.136 ± 0.056	1.608 ± 0.178	0.958 ± 0.205	1.522 ± 0.235	0.711 ± 0.047	0.593 ± 0.077	0.948 ± 0.102
4	10	0.911 ± 0.059	1.258 ± 0.014	0.675 ± 0.120	1.037 ± 0.141	0.724 ± 0.040	0.536 ± 0.092	0.824 ± 0.106
5	5	1.076 ± 0.070	1.751 ± 0.088	0.967 ± 0.055	1.163 ± 0.129	0.620 ± 0.028	0.552 ± 0.011	0.666 ± 0.048
5	3	1.053 ± 0.043	1.651 ± 0.098	0.988 ± 0.110	1.216 ± 0.069	0.638 ± 0.022	0.597 ± 0.036	0.736 ± 0.003
5	1	1.250 ± 0.372	1.670 ± 0.190	0.901 ± 0.033	1.021 ± 0.094	0.738 ± 0.149	0.543 ± 0.049	0.613 ± 0.046
6	5	1.070 ± 0.077	1.394 ± 0.148	0.701 ± 0.078	1.171 ± 0.080	0.770 ± 0.032	0.505 ± 0.056	0.844 ± 0.030
6	3	0.968 ± 0.146	1.355 ± 0.216	0.783 ± 0.177	1.198 ± 0.200	0.715 ± 0.027	0.575 ± 0.038	0.876 ± 0.006
6	1	0.843 ± 0.030	1.218 ± 0.223	0.727 ± 0.065	1.023 ± 0.205	0.706 ± 0.112	0.613 ± 0.139	0.876 ± 0.149
7	5	0.878 ± 0.043	1.380 ± 0.105	0.606 ± 0.013	0.912 ± 0.048	0.640 ± 0.076	0.441 ± 0.039	0.665 ± 0.082
7	3	0.973 ± 0.084	1.415 ± 0.239	0.625 ± 0.085	0.991 ± 0.065	0.694 ± 0.061	0.454 ± 0.119	0.716 ± 0.143
7	1	0.942 ± 0.107	1.265 ± 0.071	0.706 ± 0.043	1.052 ± 0.121	0.717 ± 0.054	0.530 ± 0.049	0.794 ± 0.073
8	5	0.939 ± 0.089	1.508 ± 0.175	1.080 ± 0.074	0.934 ± 0.030	0.618 ± 0.025	0.699 ± 0.065	0.681 ± 0.079
8	3	0.890 ± 0.071	1.295 ± 0.157	1.112 ± 0.148	1.069 ± 0.028	0.671 ± 0.028	0.876 ± 0.123	0.835 ± 0.104
8	1	0.620 ± 0.139	1.237 ± 0.172	0.999 ± 0.313	0.583 ± 0.189	0.498 ± 0.054	0.801 ± 0.178	0.502 ± 0.120

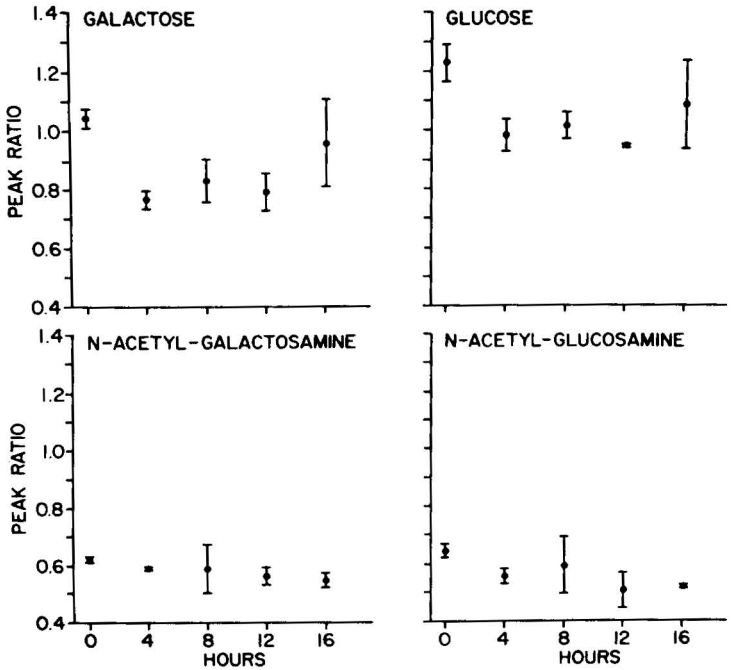

Fig. 3. Effects of hydrolysis time on standard sugars relative to xylose. A 3-μg amount of each sugar was derivatized; xylose was added after the hydrolysis step. Each time point represents a mean $\pm$ S.D. of three derivatizations. See Results (section *Effects of length of hydrolysis on sugar standards)* for significance.

Effects of length of $NaBH_4$ reduction on standard sugars

Fig. 4 graphs each sugar relative to Xyl *vs.* reduction time and shows that maximum ratios are obtained by 40 min. Relative to xylose, Glc and GlcNAc increased significantly between 30 and 40 min ($P < 0.01$) and GalNAc increased significantly between 10 and 20 min ($P < 0.05$). No hydrolysis was performed. Each time point (10, 20, 30, 40, 50 and 60 min) was run in triplicate. Acetylation was for 3 h.

Effect of acetylation temperature upon standard sugars

Fig. 5 shows that optimum acetylation in terms of maximum sugar ratios relative to xylose is achieved at 100°C. Gal:Xyl increased significantly between 80 and 90°C ($P < 0.05$) and GalNAc:Xyl increased significantly ($P < 0.01$) between 90 and 100°C. No hydrolysis was performed. $NaBH_4$ reduction was for 60 min. Each temperature point (80, 90, 100, 110, 120°C) was run in triplicate for 3 h.

Effects of length of acetylation on standard sugars

Fig. 6 shows that the optimum acetylation time in terms of maximum sugar ratios relative to xylose is 30 min. Gal:Xyl increased significantly between 5 and 10 ($P < 0.01$) and 15 and 30 min ($P < 0.05$). GlcNAc:Xyl decreased between 5 and 10 min ($P < 0.05$) while GalNAc:Xyl increased between 5 and 10 min ($P < 0.01$), 10 and 15 min ($P < 0.01$) and 15 and 30 min ($P < 0.05$). No hydrolysis was performed.

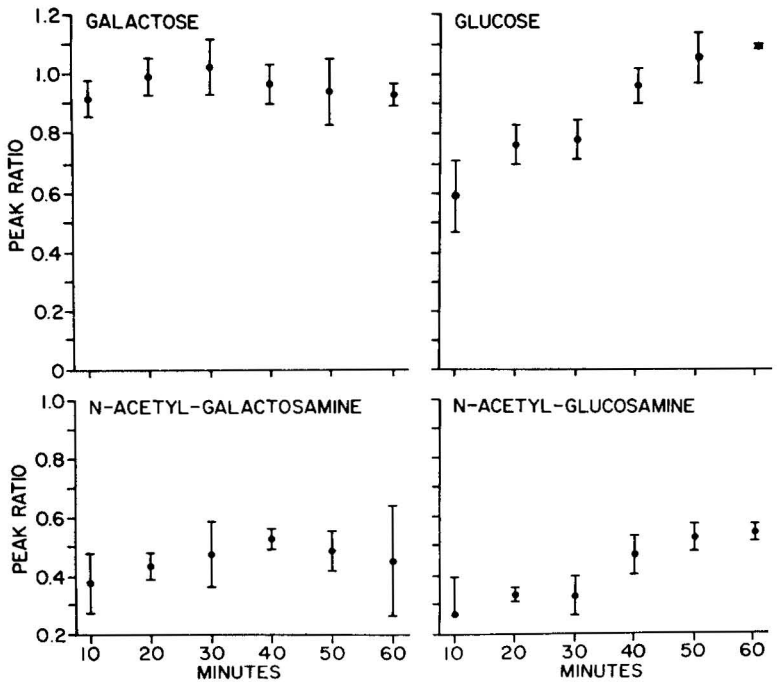

Fig. 4. Effects of varying $NaBH_4$ reduction time on peak ratios of standard sugars relative to xylose. A 3-μg amount of each sugar was derivatized with no hydrolysis step. Each time point represents the mean $\pm$ S.D. of three derivatizations. Significance was determined by a one way analysis of variance followed by Duncan's Multiple Range Test. See Results (section *Effects of length of $NaBH_4$ reduction on standard sugars)* for significance.

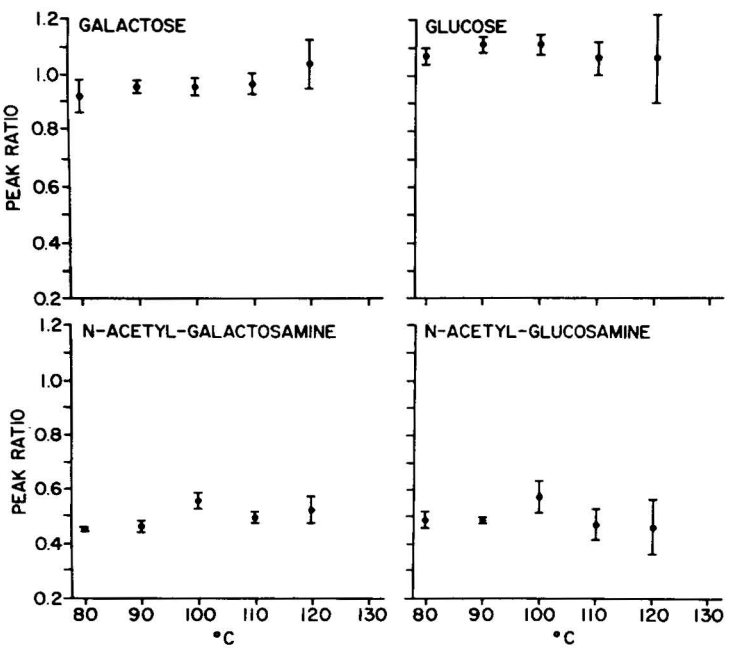

Fig. 5. Effects of varying acetylation temperatures on peak ratios of standard sugars relative to xylose. See Fig. 4 for details. Acetylation time was 3 h. See Results (section *Effect of acetylation temperature upon standard sugars)* for significance.

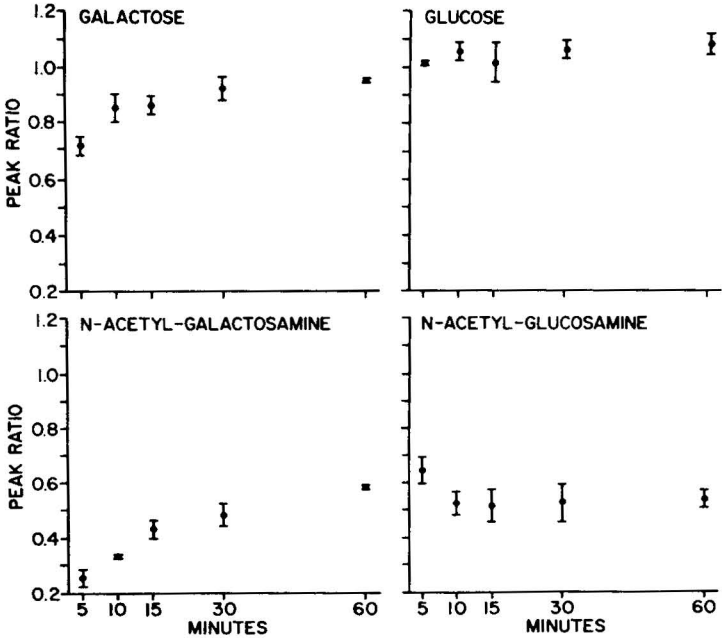

Fig. 6. Effects of varying acetylation time on peak ratios of standard sugars relative to xylose. See Fig. 4 for details. Acetylation temperature was 100°C. See Results (section *Effects of length of acetylation on standard sugars)* for significance.

$NaBH_4$ reduction was 60 min. Each time point (5, 10, 15, 30, 60 min) was run in triplicate at 100°C.

Effects of NH_4OH neutralization and hexane extraction on standard sugars

Table II shows that the only effect of neutralization and hexane extraction was a 34% decrease in GlcNAc relative to Xyl ($P < 0.005$). Three samples were treated normally (16-h hydrolysis, 60-min reduction, 3-h acetylation); three samples were neutralized with 2 drops of 7.4 *N* NH_4OH following hydrolysis and then extracted three times with 0.5 ml of hexane.

TABLE II

EFFECTS OF NH_4OH NEUTRALIZATION AND HEXANE EXTRACTION ON RATIOS OF STANDARD SUGARS RELATIVE TO XYLOSE

A 3-μg amount of each standard sugar was hydrolyzed 16 h. Xylose was added following hydrolysis. 3 samples were untreated; 3 samples were neutralized with 2 drops of 7.4 *N* NH_4OH and then extracted 3 times with 0.5 ml hexane. Significance was determined by Student's *t*-test.

Treatment	*Gal: Xyl*	*Glc: Xyl*	*GlcNAc: Xyl*	*GalNAc: Xyl*
Neutralized and extracted	0.806 ± 0.041	1.009 ± 0.055	0.471 ± 0.076*	0.570 ± 0.052
Untreated	0.823 ± 0.058	0.988 ± 0.094	0.714 ± 0.040	0.648 ± 0.046

* $P < 0.005$.

Effects of length of hydrolysis on the sugar ratios of GM_1

Table III demonstrates the phenomenon of sugar release from GM_1 *vs.* sugar degradation with time. Relative to xylose, Gal increases up to 4 h, then declines substantially between 12 and 16 h; Glc increases up to 12 h; GalNAc shows an initial increase at 2 h followed by a decline and then a rise again from 3 to 12 h.

TABLE III

EFFECTS OF LENGTH OF HYDROLYSIS ON THE SUGAR RATIOS OF GM_1

Duplicate samples per time point were prepared from an amount of GM_1 corresponding to 3 μg of its glucose content. Hydrolysis time varied between 1 and 16 h. Xylose was added following hydrolysis. Reduction was 60 min; acetylation was 3 h. Each time point represents 2 samples only, so no statistics were performed. Theoretical Gal:Glc and GalNAc:Glc ratios for GM_1 are 2:1 and 1:1, respectively.

Hydrolysis time (h)	*Gal: Xyl*	*Glc: Xyl*	*GlcNAc: Xyl*	*Gal: Glc*	*GalNAc: Glc*
1	1.080	0.171	0.500	6.384	2.924
2	1.268	0.324	0.654	4.290	2.225
3	1.322	0.418	0.638	3.186	1.538
4	1.351	0.498	0.694	2.764	1.424
8	1.302	0.638	0.756	2.040	1.205
12	1.298	0.708	0.880	1.846	1.240
16	1.180	0.685	0.770	1.725	1.112

Gal:Glc falls continuously from 1 through 16 h. It most closely approximates theoretical values of 2.00/1.00 at 8 h. GalNAc:Glc also declines between 1 and 16 h and most closely approximates theoretical values of 1.00/1.00 at 16 h. Changes from the optimized procedure included a 60-min reduction and 3-h acetylation. Duplicate samples of GM_1 were hydrolyzed for 1, 2, 3, 4, 8, 12 and 16 h.

Effects of nitrogen vs. air in the reaction mixture during GM_1 hydrolysis

Table IV shows no significant differences in the sugar ratios between samples treated normally (16-h hydrolysis, 60-min reduction, 3-h acetylation) and those having their ambient air displaced by bubbling nitrogen gas through the reactants for 30 sec before hydrolysis.

TABLE IV

EFFECTS OF AMBIENT NITROGEN *VS.* AIR DURING HYDROLYSIS UPON SUGAR RATIOS OF GM_1

Samples were treated as in Table III with 16 h of hydrolysis. Three samples had their air displaced for 30 sec by nitrogen bubbling before sealing the tube for hydrolysis; three samples were untreated. No significant differences were seen with Student's *t*-test.

Treatment	*Gal:Xyl*	*Glc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GalNAc:Glc*
GM_1 + air	1.390 ± 0.018	0.861 ± 0.030	0.789 ± 0.060	1.622 ± 0.055	0.926 ± 0.082
GM_1 + nitrogen	1.325 ± 0.118	0.855 ± 0.033	0.770 ± 0.055	1.589 ± 0.071	0.903 ± 0.056

Comparison of standard sugar ratios vs. those of GM_1

Table V shows that Gal:Xyl, Glc:Xyl and GalNAc:Glc ratios of standard sugars were significantly different from those of GM_1 ($P < 0.01$, $P < 0.001$, $P < 0.05$, respectively). In particular, the GalNAc:Glc ratio of standard sugars was 24% lower than that of GM_1. Three samples of GM_1 and three of standard sugars were hydrolyzed for 16 h, neutralized and extracted with hexane, had 60 min of reduction and a 3-h acetylation.

TABLE V

COMPARISON OF STANDARD SUGAR RATIOS WITH THOSE OBTAINED FROM GM_1

Three samples of standard sugars were prepared as described for untreated samples in Table II. Three samples of GM_1 were prepared as described in Table III. Significance was determined by a Student's *t*-test. Using these standard sugars, the corrected sugar ratios for GM_1 are Gal:Glc, 2.02:1 (theoretical 2:1) GalNAc:Glc, 1.23:1 (theoretical 1:1).

	Gal:Xyl	*Glc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GalNAc:Glc*
GM_1	$0.660 \pm 0.069^{**,\S}$	$0.744 \pm 0.040^{***}$	0.745 ± 0.077	$0.886 \pm 0.116^{\S}$	$1.010 \pm 0.114^{*}$
Sugar standards	0.860 ± 0.034	0.981 ± 0.034	0.758 ± 0.102	0.877 ± 0.023	0.772 ± 0.092

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.
§ Divided by 2 for comparison.

Sugar ratios of known gangliosides

Table VI demonstrates that use of a GM_1 standard for ratio correction of sugars from GD_{1a}, GM_2, GT_{1b} and GD_3 brings these ratios closer to theoretical values than does the use of standard sugars which yield Gal:Glc and GalNAc:Glc

TABLE VI

COMPARISON OF CORRECTED GANGLIOSIDE SUGAR RATIOS USING GM_1 AND STANDARD SUGARS

Each ganglioside was treated as in Table III. Duplicate sets were run; each set contained samples of both GM_1 and standard sugars. Ganglioside sugar ratios were corrected using both standard sugars and GM_1. These corrected ratios were compared to theoretical ratios using a paired *t*-test. Ganglioside sugar ratios corrected by using GM_1 were not significantly different from theoretical values. The GalNAc:Glc ratio varied significantly from theoretical ($P<0.005$) as did the Gal:Glc ratio ($P<0.05$) when corrected by using standard sugars.

Ganglioside	*Sugars*	*Theoretical ratio*	*Corrected ratio using GM_1*	*Corrected ratio using standard sugars*
GD_{1a}	Gal:GalNAc:Glc	2:1:1	1.9:1.0:1	2.2:1.7:1
GD_{1a}	Gal:GalNAc:Glc	2:1:1	2.0:1.2:1	2.2:1.8:1
GT_{1b}	Gal:GalNAc:Glc	2:1:1	2.0:1.1:1	2.2:1.8:1
GT_{1b}	Gal:GalNAc:Glc	2:1:1	1.9:1.0:1	1.9:1.2:1
GM_2	Gal:GalNAc:Glc	1:1:1	1.0:0.9:1	1.1:1.4:1
GM_2	Gal:GalNAc:Glc	1:1:1	1.0:0.8:1	1.2:1.3:1
GD_3	Gal:Glc	1:1	1.2:1	1.4:1
GD_3	Gal:Glc	1:1	0.9:1	0.8:1

ratios considerably different from theoretical values ($P < 0.05$ and $P < 0.005$, respectively). Variation from theoretical ratios in GM_1 corrected ratios was never more than 20% and averaged 7%. Variation from theoretical ratios corrected by using sugar standards was as high as 80% and averaged 32% due principally to the GalNAc:Glc ratios of the standard sugars being less than those of gangliosides. Changes from optimized conditions included a 16-h hydrolysis, 60-min reduction and a 3-h acetylation.

DISCUSSION

Analyses of microgram quantities of carbohydrates from glycosphingolipids requires both a sensitive and a reliable method. Sensitive techniques measuring nanogram amounts of sugars have been reported but require elaborate and expensive modifications of the simpler flame-ionization detector GLC (*e.g.* mass spectroscopy[11,12], electron-capture detectors[27,28], radiogas chromatography[29]. Reproducibility has been a persistent problem in the use of GLC to quantitate carbohydrates. The TMS method has been fraught with inconsistencies in hexosamines (from decomposition, absorption to columns and acidic resins, and de-N-acetylation during hydrolysis[14,30]). There have also been disparities in the $AgCO_3$ neutralization step with loss of the internal standard[13,15], an effect which is potentiated in the microgram range[16]. Interpretation of multiple peaks, especially the hexosamines, also enhances the difficulties.

The alditol acetate method offers a simpler spectrum. However, working in the 1–50-μg range requires some modifications of existing procedures in order to increase sensitivity of the method and to reduce baseline noise from residual reagents. Furthermore, glycosphingolipids present a special problem in that the glucose to ceramide bond is not readily cleaved[10,20] causing a situation in which there are differential rates of sugar release and destruction during hydrolysis. We have carefully investigated this alditol acetate micromethod with the goal of optimizing sugar ratios for glycolipid analysis while minimizing the length of time required for the procedure. In doing so, we have attended to anecdotal reports in the literature which speculate on causes of sugar loss.

We chose xylose as our internal standard since its peak separates well from galactose and since it is not known to be a component sugar of glycolipids (particularly gangliosides). As is clearly demonstrated by Table I and Fig. 2, xylose proves to be somewhat unstable in acid (high sugar ratios relative to xylose) especially as hydrolysis time approaches 16 h (data not shown). This confirms Albersheim's observation of low xylose stability in acid after 2 h of pinto bean cell wall hydrolysis[23]. Therefore, we have begun adding the internal standard after the hydrolysis step. Jamieson and Reid, after showing a loss of mannitol during the $AgCO_3$ neutralization step in TMS derivatization, suggested adding mannitol after hydrolysis[13] and Levvy *et al.*[14] put this suggestion into practice. We have reduced our coefficient of variation from a maximum of 32% (average of 20%) when xylose was hydrolyzed to a maximum of 15% (average 11%) when it was added following hydrolysis. Free sugars in acid are especially labile (see below).

Fucose-containing glycolipids require a different internal standard since fucose often appears as two peaks, one major peak (retention time, $t_R = 5.8$ min) and one minor peak ($t_R = 9.6$ min). The latter overlaps xylose. Therefore, we recommend

using deoxyglucose or ribose as an internal standard when dealing with fucose as these two sugars have distinguishable peaks with retention times of 11.9 and 7.0 min, respectively.

Table I shows that sugar ratios relative to glucose are consistent between 25 and 10 μg or between 5 and 1 μg. However, they vary significantly when expressed relative to xylose. Xylose was hydrolyzed in these experiments and its susceptibility to degradation is borne out by the consistency of these sugar amounts when expressed in terms of glucose rather than xylose. The 5, 3 and 1 μg results were more consistent in terms of xylose than the 50, 15 and 10 μg samples, possibly due to the closer amounts of material.

Set to set variation in sugar ratios exists as is demonstrated by Table I. This requires that each set of samples be derivatized with standards since no one set of correction terms will be reliable for every derivatization performed (due to reagent and column aging and possibly unidentified side products of the derivatization reactions).

Instability of free sugars is aptly demonstrated in Fig. 3. Xylose was added after hydrolysis so that the denominator of each ratio would remain constant with time. Large (up to 26%) decreases occurred within the first 4 h of hydrolysis with further, but insignificant, decreases in the hexosamines up to 16 h. Table III shows the results of this same experiment upon sugars released from GM_1. Here, two competing processes occur: sugar release from the lipid and sugar degradation. Glucose and N-acetylgalactosamine reach their peak values by 12 h. Kannan *et al.*[20] noted that 12 h was required for complete release of glucose from ceramide in glucocerebroside, and Zanetta *et al.*[10] claimed 16 h were required for GM_1 and GD_{1a} to completely break their glucose–ceramide bonds. However, by 16 h galactose is degrading causing a less than ideal sugar ratio for Gal:Glc (1.725:1). This could be accounted for if the values were corrected by a factor calculated from simultaneously run sugar standards to bring the Gal:Glc ratio back to the theoretical 2:1 value. However, as Table V demonstrates, there is significantly less galactose and glucose at 16 h in GM_1 than in free sugar standards. Concurrently, N-acetylgalactosamine is relatively stable. Therefore, the correction factor computed from sugar standards bring the Gal:Glc ratio to a value of 2:1 but elevates the GalNAc:Glc ratio to a value greater than 1:1 (1.31:1). These three experiments suggest that ganglioside hydrolysis differs from free sugar degradation and therefore free sugars are not legitimate standards for correcting sugar ratios of gangliosides and probably other glycolipids. Perhaps the difference is due to side products formed during lipid hydrolysis and/or micellar existence of gangliosides in aqueous HCl.

Table VI lends further support to this conclusion. Here ganglioside sugar ratios corrected by using GM_1 were not significantly different from theoretical ratios. However, ratios corrected by using standard sugars did vary significantly from theoretical values, especially in giving a faulty elevation of GalNAc:Glc ($P < 0.005$). Therefore, we suggest using pure, known gangliosides as standards when quantitating unknown gangliosides obtained from thin-layer or column chromatography. Clarke[31], Yu and Ledeen[32], and Holm *et al.*[21] have also reported using the gangliosides GM_3, GM_2, GM_1, and GD_{1a} as standards for determining correction factors in GLC analyses of ganglioside sugars (including N-acetylneuraminic acid).

The problem of choosing a hydrolysis time for gangliosides is difficult to resolve

due to the differential rates of sugar release and degradation. Our data and others'[10,20] suggest that the peak of released glucose and N-acetylgalactosamine occurs at 12 h whereas that of galactose occurs at 4 h. Thus, there could be significant breakdown of galactose by 12 h[19]. Also, the most ideal Gal:Glc ratio occurs at 8 h but the less than ideal Gal:Glc ratio at 12 or 16 h can be corrected by use of appropriate standards. This problem can be resolved in several ways. If it is convenient to hydrolyze overnight, 12 to 16 h can be utilized, but under other circumstances, 8 h should be sufficient when coupled with the use of appropriate standards.

The effects of reduction on standard sugars are shown in Fig. 4. The Glc:Xyl and GlcNAc:Xyl ratios increase with time but are not significant past 40 min. Gal:Xyl and GalNAc:Xyl tend to decrease past 40 min, but this was not statistically significant. Since both trends prove insignificant, any time between 40 and 60 min of $NaBH_4$ reduction can be considered appropriate. The best acetylation temperature (Fig. 5) appears to be 100°C since glucose, N-acetylglucosamine and N-acetylgalactosamine peak at this temperature (only GalNAc:Xyl does so significantly). We routinely use 100°C which simplifies temperature control of our heating block since both hydrolysis and acetylation can be run at the same temperature. Sugar ratios increased with the amount of time spent acetylating up to 30 min after which there were no significant differences (data for 2, 3, 4, 5 h of acetylation not shown). This differs from Niedermeier's claim that acetylation is complete within 15 min[34] although his was done in a boiling water bath which provides more uniform initial heating and thus may slightly speed up the acetylation.

Many reports have appeared claiming neutralization of the hydrolysis mixture prior to evaporation prevents sugar loss. This is especially true of TMS where $AgCO_3$ and resin have been used to remove H^+ (refs. 15, 33), but has also been reported for alditol acetates[34]. Table II shows the results of an experiment we conducted to investigate these claims. NH_4OH and hexane extraction were chosen since these are used to remove fatty acids and sphingosines from ganglioside hydrolysates before evaporation. We used NH_4OH rather than NaOH to avoid introducing a new cation into the derivatization procedure. The only significant difference between the two treatments was a 34% decrease in GlcNAc:Xyl with neutralization and hexane extraction. This could become significant when dealing with an N-acetylglucosamine-containing glycolipid and therefore an appropriate N-acetylglucosamine-containing standard should be used.

Kim *et al.*[35] advised excluding oxygen from the hydrolysis mixture of glycoproteins since oxidation side products could possibly result in a loss of sugars. We investigated this claim using GM_1. As shown in Table IV, there was no significant difference between sugar ratios obtained from hydrolysis vials containing air *vs.* those containing nitrogen. This does not exclude an effect on glycoproteins but appears to make no difference upon ganglioside sugar ratios.

We originally investigated this method with the purpose of optimizing sugar ratios and increasing the sensitivity for routine work in the 1 to 10 μg range. This required first a scaling down and an integration of pre-existing alditol acetate methods. We then established that consistent ratios can be obtained for gangliosides if an appropriate ganglioside standard is used for determining the correction factor. We found that an 8-h ganglioside hydrolysis is as appropriate as a 12- to 16-h hydrolysis based on the differential rates of sugar release and breakdown and use of standards.

Xylose, our internal standard, proved to be unstable in acid requiring its addition after hydrolysis. (We have not investigated the acid susceptibilities of ribose or 2-deoxyglucose so both may be better internal standards for future use.) Nitrogen in the hydrolysis mixture had no influence upon sugar ratios compared with air. Neutralization after hydrolysis did not prevent sugar loss and for N-acetylglucosamine actually caused a loss although the hexane extraction may also have been a factor. A 40-min reduction and a 30-min acetylation were both compatible with good ratios and 100°C proved to be the best acetylation temperature. The alditol acetate method now presents a reliable and sensitive procedure for analyzing glycolipid sugars in the 1–10-μg range.

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EVALUATION OF TRIETHYLAMMONIUM PHOSPHATE AND FORMATE–ACETONITRILE MIXTURES AS ELUENTS FOR HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY*

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SUMMARY

Trialkylammonium phosphate or formate and more specifically triethylammonium phosphate or formate buffers (pH < 3) in the presence of 15–30% acetonitrile have been shown to be compatible with the protein analysis column PAC I-125 of Waters Associates for gel permeation of peptides and proteins. A linear relationship between log molecular weight (1,000–44,000) *versus* retention time is obtained. The effect of salt and organic modifier concentrations as well as temperature and column load was studied using a mix of proteins and peptides of varying isoelectric points as well as hydrophobicity. The advantages of that system (beside being already widely used in reversed-phase high-performance liquid chromatography) include low ionic strength, UV transparence and compatibility (after elimination of the volatile acetonitrile) with most biological systems. Applications include purification of iodinated trace and natural products and precise molecular weight determination.

INTRODUCTION

Fractionation of proteins according to size utilizing cross-linked dextran or polyacrylamide gel columns was first demonstrated by Porath and Flodin[1] in 1959. This technique has become the most widely accepted method for separation and molecular-weight determination of hydrophilic as well as some hydrophobic macromolecules using aqueous buffers with or without organic modifier. While this technique might not be unique in its ability to resolve and separate proteins (*i.e.*, ion-exchange and partition chromatographies or electrophoresis on these gels are widely used) it certainly is simple and effective.

With the development of new supports which are not compressible (in contradistinction with the soft gels mentioned earlier) the basic principle of this technique

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has now been applied under higher pressures and has been given the acronym of HP-GPC for high-performance gel permeation chromatography. One major obstacle to be overcome was to find non-compressible supports compatible with the biomaterials to be chromatographed *i.e.*, (a) non-specific adsorption had to be minimized and (b) controlled pore size had to be achieved. This early work has been reviewed by Cooper and Van Derveer[2].

More recently, Hashimoto *et al.*[3] have described protein separations on chemically modified silica gel base supports, TSK-Gel PW-type columns whereas Fukano *et al.*[4] and later Rokushika *et al.*[5] have used TSK-Gel 2000 and 3000 SW columns to respectively demonstrate the usefulness of such columns for the separation and recovery of biologically active proteins and enzymes as well as the separation of saccharides and water-soluble synthetic polymers. The influence of flow-rate on plate height and retention volumes for these two sets of columns was also investigated. These investigators found a linear relationship between the logarithm of the molecular weight *versus* K (distribution coefficient) for proteins of molecular weights ranging from ferritin (mol.wt. 480,000) to insulin (mol.wt. 6000) using the TSK 3000 SW column and a phosphate buffer (0.01 M phosphate pH 6.5 containing 0.2 M sodium sulfate).

It is of interest that all these studies as well as those recently presented at the *LC Symposium III, October 1979, Boston*[6–11] agree with the fact that without addition of a detergent, the separation of proteins according to size is only possible by adjusting the pH and the ionic strength of the eluent. Consequently, relatively high concentrations of non-volatile buffers must be employed to increase ionic strength which tends to minimize electrostatic (ionic) interactions of positively charged proteins ($pI > 8$) with the negatively charged surface[9,12] resulting in a reduced elution volume. At the same time it will also decrease electrostatic repulsion of negatively charged proteins with the negative surface and result —for those proteins— in a larger elution volume than theoretically predicted. Schmidt *et al.*[12] went one step further and, using a diol phase (LiChrosorb Diol from E. Merck, Darmstadt, G.F.R.) found that at pH 5.0 this phase showed residual ionic charges below an ionic strength of 0.2 M; however, while the protein-stationary phase ionic interactions were neutralized above μ 0.20 M, unusually hydrophobic proteins (especially lysozyme and chymotrypsinogen) started to exhibit hydrophobic interactions with the support. Similar decreases in ionic interaction of proteins with Sepharose B[13] and Sephacryl S-200[14] when $\mu \geqslant 0.2$ M have been suggested. In practice low ionic strength generally results in poor resolution and recovery of proteins having widely different isoelectric points or hydrophobic character. Another approach reported by Kato *et al.*[15] using TSK-Gel SW type columns was the inclusion of sodium dodecyl sulfate in their mobile phase (ion pairing and/or detergent effect). Resolution again was highly dependent upon salt concentration (sodium sulfate: ideally 0.05–0.2 M).

In order to circumvent the problem of not being able to use successfully volatile buffers such as ammonium acetate and bicarbonate (10 mM) Waterfield and Scarce[6] chose to succinylate or citraconylate their proteins to improve elution characteristics on protein analysis columns (PAC) I-125 columns (Waters Assoc., Milford, MA, U.S.A.) as well as allow for monitoring at 254 nm.

None of these solutions to apparently basic problems (*i.e.*, those inherent to the support as well as those inherent to the proteins *i.e.*: solubility at different pH and

ionic strength) satisfied our need for a UV transparent biologically compatible and if possible voltile buffer, which would allow for good resolution and recovery of a wide range (in terms of their ionic and hydrophobic character) of proteins and peptides. We believe that the solvent system described here may have most of the ideal characteristics mentioned earlier. It represents an expansion of our work done with the triethylammonium formate (TEAF) and triethylammonium phosphate (TEAP) buffers which have proven so effective as solvents for reversed-phase high-performance liquid chromatography[16] (RP-HPLC).

The TEAP and TEAF buffers in the presence of a certain amount of acetonitrile are shown to be compatible with the PAC I-125, and to give good resolution and good recoveries for the peptides and proteins studied. Linearity of dose/response as well as calibration curve for molecules ranging from acetic acid (mol.wt. 60) or thyrotropin releasing factor (mol.wt. 363) to globulins (mol.wt. 150,000) —including proteins with quite different isoelectric points— , are presented. Concentration of the organic modifier and of the salt in the buffer is defined, and the temperature effects on resolution investigated.

MATERIALS AND METHODS

Apparatus

The apparatus consists of Waters Assoc. Models: 204 liquid chromatograph, U6K injector, two 6000A pumps, 660 programmer, Model 450 multiwave-length UV/visible detector, Infotronics Model 110 integrator, and Linear Instruments Model 445 chart recorder. Two PAC I-125 columns Nos. 093593 and 093594 were used in these studies. Full-scale absorbance is expressed in a.u.f.s. The data were analyzed with a Hewlett-Packard Model No. 9830A computer using a least-squares analysis program and graphed using Model No. 9866A printer.

Composition of the TEAP and TEAF buffer[16]

The TEAP buffer was obtained by bringing the pH of 0.25 *N* phosphoric acid to 2.25 with redistilled (over *p*-toluenesulfonylchloride) triethylamine. Several liters were made at one time which were filtered over a C_{18} cartridge in Waters Assoc. Prep LC-500. As a result, any hydrophobic UV-absorbing material was eliminated, thus allowing for clean washes at the end of gradients in our analytical system. The bulk of the buffer was kept in a cold room since bacterial contamination was observed during summer months. Prior to use, aliquots are being degassed and stirred under house vacuum for 5 min.

The TEAF buffer was obtained by bringing the pH of 0.25 *N* formic acid to 3.0 with redistilled triethylamine. It was millipore filtered before use to eliminate any solid particles that might plug the columns. For studies involving lower concentrations of TEAP buffer, simple dilutions were made from the original stock.

Conditions

The A buffer was pure or diluted TEAP whereas the B buffer was a mixture of 40% A and 60% acetonitrile. Using these two solutions both pumps delivered 0.5 ml/min for a final concentration of 30% acetonitrile.

All experiments were run at 50°C unless otherwise indicated. Conditions used

in each experiment are described in the legends of each table or figure. High temperature was controlled within 1°C by immersing the columns in a thermoregulated water bath. Flow-rate was 1.0 ml/min; chart speed was 1 cm/min; column back pressure was 100–150 p.s.i.

RESULTS AND DISCUSSION

It was shown by several groups[16–18] that peptides and even small proteins (mol.wt. $< 12{,}000$) could be eluted from reversed-phase supports using appropriate conditions (*e.g.* buffer composition and pH, organic modifier, column support, temperature, flow-rate and gradient shape have been optimized).

The reasons that larger proteins (mol.wt. $> 12{,}000$) could not be eluted include insolubility of the proteins under the chromatographic conditions, inadequate pore size of the support, and inappropriate kinetics of exchange of the proteins between the different phases.

With the availability of non-compressible hydrophilic supports (PAC I-125 in this case) which had been designed for the chromatography of large hydrophilic polymers/proteins and using our past experience in peptide/protein separation using HPLC technology, we undertook to test the compatibility of TEAP/TEAF buffers on such a column for peptide/protein separation and/or molecular-weight determination while keeping in mind our ultimate goal of developing a UV transparent, biologically compatible or volatile buffer which would give high resolution and good recoveries.

Fig. 1 shows the effect of acetonitrile concentration on the resolution of different peptides and proteins (see conditions in the legend). As could have been predicted, the separation of larger proteins is improved at lower concentration of acetonitrile (better overall solubility) whereas for smaller peptides, higher concentration of acetonitrile seem favorable. However, as is often the case for peptides and proteins exhibiting a large spectrum of solubility characteristics due to their inherent primary and tertiary structures (low to high isoelectric points, more or less hydrophobic and globular or random in conformation), no generalization is possible. For the first time however a combination of an aqueous buffer and a significant amount of an organic modifier is being successfully used for the elution of a peptide/protein mixture using a high-pressure system (see ref. 12 for effect of added ethylene glycol to mobile phase).

In the range of 15–30% acetonitrile the different components of the mixture are being separated according to size in a gel permeation mode with very little non-specific adsorption ($V_i/V_o \leqslant 1.1$, < 1.3 calculated by Regnier *et al.*[19]; (V_i = internal volume of column = $V_t - V_o$; V_t = elution volume of the excluded bovine serum albumin (BSA)). Good peak symmetry is another indication of non-specific adsorption. It is noteworthy that Sokolowski and Wahlund[20], studying peak tailing and retention behavior of tricyclic antidepressant amines and related ammonium compounds by RP-HPLC, confirmed our earlier results[16] showing that addition of alkylammonium ions to the mobile phase reduced tailing. Their extensive studies indicated that the nature and the concentration of the added alkylamines as well as the nature of the column support were critical for good peak symmetry or selectivity, respectively. Whereas triethylamine had an acceptable asymetric factor value of two, dimethyloctylamine for example, had one close to one (excellent). Whether one of

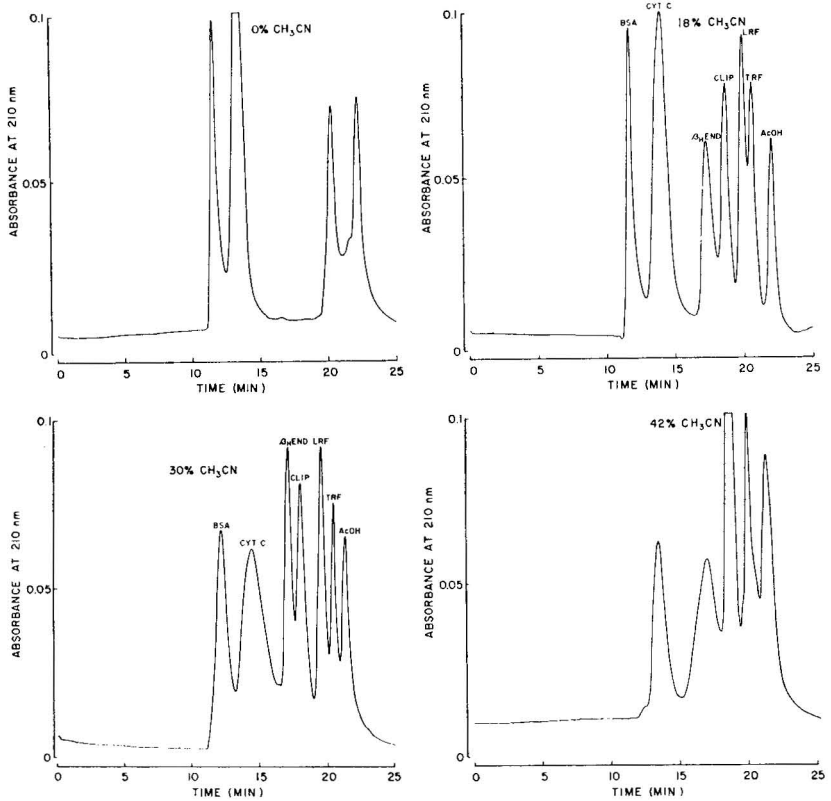

Fig. 1. Influence of acetonitrile concentration on elution pattern. Conditions: load, 50 μl protein–peptide mixture; solvent system, TEAP pH 2.25–acetonitrile as shown. Retention times (sec) for the different components are for 18% and 30% acetonitrile respectively: BSA, 695 and 729; cyt *c*, 829 and 861; β-endorphin (human), 1027 and 1011; CLIP, 1111 and 1075; LRF, 1185 and 1167; TRF, 1227 and 1221; acetic acid, 1311 and 1273.

those amines or other organic modifiers such as methanol, *n*-propanol, isopropanol, *n*-butanol, tetrahydrofuran and pyridine currently used with success in RP-HPLC of peptides will be also compatible with HP-GPC of peptides and proteins remains to be established.

Also noteworthy is the low pH (below most isoelectric points) of the aqueous buffer used for the separation shown in Fig. 1. A low pH (< 3) is recommended in RP-HPLC for most peptides[16–18] (exception: acidic peptides which are insoluble under those conditions: for example, gastrin I for which a dilute 1:1 TEAP buffer at pH $\approx$ 6.5 is recommended[21]). Obviously, proteins which would be insoluble under the conditions used, could hardly be expected to elute from any column. It is remarkable however that among the proteins present in this mix, cytochrome *c* has a p*I* of 10.6 (ref. 22) whereas BSA has a p*I* of 4.4–4.8 (ref. 23). Recovery studies using integrated areas under the peaks and different loads (5, 10, 20 and 40 μg) have shown good linearity for all components of the mixture when using 30% acetonitrile and the TEAP pH 2.25 buffer at room temperature. In that experiment, retention times

remained constant, indicating that overloading had not occurred. Day-to-day reproducibility appeared good (variability $< 1\%$ in retention times) but was not extensively studied since it is very dependent on pump performance.

Fig. 2 shows a separation similar to that reported in Fig. 1. Except for a different composition of the peptide/protein mix (see legend) which includes indole (a hydrophobic small substance: mol.wt. 117), the only variable is the concentration of the TEAP buffer in the eluting solvent. These separations are to be compared with that shown in Fig. 1 (30% acetonitrile); even though no dramatic change in resolution is observed, dilution of the TEAP buffer to 0.0625 *N* or 0.02 *M* (Fig. 2b) may give better results.

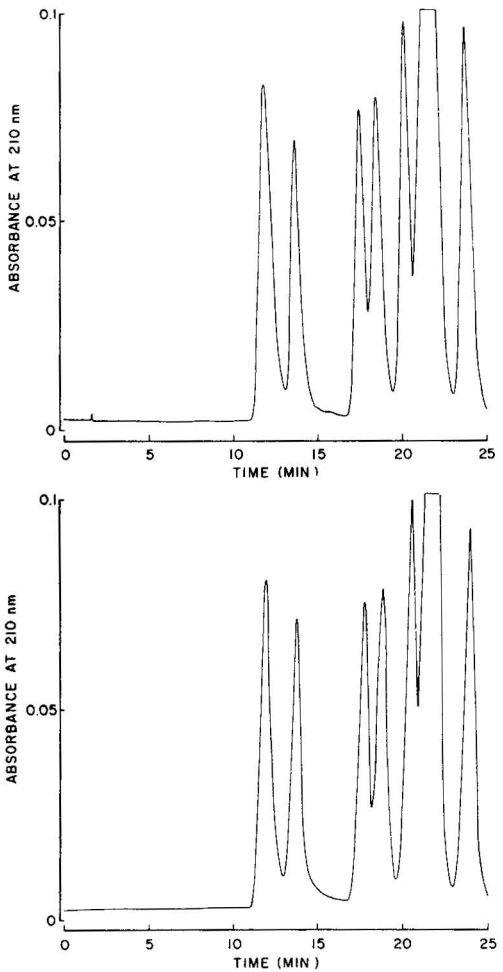

Fig. 2. Influence of TEAP concentration on elution pattern. Conditions: load, 35 μl protein–peptide mixture; solvent system top, TEAP pH 2.25–water (1:1)–30% acetonitrile; bottom, TEAP pH 2.25–water (1:3)–30% acetonitrile. Retention times (sec) for the different components are for TEAP–water (1:1) and (1:3) respectively: BSA, 710 and 700; cyt *c*, 818 and 810; β-endorphin (human), 1046 and 1048; CLIP, 1108 and 1112; LRF, 1206 and 1214; TRF + TEAF, 1300 and 1286; indole, 1424 and 1420.

TABLE I

EFFECT OF TEAP CONCENTRATION ON RETENTION TIMES OF PEPTIDES AND PROTEINS

Conditions: aqueous buffer–acetonitrile (7:3) isocratic. Clip = adrenocorticotropic hormone (18–39).

Protein	*Retention times (sec)*		
	TEAP 2.25	*TEAP 2.25–water (1:1)*	*TEAP 2.25–water (1:3)*
BSA	729	710	700
Cytochrome *c*	861	818	810
β_h-Endorphin	1019	1046	1048
Clip	1075	1108	1112
LRF	1167	1206	1214

Table I shows more accurately the effect of the TEAP concentration on retention times of peptides and proteins. Whereas proteins elute earlier at low concentration of the buffer than at higher buffer concentration, peptides have the opposite tendency. No simple interpretation of those results can be given: two phenomena may be involved: (a) a dependence of V_i upon TEAP concentration would be compatible with some interaction of the eluent and the stationary phase[20]; (b) the particular solvent system has an effect on the Stokes' radius of the peptide and protein studied. At high concentration of TEAP, proteins appear smaller than they really should be (salting out effect) whereas peptides appear larger than they really are (ion pairing and solvation effect).

The fact that indole is being unexpectedly retarded in this system —even though V_i/V_o is still < 1.3— may indicate that other compounds may also show anomalous behavior in this system (for example: very hydrophobic luteinizing hormone releasing factor (LRF) antagonist; cyclic peptides such as insulin and LRF analogs; see Fig. 4, Tables II and III.

We then investigated the effect of temperature on the standard peptide/protein mix. Fig. 3 (see legend for conditions) clearly shows the advantage of working at higher temperatures.

Using the retention times obtained from Table II (dilute TEAP 1:1), we plotted log molecular weight *versus* retention times (in sec). Fig. 4 shows a linear relationship for molecular weights ranging from 1000 to 44,000. This is somewhat different from the suppliers specifications (mol.wt. range 2000–80,000) but must be accounted for by the unusual composition of the eluting buffer. The correlation coefficient derived from linear regression analysis was found to be −0.985. BSA and in another similar experiment, human γ-globulins were excluded whereas TRF, [Met^5]-enkephalin, LRF$^{5-10}$, acetic acid, and indole also fell outside of the linear range. Insulin which was not reduced eluted with an apparent low molecular weight as expected. It is interesting to note that at this pH, peptides and proteins with a high p*I*: bradykinin, dynorphin, cytochrome *c* and soybean trypsin inhibitor appear to be larger in size than they are, probably due to some ion pairing effect or repulsive effect of the support, both already discussed.

Fig. 5 (see legend for exact conditions) shows a similar separation using the TEAF buffer, thus demonstrating that similar resolution can be achieved with a volatile buffer. It was shown that this buffer has a different selectivity under reversed-

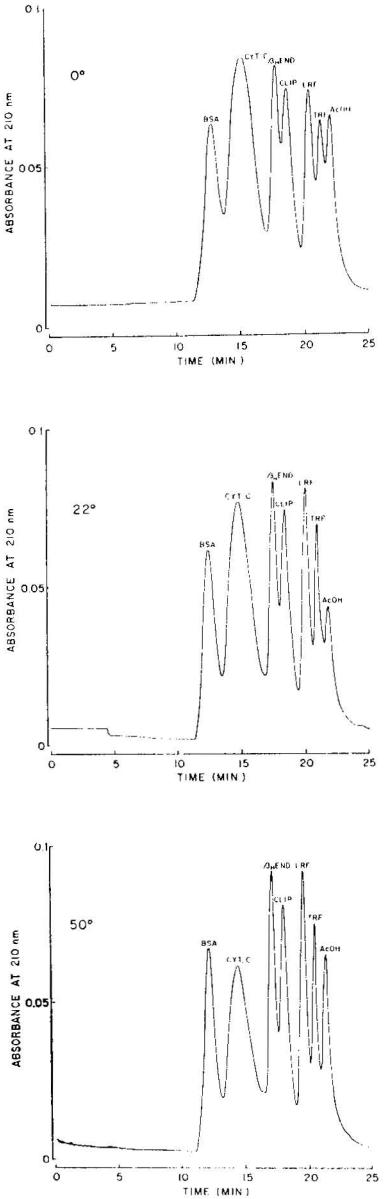

Fig. 3. Influence of temperature on elution pattern. Conditions: load; 50 μl protein–peptide mixture; solvent system TEAP pH 2.25 + 30% acetonitrile. Temperature as shown. Retention times (sec) for the different components are for 0°, 22° and 50°C respectively: BSA, 766, 740, 729; cyt *c*, 900, 878, 861; β-endorphin (human), 1060, 1042, 1011; CLIP, 1112, 1096, 1075; LRF, 1216, 1192, 1167; TRF, 1276, 1246, 1221; acetic acid, 1314, 1298, 1273.

phase conditions[29] and we do not want to exclude at this stage the possibility that it might also be the case in HP-GPC for certain compounds. Whereas 0.1% TFA, for example, is a very good solvent in RP-HPLC, it is interesting, that it has been found inappropriate with and without acetonitrile for the HP-GPC of our peptide mix.

TABLE II

PEPTIDES AND PROTEINS USED IN THIS STUDY

Retention times are averages of 3–4 runs done on different days. Values of acetic acid, indole, TRF, [Met^5]-enkaphalin, LRF (5–10), insulin and γ globulins are not introduced in calibration curve. □, values not introduced in the calibration curve; *, values used for the calibration curve.

Peptides	*Reference*	*Nos. of residues*	*Molecular weight*	*Retention time (sec)*
1□ Acetic acid			60	1370 ± 9
2□ Indole			117	1449 ± 1
3□ Thyrotropin releasing factor (TRF)*		3	363	1365 ± 2
4□ [Met^5]-enkephalin		5	573	1321 ± 5
5□ LRF (5–10) Ac-Tyr–Gly–Leu–Arg–Pro–Gly-NH_2*			718	1296 ± 3
6* Oxytocin*		9	1008	1302 ± 5
7* Bradykinin*		9	1059	1269 ± 3
8* Luteinizing hormone releasing factor (LRF)*		10	1182	1278 ± 3
9* LRF antagonist**		10	1396	1318 ± 2
10* Substance P*		11	1346	1243 ± 2
11* Dynorphin*	24	13	1602	1203 ± 5
12* α-Melanocyte stimulating hormone*		13	1663	1238 ± 4
13* Neurotensin*		13	1672	1221 ± 3
14* Bombesin*		14	1619	1250 ± 2
15* Somatostatin*		14	1638	1266 ± 7
16* CLIP (human)*		22	2463	1172 ± 2
17* β-Melanocyte stimulating hormone*		22	2658	1198 ± 4
18* ACTH (1–24)*		24	2930	1166 ± 8
19* Gastrin releasing peptide*	25	27	2785	1147 ± 2
20* Somatostatin (1–28)*	26	28	3137	1164 ± 5
21* Vasoactive intestinal peptide (VIP)**		28	3322	1148 ± 10
22* Glucagon**		29	3479	1155 ± 7
23* β-Endorphin (human)*		31	3461	1118 ± 4
24□ Insulin**		51	5700	1116 ± 3
25* Cytochrome *c**			≈12,200	898 ± 8
26* Trypsin inhibitor (soybean)**			≈14,300	857 ± 3
27* Growth hormone (human)*			≈22,000	896 ± 2
28* Trypsin (bovine)**			≈23,000	897 ± 1
29* Chymotrypsinogen (bovine)**			≈25,500	869 ± 9
30* Carbonic anhydrase**			≈29,500	775 ± 2
31* Albumin (egg)**			≈44,000	753 ± 4
32□ γ Globulins**			≈150,000	732 ± 5

* These peptides were synthesized in our laboratory using solid phase methodology[27].

** LRF antagonist: [Ac-dehydro^{3,4} L-Pro^1, *p*Cl-D-Phe^2, -D-Trp^{3,6}, N$^{\alpha}$MeLeu^7]-LRF.

VIP was a gift from Dr. S. Lavielle; glucagon, Lot 258-V016-235, gift from Eli Lilly; insulin, Lot 615-D63, gift from Eli Lilly; cytochrome *c*, Sigma (St. Louis, MO, U.S.A.) Lot 106C-7300, p*I* 10.6 (ref. 22); trypsin inhibitor, Sigma Lot Σ 51c-8130; growth hormone (human), Lot 9300[8], gift from Calbiochem; trypsine (bovine), Sigma Lot Σ 106c-8105, p*I* 10.8 (ref. 28); chymotrypsinogen (bovine), Sigma Lot A-124c-8200, p*I* 9.5 (ref. 22); carbonic anhydrase, Sigma Lot Σ 43c-8410; albumin (egg), Sigma Lot Σ 123c-8110, p*I* 4.7 (ref. 22); γ globulins, Miles Labs. (Slough, Great Britain) Lot 82-455-1, serum albumin (bovine) used in these studies was also from Miles, Lot 81-100-2 39, p*I* = 4.4–4.8 (ref. 23).

Applications

This system is being tested in our laboratory for the purification of iodinated traces of larger hormones (growth hormone, thyroid stimulating hormone) to be used in radioimmunoassays. We have also used it to determine the molecular weight of

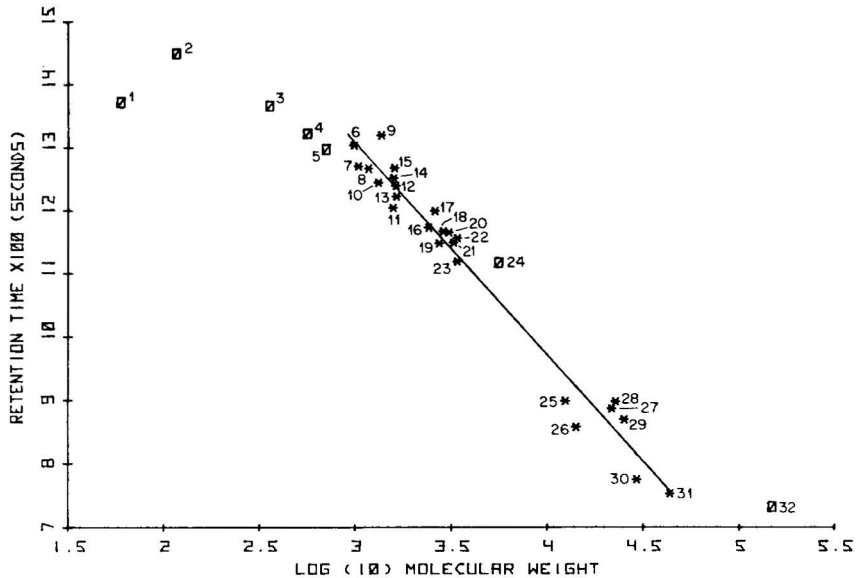

Fig. 4. Correlation of retention time and log mol.wt. in TEAP pH 2.25–water (1:1)–30% acetonitrile at 50°C. See Table II for compound identification.

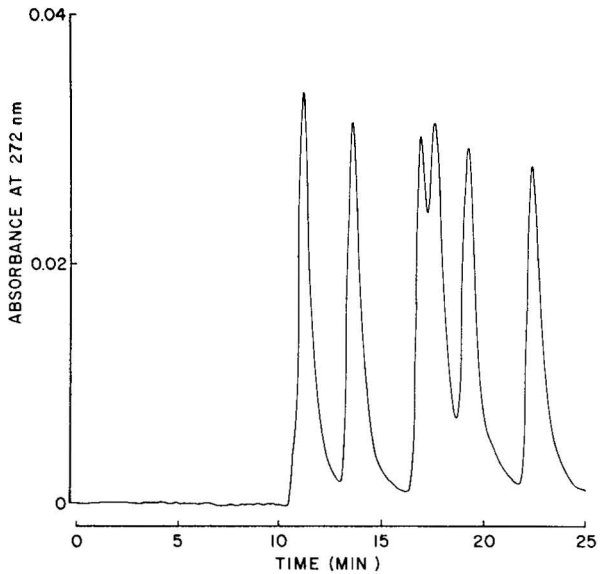

Fig. 5. Separation using TEAF–acetonitrile. Conditions: load, 25 μl protein–peptide mixture; solvent system, TEAF pH 3.0–30% acetonitrile. Retention times (sec) for the different components are BSA, 678; cyt *c*, 822; β-endorphin (human), 1026; CLIP, 1064; LRF, 1162; Indole, 1350.

several unknown biologically active molecules now being purified (ex. mammalian bombesin-like substance).

Advantages over other existing methods to determine molecular weights include high sensitivity, accuracy as well as rapidity of the method. Accurate retention

times and integrated areas measured at 210 nm allow for more precision than could be obtained routinely on soft gel columns. We have indeed been able to show reproducible and statistically significant differences in retention times for four analogous peptides of molecular weights of the order of 1350, two of which are linear, while the other two are cyclized through the backbone (Table III). The linear peptides have a molecular weight of 1367.5 and have charged N-termini at pH 2.25 whereas the cyclic peptides have a molecular weight of 1349.5 (18 less than the linear peptides) and have no end group charge. The cyclic peptide that had a smaller Stoke's radius and were uncharged, eluted later than the linear ones (see Table III).

TABLE III

Conditions: each peptide (10 μg) was eluted successively; buffer, TEAP pH 2.25–30% acetonitrile; absorbance 1.0 a.u.f.s. at 210 nm. Peptides are luteinizing hormone releasing factor (LRF) analogs synthesized in our laboratory. Primary structure of LRF is: *p*Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2.

Peptides	*Retention time (sec)*	*Molecular weight*
[Δ^3-LPro–*p*ClDPhe–DTrp–Ser–Tyr βAla–Pro–Arg–N$^{\alpha}$CH$_3$Leu–DTrp]	1293	1349.5
H_2N-Δ^3-LPro–*p*ClDPhe–DTrp–Ser–Tyr HO-βAla–Pro–Arg–N$^{\alpha}$CH$_3$Leu–DTrp	1236	1367.5
[Δ^3-DPro–*p*ClDPhe–DTrp–Ser–Tyr βAla–Pro–Arg–N$^{\alpha}$MeLeu–DTrp]	1280	1349.5
H_2N-Δ^3-DPro–*p*ClDPhe–DTrp–Ser–Tyr HO-βAla–Pro–Arg-N$^{\alpha}$MeLeu-DTrp	1241	1367.5

CONCLUSION

The volatility of TEAF or the UV transparence and compatibility of TEAP with most biological systems make both solvent systems more versatile than those which contain detergents or high salt concentration. The molecular-weight range in which this system can be used (1000–44,000) is probably dependent on the original pore size of the derivatized silica. Derivatized silica with larger pore size or from other suppliers may allow for molecular-weight determinations greater than 44,000 using these particular buffer systems.

We have not investigated the detrimental effects of both low pH and high temperature on the integrity (chemical and biological) of the peptides and proteins used in these studies. It is certain that some proteins or enzymes will be sensitive to those denaturing conditions whereas others may not. The column support, on the other hand, may be used for long periods ($>$ 300 h of operation). Retention times, however, will vary significantly with age; compare, for example, data in Table I, column 2 with data (identical in terms of conditions presented in Table II which were run three months later after the column had experienced more than 100 operating h). Even though day-to-day repeatability is seemingly good, one should not expect laboratory-to-laboratory reproducibility since performance will be dependent on the condition of the columns.

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REVERSED-PHASE ION-PAIR PARTION CHROMATOGRAPHY OF BIOGENIC CATECHOLAMINES AND THEIR α-METHYL HOMOLOGUES WITH TRIBUTYLPHOSPHATE AS STATIONARY PHASE

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SUMMARY

Ion-pair partition chromatography is applied to the separation of the biogenic catecholamines and their α-methyl homologues. A useful selectivity has been obtained using an adduct-forming organic stationary phase (tributylphosphate). The retention of the compounds can be regulated easily by means of the concentration of the counter-ion (the perchlorate ion) in the mobile phase. The selectivity for separation of amines from amino acids can be influenced by changing the pH of the aqueous phase. The phase system shows a good long-term stability and reproducibility with respect to the capacity ratios and the efficiency.

INTRODUCTION

A chromatographic phase system based on ion-pair partitioning has been developed to separate the biogenic catecholamines and their α-methyl homologues. The latter occur in biological samples after the administration of the hypotensive agent α-methyldopa. The separation and determination of the biogenic catecholamines has been described in literature. However, when the α-methyl homologues are also present, phase systems based on ion-exchange fail through lack of selectivity.[1] Phase systems based on a stationary phase of reversed-phase material and a mobile phase to which a lipophilic counter-ion, such as dodecylsulphate, is added[2] have some drawbacks. It appeared impossible to achieve a good separation of the closely related compounds adrenaline and α-methylnoradrenaline, the most important metabolite of α-methyldopa.

Moreover, such phase systems give rise to problems with respect to their stability. A gradual decrease of the capacity ratios is observed. To improve the stability of such phase systems an eluent without, or with only a very small concentra-

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tion of, the counter-ion can be used after loading of the column[3]. Separation of the catecholamines can also be achieved on octadecyl-silica and acid buffer solutions as mobile phases, but the lifetime of the columns is short under such conditions[4]. Straight phase systems based on ion-pair partition chromatography show good possibilities for the separation of catecholamines[5]. However, the organic solvent used as mobile phase is less well suited for an electrochemical detection system, which is needed to detect the small amounts of amines present in biological samples. Reversed-phase ion-pair partition systems based on the use of pentanol as the stationary phase can also separate catecholamines, but they show no selectivity towards the separation of primary amines and their N-methyl derivatives, *e.g.* noradrenaline and adrenaline[6].

This paper describes a reversed-phase ion-pair partition system based on tributylphosphate as stationary phase on LiChrosorb RP-8 as support material and aqueous phosphate buffers as mobile phases with perchlorate as counter-ion. Phase systems based on tributylphosphate as stationary phase in partition chromatography were introduced previously and applied to the separation of hydrophilic carboxylic acids[7].

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from custom-made and commercially available parts and consisted of a constant-flow pump (Constametric I, LDC, Riviera Beach, FL, U.S.A.), a thermostatted eluent reservoir, a Bourdon type manometer, an injection system (U6K, Waters Assoc., Milford, MA, U.S.A.), a stainless steel column (100 × 2.8 mm I.D.) and an amperometric detection system. The amperometric detection system consisted of a so-called wall-jet detector cell unit (E.D.T., London, Great Britain) in combination with a polarograph used as potentiostat (E-310, Bruker, Karlsruhe, G.F.R.). The detector cell was modified by replacement of the Ag/AgCl reference electrode by a home-made saturated calomel electrode. The complete chromatographic system and the detector cell were placed in an air-heated cabinet.

Chemicals and reagents

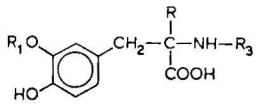
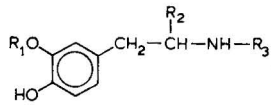
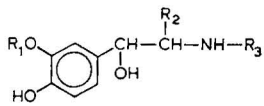
The amines used as chromatographic reference substances are listed in Table I. All other chemicals and solvents were of analytical or reagent grade and were used without further purification. Tributylphosphate was obtained from Aldrich (Milwaukee, WI, U.S.A.). The chromatographic support material LiChrosorb RP-8 (particle diameter, 10 μm) was obtained from Merck (Darmstadt, G.F.R.). Water was purified by a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). The mobile phase was prepared by mixing perchloric acid solution (11.8 *M*) and phosphoric acid solution (1.00 *M*). After dilution with water the solution was brought to the desired pH with a concentrated solution of sodium hydroxide and diluted to the desired concentration of the counter-ion and phosphate buffer. Before use the mobile phase was saturated with tributylphosphate.

Determination of the chromatographic parameters

The capacity ratio k'_i of a compound i was determined from its retention time

TABLE I

STRUCTURES OF THE CHROMATOGRAPHIC REFERENCE SUBSTANCES

Structure	R_1	R_2	R_3	*Name*	*Origin*
	H	H	H	DOPA	Fluka, Buchs, Switzerland
	H	CH_3	H	α-Methyldopa	M.S.D., West Point, PA, U.S.A.
	H	H	H	Dopamine	Fluka
	H	CH_3	H	α-Methyldopamine	Synthesized[8]*
	CH_3	H	H	3-Methoxytyramine	Aldrich, Beerse, Belgium
	H	H	CH_3	Epinine	Synthesized[9]*
	H	H	H	Noradrenaline	Fluka
	H	CH_3	H	α-Methylnoradrenaline	Sterling Winthrop, New York, NY, U.S.A.
	CH_3	H	H	Normetanephrine	Sigma, St. Louis, MO, U.S.A.
	H	H	CH_3	Adrenaline	O.P.G., Utrecht, The Netherlands
	CH_3	H	CH_3	Metanephrine	Sigma

* The syntheses were performed by the Department of Pharmacochemistry, Subfaculty of Pharmacy, Gorlaeus Laboratories.

t_{Ri} and the retention time of an unretarded compound, t_{R0}, for which potassium iodide was used. The theoretical plate height for a compound was calculated from its retention time and the peak width at 0.61 of the peak height. The porosity, ε_m, was calculated from the retention volume of the unretarded compound (V_m) and the volume of the empty column (V_0) by $\varepsilon_m = V_m/V_0$[10].

Chromatography

The columns were packed using a slurry technique with tetrachloromethane as dispersing solvent and *n*-hexane as displacing solvent. After washing the column with 25 ml of ethanol and 10 ml of water the mobile phase was pumped through the column and the column was loaded *in situ* by injection of the stationary phase[7]. The column was considered to be maximally loaded at a porosity of $\varepsilon_m = 0.46 \pm 0.01$[10]. The volume of the stationary phase (V_s) was calculated from the difference in porosity before and after loading of the column, and was usually 0.115 ± 0.005 ml. In order to obtain a stable system the mobile phase was saturated with the stationary phase, but no precolumn was used in the experiments. To prevent demixing of the mobile phase in the column caused by a slight rise of temperature the eluent reservoir was thermostatted at 298°K and the cabinet with the chromatograph and detector at 297°K. It should be noted that the solubility of tributylphosphate in water decreases with increasing temperature.

THEORETICAL

Many ion-pair extraction procedures involving adduct formation with tributylphosphate (S) are described in literature. This technique has been applied to ion-pair extractions of metal ions with perchlorate counter-ions (X^-) or of inorganic anions with hydronium ions (H_3O^+) as counter-ions, and the extraction of perchloric acid is

a well known process[11]. These ion-pairs dissociate in the organic phase[12,13]. The ion-pair distribution of a protonated amine (BH^+) with perchlorate counter-ions can be described in a similar way, taking into account ion-pair extraction and possible ion-pair dissociation in the organic phase:

$$BH^+_{aq} + X^-_{aq} + nS_{org} \overset{K_{ex}}{\rightleftarrows} BHXS_{n,org} \quad (1)$$

$$BHXS_{n,org} \overset{K_{diss}}{\rightleftarrows} BHS^+_{n,org} + X^-_{org} \quad (2)$$

where K_{ex} and K_{diss} are the equilibrium constants.

Distribution of the amine as such hardly occurs. Catecholamines such as dopamine are in ionic form in an aqueous solution at any pH. The pK_a values of dopamine are[14]: for the first phenol function 8.85; for the amine function 10.3.

The distribution of an amino acid (HBH^+), such as DOPA, will be influenced by the dissociation of the carboxylic group (pK_a 2.2), when the pH of the aqueous phase is raised:

$$HBH^+_{aq} \overset{K_a}{\rightleftarrows} {}^-HB^+_{aq} + H^+_{aq} \quad (3)$$

From the different equilibria an expression describing the distribution ratio of an amine ion-pair can be derived:

$$D = \frac{[BHSX_n]_{org} + [BHS^+_n]_{org}}{[BH^+]_{aq}} = K_{ex}[X^-]_{aq}\,[S]^n_{org}\,(1 + K_{diss}/[X^-]_{org}) \quad (4)$$

The following expression is obtained for the distribution ratio of an amino acid as ion-pair:

$$D = (K_{ex}[X^-]_{aq}\,[S]^n_{org}\,(1 + K_{diss}/[X^-]_{org}))/(1 + K_a/a_{H^+,aq}) \quad (5)$$

where K is the acid dissociation constant and $a_{H^+,aq}$ is the hydrogen activity.

Dissociation of the ion-pair in the organic phase is a common side-reaction in ion-pair partition systems[15]. According to eqn. 4, this phenomenon leads to non-linear distribution isotherms (since $[X^-]_{org}$ originates from the sample ion-pair) resulting in tailing peaks in reversed-phase chromatography unless other dissociating ion-pairs, with a common counter-ion, are extracted in high concentrations[6]. In the present case any possible dissociation is held constant due to the presence in the organic phase of perchlorate ions from extracted perchloric acid, which is known to be highly dissociated in tributylphosphate[13]. Under such conditions $[X^-]_{org}$ is governed by the perchloric acid distribution and is not influenced by the low sample concentrations (1–500 ng were injected), and a linear distribution isotherm is obtained.

The relationship between the distribution ratio (D) and the chromatographic retention is given by the expression for the capacity ratio:

$$k' = D\,(V_s/V_m) \quad (6)$$

in which V_s/V_m represents the phase ratio of the stationary and the mobile phases.

RESULTS AND DISCUSSION

In order to obtain information about the distribution phenomena and to determine optimum conditions for the required separation, the influence on the retention behaviour of the counter-ion concentration, the pH and the temperature have been investigated.

The concentration of the counter-ion in the mobile phase is an important parameter in reversed-phase ion-pair chromatography. According to eqns. 4, 5 and 6, an increase of the counter-ion concentration should lead to a linear increase of the capacity ratios, if the quotient $K_{diss}/[X^-]_{org}$ is much less than unity or is kept constant. Fig. 1 demonstrates the high increase in capacity ratios which was obtained on increasing the concentration of perchlorate ions in the mobile phase at constant pH. A deviation from linearity at low concentrations of counter-ion is observed, as well as an intercept. The intercept can be due to distribution to the stationary phase or the support material of the phosphate ion-pairs of the samples. The deviation from linearity can depend on the increase of the ionic strength, which occurs by the addition of perchlorate ions and also on a change in the degree dissociation of the sample ion-pairs, *i.e.* the factor $(1 + K_{diss}/[X^-)_{org})$, which can be caused by extracted perchloric acid. The dissociation constant, K_{diss}, for the dopamine–perchlorate ion-pair in aqueous saturated tributylphosphate has been determined by conductivity measurements and appeared to be $5 \cdot 10^{-4}$ (ref. 17).

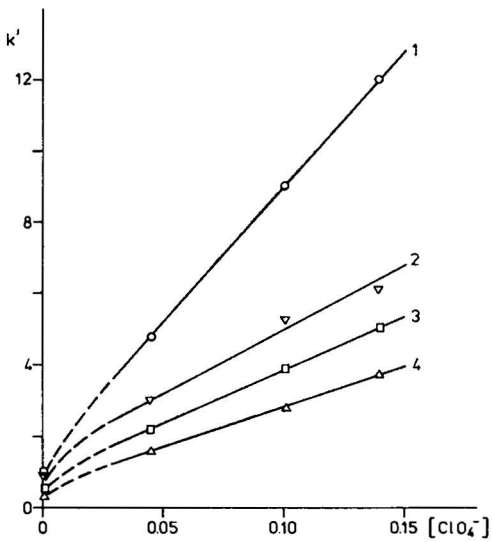


Fig. 1. Capacity ratio and counter-ion concentration. Mobile phase: phosphate buffer 0.05 *M*, pH = 2.10 saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds: 1 = dopamine; 2 = DOPA; 3 = noradrenaline; 4 = adrenaline.

A change in the degree of dissociation of the dopamine–perchlorate ion-pair in the presence of perchloric acid requires a perchlorate ion concentration in the organic phase of *ca.* $5 \cdot 10^{-3}$ *M*, which seems possible owing to the high distribution ratio and dissociation of perchloric acid in tributylphosphate[11].

In order to investigate the possibility of influencing the selectivity of the phase system, the pH of the mobile phase was varied from 1.75 to 4.85 at constant counter-ion concentration, and the capacity ratios of amines and amino acids were measured. The results are represented in Fig. 2, a plot of log k' *vs.* pH. As a consequence of the dissociation of the carboxylic group of the amino acids, the capacity ratios of these compounds decrease when the pH of the mobile phase is raised above the pK_a value (2.2). The slope of the curve of log k' *vs.* the pH of the mobile phase comes close to a value of -1. If $a_{H^+,ap} \ll K_a'$ and $[X^-]_{org} \gg K_{diss}$, eqn. 5 reduces to:

$$D = K_{ex}[X^-]_{aq}[S]_{org}^{n}\frac{a_{H^+,aq}}{K_a} \tag{7}$$

or log D (and hence log k') is proportional to $-$pH.

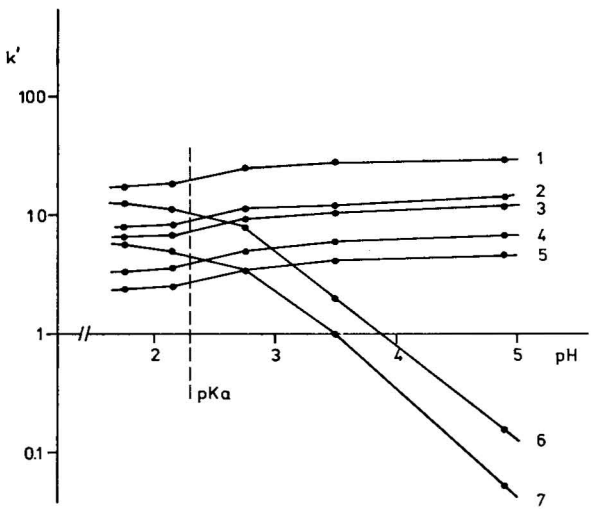


Fig. 2. Capacity ratio and pH. Mobile phase: phosphate buffer 0.05 *M*, with perchlorate as counter-ion 0.10 *M*, saturated with tributylphosphate. Stationary phase: tributylphosphate on Li-Chrosorb RP-8 (10 μm). Compounds: 1 = α-methyldopamine; 2 = dopamine; 3 = α-methylnoradrenaline; 4 = noradrenaline; 5 = adrenaline; 6 = α-methyldopa; 7 = DOPA. pK_a is the dissociation constant of the carboxylic group of the amino acids.

The increase of the capacity ratios of the amines, when the pH of the mobile phase is raised, is probably caused by an interaction with the support material. Even when no perchlorate is present in the mobile phase, a relatively large capacity ratio for the amines is observed when the pH of the mobile phase is raised. This increase is different for different comparable support materials.

As mentioned above, the temperature was kept constant at 298°K for the eluent reservoir and at 297°K for the cabinet with the chromatograph. In almost all experiments the chromatograph operated at a flow-rate of 0.50 ml/min, corresponding to a linear velocity of *ca.* 3 mm/sec, which caused a pressure drop of *ca.* 7.5 MPa. As a consequence of frictional forces, the temperature of the mobile phase will rise

one or two degrees[18]. At high linear velocities the temperature will rise above 298°K, and if no precautions are taken the mobile phase will demix. The influence of the linear velocity on the theoretical plate height has been measured, and the results for a few compounds are represented in Fig. 3.

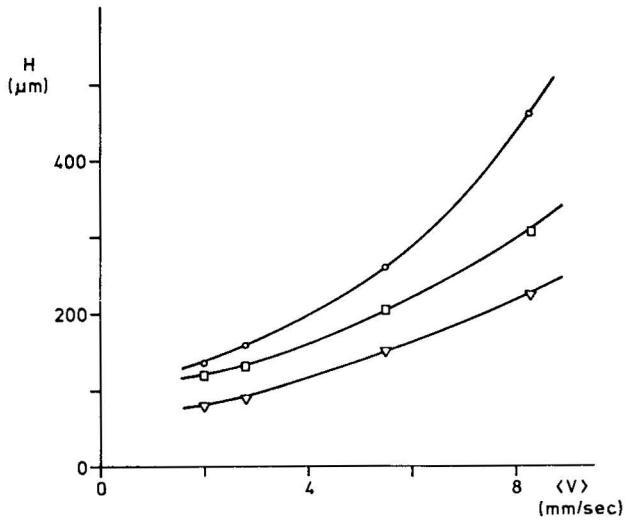


Fig. 3. Theoretical plate height and linear velocity of the mobile phase. Mobile phase: phosphate buffer 0.05 *M*, pH = 2.10 with perchlorate counter-ion 0.10 *M* saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds: ○, adrenaline; □, DOPA; △, dopamine.

Since the distribution properties in liquid–liquid phase systems are known to be dependent on temperature, the influence of an increase of 10°K on the capacity ratios has been measured. The influence is about the same for all compounds, *i.e.* there is a decrease of the capacity ratios ($k'_{298}/k'_{308} = 1.6$), so the selectivity is not influenced by temperature.

The phase system shows a remarkable selectivity with respect to the presence of an extra methyl group in the molecule. Introduction of a methyl group at the α-position of the amines results in an increase of the capacity ratio, which is expected. Introduction of a methyl group to form 3-O-methyl derivatives (the metanephrines, biotransformation products of the biogenic catecholamines) has only a small influence on the capacity ratios. Methylation of the nitrogen atom even results in a decrease of the capacity ratios (Table II).

Reversed-phase ion-pair chromatography allows the application of large volumes of samples by using a high counter-ion concentration in the sample solution. Because of the large capacity ratio during the injection the compounds are concentrated on the top of the column[16]. As a consequence of the large injection volume, the retention times of the compounds will increase. This retention time, $t_{R,\text{observed}}$, can be calculated from the injection time, the capacity ratio at high counter-ion concentration (k'_1), the capacity ratio at low counter-ion concentration (k'_2) and the distances

TABLE II

CAPACITY RATIOS OF THE BIOGENIC CATECHOLAMINES AND THE CORRESPONDING METHYL DERIVATIVES

Chromatographic conditions: stationary phase, tributylphosphate on LiChrosorb RP-8 (10 μm); mobile phase, phosphate buffer 0.05 *M*, pH 2.10 with perchlorate counter-ion 0.10 *M* and saturated with tributylphosphate.

	k' amines	*k' α-methyl derivatives*	*k' 3-O-methyl derivatives*	*k' N-methyl derivatives*
DOPA	4.9	11.0	–	–
Dopamine	8.3	17.5	7.6	5.0
Noradrenaline	3.5	7.0	3.4	2.5

migrated at these counter-ion concentrations. For the retention time of the median of the compound, the maximum of a symmetrical peak at the end of the column, eqn. 8 is valid, assuming symmetrical compound zones, a step change of the capacity ratios and a plug injection:

$$t_{R,\text{observed}} = \frac{t_{\text{inj}}}{2} + t_1 + \frac{L - z}{\langle v \rangle}(1 + k_2') \tag{8}$$

The three terms represent, in order, the time elapsed from the start of the injection until the median of the sample enters the column, the time during which the median of the compound migrates with a high counter-ion concentration from the top of the column to the point where the eluent front reaches the median, and the time during which the median of the compound migrates with the low counter-ion concentration. The symbols are as follows: z is the distance migrated by the median of the compound at high counter-ion concentration; $\langle v \rangle$ is the linear velocity of the eluent; L is the length of the column; and t_{inj} is the injection volume/flow.

For the median of the compound, t_2 is given by eqn. 9:

$$t_1 = z(1 + k_1')/\langle v \rangle \tag{9}$$

For the eluent front the same time interval is given by eqn. 10:

$$t_1 = \frac{t_{\text{inj}}}{2} + \frac{z}{\langle v \rangle} \tag{10}$$

From eqns. 9 and 10 an expression can be calculated for z:

$$z = \frac{t_{\text{inj}}}{2} \cdot \frac{\langle v \rangle}{k_1'} \tag{11}$$

Combination of eqns. 8, 9 and 11 gives a relationship for the observed retention time:

$$t_{R,\text{observed}} = 8_{\text{inj}}\left(1 - \frac{k_2'}{2k_1'}\right) + t_R \tag{12}$$

where

$$t_R = L(1 + k_2')/\langle v \rangle \tag{13}$$

The influence of the injection volume at different counter-ion concentrations on peak broadening and retention times has been investigated. The influence of the injection volume on peak broadening is represented in a plot of σ_V *vs.* injection volume (Fig. 4); σ_V was determined from half the peak width at 0.61 of the peak height and the flow-rate. The calculated (eqn. 12) and measured retention times obtained under the conditions given in Fig. 4 agree within 2%, where the k' values at high counter-ion concentration were obtained by extrapolation of the results represented in Fig. 1. The results show that peak broadening resulting from use of large volumes of samples can be reduced when using high counter-ion concentrations in the sample solution without disturbing the chromatographic system.

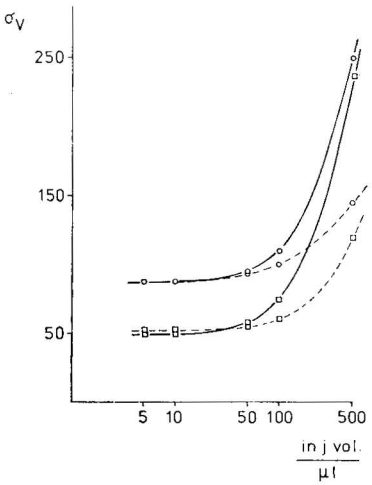


Fig. 4. Peak broadening and injection volume at different counter-ion concentrations of the sample for dopamine (○) and noradrenaline (□). Mobile phase: phosphate buffer 0.05 *M*, pH = 2.10 with perchlorate counter-ion 0.10 *M* saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 µm). Samples dissolved in phosphate buffers 0.05 *M*, pH = 2.10 and $[ClO_4^-] = 0.10$ *M* (○—○) or $[ClO_4^-] = 0.50$ mol/l (○— —○).

CONCLUSIONS

The phase system described is flexible, which means that conditions can be varied easily to obtain the required retention and selectivity. The desired separation has been achieved by proper choice of the counter-ion concentration and pH, in conformity with the proposed retention model for ion-pair distribution. The separation of the compounds of interest is shown in Fig. 5. The least retained compound (adrenaline) has a capacity ratio of 2.4, and the most retained compound (α-methyldopamine) a capacity ratio of 17.5. The system is reproducible and no problems with respect to its stability have been observed. The phase system is in use now for the determination of α-methyldopa and its metabolites after their isolation, and more than 400 biogenic samples have been run without affecting the chromatographic properties of the system. The lifetime of the support material under the conditions described is at least 6 months.

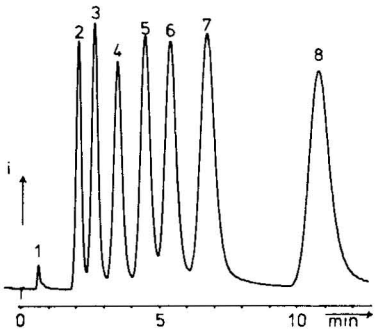


Fig. 5. Separation of a test mixture of the compounds of interest. Mobile phase: phosphate buffer 0.05 *M*, pH = 2.10 with perchlorate counter-ion 0.10 *M* saturated with tributylphosphate. Stationary phase: tributylphosphate on LiChrosorb RP-8 (10 μm). Compounds (100–200 ng injected): 1 = front peak; 2 = adrenaline; 3 = noradrenaline; 4 = DOPA; 5 = α-methylnoradrenaline; 6 = dopamine; 7 = α-methyldopa; 8 = α-methyldopamine. Flow-rate, 0.50 ml/min; pressure, 7.5 MPa; detection, amperometric at +0.75 V *vs.* saturated calomel electrode; sensitivity, 500 nA full-scale deflection.

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CHROM. 13,283

PULSED INJECTIONS OF ION INTERACTION REAGENT APPLIED TO THE LIQUID CHROMATOGRAPHIC SEPARATION OF 2,6-DISUBSTITUTED ANILINES

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(First received July 22nd, 1980; revised manuscript received August 20th, 1980)

SUMMARY

The effect of the number of pulsed 20-μl injections of 10 mM octanesulfonate on the retention times of aniline and five 2,6-disubstituted anilines was determined by fitting a model derived from the Freundlich adsorption isotherm to data collected at six different numbers of pulses. An optimization strategy based on the window diagram technique of Laub and Purnell predicted that approximately 40, 20-μl pulses of 10 mM octanesulfonate would give optimum separation using a methanol–water (45:55) mobile phase. These conditions gave baseline separation of the six components.

INTRODUCTION

Reversed-phase high-performance liquid chromatography has been highly successful in the separation and quantitation of mixtures of chemically similar compounds. Good separation can usually be achieved by judicious selection of the mobile phase composition with respect to pH, percent methanol, concentration of ion interaction reagent (IIR), etc. It is then often desireable to optimize the separation by fine adjustment of the eluent composition to aid in the quantitation of closely eluting compounds.

Laub and Purnell[1] have shown that plotting separation factor (α) curves, for all possible pairs of compounds in a mixture, *vs.* a variable chromatographic factor produces a "window diagram" that can be used to optimize a separation with respect to the variable factor.

Optimum separation is obtained when the variable factor is adjusted to the value corresponding to the top of the tallest "window" in the α plot.

In this paper we describe a method for increasing the separation power of an isocratic system by using pulsed injections of an IIR (octanesulfonate) as a variable chromatographic factor. We also give a mathematical model describing the retention behavior of the compounds studied as a function of the number of pulses of IIR and show the use of window diagrams to optimize the separation obtained with the pulse technique.

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THEORETICAL

The effect of an IIR on the retention behavior of both positively and negatively charged compounds has been investigated previously by Bidlingmeyer *et al.*[2]. They proposed an ion interaction model in which the increased or decreased retention of a solute ion is caused by the amount of charge in the primary ion layer arising from the adsorption of IIR on the stationary phase. The amount of adsorbed IIR is a function of the concentration of IIR in the mobile phase and can be described by the Freundlich adsorption isotherm[3]. Thus the capacity factor of the solute as a function of [IIR] can be described by

$$k' = \beta_0 + \beta_1 [\mathrm{IIR}]^{1/\beta_2} \tag{1}$$

where k' is the capacity factor of the charged solute, β_0 is the capacity factor in the absence of IIR, β_1 is a parameter describing the "effectiveness" of the IIR, and β_2 is a parameter of the Freundlich isotherm. We have found that when [IIR] in eqn. 1 is replaced by the number of pulses of IIR, an excellent fit is also obtained.

$$k' = \beta_0 + \beta_1 n^{1/\beta_2} \tag{2}$$

where n is the number of injections of IIR.

EXPERIMENTAL

Chromatographic system

The chromatographic system consisted of a Model 6000A solvent delivery system, a 5 cm × 4 mm I.D. Bondapak precolumn and a 30 cm × 4 mm I.D. μBondapak C_{18} main column, all from Waters Assoc. (Milford, MA, U.S.A.). An electrochemical cell equipped with glassy carbon electrodes (Bioanalytical Systems) was used in the amperometric mode at +0.9 V *vs.* an Ag/AgCl reference electrode. A computer controlled Model 70-10 automatic sample injection valve equipped with a Model 70-01 pneumatic activator (Rheodyne) was used to inject 20-μl volumes of IIR. A Model U6K variable volume injector (Waters Assoc.) was used for the introduction of samples downstream from the Rheodyne injector. Precolumn and main column temperatures were maintained at 25.0 ± 0.1°C by a Model FK constant-temperature circulating bath (Haake). The flow-rate was set at 2.0 ml/min and the time equivalent of the void volume (t_0) was 1.697 min.

Additional instrumentation

The analog electrochemical detector output was recorded by a Model 281 stripchart recorder (Soltec). Simultaneouly the signal from the detector was digitized by a Model ADC-12QZ analog-to-digital converter (Analog Devices) interfaced to a Model 9830A computer (Hewlett-Packard). The 9830A also controlled the Rheodyne injector.

Mobile phase and samples

The mobile phase consisted of methanol–water (45:55) containing 1 m*M* HCl.

A sample mixture[4] approximately 0.2 m*M* in aniline, 2-methyl-6-isopropylaniline (MIPA), 2-ethyl-6-isopropylaniline (EIPA), 2,6-diisopropylaniline (DIPA), 2-methyl-6-*tert.*-butylaniline (MTBA) and 2-ethyl-6-*sec.*-butylaniline (ESBA) (Ethyl Corp., Baton Rouge, LA, U.S.A.) was prepared in the mobile phase.

Pulsing of IIR

10 m*M* octanesulfonate (Eastman-Kodak, Rochester, NY, U.S.A.) was made up in the mobile phase. The IIR was pumped continuously through the Rheodyne valve by a peristaltic pump at approximately 3 ml/min. A specified number of 20-μl injections of IIR were made at 5-sec intervals under computer control. During an injection of IIR the Rheodyne valve was left in the inject mode for one second and then returned to fill mode. The samples were injected manually with the Waters Assoc. injector. After each chromatogram, 100% methanol was run through the column for 2 min followed by eluent for approximately 3 min before the next injection.

Experimental design

A six-level experimental design corresponding to 0, 5, 10, 20, 40 and 60 pulses, with replicates at 0 and 20, was used. The experimental order was randomized to minimize the confounding of time trends with factor effects.

RESULTS AND DISCUSSION

Fig. 1 shows a set of chromatograms obtained at 0, 5, 10, 20, 40 and 60 pulses of IIR. In the chromatogram at 40 pulses, the elution order is aniline, MIPA, EIPA,

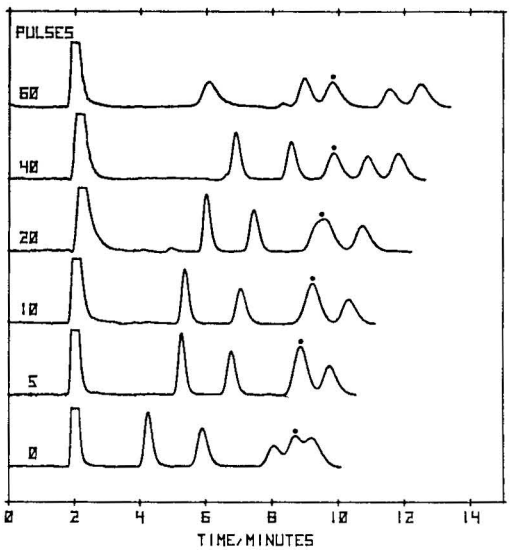

Fig. 1. Chromatograms of 10-μl injections of a mixture 0.2 m*M* in each of aniline, MIPA, EIPA, MTBA, DIPA and ESBA obtained at 0, 5, 10, 20, 40 and 60 pulses of octanesulfonate, 5 sec between pulses. Dot indicates elution of MTBA.

MTBA, DIPA and ESBA. It can also be seen in Fig. 1 that increasing the number of pulses of IIR gives a regular increase in retention of all components except MIPA at 60 pulses.

Table I contains the best non-linear least squares estimates of the parameters of eqn. 2 for each of the six samples. The small standard deviations of residuals show a good fit of the model to the data.

Fig. 2 shows a window diagram which plots the predicted separation factors (α) of all possible pairs of compounds as functions of the number of pulses. Separation

TABLE I

CAPACITY FACTORS AND PARAMETERS ESTIMATES

Pulses	*Capacity factors*					
	Aniline	*MIPA*	*EIPA*	*MTBA*	*DIPA*	*ESBA*
0	0.178	1.50	2.49	4.17	3.77	4.43
5	0.197	2.15	3.05	4.30	4.30	4.83
10	0.245	2.21	3.23	4.53	4.53	5.18
20	0.289	2.62	3.54	4.71	4.82	5.47
40	0.294	3.15	4.15	4.94	5.54	6.10
60	0.215	2.60*	4.39	4.53	5.94	6.51
	Parameter estimates					
β_0	0.178	1.51	2.49	4.15	3.78	4.43
β_1	0.047	0.237	0.226	0.128	0.171	0.165
β_2	6.18	1.92	1.90	2.182	1.61	1.61
S_r**	0.049	0.074	0.078	0.093	0.082	0.064

* Not included in least squares fit.
** Standard deviation of residuals.

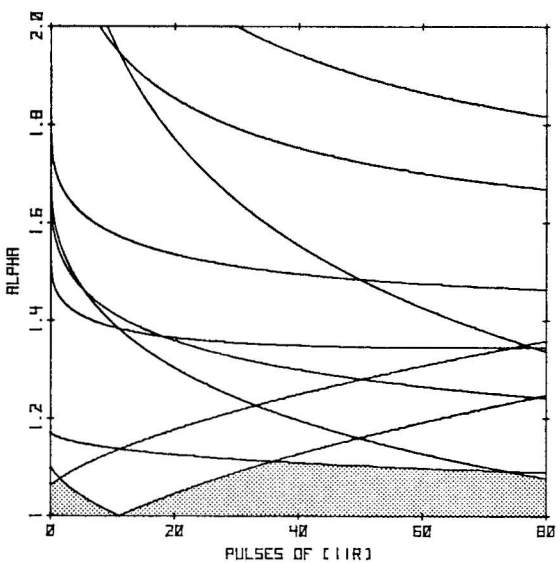

Fig. 2. α plot for the six anilines showing optimal separation conditions at 36 pulses of octanesulfonate.

factors greater than 2 are not shown. The minimum at approximately 10 pulses shows conditions where compounds coelute. The window diagram predicts optimal separation at 36 pulses; thus the excellent separation in the chromatogram at 40 pulses in Fig. 1 is very close to the optimal separation attainable with this system.

In the chromatogram at 60 pulses (see Fig. 1), MIPA elutes earlier than predicted by the trend in the other experiments. We believe this anomalous result is caused by coelution of the sample with the last of the IIR pulses. We have observed this sudden shift in retention behavior in other pulse studies; the mechanism for this behavior is under current investigation.

CONCLUSION

This study has shown that pulsed injection of IIR is an effective technique for improving separation in isocratic liquid chromatography. One advantage of the pulsed technique over conventional "ion-pair" chromatography (in which the IIR is constantly present in the eluent) is that a lengthy column equilibration period at the beginning of a set of chromatographic runs is not necessary to obtain reproducible retention times. Another advantage is forseen in the separation of a complex mixture containing positively charged, negatively charged and uncharged components: the timing and duration of the pulses can be adjusted so that the retention time of components with the same charge as the IIR would be adequately decreased, and the retention time of oppositely charged components would be adequately increased to give the desired separation[2].

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CHROM. 13,196

SIMULTANEOUS MONITORING OF LIGHT-ABSORPTION AND OPTICAL ACTIVITY IN THE LIQUID CHROMATOGRAPHY OF CHIRAL SUBSTANCES

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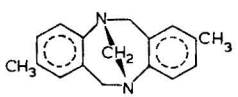
SUMMARY

A detection system for the simultaneous monitoring of the light-absorption (absorbance, A) and the optical activity as circular dichroism (differential absorbance for left- and right-circularly polarized light, $\Delta A = A_L - A_R$) of the eluent from a liquid-chromatography column is described. The optical resolution of pavine by liquid chromatography on a triacetyl cellulose column is reported.

INTRODUCTION

The optical resolution of a synthetic racemate by the traditional method of the fractional crystallisation of the two diastereomeric derivatives afforded by an enantiomerically-pure reagent is laborious and the outcome is not always certain or reproducible. The method has been described as a matter of trial and error[1] and as an art[2]. These descriptions are apt, in our experience, for the literature reports[3,4] of the optical resolution of synthetic pavine (I) through the (+)-bromocamphorsulphonic acid salts.

(I) R = H
(III) R = CH_3



(II)

In view of the versatility of column liquid chromatography for the optical resolution of both inorganic[5] and organic[6] racemates, a chromatographic alternative to the literature methods[3,4] was sought for the separation of the pavine enantiomers.

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Pavine and Tröger's base (II) have similar molecular morphologies, and the complete optical resolution of II on a column of microcrystalline triacetylcellulose (MCTC) has been reported[7], and extended to a range of organic racemates[6,8]. In reproducing the optical resolution[7] of II, and extending the method to pavine, the problem arose of monitoring the solute in the eluent from the MCTC column by light-absorption at one wavelength and the optical activity of any enantiomeric solutes with a separate instrument at a different wavelength, typically in a transparent frequency region. Inevitably, the two detection systems did not record, at a given time, the light-absorption and the optical activity of the same solute fraction.

Accordingly, an attachment has been constructed, to replace the sample and detector compartments of a standard spectrophotometer, to record simultaneously at a given monitoring wavelength the light-absorption as optical-density or absorbance (A) and the circular dichroism, *i.e.* the differential absorbance of left- (LCP) and right-circularly polarised (RCP) light, ($\Delta A = A_L - A_R$), which is the absorption counterpart of optical rotation in transparent wavelength regions. The design of the attachment is illustrated in Fig. 1, and chromatograms of I and II optically resolved on a MCTC column are recorded in Fig. 2.

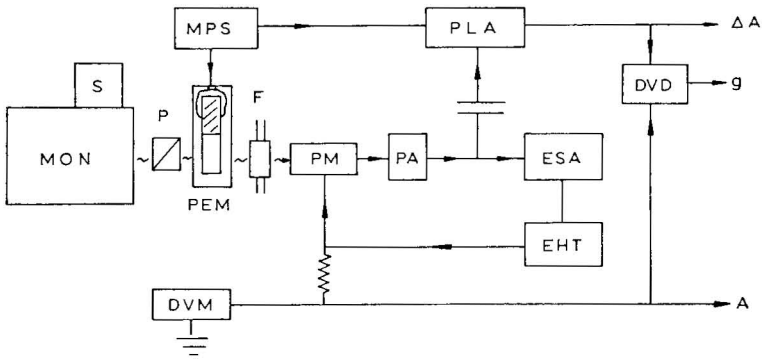

Fig. 1. The spectrophotometer modification for the simultaneous monitoring of absorbance (A) and circular dichroism ($\triangle A$) of chiral solutes in the eluent from a liquid chromatography column. The source (S) and monochromator (MON) are those of the original instrument. Radiation from the monochromator exit slit passes through a polarizing prism (P) and a photoelastic modulator (PEM) (Hinds Internat. Portland, OR, U.S.A.) to the flow-cell (F) and photomultiplier detector (PM). After the preamplifier (PA) the PM signal is fed to the error-signal servo-amplifier (ESA) which governs the extra high-tension voltage supply (EHT) to the PM registered by the digital voltmeter (DVM) in order to maintain the $V_{d.c.}$ constant at a pre-set level. A signal proportional to the change in the EHT supply is fed to one channel of a multipen recorder to measure the absorbance, A. The $V_{a.c.}$ signal from the PM after the PA is fed to the phase-lock amplifier (PLA) which receives a reference signal from the PEM power supply (MPS). The output from the PLA is fed to a second channel of the multipen recorder to measure the circular dichroism, $\triangle A = (A_L - A_R)$, and to one input of a ratiometer, the divider (DVD). The second input to the DVD is the absorbance signal, so that the DVD output measures the g ratio, $\triangle A/A$, which is also recorded as a function of the elution volume.

EXPERIMENTAL

Apparatus and method

The sample and detector compartments of a Unicam SP 600 were modified by the insertion of a Glan polarizing prism of calcite (P) and a photo-elastic

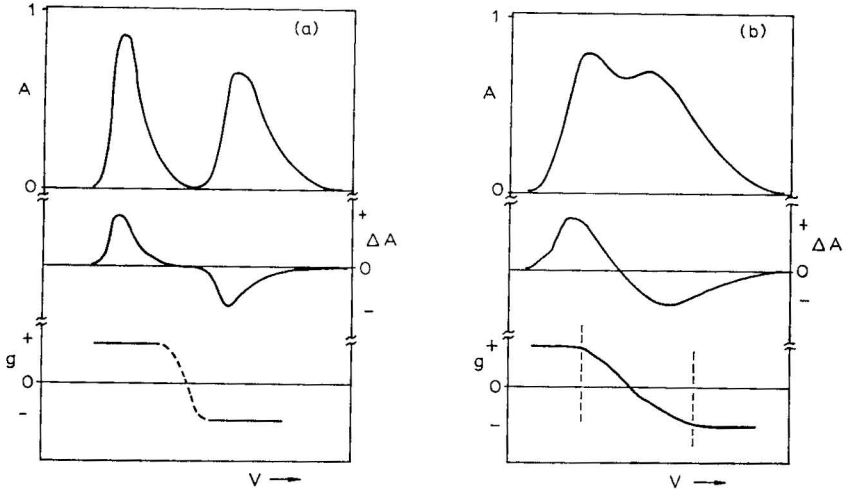

Fig. 2. Chromatograms of the optical resolution on a microcrystalline triacetylcellulose column (400 × 15 mm I.D.) of (a) Tröger's base (II), and (b) pavine (I), with a mobile phase of ethanol–water (9:1). The chromatograms record the absorbance (A), differential absorbance of LCP and RCP radiation, ($\triangle A$), and the g ratio, ($\triangle A/A$), as a function of the elution volume, (V). In (b) the fractions of pavine eluted in the volume between the vertical dashed lines are incompletely resolved, the volume being recycled to achieve a further separation.

modulator (PEM) between the monochromator and the sample chamber, and by replacing the phototube with a photomultiplier (PM) detector (Fig. 1). The photoelastic modulator consists of a transparent isotropic optical element, vitreous silica or calcium fluoride, which is periodically stressed by a single-crystal quartz transducer, to which the optical element is cemented[9,10]. The quartz crystal, some 10 × 20 × 50 mm, with electrodes on the two largest faces, has a piezoelectric resonance at ≈ 50 kHz in a uniaxial mode in the direction of the long axis, and the optical element, with dimensions ensuring acoustic resonance at the same frequency, is attached to one of the small end faces of the crystal[9,10]. The application of a sine-wave alternating voltage to the quartz crystal produces a periodic birefringence in the otherwise-isotropic optical element, and the power-level applied to the quartz transducer is adjusted so that the periodic birefringence maxima conform to the quarter-wavelength retardation condition, $d(n_x - n_y) = \lambda/4$, where d is the plate thickness, at the wavelength of interest, λ. The plane-polarized radiation emerging from the polarizing prism at the exit slit of the monochromator is thence transformed into LCP and RCP radiation alternating sinusoidally at ≈ 50 kHz.

The monitoring wavelength, λ, for the chiral solute passing through the flow-cell (F) in the eluent from the chromatographic column is chosen by reference to the absorption and circular dichroism (CD) spectrum of an enantiomer of the solute or of an analogous compound. In general the wavelength of the lowest frequency CD band maximum is adopted, or that of the major CD band where more than one CD band is associated with the lowest-energy absorption band. The CD spectrum of (−)-Tröger's base (II) has been reported[11], and that[12] of (−)-argemonine (III) which is a close analogue of (−)-pavine (I). The CD spectra of II and III (Fig. 3)

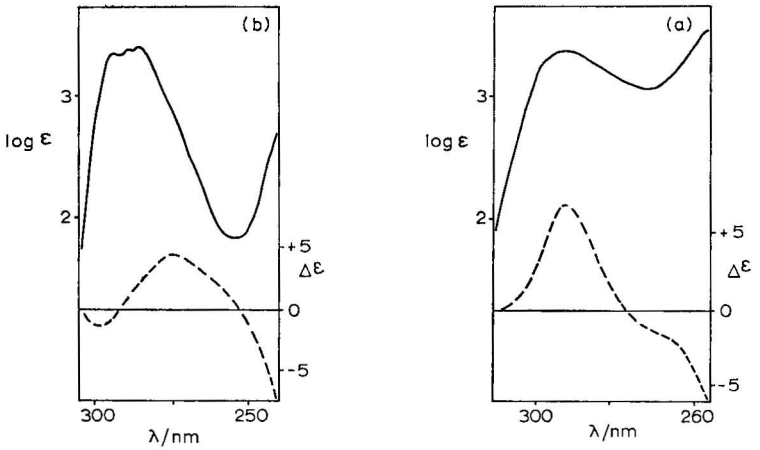

Fig. 3. The absorption spectra (upper curves) and circular dichroism spectra (lower curves) of (a), (−)-Tröger's base (II), and (b) (−)-argemonine (III) in ethanol. The corresponding spectra of (−)-pavine (I) are virtually identical to those of (III), with $g = 1.8 \cdot 10^{-3}$ at 275 nm. In the case of (II), $g = 4.5 \cdot 10^{-3}$ at 289 nm.

suggest, from the observed CD band maxima, the adoption of 280 nm for II and 275 nm for I.

With the monitoring wavelength appropriately set, the modulated LCP and RCP radiation from the PEM passes through the flow-cell without absorption or differential absorption during the initial chromatographic forerun, so that the photomultiplier registers only a direct current (d.c.) signal which provides a base-line for both the subsequent absorption (A) and differential absorption of LCP and RCP radiation ($\Delta A = A_L - A_R$). After the solute break-through from the chromatographic column, the modulated radiation transmitted through the flow-cell undergoes an overall absorption (A) and a differential absorption (ΔA), the latter being registered by the photomultiplier as a small 50-kHz a.c. signal superimposed on the now-reduced d.c. signals. Strictly the differential absorption is measured by the ratio of the two signal voltages[13], ($V_{a.c.}/V_{d.c.}$).

The ratio giving ΔA is conveniently measured, together with the overall absorption A, by maintaining the denominator ($V_{d.c.}$) at a constant level, that of the original zero-absorption d.c. signal during the forerun period, using an error-signal servo-amplifier[14] (ESA) (Fig. 1). During the absorption of radiation, the fall in the d.c. signal from the photo-multiplier is registered by the ESA, which governs the extra high-tension voltage supply (EHT) to the PM, increasing the voltage to restore the $V_{d.c.}$ from the PM to its original forerun base-line level. As the response of the PM to the applied EHT voltage is nearly logarithmic, the increase in that voltage, governed by the ESA, measures in good approximation the optical density or absorbance A of the solute passing through the flow-cell. A signal proportional to the EHT voltage-change is fed to one channel of a two-pen recorder in order to monitor the corresponding changes in absorbance A. Since the denominator of the ($V_{a.c.}/V_{d.c.}$) ratio is constant, the small 50 kHz a.c. signal now measures directly the differential absorbance ΔA. The a.c. component from the preamplifier (PA) following the photo-multiplier is fed to a phase-sensitive detector and amplifier, the phase-lock

amplifier (PLA) which receives a reference signal from the PEM (Fig. 1). The PLA discriminates the 50 kKz from other signals, and distinguishes between positive and negative differential absorbance ΔA, which is registered by the second channel of the two-pen recorder.

A refinement allows the enantiomeric purity of the solute passing through the flow-cell to be recorded. The dissymmetry ratio, $g = \Delta A/A$, introduced by Kuhn[15], is a constant at a given wavelength with an optimum value for an optically-pure enantiomer. The individual absorbance A and differential absorbance ΔA signals fed to a divider (DVD) give an output measuring the g ratio. For a racemate which is completely resolved into its enantiomers chromatographically, as in the case of Tröger's base (II) on a MCTC column[7], the g ratio has its constant optimum value throughout the elution band of each enantiomer, the g ratio at 289 nm being positive for the first-eluted (−)-isomer of II and negative for the second, the (+)-isomer with $|g| = 4.5 \cdot 10^{-3}$ (Fig. 2). For a racemate which is incompletely resolved chromatographically, giving a single absorbance elution band A, but a bisignate double differential-absorbance band ΔA, as in the case of pavine on a MCTC column, the g ratio *versus* elution-volume record has positive and negative stationary sections at the beginning and at the end of the elution band, joined by an intermediate sectionin which the value of $|g|$ progressively diminishes from the first optimum and increases to the second (Fig. 2). The g ratio trace identifies the enantiomerically-pure fractions, and provides a measure of the optical purity of the intermediate fractions. The lower limit of the enantiomeric purity which may be determined by the method is 1% or less, as the instrumental limitation is $|g| \approx 10^{-5}$. and the optimum g ratio at a wavelength within the range of the lowest-energy absorption band is generally $\geqslant 10^{-3}$ for a wide range of chiral molecules[16].

Materials

Pavine was prepared from papaverine (BDH, Poole, Great Britain) by the method of Pyman[17], and the Tröger's base from Aldrich (Milwaukee, WI, U.S.A.) was recrystallised from ethanol. MCTC was prepared by the heterogeneous acetylation procedure of Hesse and Hagel[7,8]. As the optical isomers of Tröger's base racemise under even weakly acidic conditions[18], the washed MCTC product (80 g) was suspended in a mixture of ethanol (250 ml), water (30 ml) and 0.88 sp. gr. ammonia (10 ml), and stirred under reflux for 1 h. The treatment not only removes acidic residues, but also swells the MCTC. The slurry obtained was used to pack a glass chromatography column, 400 × 15 mm I.D. The mobile phase employed was ethanol–water (9:1) under a positive pressure of 1.7 atmosphere of nitrogen, which gave a flow-rate of 25 ml/h. Samples of up to 50 mg of racemic I or II were separated into their respective enantiomers on the column. In the case of I the intermediate fraction, where the g ratio declines from the positive optimum and subsequently increases to the negative optimum, gave further enantiomerically-pure fractions on recycling through the column.

CONCLUSION

The system described has the advantage, over the sequential monitoring of the absorbance and the optical rotation of enantiomeric solutes in the eluent from a

chromatographic column for the optical resolution of a racemate, not only of recording simultaneously the concentration, as the absorbance A, and the optical activity, as ΔA, of a given fraction with a single detection system, but also of measuring the enantiomeric purity of the solute in that fraction. In case where the g ratio, $\Delta A/A$, of the enantiomers at the analytical wavelength employed is not previously determined, the observation of two segments, in the g ratio *versus* elution volume relation, where the g ratio is invariant for a limited range, and equal in magnitude but opposite in sign for the two segments, affords an indication that the optical resolution is complete over those limited ranges, and that the corresponding fractions are enantiomerically pure.

The optical purity of products given by asymmetric syntheses, which generally provide less than 100% of a single enantiomer, is estimated chromatographically with the detection system described more readily than by the corresponding optical-rotation procedure[19]. The chromatographic estimation of the optical purity of an enantiomeric mixture is problematic only when the elution peaks of the two enantiomers overlap or are fused[19]. Provided that the elution fractions of the more abundant enantiomer in the mixture give a segment in the g ratio *versus* elution volume relation which is invariant over a limited range, and thus is optimum, $|g|_{opt}$, the optical purity of the mixture is given by the ratio of the mean g-ratio over the chromatogram of the enantiomer mixture to its optimum value, $|\bar{g}|/|g|_{opt}$.

The principal limitation of the simultaneous absorbance, A, and circular dichroism, ΔA, detection system in its present form is the use of a calcite polarizing prism, which has a shorter-wavelength transmission limit of $\approx$ 240 nm. The limit is reducible to $\approx$ 185 nm by replacing the calcite prism with a Rochon quartz polarizing prism. The replacement involves the introduction of a fused silica lens or, better, a silica–fluorite achroamt, to bring to a focus the images of the exit slit of the monochromator given by the ordinary and the extraordinary polarized rays transmitted by the Rochon prism, so that the unwanted extraordinary beam may be masked off. Even with the replacement envisaged, the monitoring of chiral hydrocarbons in the eluent from a chromatography column would not be practicable by the simultaneous absorption and circular dichroism procedure, which was designed essentially for the detection of chiral benzenoid and larger aromatic derivatives absorbing at wavelengths longer than $\approx$ 250 nm.

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* *Editor's Note:* See also S. A. Westwood, D. E. Games and L. Sheen, *J. Chromatogr.*, 204 (1981) 103, which had not been published when this paper was received.

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CHIRAL RECOGNITION IN GAS CHROMATOGRAPHY BY DIAMIDE–DIAMIDE SOLUTE–SOLVENT INTERACTION

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SUMMARY

The resolution of N-trifluoroacetyl (N-TFA) *tert.*-butylamides of Ala, Val, Leu and Pro by gas chromatography on the N-lauroyl *tert.*-butylamide derivatives of L-alanine (I), L-leucine (II), D-phenylglycine (III) and L-phenylalanine (IV) as stationary phases was investigated, and the results compared with those obtained for the corresponding N-TFA isopropyl esters.

Efficiency of resolution of the two classes of compounds and the differences between them depend greatly on the nature of the phase. N-Lauroyl-L-Phe-*tert.*-butylamide (IV), which is highly efficient for the resolution of the N-TFA esters of α-amino acids, shows even more selectivity for the N-TFA *tert.*-butylamides.

Proline, which is resolved only with great difficulty as its N-TFA ester, behaves distinctly differently from the amino acids having a primary amine group, and on all phases shows high resolution factors, ranging from 1.173 to 1.384.

The chromatography of α-amino acids as N-TFA *tert.*-butylamides would appear to have considerable interest from the analytical point of view. Some ideas on the possible mechanism of chiral recognition, involving, in particular, a diamide–diamide intercalation model, are discussed.

INTRODUCTION

Much of the research carried out in our laboratory in recent years on the resolution of optical isomers has centred around systems in which the solute–solvent association necessary for chiral recognition is effected through hydrogen bonding. In particular, we have studied extensively the highly selective diamide phases derived from α-amino acids, R'''CONHCH(R'')CONHR', where R'' = isopropyl. These investigations have dealt with the effects[1–3] of R' and R''' on selectivity and performance of these phases, and have recently been extended to the study of the influence of the R'' group[4,5]. These diamides permit the resolution of different classes of compounds. However, their most important application lies in the area of α-amino acids, which were chromatographed hitherto exclusively as N-perfluoroacyl ester derivatives.

The mechanism of resolution of N-acyl-α-amino acid esters on diamide phases

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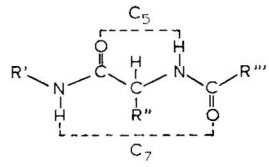
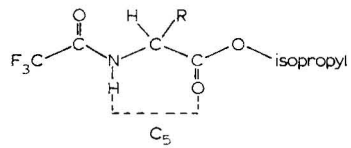
has been discussed before[2,3,6–8]. It suffices here to recall that the interaction between pertinent solute and solvent molecules is assumed to occur through hydrogen-bonded association of the "C_5–C_5" or "C_5–C_7" type. Recently[8], arguments have been produced for the possible larger contribution of the "C_5–C_7" solute–solvent association to chiral recognition:

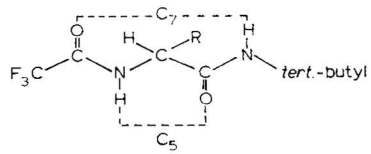


DIAMIDE

N-Acyl-α-amino acid esters have only a C_5 conformation:



It seemed of great interest to study solutes derivatized in such a way as to have both a C_5 and a C_7 side, for instance, the N-trifluoroacetyl (N-TFA) α-amino acid *tert.*-butylamides:



In the present paper we report on the stereoselectivity observed in the interaction of N-TFA-*tert.*-butylamide derivatives of a number of α-amino acids with several diamide phases, and compare the results with those obtained for the corresponding N-TFA-isopropyl esters.

EXPERIMENTAL

Materials

Chromatographic resolution was carried out on the following stationary phases: N-lauroyl-L-alanine *tert.*-butylamide (I)[4]; N-lauroyl-L-leucine *tert.*-butylamide (II)[5]; N-lauroyl-D-phenylglycine *tert.*-butylamide (III)[5] and N-lauroyl-L-phenylalanine *tert.*-butylamide (IV)[5]. These compounds were synthesized according to the general procedure described previously[3]; full details will be reported elsewhere[5].

The N-TFA *tert.*-butylamides of Ala, Val, Leu and Pro were prepared from the N-TFA-amino acids, synthesized according to Weygand and Geiger[9], by one of the following procedures.

(1) To a solution of N-TFA-α-amino acid in chloroform or ethyl acetate, kept at −5 to −10°C, N-hydroxysuccinimide (1.1 equiv.) and dicyclohexylcarbodiimide (1.0 equiv.) were added. After 24 h the dicyclohexylurea formed was filtered off, and into the stirred solution, cooled as above, was added dropwise a mixture of *tert.*-butylamine and N-methylmorpholine (1.0 equiv. each); stirring was then continued in the cold (48 h). The reaction mixture was washed successively with 2% HCl, water, 5% $NaHCO_3$, water and then dried over $MgSO_4$. The residue left on evaporation of the solvent was the desired compound, as checked by NMR spectroscopy. During this reaction no racemization was observed to occur in analogous cases[5].

(2) First, the acid chloride of the N-TFA-α-amino acid in dry dichloromethane was formed by reaction with a slight excess of thionyl chloride at room temperature for 1 h. The solvent was removed by evaporation with a nitrogen stream. The residue was redissolved in dry dichloromethane, cooled in a water–ice bath and *tert.*-butylamine (2 equiv.) was added dropwise. The solution was then stirred for another hour. The reaction mixture was washed as above, and the desired compound obtained on evaporation of the solvent.

The second procedure is more rapid and convenient than the first. However, some racemization of the α-amino acids with a primary amine group (but not of proline) occurs under the above conditions. For the purpose of the present study, where non-racemic mixtures were prepared only for peak identification, both methods can be used.

Chromatographic conditions

Stainless-steel capillary columns (100 ft. × 0.02 in.) were coated by the plug method with I and II (mounted in a Varian Series 2700 chromatograph) and (150 ft. × 0.02 in.) with III and IV (mounted in a Varian Series 1200 chromatograph). Both instruments were provided with a splitter and a flame ionization detector. The temperatures of the injector and detector were 240°C; column temperatures used are given in Table I. The helium flow-rate was 3 ml/min for all columns. The order of elution of the peaks was established for Ala and Pro by operating with mixtures enriched in the D and the L enantiomer, respectively. The result was extrapolated to Val and Leu.

DISCUSSION

The results are listed in Table I and some typical chromatograms are given in Figs. 1–4.

The order of elution for both classes of compounds is the same on I–IV. As expected, on the D-phenylglycine phase (III), the order is reversed with respect to I, II and IV, which are derived from L-α-amino acids.

As to the magnitude of the resolution factors, it is advantageous to discuss the data for the α-amino acids having a primary group separately from those for proline. Although the temperature at which the isopropyl esters and the corresponding diamide derivatives were chromatographed is not the same, there is no difficulty in judging the differences in selectivity for the two classes of compounds.

As can be seen in Table I, Ala, Val and Leu show different behaviour on the four phases. Thus, on I (derived from alanine) the two classes of derivatives show

TABLE I

RESOLUTION OF N-TFA ISOPROPYL ESTERS AND OF N-TFA *tert.*-BUTYLAMIDES OF α-AMINO ACIDS ON DIAMIDES, R'''CONHCH(R'')CONHR', AS STATIONARY PHASES

Optical purity: 98% (I), 99% (II), 81% (III) and 99% (IV). For chromatographic conditions, see Experimental.
r = Corrected retention time (min). $r_{L/D}$ = resolution factor = ratio of the corrected retention time of the enantiomer eluting last over that of the enantiomer eluting first, calculated with r values expressed to the second decimal.

Phase	*N-TFA-alanine*						*N-TFA-valine*		
	Isopropyl ester			*tert.-Butylamide*			*Isopropyl ester*		
	r	$r_{L/D}$	T(°C)	r	$r_{L/D}$	T(°C)	r	$r_{L/D}$	T(°C)
I. N-Lauroyl-L-alanine *tert.*-butylamide	D 5.00	1.080	130	46.6	1.083	140	6.60	1.061	130
	L 5.40			50.5			7.00		
II. N-Lauroyl-L-leucine *tert.*-butylamide	D 8.46	1.163	150	48.2	1.143	160	12.50	1.138	150
	L 9.84			55.1			14.22		
III. N-Lauroyl-D-phenylglycine *tert.*-butylamide*	L 8.60	(1.140)	130	65.20	(1.055)	150	12.00	(1.117)	130
	D 9.80			68.80			13.40		
IV. N-Lauroyl-L-phenylalanine *tert.*-butylamide	D 4.82	1.212	130	30.24	1.223	150	7.42	1.207	130
	L 5.84			37.00			8.96		

* III being a D phase, the order of elution is reversed. Resolution factors listed are $r_{D/L}$.

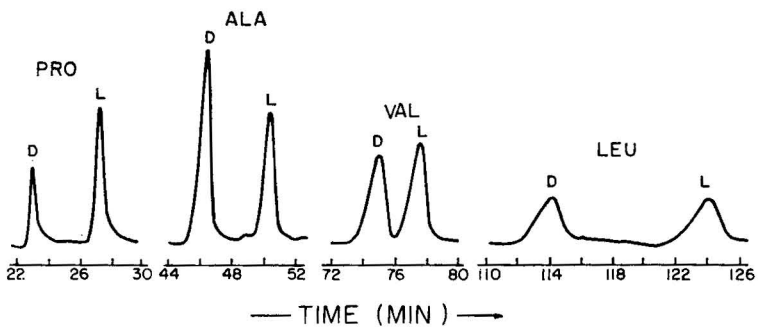


Fig. 1. Chromatogram of N-TFA *tert.*-butylamides of L-enriched Pro, D-enriched Ala, D,L-Val and D,L-Leu on a stainless-steel capillary column (100 ft. × 0.02 in.) coated with N-lauroyl-L-alanine *tert.*-butylamide. For chromatographic conditions, see Experimental. Temperature, 140°C.

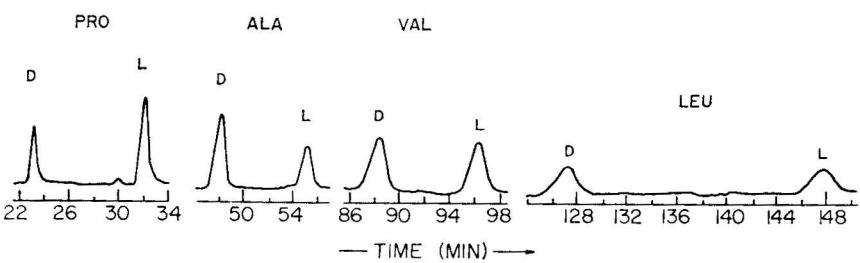


Fig. 2. Chromatogram of N-TFA *tert.*-butylamides of L-enriched Pro, D-enriched Ala, D,L,Val and D,L-Leu on a stainless-steel capillary column (100 ft. × 0.02 in.) coated with N-lauroyl-L-leucine *tert.*-butylamide. For chromatographic conditions, see Experimental. Temperature, 160°C.

N-TFA-leucine						*N-TFA-proline*								
tert.-Butylamide			*Isopropyl ester*			*tert.-Butylamide*			*Isopropyl ester*			*tert.-butylamide*		
r	$r_{L/D}$	*T*(°C)	*r*	$r_{L/D}$	*T*(°C)	*r*	$r_{L/D}$	*T*(°C)	*r*	$r_{L/D}$	*T*(°C)	*r*	$r_{L/D}$	*T*(C°)
75.20	1.034	140	15.70	1.095	130	114.30	1.087	140	17.00	1.000	130	23.10	1.186	140
77.80			17.20			124.20			17.00			27.40		
88.40	1.089	160	24.30	1.216	150	127.20	1.162	160	29.02	1.036	150	23.20	1.384	160
96.30			29.54			147.80			30.06			32.10		
90.80	(1.066)	150	11.00	(1.145)	150	140.80	(1.020)	150	40.40	(1.000)	130	43.80	(1.173)	150
96.80			12.60			143.80			40.40			51.40		
53.00	1.216	150	6.50	1.215	150	78.32	1.343	150	22.42	1.000	130	17.18	1.314	150
64.48			7.90			105.20			22.42			23.40		

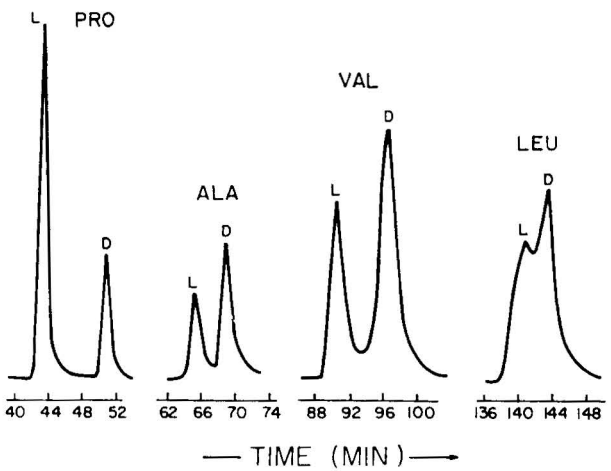


Fig. 3. Chromatogram of N-TFA *tert.*-butylamides of L-enriched Pro, D-enriched Ala, D,L-Val and D,L-Leu on a stainless-steel capillary column (150 ft. × 0.02 in.) coated with N-lauroyl-D-phenylglycine *tert.*-butylamide. For chromatographic conditions, see Experimental. Temperature, 150°C.

approximately the same *r* values. On the leucine phase (II) the resolution for the *tert.*-butylamines is approximately equal for the alanine derivative and lower for valine and leucine, as compared with the corresponding N-TFA isopropyl esters. With the D-phenylglycine phase (III) the diamide solutes have considerably lower resolution factors. On the other hand, on the highly selective phase IV (derived from Phe), the diamide solutes have even higher resolution factors than the isopropyl esters.

Diamides can interact through hydrogen bonding in a number of ways to form structures of the pleated sheet type[10,11]. The diamide molecules can, for instance, be aligned parallel or antiparallel to each other as illustrated in Fig. 5. As in the chro-

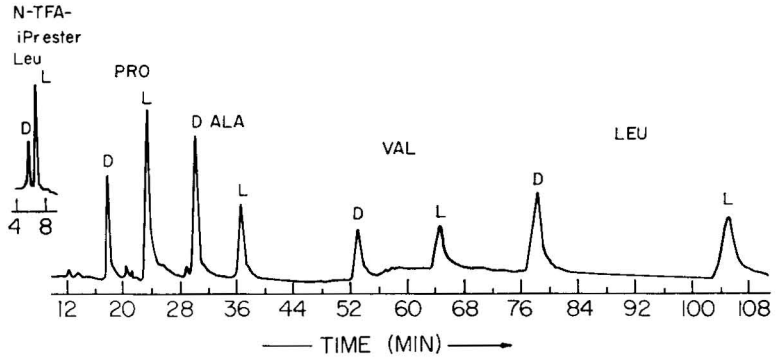


Fig. 4. Chromatogram of N-TFA *tert.*-butylamides of L-enriched Pro, D-enriched Ala, D,L-Val and D,L-Leu and of the N-TFA isopropyl esters of L-enriched Leu on a stainless-steel capillary column (150 ft. × 0.02 in.) coated with N-lauroyl-L-phenylalanine *tert.*-butylamide. For chromatographic conditions, see Experimental. Temperature, 150°C.

matographic process the solute is always present in a very low concentration in the liquid phase, its potential for hydrogen bonding will be fully implemented by interaction with surrounding solvent molecules, resulting in intercalation (Fig. 5). The stereoselectivity observed should result from the differences in interaction between

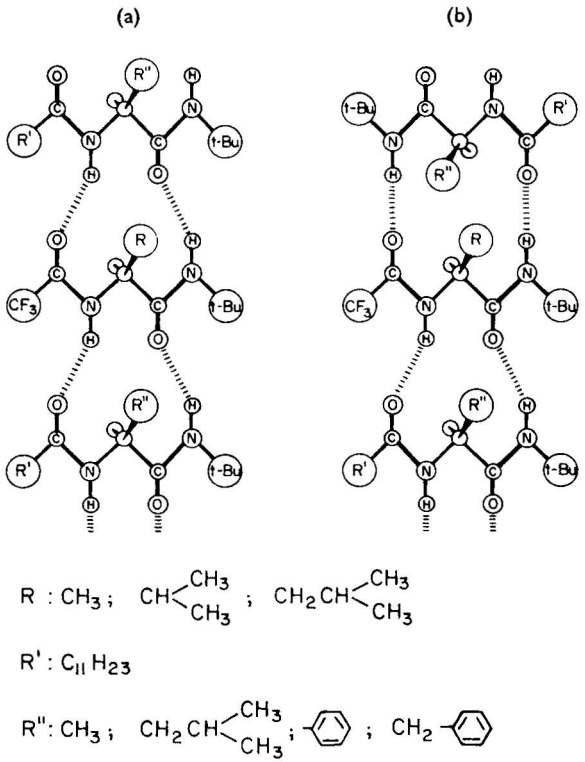
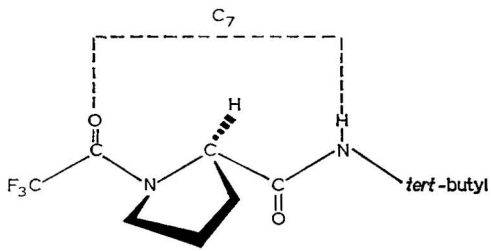


Fig. 5. Schematic representation of the intercalation of a diamide solute molecule in the diamide solvent aligned parallel (a) or antiparallel (b).

the substituents at the asymmetric centres in the diastereomeric intercalation complexes.

For proline the picture is different than for Ala, Val and Leu. Indeed, N-TFA-proline isopropyl ester does not possess a C_5 side. Its r values are small on all diamide phases, and the mechanism of chiral recognition differs from that of the other N-TFA-amino acids which have a primary amine group. In contrast, for the diamide of proline a C_7 conformation is available:



Association with the solvent through a C_7–C_5 or a C_7–C_7 hydrogen-bonded ring is possible. The relatively large resolution factors are ascribed to these associations, with the rigidity imparted to the solute molecule by the ring structure of proline presumably also playing a role. It should also be pointed out that the diamides of proline have lower retentions than those of Ala, Val and Leu. For the isopropyl esters, on the other hand, the inverse is true: their retention is approximately the same as that of the leucine derivatives. This behaviour is ascribed to the fact that, in contrast to the other α-amino acid derivatives, the N-TFA-proline *tert.*-butylamide can interact with neighbouring solvent molecules only by three and not by four hydrogen bonds. The proline derivatives cannot form an intercalation complex of the type shown in Fig. 5, and hence their total interaction with the phase is smaller.

From an analytical point of view the use of diamide solutes has great interest for the determination of the enantiomeric composition of proline and its analogues. The data for phase IV further indicate that the chromatography of N-TFA *tert.*-butylamides of α-amino acids having a primary amine group may lead to more efficient resolution than that of the corresponding N-TFA isopropyl esters. The methods used for the formation of the N-TFA *tert.*-butylamides are described in the Experimental. Further work is required to develop procedures which are rapid and which also completely avoid racemization during derivatization.

It has been shown[12,13] that an intercalation mechanism can explain satisfactorily the chiral recognition observed for the interaction of aromatic monoamides of the type $ArCH(CH_3)NHCOR$. In the present paper we suggest that also in the case of chiral diamides derived from α-amino acids, intercalation of N-TFA *tert.*-butylamides of α-amino acids between the solvent molecules leads to the observed stereoselective effects. It will be the objective of future studies to ascertain by spectroscopic methods, as well as by calculations, the modes of alignment between solvent molecules in the different phases in the liquid state. Subsequently, it would be necessary to estimate the differences in energy of the diastereomeric intercalation products in an attempt to interpret the experimental data.

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CHROM. 13,231

GAS CHROMATOGRAPHY OF SIMPLE MONOCARBONYLS IN CIGARETTE WHOLE SMOKE AS THE BENZYLOXIME DERIVATIVES

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SUMMARY

A qualitative and semi-quantitative method was established for the investigation of low-molecular-weight volatile carbonyl compounds in cigarette whole smoke. The carbonyls were trapped on a silica gel "column" and eluted with water. The aqueous solution was then treated with benzyloxyamine to form the corresponding oximes, which were then separated on a short (12 m) FFAP glass-capillary gas chromatographic column with temperature programming, and detected by a nitrogen selective detector. An internal standard was added both as a reference for retention time determinations, and as an aid in estimating the amounts of the individual carbonyls in the smoke samples.

INTRODUCTION

The detection and analysis of volatile carbonyl compounds in complex matrices have been investigated rather extensively and reported in the literature. Usually, these carbonyls have been analyzed as their 2,4-dinitrophenylhydrazones (2,4-DNPHs), using paper, column, thin-layer, gas and liquid chromatography as separation techniques.

Shibasaki and Iwabuchi[1] have determined these compounds in "miso" aroma; Shimizu *et al.*[2] in roasted starch; Kallio and Linko[3] in arctic bramble, Pyysalo[4] and Hirrsalmi *et al.*[5] in hybrids between raspberry and arctic bramble; Linko *et al.*[6] in carrots; and Bachmann *et al.*[7] in urine. The analysis of simple carbonyl compounds in tobacco smoke has also been the subject of much attention, for about the last 25 years[8–18], again with the majority of determinations made using the 2,4-DNPHs. More recently, a method was described for the preparation of benzyloxime derivatives of simple monocarbonyls[19], and the work described here applies that method for the use of benzyloxime derivatives to the analysis of carbonyls in cigarette smoke.

Briefly, the cigarette whole smoke was passed through a silica gel column to trap the carbonyls, followed by elution with water. The carbonyls were then converted to the corresponding oximes, which were extracted into diethyl ether. Separation of the oximes was accomplished by temperature-programmed glass-capillary gas chromatography (GC) on a short (12 m) FFAP column. An internal standard was added

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both as a reference for retention time determinations, and as an aid in estimating the amounts of the individual carbonyls in the smoke samples.

EXPERIMENTAL

Smoke trapping

Smoke absorption traps were constructed as in Fig. 1. Part B was filled with 5.5 g of silica gel (Grade 408, 12–28 mesh, Davison Chemical, Baltimore, MD, U.S.A.), gently tamped to a 100-mm column and held in place by two pieces of glass wool. The silica gel was positioned about 5 mm from the open end of the tube, where the cigarette was fitted for smoking. The assembled trap (Fig. 2) was connected in a horizontal alignment to a syringe-type smoking machine in place of the Cambridge filter pad. In this manner, the whole smoke was drawn through the silica gel trap.

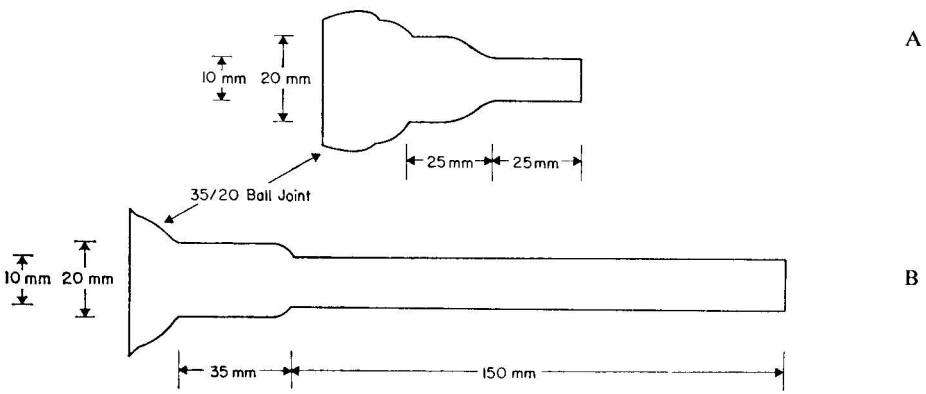

Fig. 1. Smoke absorption trap, dimensions.

Smoking conditions

Standard smoking conditions were used: The cigarettes were conditioned at 25°C and 60% relative humidity and smoked, taking a 35-ml puff of 2 sec duration each minute, to a 30-mm butt length. Three cigarettes were smoked into each trap.

Internal standard solution

An aqueous solution was prepared to contain about 80 μg of hexanal per milliliter of solution.

Sample preparation

Following smoking, part B of the trap was disconnected and clamped in a vertical position with the ball joint end up; in this manner it functioned as a column for the elution of the carbonyls (Fig. 3). A 1-ml volume of the hexanal internal standard solution was added to the top of the column and the carbonyls eluted with water. About 15 ml were collected in a screw-capped bottle, and the benzyloximes prepared as reported earlier[19], whereby the oximes are isolated in diethyl ether.

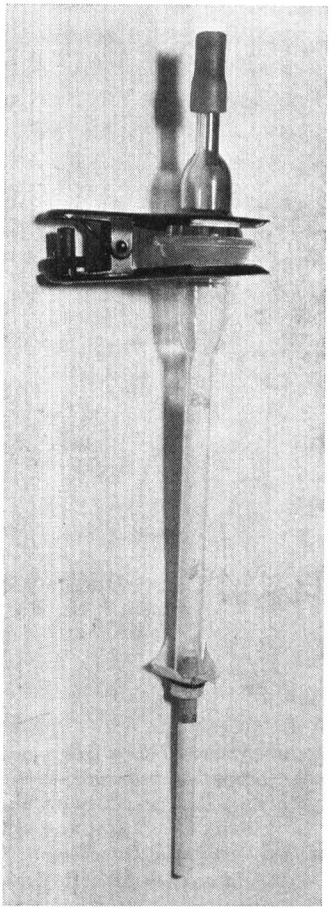

Fig. 2. Smoke absorption trap, assembled for smoke collection.

Gas chromatography

The GC conditions were identical to those previously reported[19] for the benzyloxime derivatives of short-chain carbonyls.

RESULTS

Fig. 4 shows a representative scan of the carbonyls of whole smoke from three Kentucky Reference 2R1 cigarettes (research cigarettes produced for the University of Kentucky Research Foundation). (A more extensive listing of retention times of carbonyl benzyloximes can be found in ref. 19.)

Table I illustrates experimental values determined for several carbonyls in the smoke of three commercially available cigarettes, as determined as the benzyloximes. The average as well as the range of values obtained are given; each average is the result of at least four, and in some cases as many as eight, determinations.

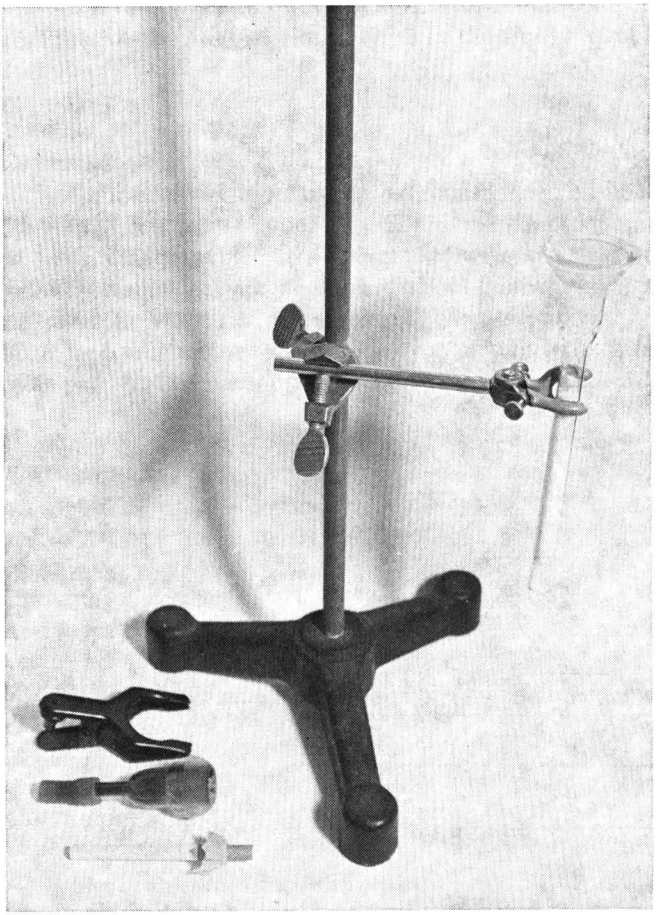

Fig. 3. Smoke absorption trap, assembled for elution.

TABLE I

LEVELS OF SELECTED CARBONYLS IN THE WHOLE SMOKE OF SOME CIGARETTES

Levels are tabulated as average; values in parentheses indicate the range. The values are given in μg/cigarette.

	Cigarette A (filter)	*Cigarette B (filter, low delivery)*	*Cigarette C (non-filter)*
Formaldehyde	31(10–50)	10(9–10)	21(12–30)
Acetone	400(325–475)	137(130–144)	330(310–350)
Propanal	61(37–100)	37(30–40)	50(50–53)
Acrolein	23(13–37)	3(3–4)	22(20–25)
Methacrolein	17(14–38)	18(18–19)	27(20–32)
Butanal	20(9–29)	13(12–13)	18(17–20)

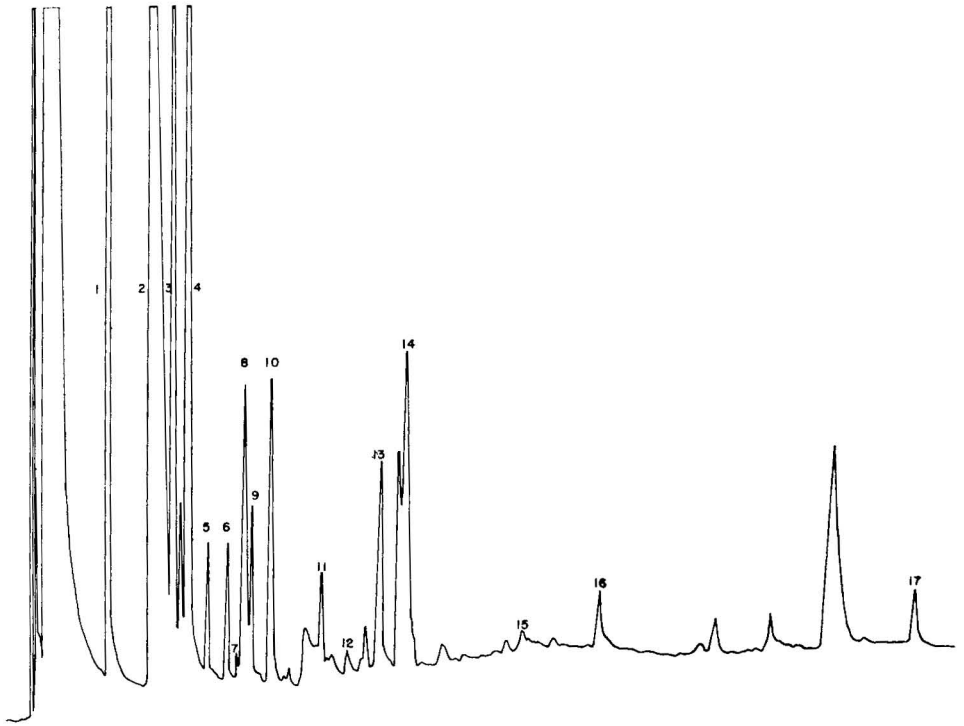

Fig. 4. GC scan of benzyloximes of some carbonyls in Kentucky Reference 2R1 cigarette whole smoke. For chromatographic conditions, see ref. 19. Peak identification is based on retention time relative to the hexanal compound. Peaks: 1 = formaldehyde; 2 = acetaldehyde; 3 = propanal; 4 = acetone; 5 = butanone; 6 = propenal (acrolein); 7 = pentanone (2- and/or 3-); 8 = butanal; 9 = methacrolein; 10 = isopentanal; 11 = pentanal; 12 = 5-hexen-2-one; 13 = 2-butenal (crotonaldehyde); 14 = hexanal; 15 = cyclohexanone; 16 = 2-hexenal and/or cycloheptanone; 17 = benzaldehyde.

DISCUSSION

Optimization of conditions

In the development of the method, several variables were evaluated to optimize conditions.

Collection of carbonyls from smoke. Several methods of collecting the carbonyls from cigarette smoke were investigated, including bubbling the smoke through liquid traps similar to those described by Mansfield *et al.*[18]. The silica gel traps described in Experimental were found to be the method of collection giving the highest efficiency while still retaining the puff characteristics best in terms of least-pressure drop during smoking.

Number of cigarettes per trap. It was found that the amount of the individual carbonyls recovered was linear and proportional to the number of cigarettes smoked onto one trap, up to about five cigarettes. After that point, the recovery leveled off, indicating consistent recovery of the carbonyls for no more than five cigarettes per trap. The level chosen for this work was three cigarettes per trap.

Elution of carbonyls from the silica gel trap. Two approaches were investigated: elution with solvent through the silica gel column, *versus* extraction by slurrying the gel in solvent. It was found that the elution technique removed virtually all of the carbonyls of interest while the slurry technique did not. This was determined using a standard acrolein solution and derivatizing following either elution or slurrying. Comparisons were then made to the same derivative prepared by treating the standard solution directly.

Choice of eluting solvent. The most convenient method would be to perform the derivatization in the eluting solvent itself; this limited the choice to either methanol or water, with subsequent isolation of derivatives by either evaporation or extraction. Both solvents eluted the carbonyls equally well, but elution with water was chosen because the subsequent extraction with ether introduced a useful clean-up step, in that the unreacted reagent could be held in the aqueous phase simply by acidifying before extraction.

Volume of eluting solvent. A 15-ml volume of water was found to be sufficient for complete elution of the carbonyls of interest. Increasing the volume to 40 ml made no difference in the results, and no attempt was made to decrease the volume below 15 ml.

Problems with quantitation

One difficulty with this method was that the most prevalent carbonyl in cigarette smoke, acetaldehyde, could not be determined. For some as yet unexplained reasons, peaks corresponding to the benzyloxime derivative of acetaldehyde appear in the reagent blanks of the respective preparations. Mass spectra of these peaks are identical to the spectra of the authentic acetaldehyde derivatives, but as stated, their source in the blanks is at present unknown.

Another problem encountered was the lack of reproducibility from run to run. Although the reproducibility of the GC step was very good (as measured by repeated injections of the same solution), a fairly wide range of values were obtained for some of the oximes following replicate analyses. For this reason, the method is described as semi-quantitative.

A third difficulty was that several of the carbonyls form two geometric isomers (*syn* and *anti*) of the oximes. This was not a major problem, since the ratio of major to minor peaks was found to be constant under a given set of conditions. Also, the only compounds for which the minor peak was substantial (*i.e.*, $>10\%$ of the major peak) were acetaldehyde and hexanal. As stated above, acetaldehyde could not be determined by this method, and hexanal is present in only very small amounts (if any) in the smoke of the cigarettes studied, and was in fact added as the internal standard.

The values obtained for the carbonyls conform reasonably well with the published figures[8–18]. One of the exceptions is acrolein for which the values are significantly lower than has been generally reported.

CONCLUSIONS

Described is a procedure for the collection of low-molecular-weight carbonyls from cigarette whole smoke, and the subsequent separation of their benzyloxime derivatives by glass-capillary GC. In addition, the use of a nitrogen selective detector

results in enhancement of sensitivity and considerable simplification of the chromatogram.

The utility of the procedure described in this paper lies in the fact that good separation of many carbonyls found in cigarette smoke is achieved chromatographically, thus making possible the generation of useful qualitative and semi-quantitative information about this very important class of compounds in cigarette smoke, or in other complex matrices.

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The previous work of Robert Esperdy with silica gel as a trapping medium for smoke was also very helpful.

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CHROM. 13,205

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ACID-STABLE AND ACID-LABILE PHOSPHOAMINO ACIDS

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(Received August 1st, 1980)

SUMMARY

A high-performance liquid chromatographic system has been developed which permits the separation of both acid-stable and acid-labile phosphoamino acids. An anion-exchange resin and two buffers of different ionic strength and near neutral pH are used. A low-ionic-strength buffer is used for the separation of N-ω-phosphoarginine and N-ε-phospholysine, while the higher-ionic-strength buffer permits the clear separation of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine. An in-stream fluorometric detection system using *o*-phthalaldehyde permits the rapid analysis of samples containing as little as 25 pmoles of phosphoamino acid. This method has been applied to the detection of τ-phosphohistidine from alkaline digests of chemically phosphorylated calf thymus histone 4 and bovine myelin basic protein.

INTRODUCTION

Phosphorylation of proteins on specific amino acid residues has been found to be an important modification that often results in altered catalytic activity or other biological property[1], and some phosphorylated proteins are formed as catalytic intermediates during enzymatic reactions[2,3]. Demonstration of the existence of phosphoproteins has relied on isolation of the phosphorylated form, hydrolysis of the protein by chemical or enzymatic means, and identification of the specific phosphoamino acid. Methods of identification most often used have included ion-exchange chromatography[4], paper and thin-layer chromatography[5], and high-voltage electrophoresis[6].

Many phosphorylated proteins have been found to contain *o*-phosphoserine and/or *o*-phosphothreonine which are relatively acid-stable and usually base-labile phosphomonoesters. In addition to these, phosphorylation has been shown to occur on the basic amino acids, histidine[2], and lysine[4,7], and on the sulfur of cysteine[8]. Unlike *o*-phosphoserine and *o*-phosphothreonine the phosphorylated basic amino acids are extremely acid-labile and base-stable[9]. During the isolation and characterization of proteins containing phosphoramidate (P–N) bonds, careful effort must be made to maintain neutral or slightly basic conditions in order to avoid hydrolysis.

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Conventional techniques for amino acid analysis using both low[10] and high-pressure systems[11] use acidic buffers which would be unsuitable for the detection of P–N-containing phosphoamino acids. A new high-performance liquid chromatography (HPLC) method was developed which permits the separation of both types of phosphoamino acids under conditions which maintain their stability. A polystyrene-type anion-exchange resin is used along with two buffers of near neutral pH and different ionic strength.

METHODS

Chemicals

o-Phthalaldehyde (OPT) was purchased from Durrum (Palo Alto, CA, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.). A protein hydrolysate standard was obtained from Pierce (Rockford, IL, U.S.A.). Phosphoserine and phosphothreonine were obtained from Sigma. N-ω-Phosphoarginine (Sigma) was found to be about 80% pure by HPLC analysis. It was purified by Dowex 1 chromatography using a 0 to 0.25 *M* $KHCO_3$ linear gradient. Purification yielded a single product with an identical elution volume to the enzymatic product formed by arginine kinase[12] with ATP and arginine. τ-Phosphohistidine* was prepared from phosphoramidate and histidine by described methods[6] and purified by Dowex 1 chromatography. Phospholysine was prepared by the method of Zetterqvist[4], and yielded both N-α-phospholysine and N-ε-phospholysine and a number of other products. Dowex 1 chromatography did not adequately separate the two isomers. An improved synthetic method was later developed which gave N-ε-phospholysine as the sole product and will be published elsewhere[13].

Buffers and OPT reagent

The low-ionic-strength buffer, A (15 mM KH_2PO_4), was prepared by dissolving reagent grade potassium dihydrogen phosphate in deionized water and titrating to pH 7.5 with concentrated potassium hydroxide. The high-ionic-strength buffer, B (250 m*M* KH_2PO_4), was prepared in a similar fashion but was brought to a final pH of 6.3. Phenol was added to a final concentration of 1 g/l. Solutions were routinely filtered through 0.2-μm filters (Millipore) before use. The OPT solution was prepared as described[11], but the final concentration of OPT was reduced to 500 mg/l.

HPLC

The arrangement of HPLC equipment is similar to that described by Voelter and Zech[14]. The column effluent enters a tee connector and mixes with a stream of OPT reagent driven by a separate pump. The mixture then travels through a piece of PTFE tubing which serves as a reaction coil before entering the flow cell of a fluorometer. An Altex Model 100 solvent metering pump was used in combination with a 250 mm × 4.6 mm I.D. column packed with Chromex DA-X12-11 anion-exchange resin (polystyrene quarternary amine-type, 12% cross-linking, Durrum). The column was held at 50°C in a water bath with a flow-rate of 0.5 ml/min. The

* The nomenclature τ-phosphohistidine (3-phosphohistidine) and π-phosphohistidine (1-phosphohistidine) following IUPAC–IUB recommendations (*cf. Arch. Biochem. Biophys.* 160 (1972) 1–8).

column eluate passes through a short piece of PTFE tubing (200 × 0.33 mm) before entering the PTFE mixing tee (0.8 mm I.D., Altex). The OPT solution was pumped into the other inlet on the tee by a Beckman Accuflo pump at a rate of 0.5 ml/min. The combined eluates pass through a reaction coil of PTFE tubing (720 cm × 0.3 mm I.D.) held at ambient temperature prior to entering a 15-μl flow cell of the fluorometer (Spectra Glo, Gilson). The manufacturer's OPT excitation and emission filters were used. The column was equilibrated with buffer A or B prior to use and according to the separation contemplated.

Chemical phosphorylation of histone 4 and bovine myelin basic protein

Phosphoramidate, prepared by the method of Stokes[15], was used to phosphorylate calf thymus histone 4 [prepared from whole histone, Type II-A, (Sigma) according to the procedure of Böhm *et al.*[16]] or bovine myelin basic protein (gift of Dr. Fred Westall) under the conditions modified from Rathlev and Rosenberg[17] used to phosphorylate insulin. In some instances, [^{32}P]phosphoramidate, prepared by the method of Sheridan *et al.*[18], was used. A 5-mg amount of protein was incubated with either unlabeled (20 mg) or [^{32}P]phosphoramidate (30,000 cpm/μmole) for 4 days at room temperature in 200 m*M* Tris, pH 7.4, containing 6 *M* urea. Unreacted phosphoramidate was removed by dialysis or by passage over Sephadex G-25. Radioactivity incorporated into the protein was shown by running a portion of the sample on an 18% SDS-polyacrylamide gel prepared as described by Laemmli[19]. Neutral staining (0.25% Coomassie blue in 25% isopropanol) and destaining (10% isopropanol) procedures were used followed by autoradiography.

Alkaline hydrolysis of phosphorylated proteins

A sample of phosphorylated protein (*ca.* 1 mg) was dialyzed against 100 m*M* $NaHCO_3$,pH 8.2, and lyophilized overnight. The residue was dissolved in 3 *M* KOH, sealed in a glass tube, and held at >115°C for 3 h. The hydrolysate was very carefully neutralized to pH 7.5 with 2 *N* $HClO_4$ and the insoluble salts removed by centrifugation. The supernatant was concentrated by lyophilization and resuspended in water. Samples were filtered through 0.2-μm filters before use.

RESULTS AND DISCUSSION

The elution profile of a standard protein hydrolysate mixture (100 pmoles of each amino acid) and 100 pmoles each of *o*-phosphoserine, *o*-phosphothreonine and τ-phosphohistidine is shown in Fig. 1. At pH 6.3, a high concentration of salt (buffer B, 250 m*M* KH_2PO_4) was necessary for the elution of the phosphoamino acids within a reasonable period of time. However, this buffer was unsuitable for the separation of N-ω-phosphoarginine and N-ε-phospholysine since, at this concentration, they tended to coelute with the other amino acids, which is consistent with their lower overall negative charge. As shown ahead, a lower-ionic-strength buffer was used to separate N-ε-phospholysine and N-ω-phosphoarginine from one another and from the other amino acids. τ-Phosphohistidine elutes more rapidly than *o*-phosphoserine which most likely reflects the lower overall charge on the phosphorylated histidine due to the partial protonation of the imidazole nitrogen [pK_a 6.4 (ref. 6)]. Hydrophobic interaction between the imidazole ring and backbone of the resin may contrib-

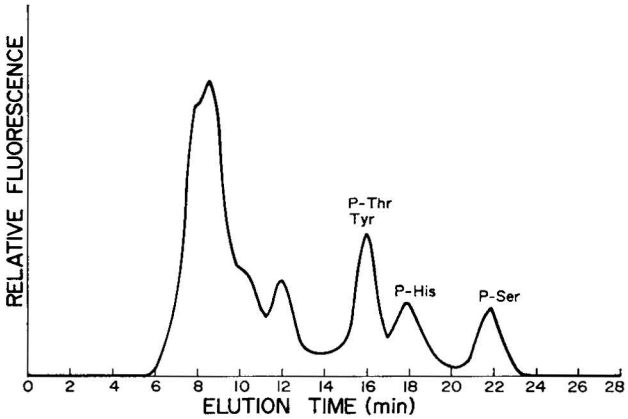

Fig. 1. HPLC separation of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine. A 25-μl volume of a mixture of protein hydrolysate standard (100 pmoles each amino acid) containing 100 pmoles of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine was injected. Fluorometer sensitivity R = 10. Elution buffer is 250 m*M* KH_2PO_4 pH 6.3 (buffer B), 50°C.

ute to its greater retention time relative to *o*-phosphothreonine. Using buffer B, τ-phosphohistidine and π-phosphohistidine coelute. A higher pH buffer, 250 m*M* KH_2PO_4, pH 7.2, resolved the two, giving elution times of 25 and 18 min, respectively.

Using buffer B, *o*-phosphothreonine and tyrosine coelute. The long retention time of tyrosine is presumably due to its hydrophobic interaction with the resin. Therefore, conditions were sought which would give a clear separation between tyrosine and *o*-phosphothreonine as well as between *o*-phosphoserine and τ-phosphohistidine. Slight variations in pH, temperature and salt concentration failed to give a clear separation of all four of these amino acids. However, employing a higher salt concentration (300 m*M* KH_2PO_4, pH 6.5, buffer C), *o*-phosphothreonine and tyrosine are separated, but τ-phosphohistidine now coelutes with tyrosine. Fig. 2 shows the elution profile using buffer C. Clearly, if ^{32}P-labeled phosphoamino acids are analyzed, the coelution of *o*-phosphothreonine and tyrosine (using buffer B) or τ-phosphohistidine and tyrosine (using buffer C) is not critical, as the identity of the [^{32}P]phosphoamino acid can be determined by measuring the radioactivity of collected fractions.

Separation of N-ω-phosphoarginine and N-ε-phospholysine was achieved using the lower-ionic-strength buffer, A (Fig. 3). 15 m*M* KH_2PO_4 was found to be the optimal salt concentration for the temperatures and pH values tested. At higher salt concentrations, N-ω-phosphoarginine and N-ε-phospholysine coelute and migrate into the region of the other amino acids. Using buffer A, N-ε-phospholysine elutes at about 20 min, N-ω-phosphoarginine elutes at 22.5 min, while N-α-phospholysine elutes at a much later time. In preliminary experiments using 75 m*M* KH_2PO_4 buffer, pH 7.5 (buffer D), a mixture of the two isomers of phospholysine separated well, showing a peak at 11.0 min (N-ε-phospholysine) and 17.0 min (N-α-phospholysine). Buffer D was most useful for the rapid evaluation of the reaction products obtained during the synthesis of N-ε-phospholysine. Phospholysine, prepared by the method of Zetterqvist[4], yielded several products. However, the product formed using a new synthetic

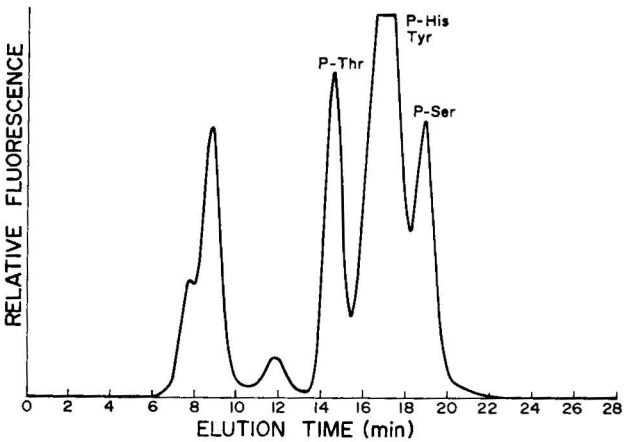

Fig. 2. HPLC separation of τ-phosphohistidine, *o*-phosphoserine and *o*-phosphothreonine. A 25-μl volume of a mixture of τ-phosphohistidine (400 pmoles), *o*-phosphoserine (450 pmoles), *o*-phosphothreonine (380 pmoles) and tyrosine (890 pmoles) was injected. Fluorometer sensitivity R = 10. Peaks eluting at 7–10 min are minor impurities in the standards. Elution buffer is 300 m*M* KH_2PO_4 pH 6.5 (buffer C), 50°C.

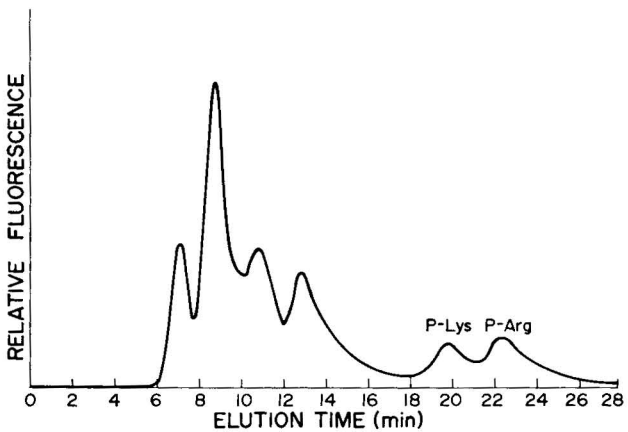

Fig. 3. HPLC separation of N-ε-phospholysine and N-ω-phosphoarginine. A 25-μl volume of a mixture of protein hydrolysate standard (100 pmoles each amino acid) containing 100 pmoles of N-ε-phospholysine and N-ω-phosphoarginine was injected. Fluorometer sensitivity R = 10. Elution buffer is 15 m*M* KH_2PO_4 pH 7.5 (buffer A), 50°C.

method for N-ε-phospholysine, starting from the copper chelate of lysine, gave only the 11.0 min peak using buffer D.

Table I shows the fluorescence yield of the phosphoamino acid standards relative to tyrosine. These determinations were performed in the absence of the detergent Brij-35 which was found to have little or no effect on the fluorescence of the phosphorylated amino acids, including N-ε-phospholysine, although it is known to dramatically increase the fluorescence of lysine[11].

TABLE I

RELATIVE FLUORESCENCE OF PHOSPHOAMINO ACIDS

Fluorescence is expressed relative to tyrosine. A plot of relative fluorescence *vs.* amount of phosphoamino acid was determined for each, and the slope divided by the slope obtained for tyrosine.

Phosphoserine	1.34
Phosphothreonine	1.37
N-ω-Phosphoarginine	2.61
N-ε-Phospholysine	1.00
τ-Phosphohistidine	1.63

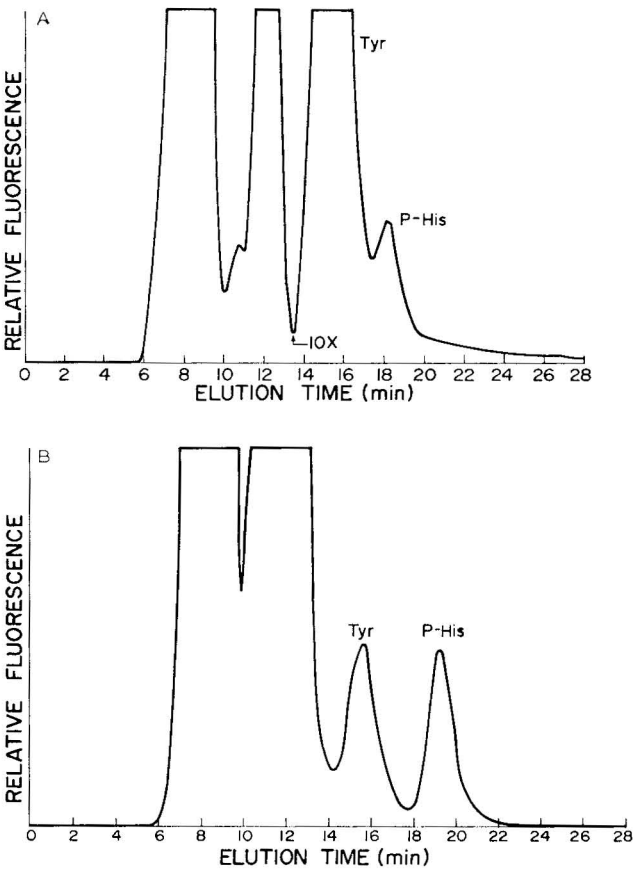

Fig. 4.A, Identification of τ-phosphohistidine from potassium hydroxide digest of chemically phosphorylated histone 4. A 50-μl volume representing 50 μg of phosphohistone 4 was injected. Initial fluorometer sensitivity was R = 1, at 13.5 min the sensitivity was increased ten fold (R = 10). Elution buffer is 250 m*M* KH_2PO_4 pH 6.3 (buffer B), 50°C. B, Identification of τ-phosphohistidine from potassium hydroxide digest of chemically phosphorylated bovine myelin basic protein. 10 μl representing 50 μg of phosphorylated protein were injected. Fluorometer sensitivity R = 5 (1/2 less than R = 10). Elution buffer is 250 m*M* KH_2PO_4 pH 6.3 (buffer B), 50°C.

It has been reported that histone 4 and myelin basic protein are enzymatically phosphorylated on histidine residues[5,7] as well as serine residues[20]. Chemical phosphorylation of these proteins on histidine residues was performed using phosphoramidate as a phosphoryl donor. Fig. 4A and B shows an HPLC analysis of a KOH digest of phosphoramidate-treated histone 4 and myelin basic protein. Acid treatment (0.5 *N* HCl, 1 min at 100°C, followed by reneutralization) of the KOH digests caused the disappearance of the OPT-reactive peak at 19 min.

Due to the extreme acid lability of P–N compounds, neutral or basic conditions must be maintained during chemical analysis. Present HPLC methods for amino acid analysis[11,14] are unsuitable for P–N compounds due to the common use of acidic buffers. This places a large constraint on the parameters which may be varied for effective resolution of both acid-stable and acid-labile modified amino acids. Use of high pH buffers (pH >8) for phosphoamino acid analysis was unsuitable, as an extremely high salt concentration was required for elution. Resolution was also quite poor under these conditions. The only conditions that varied in this work were slight modifications in pH around neutrality and rather large variations in ionic strength and temperature. The use of a gradient of ionic strength and/or pH may permit the resolution of all phosphoamino acids during a single analysis.

ACKNOWLEDGMENTS

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USE OF CATION-EXCHANGE RESIN FOR THE DETECTION OF ALKYLPYRIDINES IN BEER

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SUMMARY

A method has been devised whereby trace amounts of certain basic compounds, such as pyridines, may be detected and semi-quantified in beer in the presence of an excess of other flavour constituents including pyrazines. The method involves steam distillation of beer under reduced pressure and subsequent passage of the distillate through a column of weakly acidic Zerolit cation-exchange resin. The resin is eluted with aqueous sodium chloride, the eluate extracted with organic solvent and the concentrated extract analysed by gas chromatography coupled with mass spectrometry. Using this technique with multiple ion detection, a series of alkylpyridines was readily detectable in beers and worts at levels below 1 ppb*.

INTRODUCTION

Many investigators have recognised the important contribution which certain volatile basic compounds can make towards the aroma and flavour of some beers[1–5]. However, of the components which have been chemically characterised in basic extracts of beers, pyrazines are the group which have received most attention[3–5]. This is presumably because they are the predominant class of compounds occurring in basic extracts of beer obtained using techniques based upon solvent extraction[3–5], and because they are well-known to be a highly flavour-potent group of substances[6]. Other volatile basic constituents of beer which have been identified include amines[1], amides[5] and traces of a small number of pyridines[2,4,5] and thiazoles[4,5]. However, detection of the latter two groups of compounds in beer is very difficult because they tend to be masked by the relative excess of pyrazines which are present. This investigation set out to devise a method for detecting trace volatile basic compounds, other than pyrazines, which occur in beer.

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

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EXPERIMENTAL

Reagents and standards

Zerolit 236 SRC 41 cation-exchange resin (7 g dry weight) was washed with 0.6 *M* hydrochloric acid (100 ml) and deionised water (200 ml) prior to use.

Pentane (Koch-Light, Colnbrook, Great Britain) and diethyl ether (BDH, Poole, Great Britain) were both redistilled prior to use. Antifoam was a solution of 10% silicone DC antifoam RD emulsion (Hopkin and Williams, Chadwick Heath, Great Britain) in water. Authentic beer flavour constituents were mostly obtained from commercial suppliers, and were homogeneous according to analysis by GC. The amino acid ethyl esters[7] were obtained from a commercial supplier or were synthesised according to a literature procedure. A sample of 2,3-dimethylpyridine was the generous gift of Dr. M. Novotný of Indiana University, Bloomington, IN, U.S.A.

No interfering pyridines were detected in any of the reagents described above.

Preparation of wort and beer

Wort and beer were prepared on the pilot scale using a standard procedure[8].

Preparation of basic extracts of wort and beer

Wort or beer (4 l) containing antifoam solution (10 ml) was vacuum steam distilled at 25°C and 0.02 mmHg pressure, using the method of Pickett *et al.*[9]. The distillate was then passed through a column of Zerolit 236 SRC 41 cation-exchange resin (110 × 12 mm, 7 g dry weight) at *ca.* 7 ml/min. The resin was washed with deionised water (200 ml) and subsequently eluted with 2 *M* aqueous sodium chloride (230 ml). The pH of the eluate was adjusted to *ca.* 8.5 by addition of 2.5 *M* aqueous sodium hydroxide, and the eluate then continuously extracted for 6.5 h with a 1:2 mixture of pentane and diethyl ether (40 ml). After drying over anhydrous sodium sulphate (5 g), the organic extract was concentrated to 0.5 ml, by gentle warming on a water bath (40°C) using the technique of Junk *et al.*[10], spiked with 3 μl of a 1% solution of ethyl octanoate in ethanol, further concentrated to 0.2 ml and finally examined by gas chromatography (GC) and GC–mass spectrometry (MS).

Recoveries of authentic compounds from 4% aqueous ethanol using the Zerolit procedure

Dilute solutions, containing mixtures of authentic compounds (1 ppm each) in 4% aqueous ethanol, were passed through a column of Zerolit 236 SRC 41 resin (110 × 12 mm). The compounds were eluted from the resin and concentrated using the same technique as described above for beer extracts. Concentrates were then examined by GC (using a conventional packed column) and peak heights (obtained by flame-ionisation detection) compared to those obtained for standard mixtures of of the appropriate compounds.

Instrumental analyses

Gas chromatography. GC of beer extracts was carried out using a support-coated open tubular column coated with Carbowax 20M[7].

GC of mixtures of authentic beer flavour compounds was carried out using a Pye GCV gas chromatograph, equipped with a linear temperature programmer and synchronous flame-ionisation, flame-photometric (394 nm filter) and alkali flame-

ionisation detection. The GC column, 2.8 m × 4 mm I.D. glass packed with 20% Carbowax 20M on Chromosorb W AW DMCS (80–100 mesh), was operated with a nitrogen carrier gas flow-rate of 78 ml/min and a temperature programme of 50–200°C at 3°C/min.

Combined gas chromatography–mass spectrometry. This was carried out using a Finnigan 1020 GC–MS system. Chromatographic separations were performed with a 50 m × 0.3 mm I.D. glass capillary wall-coated open tubular column coated with Carbowax 20M (GC² Chromatography, Northwich, Great Britain). Helium (CP grade, B.O.C., London, Great Britain) was used as carrier gas at a linear flow-rate of 26.5 cm/sec, and the oven temperature programme employed was 60°C for 5 min followed by an increase of 3°C/min to 200°C.

Effluent from the capillary column was passed directly into the source of the mass spectrometer via a heated 0.1 mm I.D. glass capillary transfer line. The source temperature was 80°C and the transfer line was maintained at 230°C. Mass spectra, measured with an ionisation energy of 70 eV, were either scanned from *m/e* 45 to 250 every 1 sec, or were scanned in the multiple ion detection (MID) mode as described below. All data acquired were stored on disk for later recall.

RESULTS AND DISCUSSION

In preliminary work basic extracts of beers were prepared using a variation of the commonly employed solvent extraction technique. The pH of the beer was adjusted to *ca.* 8.5, prior to steam distillation under reduced pressure at ambient temperature according to the method of Pickett *et al.*[9]. The distillate (after pH adjustment to *ca.* 8.5) was then continuously extracted with organic solvent, followed by by acid extraction of the organic phase. After washing of the acid phase and subsequent pH adjustment to *ca.* 8.5 it was re-extracted with organic solvent. The majority of the solvent (pentane–diethyl ether, 1:2) was then removed by gentle warming on a water bath (40°C), using the technique of Junk *et al.*[10]. Examination by GC–MS, of extracts produced in this way, confirmed that pyrazines were the major class of volatile basic compound present. Several pyridines (of molecular weights 93 and 107) could be detected in extracts prepared from a commercial ale and a commercial stout, but the mass spectra were not of sufficiently high quality to allow the exact nature of the isomers to be determined. It was noticeable that pyridines were detected only in those sections of the chromatogram which were relatively free of pyrazines and other major components. Subsequently beers with known additions of both pyrazines and pyridines were extracted and concentrated. Recoveries of the added compounds were actually found to be rather higher for pyridines (*ca.* 60–90%) than for pyrazines (*ca.* 40–70%), showing that the solvent extraction method itself was not responsible for the relative levels of pyrazines and pyridines noted in beer extracts.

Peppard and Douse[11] recently reported the use of Amberlite XAD-2 resin in preparing concentrates of beer flavour compounds. The technique was therefore investigated in the present studies connected with basic beer constituents. Using mixtures of authentic compounds (0.5 ppm each) in 4% aqueous ethanol, the recoveries of 2,5- and 2,3-dimethylpyrazines and 2-, 2,4- and 2,4,6-methylpyridines were all poor (8–20%) as was the recovery of thiazole (2%). The recoveries of more heavily substituted compounds using the resin were considerably better, *e.g.* 2-methyl-3-

acetylpyrazine (68%), 6,7-dihydro-2,5-dimethyl-5H-cyclopentapyrazine (83%) and 2-ethyl-4,5-dimethylthiazole (84%), but the method clearly was not as efficient for concentrating the lower molecular weight pyridines from beer as the previously described solvent extraction technique and so was not investigated further.

TABLE I

p*K*a VALUES OF SOME COMMON PYRAZINE, PYRIDINE AND THIAZOLE DERIVATIVES[12,13]

Compound	*pK_a Value*
Pyrazine	0.65
2-Methylpyrazine	1.45
2,5-Dimethylpyrazine	1.85
Pyridine	5.20
2-Methylpyridine	5.95
2-Ethylpyridine	5.90
2,5-Dimethylpyridine	6.45
Thiazole	2.44

Pyrazines, being weaker bases than either pyridines or thiazoles, generally have lower pK_a values, as indicated by the examples given in Table I. The possibility of exploiting such large differences in basicity in order to separate these classes of compounds was therefore investigated. In the first instance the strongly acidic cation-exchange resin Dowex 50W-X8 and the weakly acidic cation-exchange resin Zerolit 236 SRC 41 were tested using mixtures of authentic compounds (1 ppm each) in 4% aqueous ethanol and eluting the resin columns with 2 *M* aqueous sodium chloride. The compounds used included pyrazines, pyridines, amines and thiazoles as well as several neutral and acidic volatile beer flavour constituents.

Some typical recoveries achieved using the Dowex resin are given in Table II. All of the basic compounds tested were recovered to some extent and were thus separated from non-basic compounds. However, recoveries of certain basic compounds were extremely poor, and in any case the results indicated that the resin was not suitable for separating pyridines and/or thiazoles from pyrazines.

TABLE II

RECOVERIES OF COMPOUNDS FROM 4% AQUEOUS ETHANOL USING DOWEX 50W-X8 RESIN (62 × 9 mm)

Compound	*Recovery (%)*
2,5-Dimethylpyrazine	41
2,3-Dimethylpyrazine	44
Thiazole	44
2-Butyl-4,5-dimethylthiazole	19
2-Methylpyridine	57
2-Ethylpyridine	55
Ethyl octanoate	0
l-Carvone	0

TABLE III

RECOVERIES OF COMPOUNDS FROM 4% AQUEOUS ETHANOL USING ZEROLITE 236 SRC 41 (110 × 12 mm)

Compound	*Recovery (%)*
2-Methylpyrazine	0
2,3-Dimethylpyrazine	0
Thiazole	0
4-Methylthiazole	4
2-Isobutylthiazole	25
4-Ethyl-5-methylthiazole	68
2-Isopropyl-4-methylthiazole	67
2-Butyl-4-methylthiazole	62
2-Butyl-4,5-dimethylthiazole	56
Pyridine	64
2-Methylpyridine	53
2-Ethylpyridine	88
2,4-Dimethylpyridine	80
2,4,6-Trimethylpyridine	87
Ethyl valine	70
Ethyl isoleucine	75
2-Acetylpyrrole	0
2-Acetylfuran	0
2-Acetylthiophene	0
Isoamyl acetate	0
Ethyl octanoate	0
Isoamyl alcohol	0
2-Phenylethanol	0
l-Carvone	0
Octanoic acid	0

Table III shows some recoveries achieved in similar experiments using the Zerolit resin. In contrast to the results obtained using the Dowex resin, the results indicated in Table III clearly show that the Zerolit resin may be successfully used to separate pyridines and certain alkylthiazoles from pyrazines. Experiments in which the Zerolit resin was eluted successively with several batches of 2 *M* aqueous sodium chloride revealed that, whilst pyridines were relatively easily eluted from the resin under these conditions, some of the thiazoles were bound more strongly to the resin and thus required a much larger volume of eluent.

The potential use of the Zerolit resin for concentrating preferentially, small quantities of pyridines and certain thiazoles from an aqueous medium containing several per cent of ethanol as well as relatively large amounts of many other volatile flavour constituents including pyrazines, was therefore established. A method for concentrating such pyridines and thiazoles from beer, based on the use of the resin, was thus designed (see Experimental) and subsequently tested.

A commercial stout was chosen for the initial study, since this type of beer generally has high levels of nitrogen-containing heterocyclic compounds. Examination of the extract by GC, employing the use of a capillary column, revealed it to be extremely complex in composition although subsequent analysis by GC–MS showed neither pyridines nor thiazoles to be amongst the major components present. However,

application of the technique of mass fragmentography[14] did reveal a series of pyridines to be present in the extract at relatively low levels. These included pyridine itself, three compounds of molecular weight 93 (2-, 3- and 4-methylpyridines) and nine compounds of molecular weight 107 (2-, 3- and 4-ethyl- and 2,3-, 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dimethylpyridines). Whilst some of these compounds have distinctive mass spectra which allow tentative structural assignment to be made, the majority have spectra which are rather similar[15,16]. However, employing the technique of peak enhancement with authentic compounds, positive structural assignment was made in all cases. Other compounds detected in the basic extract of stout include a group of amino acid ethyl esters, namely ethyl valine, ethyl leucine and ethyl isoleucine, which were identified for the first time in beer[7]. There were also indications that the extract contained a series of aliphatic amines, a class of compounds which have previously been detected in beer[1]. However, mass fragmentography confirmed the expected absence from the extract of several alkylpyrazines which had previously been readily detected in the same stout using the solvent extraction procedure mentioned above. Application of mass fragmentography, using ions characteristic of alkylthiazoles[17], also failed to reveal the presence of any of this class of compound in the stout extract. Harding *et al.*[18] have detected a series of ten thiazoles in roasted barley, which is one of the raw materials used in brewing stouts as well as some ales. However, the absence of these compounds in beer extracts prepared in the present studies is to be expected at least for the simplest alkylthiazoles which, as pointed out above, are very poorly retained by the Zerolit ion-exchange resin; presumably the more highly substituted thiazoles do not survive the brewing process in amounts sufficient for detection by this method.

When basic extracts of a series of other beers (including ales and a lager) were prepared and analysed in the same way, some pyridines were detected but these were generally at much lower levels than had been detected in the extract from the commercial stout. It was apparent that in some of these extracts the compounds, if present at all, must be at levels well below the detection limit of the method. It was therefore decided to re-examine all of the extracts prepared using the much more highly sensitive method of MID[14]. Thus effluent from the capillary column was scanned (at 1-sec intervals) for components having mass spectra exhibiting ions of *m/e* 92, 106 and 107 (residence time per ion per scan = 0.13 sec) and *m/e* 101 and 127 (residence time per ion per scan = 0.01 sec). In this way ethyl- and dimethylpyridines (whose identities were confirmed by means of retention time and *m/e* 92:106:107 pattern) were quantified by measuring their *m/e* 107 response relative to the *m/e* 101 response of ethyl octanoate which was added to each sample as an internal standard. Relative response factors for the pyridines were determined by chromatographing a mixture containing 1 ppm each of the ethyl- and dimethylpyridines and again using MID with quantification based on measurement of the *m/e* 107 peak areas. Table IV lists the relative levels of ethyl- and dimethylpyridines which were measured in the range of commercial and experimental beers examined. Fig. 1 shows a chromatogram, reconstructed from the *m/e* 107 ion current, obtained from a basic extract of the commercial stout to which had been added 1, 0.5 and 0.1 ppb, respectively, of 2,5-dimethylpyridine, 4-ethylpyridine and 3,5-dimethylpyridine. The magnitudes of the increases in sizes of the responses for these three compounds indicate that all of the pyridines listed in Table IV were present in the untreated stout at levels below 1 ppb,

TABLE IV

RELATIVE LEVELS OF ETHYL- AND DIMETHYLPYRIDINES OCCURRING IN A RANGE OF BEERS

The figures in this table refer to corrected peak areas, the highest level being arbitrarily set to 1000. n.d. = not detected.

Pyridine	*Beer*			
	Commercial stout	*Experimental beer**	*Commercial ale*	*Commercial lager*
2,6-Dimethyl	112	29	31	1
2-Ethyl	93	3	1	0.3
2,5-Dimethyl	143	15	1	n.d.
2,4-Dimethyl	302	134	11	0.6
3-Ethyl	1000	74	36	2
4-Ethyl	120	26	n.d.	n.d.
3,5-Dimethyl	26	4	n.d.	n.d.
3,4-Dimethyl	797	196	5	n.d.

* Beer brewed with a grist containing 30% crystal malt.

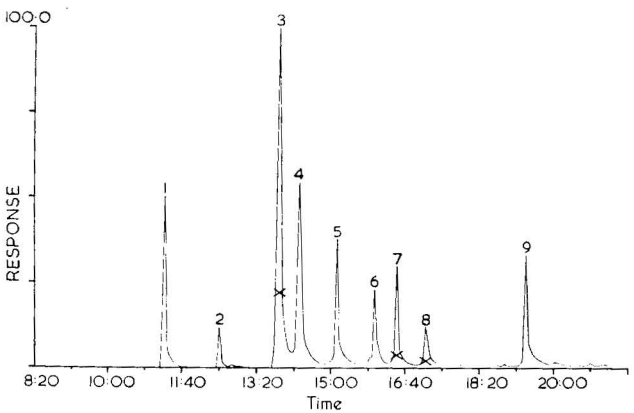

Fig. 1. Section of *m/e* 107 ion current chromatogram of extract from commercial stout spiked with alkylpyridines. Peaks: 1 = 2,6-dimethylpyridine; 2 = 2-ethylpyridine; 3 = 2,5-dimethylpyridine; 4 = 2,4-dimethylpyridine; 5 = 2,3-dimethylpyridine; 6 = 3-ethylpyridine; 7 = 4-ethylpyridine; 8 = 3,5-dimethylpyridine; 9 = 3,4-dimethylpyridine. X signifies height of peaks from beer without additions.

and in the other beers at even lower levels, becoming barely detectable in the lager. In the present studies the presence of alkylpyridines in wort prior to fermentation was also confirmed, similar levels being detected in an experimental beer (see Table IV) and the wort from which the beer was prepared.

Alkylpyridines have been detected in a wide range of food products, including whisky[19], cooked rice[20], wheaten bread[21], roasted lamb fat[22], black tea[23], Soy sauce[24], roasted cocoa[25], etc. A limited number of alkylpyridines have also been detected in roasted barley[18], but the majority of identifications reported in this case were tentative only. In addition to the occurrence, in food products, of alkylpyridines derived from natural precursors, these substances are sometimes deliberately added as flavouring

agents, often at levels well in excess of 1 ppm[26–28]. The method described here could well be applicable to the study of food products other than beer which contain the products of non-enzymic browning.

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Note

The possibility of using osmotic phenomena in an eluent pump for column liquid chromatography

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Advances in liquid chromatography (LC) over the past few years have been dramatic, but its wider implementation is hindered by the complexity of the apparatus involved. One of the most complex and essential units of a liquid chromatograph is the device for initiating an eluent flow (pump). Many investigators have been involved in pump design and much has been published, the excellent review by Martin and Guiochon[1] being a good example. An LC pump must obviously meet the following requirements: (1) simple design, (2) practically complete absence of pressure fluctuations, (3) reliability and (4) moderate cost. Naturally, the pump must not be a source of impurities that may contaminate the eluent.

In order to assess the feasibility of new pump designs, we have tried a pump based on a principle never before used in liquid chromatography, namely osmosis[2]. A paper by Pretorius *et al.*[3] dealt with the use of electroosmosis and not osmosis; the technique described influenced the separation process in the column.

The performance of the proposed pump is based on providing an eluent flow in LC by means of osmotic pressure. The pressure is created by an osmotic system in which the concentration of the solution is maintained constant by keeping it in contact with the solid phase of the solute. The temperature of the system is also constant. As is well known[4], osmosis depends on the phenomenon that contact between a solute and a solvent through a semi-permeable membrane gives rise to an increase in pressure, called the osmotic pressure, as a result of penetration of the solvent into the solution. As far as concentrated solutions are concerned, the parameters on which the osmotic pressure is dependent are not yet well known, nor is the functional relationship between them. The equilibrium osmotic pressure is a function of solute concentration and temperature:

$$P = \varphi(C,T) \tag{1}$$

where C is the solute concentration and T is temperature.

In known osmotic systems the solute concentration tends to decrease because of dilution of the solution by the solvent flow, which results in a pressure drop. However, by maintaining the solute concentration in the solution constant one can attain a constant osmotic pressure at an appropriate flow-rate. In particular, the solute concentration can be maintained constant by keeping the solution of a sub-

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stance in contact with its solid phase. This is precisely what is used in the first version of our osmotic cell as a source of pressure for LC.

Fig. 1 shows as an example a version of the device embodying the proposed method for creasing an eluent flow in LC[4]. The eluent occupies a compressible volume (1) confined by a flexible partition (2) (or by the eluent and immiscible solution interphase) inside a rigid container (3) communicating with that space (4) of the osmotic system which accommodates a saturated solution of the substance and the solid phase (5) of the latter, separated by a semi-permeable membrane (6) from the solvent occupying another space (7) of the osmotic system.

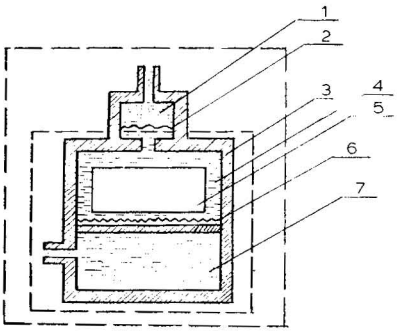

Fig. 1. Osmotic cell. 1 = Eluent; 2 = flexible partition; 3 = rigid container; 4 = saturated solution; 5 = solid phase of the substances; 6 = semi-permeable membrane; 7 = solvent (water).

The operation of the device can be described as follows. Contact between the solution and solvent through the membrane, which is permeable to the solvent and impermeable to the solute, initiates a solvent flow which gives rise to an osmotic pressure. Under the effect of osmotic forces the solvent passes through the semi-permeable membrane into a vessel containing the solvent. To maintain the solution concentration constant at the saturation level at a given temperature, the solution is made to contact an excess amount of the solute which, in a particular instance, is a salt in the solid phase. The osmotic pressure arising in the solvent container is transmitted to the vessel with the eluent. Thus, the eluent flows at a constant rate. To corroborate this finding we recorded, for comparison, the pressure fluctuations in the proposed osmotic pump and in a commercially available pump. In the former the pressure fluctuations were virtually nil, whereas in the MP-2C pump the fluctuations in flow constitute 1–2% of the nominal value.

EXPERIMENTAL

The test the proposed method for providing a uniform eluent flow in LC we developed a special device consisting of two compartments separated by a semi-permeable membrane of acetate cellulose on a cermet substrate. Distilled water was used as the solvent. A saturated solution of magnesium sulphate in water was placed above the membrane and magnesium sulphate (solid phase) was used to maintain the solution concentration constant. Water was fed from the cermet substrate side. The membrane area was 25 cm^2, the cell temperature was 22°C and *n*-hexane was used as the eluent.

Fig. 2 shows schematically the arrangement of the osmotic system for studying the effect of cell parameters on the eluent flow-rate.

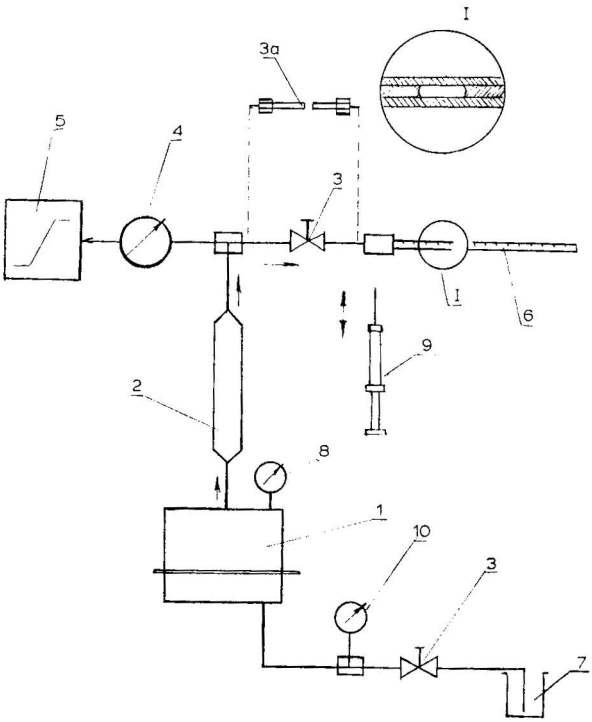

Fig. 2. Osmotic system for studying the effect of cell parameters on the eluent flow-rate. 1 = Osmotic cell; 2 = eluent; 3 = fine-adjustment valve; 3a = liquid column; 4, 5 = automatic pressure recorder; 6 = metering capillary; 7 = distilled water; 8, 10 = pressure gauges; 9 = syringe.

RESULTS AND DISCUSSION

Fig. 3 shows the eluent flow-rate *versus* pressure in the osmotic cell.

The functional relationship between the eluent flow-rate and pressure can be explained with the aid of the equivalent shown in Fig. 4.

By analogy with an equivalent electric circuit diagram where the osmotic cell

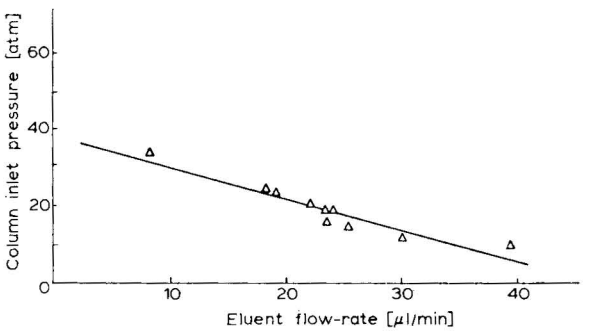

Fig. 3. Eluent flow-rate *versus* osmotic cell pressure.

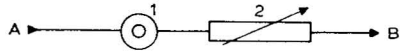

Fig. 4. Diagram to illustrate the relationship between eluent flow-rate and osmotic pressure. A and B = inlet and outlet, respectively of the system at a pressure of about 1 atm; 1 = osmotic cell; 2 = flow resistance, including the variable resistance used for controlling the flow through the chromatographic column plus the constant flow resistance.

may be regarded as equivalent to a battery, we can write

$$P_0 = WR_0 + WR \tag{2}$$

where P_0 is the total pressure in the system, W is the eluent flow-rate in the system, R_0 is the flow resistance of the osmotic cell and R is the variable flow control resistance plus the column resistance. As can be inferred from eqn. 2, the column inlet pressure

$$P_{in} = P_0 - WR_0 \text{ (where } P_{in} = WR) \tag{3}$$

Eqn. 3 shows that P_{in} decreases linearly with increasing eluent flow-rate, W, in the system. The derived equation agrees well with experiment (see Fig. 3).

A chromatogram obtained with the above-described osmotic system is shown in Fig. 5.

We believe that such pumps will be particularly useful in microcolumn chromatography.

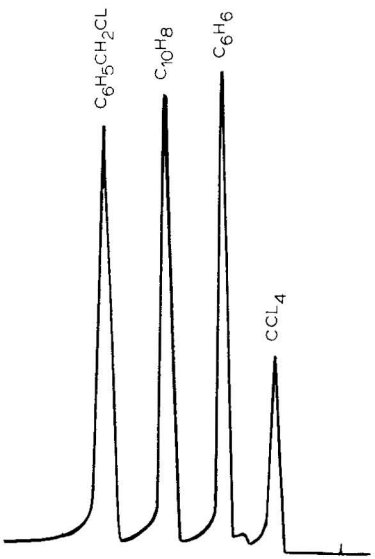

Fig. 5. Chromatogram taken on a VKS-11 liquid chromatograph incorporating the proposed osmotic pump. Column, silica gel Separon SI VSK (5 μm), packing, length 15 cm, I.D. 4 mm; eluent, *n*-hexane, flow-rate 3 ml/h; sample, CCl_4 + C_6H_6 + $C_{10}H_8$ + $C_6H_5CH_2Cl$ (2.5 l).

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Note

Effect of ammonium hydroxide concentration on the recoveries of amino acids during preparation for gas–liquid chromatography

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In preparation for analysis by gas–liquid chromatography (GLC), amino acids on biological samples, or hydrolysates, are separated from potential interfering compounds using a strong cation-exchange resin (sulfonated polystyrene). Ammonium hydroxide at a concentration of 2 N[1,2], 3 N[3], or 7 N[4–6] is used to elute amino acids from the cation-exchange resin. During the initial stages of adapting a cation-exchange procedure for isolation of amino acids from biological substances, it was found that upon subsequent GLC measurement, recoveries of lysine and arginine were less than 50% and highly variable when ammonium hydroxide at concentrations greater than 3 N was used as the eluant. Recovery of arginine was always higher than that of lysine, since NH_4^+ displaces lysine from a resin of sulfonated polystyrene more readily than it displaces arginine[7,8], the recovery of lysine rather than arginine was expected to be higher. This note reports the effects of high concentrations of ammonium hydroxide upon the GLC measurement of lysine and arginine in a solution of amino acids of known concentration, and an improved recovery of amino acids in the presence of dilute ammonium hydroxide solutions.

EXPERIMENTAL

Amino acid, and internal and external standards were dissolved in aqueous 0.1 N hydrochloric acid. An amino acid (amino acids purchased as kit LAA-21 from Sigma, St. Louis, MO, U.S.A.) solution containing 2.5 mM L-alanine, glycine, L-valine, L-threonine, L-serine, L-leucine, L-isoleucine, L-proline, L-methionine, L-aspartic acid, L-phenylalanine, L-glutamic acid, L-lysine–HCl, L-tyrosine, L-arginine–HCl, and L-histidine–HCl and 1.25 mM L-cystine was prepared. Cycloleucine (Aldrich, Milwaukee, WI, U.S.A.) was used (2.5 mM) as an internal standard. Pipecolic acid–HCl (Sigma) was used (2.5 mM) as an external standard.

The solvents under study included distilled deionized water and 1, 2, 3, 4 and 5 N ammonium hydroxide. To triplicate 3-ml samples of each solvent in 13 × 100 mm

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TABLE I

MEAN RELATIVE MOLAR RATIOS AND COEFFICIENTS OF VARIATION FOR AMINO ACIDS PREPARED FOR GAS–LIQUID CHROMATOGRAPHY FROM SOLUTIONS OF VARIOUS CONCENTRATIONS OF AMMONIUM HYDROXIDE

$$RMR = \frac{\text{(peak area amino acid/molar concentration amino acid)}}{\text{(peak area internal standard/molar concentration internal standard)}}$$

C.V. = (Standard deviation/mean) × 100.

Amino acid	*Treatment*											
	Water		*Ammonium hydroxide (N)*									
			1		*2*		*3*		*4*		*5*	
	RMR	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*	*RMR*	*C.V.*
Alanine	0.69	3.9	0.71	1.4	0.67	4.0	0.69	4.7	0.64	15.9	0.50	25.8
Glycine	0.64	0.9	0.63	1.6	0.56	9.3	0.59	4.4	0.60	5.8	0.55	8.5
Valine	0.95	3.3	0.94	2.1	0.97	4.1	0.96	3.6	0.90	16.7	0.78	19.7
Threonine	0.96	3.3	0.95	1.0	0.88	8.9	0.91	4.0	0.97	4.1	0.91	6.4
Serine	0.86	4.7	0.86	1.2	0.79	8.6	0.80	3.8	0.89	3.9	0.85	10.0
Leucine	1.06	0.9	1.09	0.9	1.12	3.6	1.10	2.7	1.11	2.5	1.05	1.9
Isoleucine	0.98	1.0	0.95	3.8	1.03	6.5	0.96	3.6	0.93	17.4	0.86	16.5
Proline	0.86	0.0	0.90	0.7	0.89	3.6	0.89	1.9	0.96	5.7	0.94	9.8
Methionine	0.89	1.1	0.90	1.7	0.88	10.8	0.87	2.3	0.79	15.2	0.75	14.4
Aspartic acid	1.14	0.0	1.12	1.0	1.19	0.9	1.13	1.2	1.28	11.6	1.26	15.3
Phenylalanine	1.34	1.1	1.35	2.3	1.59	22.3	1.40	3.9	1.47	8.0	1.45	12.4
Glutamic acid	1.16	0.5	1.14	0.5	1.14	5.1	1.14	1.8	1.32	16.2	1.32	16.7
Lysine	1.04	6.7	1.02	5.4	0.78	41.8	0.91	7.3	1.16	26.4	1.21	19.8
Tyrosine	1.36	0.8	1.37	2.8	1.37	2.9	1.40	2.1	1.61	13.6	1.54	17.7
Arginine	1.01	4.7	0.96	4.6	0.53	74.9	0.86	10.8	0.78	20.6	0.87	7.6
Cystine	0.89	2.6	0.85	3.6	0.62	47.5	0.83	11.3	1.69	53.1	1.31	—

culture tubes, were added 100 μl of the amino acid solution and 100 μl of the internal standard. Culture tubes were kept in an ice bath and all solutions were stored at 4°C before use. The culture tubes were capped with PTFE-lined screw-caps and the solutions were thoroughly mixed, frozen immediately at −30°C, and lyophilized.

The lyophilized amino acid residues were prepared as the isobutyl-N(O)-heptafluorobutyryl esters[9,10]. A co-injection of heptafluorobutyric anhydride (HFBA) with each preparation of amino acid esters (dissolved in methylene chloride) was necessary to obtain repeatable relative molar ratios (RMR) for the esters of serine, arginine and cystine. With a co-injection of HFBA, the histidine ester appeared between the esters of phenylalanine and glutamic acid with a low recovery relative to the internal standard. Results for the histidine ester are, therefore, not reported. RMR, as defined by Gehrke and Stalling[11], and noted in the footnote of Table I, were calculated ($n = 3$) with cycloleucine as the internal standard. Coefficients of variation (C.V.) for the mean RMR of each amino acid were calculated according to Snedecor and Cochran[12].

The absolute, rather than relative, recoveries of individual amino acids from a cation-exchange resin (AG 50W-X8, H^+, 200–400 mesh; Bio-Rad Labs. Richmond, CA, U.S.A.) were determined in triplicate with 1 *N* ammonium hydroxide as the eluent, using the procedure of Boila and Milligan[9]. A 100-μl volume of the solution of amino acids plus 100 μl of internal standard solution were each added to a column (155 × 10 mm I.D.) of cation-exchange resin (bed-volume of 1 ml) and eluted with 1 *N* ammonium hydroxide. Recoveries of amino acids and cycloleucine from the column of cation-exchange resin were calculated using pipecolic acid as the external standard (100 μl) added to the combined fractions eluted with 10 ml 1 *N* ammonium hydroxide plus a 5-ml water wash. The combined eluate was frozen to −30°C, lyophilized and prepared fro chromatography.

RESULTS AND DISCUSSION

At a concentration of 2 *N* ammonium hydroxide or higher, mean RMR of several amino acids were different than for the treatments entailing distilled deionized water or 1 *N* ammonium hydroxide (note phenylalanine, lysine, tyrosine, arginine and cystine, Table I). A reduced RMR indicates a lower recovery of an amino acid relative to the internal standard, while an increased RMR indicates a higher recovery of an amino acid relative to the internal standard. In addition to changes in RMR, C.V. of RMR for each amino acid listed in Table I were increased which indicates that there was a greater variability in the recovery of amino acids at higher concentrations of ammonium hydroxide. This increased variability for RMR, at 2 *N* ammonium hydroxide or higher, precludes precise quantitative measurement of the concentration of amino acids in biological samples.

The amounts of the amino acids recovered from the ion-exchange chromatography using 1 *N* ammonium hydroxide as the eluant were 90%, or more, of the amounts added with the exception of lysine and arginine. Recoveries of lysine and arginine were approximately 80% with C.V. of 2.5% and 3.2%.

Clearly, the variability for RMR of individual amino acids, among triplicate analyses, can be reduced by using 1 *N* ammonium hydroxide to elute the amino acids from a sulfonated polystyrene cation-exchange resin in preparation for GLC.

At this low concentration of ammonium hydroxide the recovery of amino acids and internal standard from the resin are maximized and a repeatable RMR is obtained.

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Note

Identification of a new metabolite after incubation of N-benzylaniline with rabbit liver microsomes

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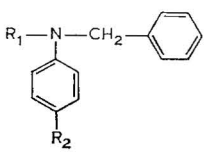
Previous workers reported the *in vitro* metabolism of N-benzylaniline in an investigation of the toxicity of alkyl- and aryl-substituted anilines[1]. Aniline and nitrobenzene were obtained as metabolites after intravenous injection of N-benzylaniline (1) into cats.

Primarily, we were studying the metabolism of the antihistaminic drug antazoline (3). We have shown that N-benzylaniline was one of the major metabolites of antazoline[2]. We therefore found it necessary to examine the metabolic fate of N-benzylaniline (1) using rabbit liver microsomal fraction. The stability and extractability of the metabolic product under the conditions of incubation and analysis were also examined. Structures of the compounds concerned are shown in Fig. 1.



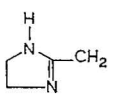
R_1	R_2	Compound
	H	Antazoline, 2-(N-benzylanilinomethylimidazoline)
H	H	N-Benzylaniline
H	OH	p-Benzylaminophenol

Fig. 1. Structures of antazoline (3), N-benzylaniline (1) and *p*-benzylaminophenol (2).

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EXPERIMENTAL

Compounds and reagents

Antazoline·HCl (3) was supplied by Ciba-Geigy (Basle, Switzerland). N-Benzylaniline (1) was obtained from BDH (Poole, Great Britain), *p*-benzylaminophenol (2) from Eastman (Rochester, NY, U.S.A.), $NADPNa_2$, glucose-6-phosphate, disodium salt, and glucose-6-phosphate dehydrogenase from Boehringer (Mannheim, G.F.R.), NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.), acetonitrile from Fisons (Loughborough, Great Britain) (redistilled and kept over calcium chloride at room temperature), AnalaR diethyl ether from BDH (freshly distilled), 2,3,5-triphenyl-2*H*-tetrazolium chloride monohydrate (98%) from Aldrich (Milwaukee, WI, U.S.A.), *n*-pentane from Fisons, iron(III) chloride from May and Baker (Dagenham, Great Britain) and sodium metabisulphite ($Na_2S_2O_5$) from BDH.

Thin-layer chromatography (TLC)

Glass plates (20 × 20 cm) were sprayed to a thickness of 0.25 mm with a mixture of silica gel G (Merck, Darmstadt, G.F.R.) and water (1:2). The plates were allowed to dry at room temperature for 1 h at 110°C before use. The solvent system was chloroform–acetone (9:2). The various spots were revealed by spraying with (a) ammoniacal silver nitrate solution; (b) triphenyltetrazolium chloride (TTC); (c) iron (III) chloride solution (5% in 0.5 *N* hydrochloric acid) and (d) Dragendorff's reagent. These spray reagents were prepared and used according to the methods described by Merck[3].

Gas–liquid chromatography (GLC)

A Perkin-Elmer Model F33 gas chromatograph equipped with a flame-ionization detector and a 1.0-mV Perkin-Elmer recorder was used. All columns were acid-washed and treated with dimethyldichlorosilane. The conditions were as follows. System A: 2-m glass column packed with Chromosorb Q (100–120 mesh) coated with OV-17 (3%, w/w) and operated with a hydrogen pressure of 1.12 kg/cm², air 1.68 kg/cm² and nitrogen 2.1 kg/cm². System B: 2-m glass column packed with Chromosorb W (80–100 mesh) coated with OV-17 (5%, w/w) and operated under the conditions described under system A. System C: 1-m glass column packed with Chromosorb W (80–100 mesh) coated with UCW-98 (10%, w/w) and operated with a hydrogen pressure of 2.1 kg/cm², air 0.7 kg/cm² and nitrogen 1.4 kg/cm². All of the columns were conditioned at 250°C for 24 h before use and the injection port temperature was 250°C. Each of these columns was silanized with 2 × 5 ml of hexamethyldisilazane (HMDS) before use.

Combined gas–liquid chromatography and mass spectrometry

All mass spectra were obtained using a Perkin-Elmer Model 270 gas chromatograph–mass spectrometer at an electron energy of 70 eV. A 1.0-m × 0.64 cm O.D. glass column packed with UCW-98 (10%, w/w) on Chromosorb W (80–100 mesh) was used at 190°C (oven temperature); helium (1.4 kg/cm²) was used as the carrier gas.

Metabolism

Incubation procedure. N-Benzylaniline·HCl (1) (10 μmol/ml; 1 ml) was in-

cubated at 37°C for 40 min with the microsomal fraction from a liver homogenate of a New Zealand white rabbit. Each incubation mixture contained 1 ml of substrate, 1 ml of cofactor solution made of glucose-6-phosphate, disodium salt (6 mg, 20 μmol), 0.6 *M* nicotinamide (0.1 ml, 60 μmol), $NADPNa_2$ (3.4 mg, 4 μmol), distilled water (0.7 ml); phosphate buffer, pH 7.4 (3 ml) and liver microsomal fraction (1 ml). Glucose-6-phosphate dehydrogenase (2 units) was added to the microsomal fraction preparation. Control experiments were carried out at the same time. In all instances, the incubation mixtures were incubated for 5 min at 37°C with shaking before the addition of the substrate. The incubation reactions were stopped by putting the flasks in ice and extracted as described.

p-Benzylaminophenol (2). To the incubation mixture (6 ml) was added $Na_2S_2O_5$ solution (10%, 1 ml) and sodium chloride (2 g). The pH was adjusted to 8.0–8.2 with ammonia solution (10%) and the mixture was extracted with freshly distilled diethyl ether. The concentrated ethereal extracts were examined by GLC systems A, B and C and by TLC. A separate portion of the concentrated ethereal extracts was allowed to dry under nitrogen gas; dry acetonitrile (10–15 μl) and BSTFA (10 μl) were added and the mixture was allowed to stand at room temperature for 5 min. The trimethylsilyl derivative of *p*-benzylaminophenol (4) was then examined by GLC system A.

Extractability of p-benzylaminophenol (2) from buffer and microsomal preparations. Two solutions (a, b) of *p*-benzylaminophenol (2, 20 μg/ml, base) were freshly prepared in phosphate buffer (pH 7.4)–methanol (9:1, v/v) (a) and in freshly distilled diethyl ether (b). To two tubes each containing 5 ml of solution (a) were added rabbit liver microsomes (0.25 g/ml, 1 ml), sodium chloride (2 g) and $Na_2S_2O_5$ solution (10%, 1 ml). To another set of tubes [2 × 5 ml, (a)] was added phosphate buffer (pH 7.4, 1 ml) instead of the liver microsomes. All of the samples were adjusted to pH 8.0 with ammonia solution (10%) and extracted with freshly distilled diethyl ether (4 × 5 ml). N-Benzoylaniline (5) as an external standard (50 μg/ml, base in *n*-pentane, 1 ml) was added to each of the ethereal extracts and also to each of two samples (2 × 5 ml) of solution (b). The ethereal solutions were concentrated and analysed by GLC system B. The average peak-height ratios (*p*-benzylaminophenol/N-benzoylaniline) were compared and the value obtained for solution (b) was arbitrarily taken as 100%.

Stability of p-benzylaminophenol (2) in phosphate buffer (pH 7.6). A freshly prepared solution of *p*-benzylaminophenol (2, 20 μg/ml, 5 ml) in phosphate buffer (pH 7.4)–methanol (4:1, v/v) was placed in each of two 20-ml centrifuge tubes (a and b). Phosphate buffer (1 ml) was added to tube (a) and $Na_2S_2O_5$ solution (10%, 1 ml) to tube (b). Duplicate samples were taken in each instance. Both tubes were stored at room temperature for 40 h and the samples were then extracted and analysed by GLC system B as described above. Two control samples (1 and 2) of *p*-benzylaminophenol (2, 20 μg/ml, in diethyl ether, 5 ml each) were also prepared. The average peak-height ratios (*p*-benzylaminophenol/N-benzoylaniline) were compared and the value obtained for the control samples (1 and 2) was arbitrarily taken as 100%.

Quantitative analysis of p-benzylaminophenol (2). Standard solutions were always freshly prepared. Calibration graphs (in the appropriate biological fluid or buffer) based on the ratios of the peak heights of the compounds to be quantitated to

those of their GLC reference standards using the methods described were obtained representing six different concentrations (50–5 μg/ml, base). The results were subjected to linear regression analysis to give the appropriate calibration factors. In all metabolic studies *p*-benzylaminophenol (2) was analysed as its trimethylsilyl (TMS) derivative with methadone as the external standard using GLC system A.

RESULTS AND DISCUSSION

Stability and extractability of p-benzylaminophenol (2)

Complete decomposition of *p*-benzylaminophenol (2) occurred when it was kept in phosphate buffer (pH 7.4) for 40 h. However, 62% of *p*-benzylaminophenol was recovered from a similar buffer solution containing sodium metabisulphite (10%, Table I). Sodium metabisulphite, a known antioxidant, has been used to stabilize adrenaline injectable solutions (BP 1973)[4]. Sodium metabisulphite was therefore used to stabilize *p*-benzylaminophenol (2) aqueous solution throughout these studies.

TABLE I

STABILITY OF *p*-BENZYLAMINOPHENOL (2) STORED (a) IN PHOSPHATE BUFFER (pH 7.4) AND (b) IN PHOSPHATE BUFFER CONTAINING $Na_2S_2O_5$ (10%, 1 ml) FOR 40 h AT ROOM TEMPERATURE

Results are expressed as a percentage of the quantitative recovery of a control (samples 1 and 2, as described under Experimental). Duplicate samples were analysed in each instance.

Sample	*Average peak-height ratio*	*Recovery (%)*
Control	2.4	100
a	0	0
b	1.5	62

When *p*-benzylaminophenol (2) was extracted from phosphate buffer (pH 7.4) and phosphate buffer containing liver microsomes (the final microsome concentration was the same as that used in incubation experiments) at pH 8.0 with diethyl ether, the recoveries were 98 and 93%, respectively (Table II). The extraction method was thus demonstrated to be satisfactory. Extraction at pH values above 8.5 or the use of strong alkalis for pH adjustment is to be avoided as such conditions may lead to

TABLE II

RECOVERY OF *p*-BENZYLAMINOPHENOL (2) (a_1) IN PHOSPHATE BUFFER pH 7.4 AND (a_2) IN PHOSPHATE BUFFER CONTAINING RABBIT LIVER MICROSOMAL FRACTION AT THE SAME DILUTION AS USED IN INCUBATION EXPERIMENTS

Results are expressed as a percentage of quantitative recovery of a control (sample b, as described under Experimental). Duplicate samples were analysed in each instance.

Sample	*Average peak-height ratio*	*Recovery (%)*
Control	2.3	100
a_1	2.2	98
a_2	2.1	93

chemical breakdown of *p*-benzylaminophenol (2). It is important to check the recovery of compounds from liver homogenate preparations, as binding to such homogenates may lead to large errors in analytical procedures[5,6].

Quantitative analysis of p-benzylaminophenol (2)

Column A (Table III) was chosen for the quantitative analysis of *p*-benzylaminophenol (2) produced metabolically because of the shorter retention time obtained for *p*-benzylaminophenol (2). Both *p*-benzylaminophenol (2) and N-benzoylaniline (5) (external standard) gave tailing peaks and were not well separated on column A. A sharp and symmetrical peak was obtained for *p*-benzylaminophenol (2) after trimethylsilylation with BSTFA. Methadone (6) was found to be a suitable reference compound on the same column (A), giving sharp, symmetrical and well separated peaks. However, it was not possible to extract methadone (6) completely in the pH range (8.0–8.2) used for the *p*-benzylaminophenol (2) extraction. Therefore, methadone (6) was used as an external standard, to be added to the ethereal extract containing *p*-benzylaminophenol (2). Straight-line calibration graphs were obtained for *p*-benzylaminophenol (2) using the above method. In all instances the calibration graphs were constructed using the appropriate liver homogenate preparation.

Identification of the metabolic product

p-Benzylaminophenol (2) was identified as a metabolic product of N-benzoylaniline (1) as follows: (i) TLC of the ethereal extract (pH 8.0) gave a spot with an R_F value of 0.39. Black, violet and yellow spots were obtained upon spraying with ammoniacal silver nitrate, iron(III) chloride and triphenyltetrazolium chloride (TTC) reagents, respectively (Table III). (ii) GLC of the ethereal extract (pH 8.0) gave peaks with retention times of 6.0, 17.0 and 15.0 min on columns A, B and C, respectively (Table III). Derivatization with BSTFA gave the trimethylsilyl derivative of *p*-benzylaminophenol (4), with a retention time of 7.0 min (column A, Table III). (iii) The GLC–mass spectrum of the ethereal extract (pH 8.0) showed the presence of fragment ions at *m/e* 91 (100%) and 199 (50%), corresponding to the base peak and the molecular ion, respectively (Fig. 2a and b). The mass spectrum of the trimethyl-

TABLE III

GLC AND TLC CHARACTERISTICS OF ANTAZOLINE (3) N-BENZYLANILINE (1), *p*-BENZYLAMINOPHENOL (2) AND RELATED COMPOUNDS

*Compound**	*GLC retention times (min)**			*TLC R_F values*
	Column A	*Column B*	*Column C*	
Antazoline	11.0(240°C)	—	—	On the baseline
N-Benzylaniline	1.5(220°C)	5.0(230°C)	4.0(190°C)	0.96
AC N-benzylaniline	—	—	8.0(190°C)	—
p-Benzylaminophenol	6.0(220°C)	17.0(230°C)	15.0(190°C)	0.66
TMS *p*-benzylaminophenol	7.0(215°C)	—	—	—

* TMS = trimethylsilyl derivative; AC = acetyl derivative.

** The appropriate GLC oven temperatures are given in parentheses.

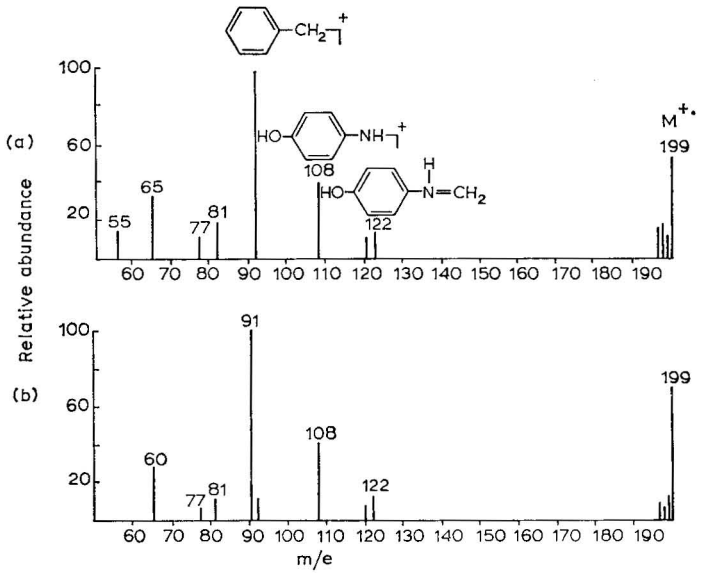

Fig. 2. Mass spectra of (a) synthetic *p*-benzylaminophenol (2) and (b) metabolically produced *p*-benzylaminophenol from N-benzylaniline (1).

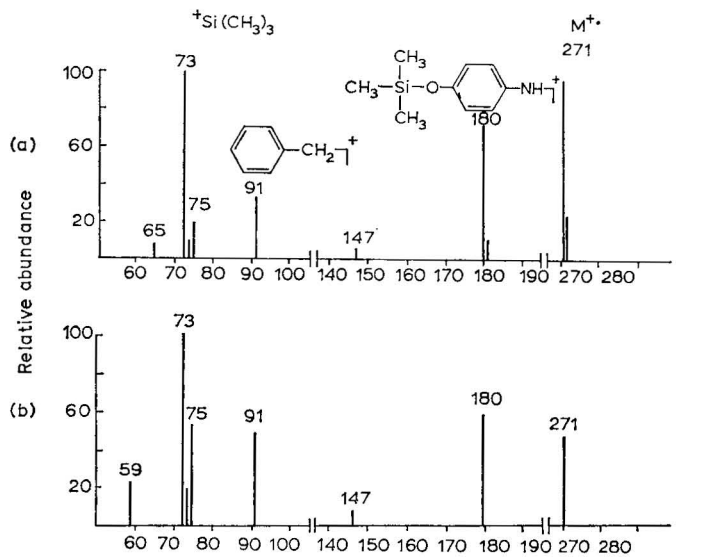

Fig. 3. Mass spectra of (a) synthetic *p*-benzylaminophenol and (b) metabolically produced *p*-benzylaminophenol from N-benzylaniline (1) as their trimethylsilyl derivatives.

silyl derivative (4) gave fragment ions at *m/e* 73 (base peak), 271 (molecular ion), 180, 91 and 75 (Fig. 3a and b). The fragment ions at *m/e* 73 and 75 are indicative of the trimethylsilyl derivatives[7,8].

The characteristics of the metabolically produced *p*-benzylaminophenol described above are identical with those of the authentic reference compound (2).

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Note

Inadvertent formation of polynuclear aromatic hydrocarbons by the flame sealing of glass ampules

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In the course of general chemical analysis of organic materials or as a method of storage or shipment of high purity reagents it is common practice to seal liquid samples in glass ampules. Borosilicate glass ampules are sealed with a small flame applied to the external surface of the glass neck. During the course of mass spectroscopic investigations of certain hexane extracts of fish oil which had been sealed in ampules for transport, a number of polynuclear aromatic hydrocarbons (PAHs) were found in these extracts. Further investigations showed that the flame sealing of the glass ampules led to PAH formation. We wish to alert other investigators to this problem and describe how the formation of PAHs during sealing can be avoided.

EXPERIMENTAL

Apparatus

A Waters Scientific high-performance liquid chromatograph consisting of two M-6000 A pumps, a No. 660 solvent programmer and a U6-K injector, was used throughout. Schoeffel SF 770 fluorescence and FS 970 UV detectors were used coupled to a Spectra-Physics System I computing integrator. UV and fluorescence chromatograms were recorded on Omniscribe and Linear recorders respectively at 10 mV full scale. The analytical column used was a VYDAC 201 TP reversed phase (25 cm × 3.2 mm I.D.; 10 μm).

Chromatographic analysis

Linear gradient elution from 70–100% (v/v) acetonitrile (Caledon Labs., Georgetown, Canada, HPLC grade) in glass-distilled water was used throughout. Inlet pressure was 1200 p.s.i.g.; flow-rate used was 1 ml/min. Wavelengths used were: UV, 265 nm; fluorescence, excitation at 280 nm, emission at >389 nm. Injection volumes varied from 1–10 μl. Individual PAHs were identified and quantitated by external and internal standardization as well as by the method of standard additions

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when necessary. Peak areas and retention times were obtained from the System I computing integrator.

Sample preparation

Pyrex glass ampules, 10 cm × 18 mm O.D., were washed with glass-distilled acetone, then hexane (Caledon, distilled-in-glass) and dried in a stream of dry nitrogen (Canadian Liquid Air, Halifax, Canada). A 2-ml volume of the test solvent was carefully placed in the bottom of the ampule, avoiding any wetting of the neck of the ampule. The ampule was then sealed using a propane/oxygen blowtorch (Fisher Scientific, Pittsburgh, PA, U.S.A.; No. 2-716), care being taken to avoid flaming the open end. After sealing, the ampules were allowed to cool standing with the sealed end uppermost (*ca.* 15 min). They were then shaken to rinse the walls with the enclosed solvent and, after opening, 100 μl of dimethyl sulfoxide (DMSO) (purified) were added to the ampule and the test solvent was evaporated under a stream of dry nitrogen into the 100 μl DMSO prior to HPLC analysis. Appropriate blanks were run throughout.

RESULTS AND DISCUSSION

The extremely heterogeneous mixture of PAHs formed during the sealing of hexane in a borosilicate ampule at room temperature is shown in Fig. 1A. It has been shown that combustion of organic materials results in the formation of very complicated mixtures of alkylated and non-alkylated PAHs, the relative amount of non-

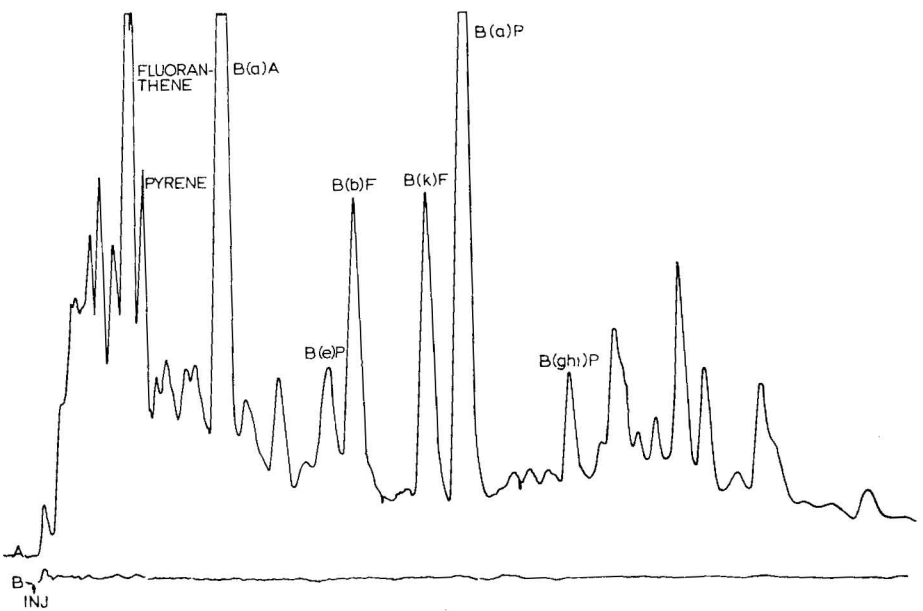

Fig. 1. A, chromatogram of a heterogeneous mixture of PAHs formed during the sealing of hexane in a borosilicate ampule at room temperature. B, chromatogram when ampule and contents are chilled before flame sealing.

TABLE I

POLYNUCLEAR AROMATIC HYDROCARBON CONCENTRATION, g/ml TEST SOLVENT

Abbreviations used: fluor = fluoranthene; chry = chrysene; B(*a*)A = benz[*a*]anthracene; B(*e*)P = benzo[*e*]pyrene; B(*b*)F = benzo[*b*]fluoranthene; B(*k*)F = benzo[*k*]fluoranthene; B(*a*)P = benzo[*a*]pyrene; B(*ghi*)P = benzo[*g,h,i*]perylene; ND = none detected.

PAH	*Pentane*	*Hexane*	*Heptane*	*Nonane*	*Benzene*	*Acetone*	*Dichloro-methane*	*Ethanol*	*Methanol*	*n-Propanol*	*n-Butanol*	*Aceto-nitrile*
Fluor	3994	2368	202	ND	606	22	13	ND	ND	ND	ND	ND
Pyrene	11,358	10,332	ND	59	ND	161	ND	ND	ND	ND	ND	ND
Chry	4867	3900	247	21	1125	55	43	ND	ND	ND	ND	ND
B(*a*)A	16,422	12,082	64	ND	272	27	25	ND	ND	ND	ND	ND
B(*e*)P	6641	6166	417	19	1115	98	ND	ND	ND	ND	ND	ND
B(*b*)F	723	690	65	2	126	13	ND	ND	ND	ND	ND	ND
B(*k*)F	164	119	12	0.3	12	2	ND	ND	ND	ND	ND	ND
B(*a*)P	662	630	53	0.4	38	15	3	ND	ND	ND	ND	ND
B(*ghi*)	3879	1523	193	1.5	120	65	ND	ND	ND	ND	ND	ND

alkylated PAHs increasing with increasing combustion temperature[1]. We have quantitatively determined nine common PAHs which are formed in the flame sealing process. We do not mean to imply that only non-alkylated PAHs are formed. In fact, due to the relatively low temperature of the melting glass, a relatively large percentage of alkylated PAHs should be formed. It is likely that oxygenated PAHs as well as a variety of other organic compounds are present in these ampules. (Fig. 18 illustrates also that chilling of the ampule and contents before flame sealing prevents PAH formation during sealing.)

Table I shows the concentrations in μg/ml of test solvent of nine individual non-alkylated PAH compounds formed when sealing various solvents in ampules at room temperature. It is evident that the concentration, *i.e.* formation, of PAHs decreases as solvents vary from pentane (low boiling) to nonane (high boiling). This decrease in PAH formation is probably due to the decreased volatility of the solvent as the chain length of the alkane increases. The situation is obviously more complicated than this since each alkane solvent tested formed a characteristic PAH distribution. Benzene, a volatile aromatic solvent also forms PAHs when flame sealed in ampules at room temperature. The amount formed fell between hexane and heptane, as did the boiling point, suggesting that aromaticicity of the solvent did not play an important role in PAH formation. Acetone and dichloromethane formed lesser amounts of PAHs presumably due to the presence of oxygen or chlorine in the starting solvent. The decomposition of the test solvents was also obvious from the discoloration that occurred. The nature of these decomposition products, particularly those from dichloromethane, is being further investigated. A number of solvents (acetonitrile, *n*-butanol, *n*-propanol, ethanol and methanol) did not form PAHs when sealed at room temperature. This is somewhat surprising since the boiling points of these solvents are in the range of those of the solvents forming PAHs.

We have been unable to find any report documenting formation of PAHs from organic solvents under the conditions described here. The amount of PAHs formed (up to 50 μg PAH/ml pentane) is surprising, especially when contrasted with the ease at which their formation can be prevented, by simply prechilling the ampule and contents in wet ice prior to sealing. This discovery has importance in at least three areas of chemistry:

(1) Trace residue analysis of foods, etc., at mg/kg or μg/kg levels, since it is common to store cleaned up extracts in such ampules. These extracts are often in hexane, pentane or benzene, all solvents which formed PAHs in our study. Reanalysis of these stored extracts would show higher PAH levels than the original analysis and could lead to confusion if tolerance levels are approached. For example, the allowable level of benzo[*a*]pyrene in meat and cheese in the Federal Republic of Germany is 1 μg/kg (ref. 2). Analysis of sealed extracts for certain organochlorine compounds such as polychlorinated biphenyls could be in error since many PAHs are electron-capture sensitive and depending on chromatographic conditions, could co-elute with the organochlorine compounds.

(2) Environmental baseline and monitoring levels. Contaminants in environmental samples such as seawater are often present at below ppb (10^9) levels. Formation of PAHs at the levels reported here can exceed environmental levels and erroneous reporting of PAHs could occur.

(3) Pure chemical and intercalibration studies. Since it is common to ship

high-purity chemicals, check sample standards and samples, sealed in glass ampules, the formation of PAHs could result in contamination of high-purity compounds and apparent changes in levels of organics in check sample standards and samples, especially those concerned with determination of trace amounts of PAHs (*e.g.* petroleum hydrocarbon intercalibration programs).

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Note

Gas chromatographic separation of amino acid amide enantiomers on optically active stationary phases

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The gas chromatographic (GC) resolution of N-acyl-DL-amino acid esters on optically active stationary phases has been studied in considerable detail since the first success by Gil-Av *et al.*[1]. Many workers tried mainly the variation of chemical structure in chiral stationary phases to obtain high efficiency[2,3], but none used chemical variations of the solutes other than different ester groups or N-acyl groups. It was the purpose of the present research to examine the effect on the separation of amino acid enantiomers of converting the carboxyl group of the solute into amide.

EXPERIMENTAL

N-Trifluoroacetyl-(N-TFA)amino acid isopropylamides were prepared from the N-TFA-amino acid chlorides by treatment with isopropylamine.

GC was carried out with a Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector. Chromatographic conditions used for the separation of optical isomers are summarized in Table I. N,N′-[2,4-(6-ethoxy-1,3,5-triazinediyl)] bis(L-valyl-L-valyl-L-valine isopropyl ester) (OA-300) and N,N′,N″-[2,4,6-(1,3,5-triazinetriyl)] tris(N^{α}-lauroyl-L-lysine-*tert*.-butylamide) (OA-400) were prepared as described previously[4,5].

RESULTS AND DISCUSSION

The results of the GC separation are given in Table I. In alanine, valine, leucine, methionine and phenylalanine, the separation factors for N-TFA-DL-amino acid isopropylamides were lower than those of N-TFA-DL-amino acid isopropyl esters. In contrast, it was noted that N-TFA-DL-proline isopropylamide was resolved with a high separation factor in spite of the fact that the same chromatographic conditions gave no detectable separation for N-TFA-DL-proline isopropyl ester. A typical chromatogram of N-TFA-DL-proline isopropylamide is shown in Fig. 1.

As is well known proline shows the lowest separation factor of all racemic amino acids in their N-acyl ester form, and this behaviour is thought to be due to the secondary amide group which has no hydrogen left on its nitrogen atom after acylation. The separation factor for N-TFA-DL-proline methyl ester is only 1.057 at 130°C on

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TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF SOME AMINO ACID ENANTIOMERS

Glass capillary column, 40 m × 0.25 mm I.D. Carrier gas (helium) flow-rate, 0.6 ml/min. A = OA-300; B = OA-400.

Amino acid	*Temperature (°C)*	*Stationary phase*	*N-TFA isopropyl ester*			*N-TFA isopropylamide*		
			Retention time (min)*		$\alpha_{L/D}$	*Retention time* (min)*		$\alpha_{L/D}$
			D	*L*		*D*	*L*	
Alanine	180	A	1.00	1.07	1.07	13.80	14.30	1.036
Valine	180	A	1.12	1.19	1.06	18.02	18.67	1.036
Leucine	180	A	1.93	2.05	1.06	24.40	24.71	1.013
Proline	180	A	3.40	3.40	1.00	9.60	10.77	1.122
	130	B	6.85	6.95	1.02	27.53	35.10	1.275
	100	B	25.56	26.32	1.030	134.9	191.3	1.418
Methionine	180	A	9.88	10.50	1.063	92.50	95.10	1.028
Phenylalanine	180	A	12.48	13.20	1.058	118.0	120.0	1.017

* Time from solvent peak.

N-lauroyl-L-valine-*tert.*-butylamide[6], which is the most powerful chiral stationary phase known for GC separation of enantiomeric amino acid esters. In this study the excellent separation factor for N-TFA-DL-proline isopropylamide was observed on OA-300 although the column temperature was rather high. When a moderate temperature was used, very high separation factors were obtained on OA-400. Hitherto it has been necessary to use long capillary columns for resolution of DL-proline in the ester

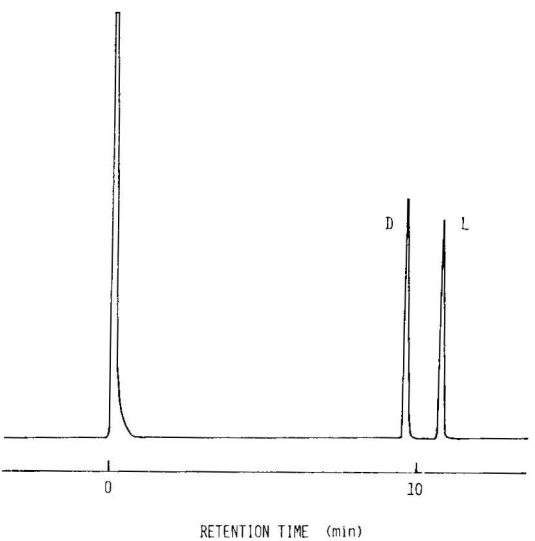

Fig. 1. Gas chromatogram of N-TFA-DL-proline isopropylamide. Column: glass capillary (40 m × 0.25 mm I.D.) coated with OA-300. Temperature: 180°C.

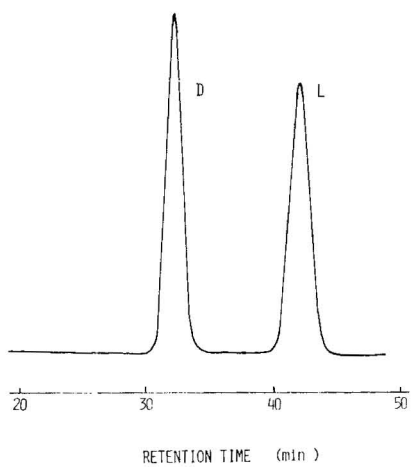

Fig. 2. Gas chromatogram of N-TFA-DL-proline isopropylamide. Column: 2 m × 3 mm I.D., containing Chromosorb W AW DMCS (100–120 mesh) coated with 5% of OA-400. Temperature: 130°C.

form, but such high separation factors allow the separation with a packed column as shown in Fig. 2.

Proline is used as a chiral reagent for conversion of the enantiomers of various alcohols, amines and amino acids into diastereomers and to resolve the optical isomers by GC using usual optically inactive stationary phases[7]; therefore, it is important to estimate its optical purity. This study enabled us to develop a new analytical method for the determination of optical isomers of proline. These results will be reported elsewhere.

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Note

Gas chromatographic separation of enantiomers of some dipeptides on an optically active stationary phase

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(Received August 11th, 1980)

The advantage of gas chromatographic (GC) separation of diastereomeric dipeptides has been demonstrated by Weygand *et al.*[1] in their study on racemization occurring in peptide syntheses. Halpern and Westley[2] subsequently resolved many amino acids as N-trifluoroacetyl (TFA)-L-prolyl peptide methyl esters. Westley *et al.*[3] have also resolved a series of cyclic dipeptides (diketopiperazines) for the determination of optical purity and absolute configuration of diastereoisomers.

As the GC separation in these studies was achieved with ordinary optically inactive stationary phases, the enantiomers of dipeptides were not resolved. Therefore it was necessary to hydrolyse the dipeptides and determine the configuration of the resulting amino acids in order to distinguish DL from LD or DD from LL diastereoisomers.

In this paper we report the direct GC separation of enantiomers of some linear and cyclic diastereomeric dipeptides with an optically active stationary phase.

EXPERIMENTAL

The dipeptides alanylalanine (I), valylvaline (II), prolylalanine (III) and prolylvaline (IV), were prepared from the N-TFA- or N-pentafluoropropionyl (PFP)-amino acid chlorides by treatment with the corresponding amino acid esters. Cyclic alanylalanine (V) was kindly provided by Dr. Y. Yamamoto of Kyoto University, Kyoto, Japan.

GC was carried out with a Shimadzu GC-7A gas chromatograph equipped with a flame ionization detector. Chromatographic conditions used for the separation of optical isomers are summarized in Table I. A thermostable optically active stationary phase, N,N'-[2,4-(6-ethoxy-1,3,5-triazine)diyl]bis(L-valyl-L-valyl-L-valine isopropyl ester) (OA-300) was prepared as described previously[4].

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TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS OF DIPEPTIDES*

	Dipeptide	*Column temp. (°C)*	*Retention time** (min)*		*Separation factor, α (DL/LD)*	*Retention time** (min)*		*Separation factor, α (LL/DD)*
			LD	*DL*		*DD*	*LL*	
I	DL-Alanyl-DL-alanine***	180	37.1	38.5	1.038	39.7	45.4	1.144
II	DL-Valyl-DL-valine§	180	34.6	37.2	1.075	37.2	38.6	1.038
III	DL-Prolyl-DL-alanine***	180	34.2	32.8	0.959	43.4	50.1	1.154
IV	DL-Prolyl-DL-valine***	180	41.1	39.5	0.961	49.3	55.9	1.134
V	Cyclo-DL-alanyl-DL-alanine	185	183.4		—	166.0	170.8	1.029

* Chromatographed on a glass capillary column (40 m × 0.25 mm I.D.) coated with OA-300; carrier gas, helium at a flow-rate of 0.5–0.7 ml/min.

** Time from solvent peak.

*** Resolved in the form of N-TFA-isopropyl ester.

§ Resolved in the form of N-PFP-isopropyl ester.

RESULTS AND DISCUSSION

The results of the GC separation of optical isomers of dipeptides are given in Table I. The LD, DL, DD and LL isomers of I, III and V were resolved as their N-TFA-dipeptide isopropyl esters. A typical chromatogram is shown in Fig. 1. The peaks of DL and DD isomers of II were superimposed under these conditions. It is notable that the LD and DL, and DD and LL isomers which have not been separated on ordinary optically inactive stationary phases, are resolved with good separation factors (1.038–1.154).

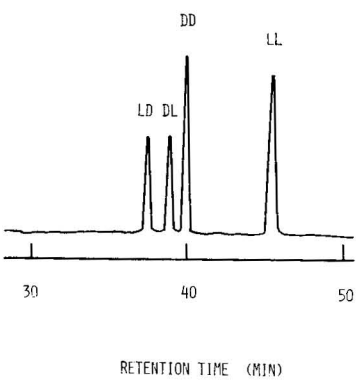

Fig. 1. Gas chromatogram of N-TFA-DL-alanyl-DL-alanine isopropyl ester. Conditions: see Table I.

Peak identifications were made by chromatographing successively racemic and some synthetic mixtures of enantiomers of dipeptides with known configuration; DD isomers eluted prior to LL isomers in every instance. This indicates that there is a greater interaction between LL isomers and the optically active stationary phase with

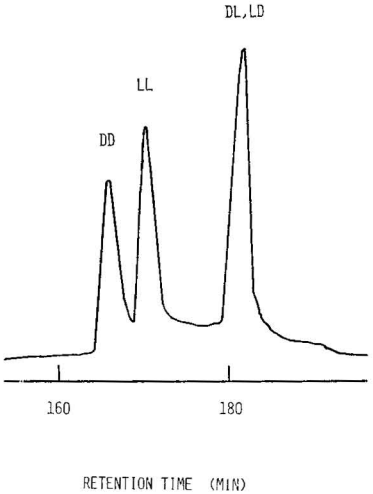

Fig. 2. Gas chromatogram of cyclo-DL-alanyl-DL-alanine. Conditions: see Table I.

an L-amino acid moiety than is the case for DD isomers. These findings are supported by the fact that D-isomers of both N-acylamino acid esters and amides have shorter retention times than L isomers on the same chiral stationary phase, OA-300.

The order of elution of the four isomers of I and II was LD > DL > DD > LL, and for III and IV was DL > LD > DD > LL. It is interesting that the elution sequence of the LD and DL isomers is reversed. It is considered that the alanine ester part of I, the valine ester part of II, and the proline amide part of III and IV make a greater contribution to the separation, and the enantiomers with the L configuration in these moieties are more retained in the column. This view is compatible with the findings that the separation factor of N-TFA-alanine amide is smaller than that of N-TFA-alanine ester, and that of N-TFA-proline amide is much larger than those of N-TFA-alanine and valine esters[5].

Cyclic alanylalanine (2,5-dimethyldiketopiperazine) was resolved into three peaks (Fig. 2). As the DL- and LD-isomers are identical in this dipeptide, their peaks are superimposed, and the ratio of the three peak areas is 1:1:2. The DD isomer eluted again before the LL isomer.

In conclusion, we have succeeded in the direct resolution of the enantiomers of some linear and cyclic dipeptides by use of an optically active stationary phase. This technique is suitable for the determination of optical purity and for the configurational assignment of dipeptides.

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Note

Direct separation of α-hydroxycarboxylic acid ester enantiomers by gas chromatography with optically active copper(II) complexes

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Hitherto gas chromatographic (GC) separation of enantiomers of hydroxycarboxylic acids, including lactic acid which is an important biological substance, has been achieved by forming derivatives with some optically active reagents, such as *l*-menthol, and separating the resulting mixture of diastereoisomers with the usual optically inactive stationary phases[1,2]. However, using this method, α-hydroxycarboxylic acid ester enantiomers must be hydrolysed before the derivatization to diastereoisomers.

The enantiomers of α-hydroxycarboxylic acid esters have never been resolved as such on optically active stationary phases. It is considered that the reason is that almost all known optically active stationary phases are derivatives of amino acids or dipeptides for which resolution has been attributed to diastereoisomeric interactions involving hydrogen bonds between CONH groups. Therefore, in order to resolve the enantiomers of α-hydroxycarboxylic acids, which have no NH group, it is necessary make the corresponding acid amides. For example, the enantiomers of O-pentafluoropropionyl lactic acid cyclohexyl amide were resolved with a high resolution factor on Chirasil-Val[3].

In the present paper we report the direct resolution of some chiral lactic acid esters by GC on copper(II) complexes of optically active Schiff's bases[4].

EXPERIMENTAL

Chemicals

The methyl, ethyl and isobutyl esters of DL-lactic acid were purchased from Wako (Osaka, Japan). L- and DL-lactic acid isopropyl ester and DL-α-hydroxybutyric acid isopropyl ester were synthesized by esterfication of each carboxylic acid.

The binuclear copper(II) complexes of N-salicyliden-(*R*)-2-amino-1,1-bis-(5-*tert*.-butyl-2-octyloxyphenyl)-propan-1-ol and N-salicyliden-(*S*)-2-amino-1,1-bis-(5-*tert*.-butyl-2-heptyloxyphenyl)-3-phenyl-propan-1-ol (I and II) were prepared by Dr. T. Nagase as reported previously[5].

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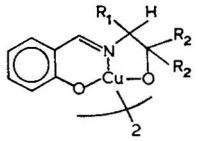

I : $R_1 = CH_3$, $R_2 = tert\text{-}C_4H_9$-(C$_6$H$_4$)-O-n-C_8H_{17}

II : $R_1 = CH_2$-(C$_6$H$_5$), $R_2 = tert\text{-}C_4H_9$-(C$_6$H$_4$)-O-n-C_7H_{15}

Gas Chromatography

The experiments were carried out with a Shimadzu Model GC-7A gas chromatograph equipped with a flame ionization detector. Glass capillary columns (40 m × 0.25 mm I.D.) coated with the mixture of the copper complex (I or II) and silicone OV-101 (1:1), and packed columns (2 m × 3 mm I.D.) filled with Chromosorb W AW DMCS (80–100 mesh) coated with 6% of the mixture of I and silicone OV-101 (5:1), were used.

GC mass spectrometric (MS) measurements were performed on a Shimadzu Model LKB-9000 GC–MS instrument.

RESULTS AND DISCUSSION

The results of the GC separation are given in Table I. The DL-lactic acid esters and a DL-α-hydroxybutyric acid ester were resolved into their antipodes with good separation factors. An example of a chromatogram is shown in Fig. 1.

As the optically active copper complex (I) shows a relatively large selectivity for the lactic acid esters, their enantiomers can be also separated on packed columns, as shown in Fig. 2. In these chromatograms of racemic isobutyl lactate, peak areas

TABLE I

GAS CHROMATOGRAPHIC SEPARATION OF α-HYDROXYCARBOXYLIC ACID ESTER ENANTIOMERS

Glass capillary columns, 40 m × 0.25 mm I.D. Carrier gas, helium at a flow-rate of 0.6 ml/min. Stationary phases: A, Mixture of the copper complex of I and silicone OV-101 (1:1). B, Mixture of the copper complex of II and silicone OV-101 (1:1).

Compound	*Stationary phase*	*Column temp. (°C)*	*Retention time* (min)*		*Ratio of retention times*
			First peak	*Second peak*	*(second/first)*
Lactic acid esters, $CH_3CH(OH)COOR$:					
R = CH_3	A	70	3.6	4.2	1.17
C_2H_5	A	70	6.3	7.1	1.13
iso-C_3H_7	A	70	7.3(D)	8.5(L)	1.16
	B	110	2.4(L)	3.2(D)	1.33
iso-C_4H_9	A	70	21.9(D)	24.8(L)	1.13
	B	110	4.3(L)	5.8(D)	1.35
α-Hydroxybutyric acid isopropyl ester	A	70	15.7	16.8	1.07

* Measured from solvent peak.

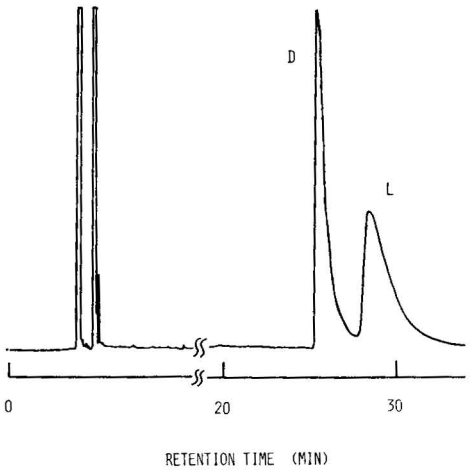

Fig. 1. Gas chromatogram of DL-isobutyl lactate. Conditions: see Table I.

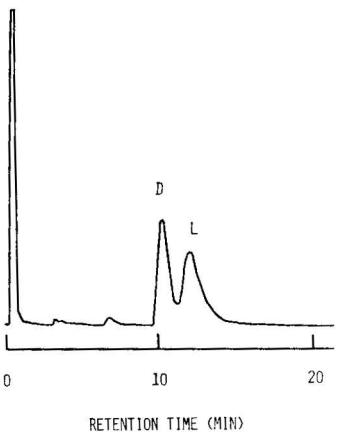

Fig. 2. Gas chromatogram of DL-isobutyl lactate. GC conditons: glass column (2 m × 3 mm I.D.) containing 80–100 mesh Chromosorb W AW DMCS coated with 6% of the mixture of the copper complex I and silicone OV-101 (5:1); temperature, 70°C; carrier gas, nitrogen at a flow-rate of 30 ml/min.

of enantiomers were nearly equal, although the longer-retained L enantiomer had a much broader peak, accompanied by tailing, than the D enantiomer. Such an anomalous peak shape shows that the numbers of theoretical plates for enantiomers are not equal. Similar results have been reported by Lochmuller and Souter[6] for the GC resolution of enantiomers with optically active mesophases. The mass spectra obtained from two separated peaks were identical (Fig. 3), and consistent with the standard spectrum of the racemic lactic acid ester.

Peak identifications were made by chromatographing successively racemic and 1:3 mixture of enantiomers of lactic acid isopropyl and isobutyl esters. On the copper complex I, the D isomers eluted before the L isomers. Supporting evidence for the

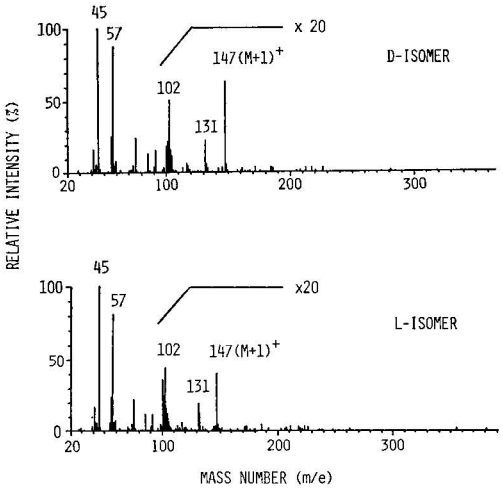

Fig. 3. Mass spectra of D- and L-isobutyl lactate. Operational conditions: electron energy, 70 eV; trapping current, 60 μA; inlet system, GC on a glass column as described in Fig. 2.

successful resolution of DL-lactic acid ester was produced by use of the copper complex II, which possesses the opposite configuration (*S*) from I; the order of appearance of the peaks was reversed when the complex II was used.

It is convenient for practical purposes that these racemic esters can be resolved without any pretreatment such as acylation or hydrolysis. It is notable that the optically active copper complexes, which were found to be useful primarily as catalysts for asymmetric synthesis[5], are also valuable as optically active stationary phases for the separation of enantiomers by GC .

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Note

Investigation of the gas–liquid chromatographic separation of phencyclidine and some heterocyclic analogues by combined gas–liquid chromatography–mass spectrometry

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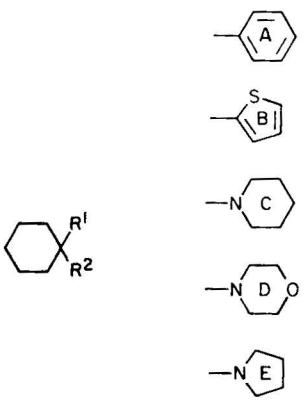
The widespread abuse of phencyclidine and an increasing number of its analogues[1,2] creates the need for rapid unambiguous identification. Two recent papers give gas chromatographic (GC) data for the separation of some analogues[3,4]. The two papers overlap on the description of five of these, namely: phencyclidine (I), 1-[1-(2-thienyl)cyclohexyl]piperidine (II), 1-(1-phenylcyclohexyl)morpholine (III), 1-[1-(2-thienyl)cyclohexyl]morpholine (IV), and 1-(1-phenylcyclohexyl)pyrrolidine (V). To complete the series, this paper will also include 1-[1-(2-thienyl)cyclohexyl]pyrrolidine (VI) (Fig. 1).



	R^1	R^2
I	A	C
II	B	C
III	A	D
IV	B	D
V	A	E
VI	B	E

Fig. 1. Structures of phencyclidine and its analogues.

Both Bailey *et al.*[3] and Cone *et al.*[4] give comparable data for OV-17 and SE-30 but results differ for OV-225. It is interesting to note that the systems that seem to separate best the thienyl analogues from the phenyl ones (OV-7 and SE-30 (ref. 3); SE-30, OV-225 and Sílar 5 CP (ref. 4), give relatively short, equal retention times for all three thiophenes. On systems where the thienyl analogues have long retention times [OV-17 and OV-225 (ref. 3); OV-17 (ref. 4)], they are distinguishable from one another but hardly from their phenyl counterparts. It was mentioned by Bailey *et al.*[3] that some of their results are indicative of decomposition.

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This paper will attempt to demonstrate using GC–mass spectrometric (MS) techniques that the short retention times observed for the thiophene analogues on some phases were in fact due to on-column decomposition. No such decomposition was obtained on these phases until it was induced by contaminating the phases prior to their use.

EXPERIMENTAL

A Hewlett-Packard 5985-A GC–MS data system was used. Columns were of glass, 4–8 ft. × 2 mm I.D. Injection port temperature was 250°C, and the detector and interface temperatures were 275°C. The carrier gas was helium with a flow-rate of 30 ml/min. Retention times were determined using a mixture of the bases of all six compounds dissolved in methanol. Source of samples were as previously described[3]. Silyl 8 is a trade name of Pierce (Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Results of the aforementioned papers were reproduced, except where the reported retentions times of the thiophene analogues were much shorter than their phenyl counterpart. It can be seen (Table I) that the phenyl analogues and their thienyl counterparts (I *vs.* II, III *vs.* IV, V *vs.* VI) exhibit little difference in chromatographic behaviour. Both the SE-30 and the OV-101 were found to give some separation, however the resolution of paired analogues was incomplete (Fig. 2). Analysis of the mass spectra of the emerging peaks revealed them to be almost identical with those published by Bailey *et al.*[3]. No evidence of decomposition could be found.

TABLE I

RETENTION TIMES OF PHENCYCLIDINE AND ANALOGUES

Conditions: A, 3% SE-30 on 80–100-mesh Chromosorb W, 150°C, 4 ft.; B, 5% OV-101 on 100–120-mesh Chromosorb W, 150°C, 6 ft.; C, 3% OV-7 on 80–100-mesh Chromosorb W, 150 °C, 4 ft.; D, 3% OV-7 (acid treated) on 80–100-mesh Chromosorb W, 150°C, 4 ft.; E, 3% OV-17 on 100–120-mesh Gas-Chrom Q, 160°C, 6 ft.; F, 2% OV-25 on 80–100-mesh Chromosorb W, 150°C, 8 ft.; G, 3% OV-225 on 100–120-mesh Chromosorb W, 140°C, 4 ft.; H, 3% Silar 10C on 100–120-mesh Gas-Chrom Q, 150°C, 4 ft.

Compound	*Retention time (min)*							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
I	5.3	10.7	12.5	18.3	14.9	9.7	12.9	3.6
II	5.1	10.2	12.5	18.3	14.9	9.7	12.9	3.6
III	6.8	13.3	18.3	26.3	23.6	15.6	28.9	14.6
IV	6.4	12.6	17.6	25.7	23.6	15.6	28.9	14.6
V	3.8	7.6	8.7	12.6	10.5	7.0	9.8	3.0
VI	3.6	7.0	8.2	11.9	10.3	7.0	9.8	3.0
VII	—	—	—	2.3	—	—	—	—

Much shorter retention times for II, IV and VI, could be produced by injecting small quantities (10–15 μl) of dilute aqueous hydrochloric acid onto a 3% OV-7 column prior to its use. This had the effect of producing a new early eluting peak with

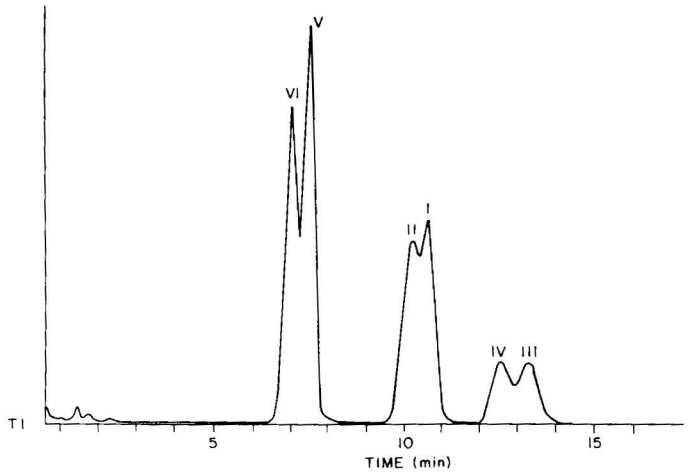

Fig. 2. Separation of phencyclidine and its analogues on 5% OV-101 at 150°C.

the same retention time (2.3 min) for all three thienyl analogues (Table I). Analysis of the mass spectra of the compounds giving rise to this peak (Fig. 3), suggested that this new compound is 1-(2-thienyl) cyclohexene (VII), formed by acid catalysed loss

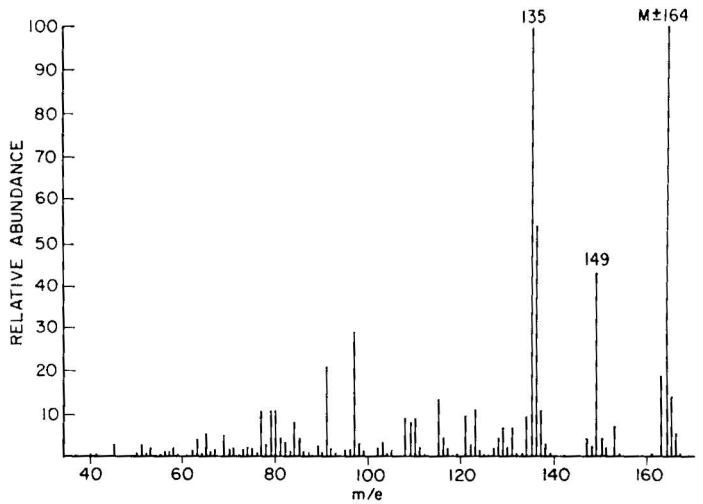

Fig. 3. Electron impact spectra of the decomposition product of compounds II, IV and VI.

of the R^2 ring substituent (Fig. 4). The five major peaks (*m/e* 79, 135, 136, 149, 164) of the spectrum, are also found to be amongst the predominant peaks below *m/e* 165

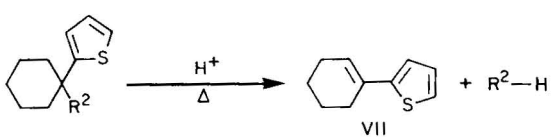

Fig. 4. Decomposition scheme of thienyl analogues on acid-treated OV-7.

in the spectra of the three thiophene analogs published by Bailey *et al.*[3]. The peaks at *m/e* 97 along with those at M −15 (*m/e* 149) and M −28 (*m/e* 136) are characteristic of thiophene and cyclohexene derivatives, respectively[5]. The small ion at *m/e* 153 is likely due to an impurity as it is the base peak in a scan of the area immediately following the GC peak corresponding to VII. At 150°C decomposition was not complete since vestiges of the thienyl analogues could still be seen. However if the temperature of the column is raised to 200°C, decomposition of the thiophenes becomes virtually complete. It would seem that injector temperature is not the source of the decomposition as it was maintained at 250°C at all times and only a raise in column temperature afforded increased decomposition, thus indicating that the column change generated by treatment with the hydrochloric acid was responsible for the decomposition relative to the results obtained prior to acid treatment (Table I). It is also noteworthy that the retention times of the phenyl and the undecomposed thienyl analogues were noticeably lengthened. The original performance of the column was restored by treatment with Silyl 8.

It is now obvious that the data previously reported[3,4] that gave short retention times for the thienyl analogues in relation to their phenyl counterpart, cannot be used for identification purposes. However deliberate selective decomposition of the thienyl compounds could be carried out to facilitate their differentiation from their phenyl analogues.

It is therefore recommended that columns that have been used with acid salts of organic compounds should be treated with Silyl B before the analysis of phencyclidine and its analogues is attempted.

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CHROM. 13,140

Note

Efficient lipoxygenase assay by gas–liquid chromatography

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Lipoxygenase (E.C. 1.13.1.13) catalyzes the aerobic oxidation of unsaturated fatty acids with a *cis*-1-*cis*-4-pentadiene system to optically active conjugated *cis,trans*-diene hydroperoxides[1,2]. The enzyme has been isolated from a variety of plant sources[3–5] and has been implicated in rancidity and general food decay[6].

Many assay techniques have been adapted to the study of this enzyme, including manometry and iodiometry[7], Cupplate diffusion[8], polarography[9] and spectrophotometry[10]. The last two methods are more widely used and have been the subject of a recent assessment[11]. The authors concluded that, although the polarographic method is to be preferred, it is not sufficient to fully characterize the reaction mechanism.

The present report shows that gas–liquid chromatography (GLC) can be adapted to measure the enzyme activity. It is much more sensitive and devoid of many of the limitations of the other techniques. It may also be comparable in rapidity to the spectrophotometric method.

MATERIALS AND METHODS

Materials

The boron trifluoride–methanol reagent was supplied by Applied Science Labs. (State College, PA, U.S.A.). Linoleic acid (*cis*-9-*cis*-12-octadecadienoic acid) and soy bean lipoxygenase Type II were obtained from Sigma (St. Louis, MO, U.S.A.).

The substrate was 3.5 mg/ml sodium linoleate in a 0.5 *M* NH_4OH–NH_4Cl buffer, pH 7.0, plus 0.5% (w/v) sodium cholate as emulsifier.

Enzyme assay

A preparation containing 0.1 mg protein per ml in 50 m*M* phosphate buffer, pH 7.0 was used as the enzyme solution. A 7-ml volume of the substrate solution was placed into each of two 25-ml volumetric flasks. These were then placed in a water-bath at 30°C and saturated with clinical grade oxygen, bubbled in for 5 min. A 0.2-ml volume of the enzyme solution was then added to flask 1 and to the second flask (blank) were added 0.2 ml of the phosphate buffer. The time was noted and, at 15-sec intervals, 1 ml was withdrawn from each flask and placed into separate round-bottomed flasks containing 5 ml of chloroform–methanol (1:1, v/v) acidified with

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0.01 ml of 10% H_2SO_4. The chloroform layer was separated and reduced to dryness in a rotary evaporator.

Methylation and GLC

Fatty acid methyl esters (FAME) were prepared by a slight modification of the method of Metcalfe *et al.*[12] using a boron trifluoride–methanol mixture. Typically, 0.5 ml of 0.5 *N* methanolic sodium hydroxide was added to the dried extract and warmed gently on a steam-bath for 1 min. A 1-ml volume of the boron trifluoride–methanol mixture was then added and the solution boiled for 2 min. One millilitre of a saturated solution of sodium chloride was added and 2 ml of diethyl ether were then used to extract the FAME. After successive washings with water, the extracts were dried with Na_2SO_4 and used directly for GLC.

A 0.5-μl volume was injected into a Model GCD Pye Unicam gas chromatograph, equipped with a glass column (1.5 m × 4 mm I.D.) packed with 8% HI-EFF 1,4-butanediol succinate polyester (Applied Science Labs.) on Chromosorb W (80–100 mesh) and with dual hydrogen flame ionization detectors. The carrier gas (nitrogen) flow-rate was maintained at 40 ml/min. The detector and column temperatures were kept at 200°C and 190°C respectively.

RESULTS AND DISCUSSION

Fig. 1 shows the GLC profiles obtained from the sample as a function of time. A pilot spectrophotometric assay (inset of Fig. 1) showed that, although linearity exists in the initial 3 min of reaction, the reliability of the curve is doubtful owing to the high noise level. At 234 nm, which is the $\lambda_{max.}$ of the hydroperoxides present in the spectrophotometric assay, there are "ill-defined" chromophore interferences. This together with the lipophilic nature of the substrate (solubilizing agents required) can lead to considerable spectral fluctuations. Furthermore, the hydroperoxides have been shown to be immediately converted into secondary oxidation products[13], complicating their use as a basis of the enzyme assay. Because of the high

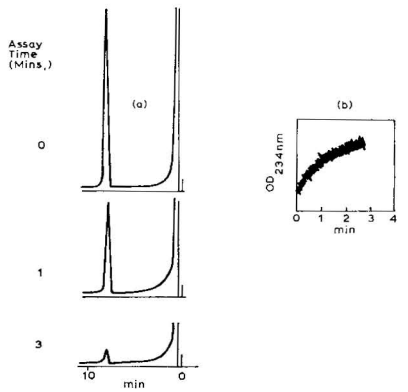

Fig. 1. GLC profiles (scan rate = 60 sec/cm) of linoleate as a function of time: (a) enzyme assay, conditions are described in Materials and methods; (b) activity profile of the enzyme assay at $\lambda_{max.}$ 234 nm.

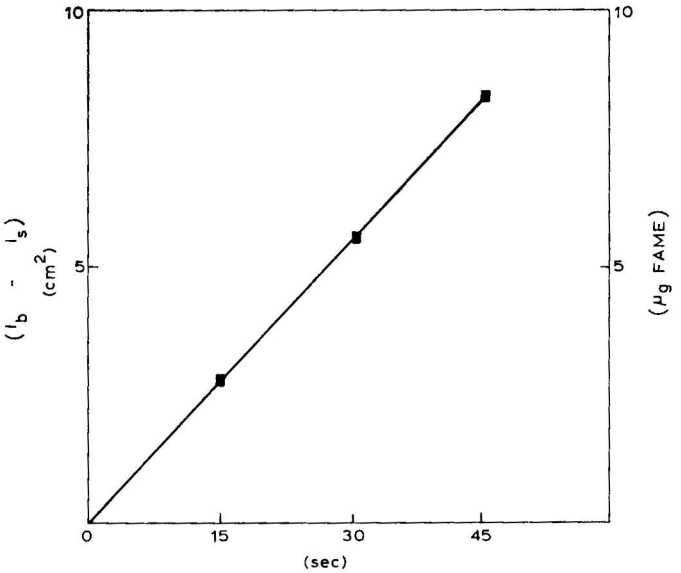

Fig. 2. Amount of linoleate consumed as a function of assay time. $I_b - I_s$ = Intensity of blank minus intensity of sample.

sensitivity of gas chromatography, even microgram quantities may be employed in the assay, thus obviating the problems posed by the poor solubility in water of the substrates.

The peak intensities were estimated by the triangulation method, and a plot of the amount of fatty acid consumed against time is linear over the period studied (Fig. 2). This method suffers from the disadvantage that comparatively longer times are required to conclude the assay. If, however, appropriate conditions are selected, a considerable time reduction can be achieved. For instance, a reduction of the percentage of stationary phase coating and an increase in operating temperature will presumably further shorten the retention times (8.6 min) observed here.

The enzyme has also been known to act directly on triglycerides, especially in the absence of lipolytic enzymes[14]. If it is desired to monitor such activity, it may therefore be necessary to employ a transesterification procedure in the preparation of the FAME. In such cases, longer reflux times with the methylating agent, such as in the method of Stoffel *et al.*[15] may be required. We have observed that there is no apparent decomposition of the substrate, especially when nitrogen is continually bubbled through the refluxing mixture. We have also observed that sodium cholate is preferred to many other dispersing agents, although at concentrations higher than 0.5% (w/v) in the assay medium the enzyme is inhibited[16].

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Note

Analysis of mimosine and 3-hydroxy-4(1H)-pyridone by high-performance liquid chromatography

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Mimosine, β-[N-(3-hydroxy-4-oxopyridyl)]-α-aminopropionic acid, is a non-protein amino acid that occurs in the tropical plants, *Mimosa pudica* and more importantly, in *Leucaena leucocephala*. Its presence in *Leucaena* has prevented the widespread use of this legume for intensive animal feeding as mimosine induces the depilatory and other toxic effects in ruminants and monogastric animals[1,2]. 3-Hydroxy-4(1H)-pyridone (DHP), a metabolite of mimosine in both plants[3] and animals[4], has also been associated with the development of various abnormal growth or metabolic effects in ruminants[5,6].

A range of methods has been developed for the analysis of mimosine and/or DHP utilising ion-exchange and paper chromatography[7], gas chromatography[8], an amino acid analyser[9], and colorimetry with an auto-analyser[10]. All these methods are unsatisfactory for use as a standard routine method as they are either specific for only mimosine or DHP, are not suitable for analysis of both plant and animal extracts, are tedious and time-consuming, or are subject to variable losses during analysis. This paper describes a sensitive and simple method for the simultaneous analysis of mimosine and DHP in plant material and urine by high-performance liquid chromatography (HPLC).

METHODS AND RESULTS

HPLC analyses were performed on a μBondapak C_{18} column in a Waters liquid chromatograph (Model No. ALC/GPC 244) using a single wavelength UV (280 nm) absorbance detector. Rapid elution and good separation of mimosine and DHP in standard solutions was obtained using a solvent system of 0.2% (w/v) orthophosphoric acid in double distilled water at a flow-rate of 1 ml/min (Fig. 1a). There was a linear response of both peak height and peak area to concentration of mimosine and DHP with the limits of detection being 1 ng mimosine and 2 ng DHP.

Leaf samples of *Leucaena* were prepared for analysis by initially holding the leaf at 20°C for 24 h, to ensure the production of some DHP[3], and then freeze dried. Dried leaf (25 mg) was ground in a mortar with 0.1 *N* hydrochloric acid (10 ml) to extract mimosine and DHP[7], the mixture was then centrifuged for 10 min at 7500 *g* and the supernatant was filtered under nitrogen (60 p.s.i.) through a membrane ultrafilter. Analysis of the leaf extract (10-μl aliquot) showed that sharp resolution of mimosine and DHP was retained and there were no major components in the extract that interfered with the analysis (Fig. 1b).

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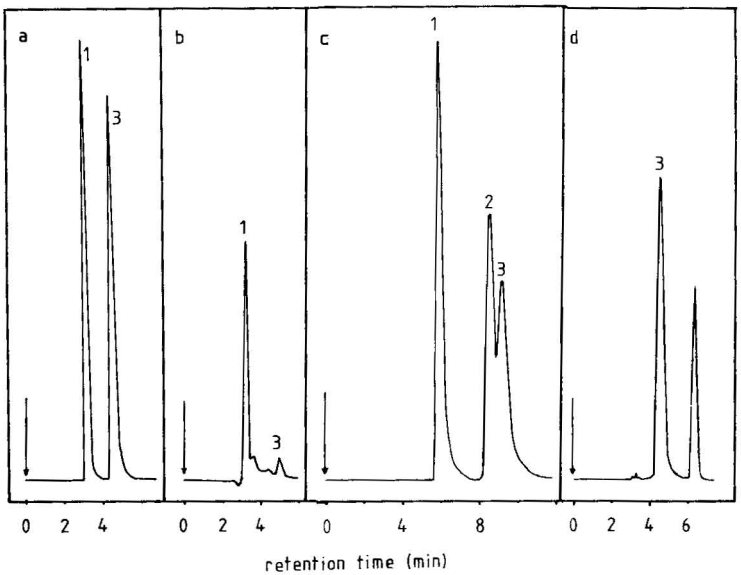

Fig. 1. Chromatograms of the separation of mimosine and DHP. (a): Standards; (b): extract from *Leucaena* leaf; (c): standards at reduced flow-rate (0.5 ml/min); (d): hydrolysed urine. Sample composition: 1 = mimosine; 2 = DHP-glucoside; 3 = DHP.

Urine obtained from ruminants that have been fed *Leucaena* can contain mimosine, DHP and DHP-glucoside[4]. DHP and DHP-glucoside in a standard solution were partially resolved when the solvent flow was reduced to 0.5 ml/min (Fig. 1c). However for most studies only a total estimate of DHP is required and a quantitative conversion of glucoside to DHP can be achieved by acid hydrolysis[7]. Fresh urine was mixed with an equal volume of 10 *N* hydrochloric acid and heated at 110°C for 4 h, the pH was adjusted to pH 3 with sodium hydroxide, the solution filtered and made up to volume. Analysis of the urine extract (10-μl aliquot) showed that DHP gave a sharp peak with only a small amount of mimosine present and no DHP-glucoside (Fig. 1d). There were no major interfering compounds in the extract.

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Note

High-performance liquid chromatographic method for the analysis of D-arabino-2-hexosulose (D-glucosone)

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D-Glucosone is the key chemical intermediate in a new process for manufacturing crystalline fructose from glucose. This process involves a combined enzymatic oxidation and chemical reduction reaction run in an aqueous system[1].

Reliable measurement of glucosone has been limited. A colorimetric assay —the reaction of triphenyltetrazolium chloride— has been developed but interference from D-glucose, D-gluconic acid and other reductants occurs[2]. A gas chromatographic assay —analysis of the trimethylsilylated derivative— has also been developed but it is time consuming (rigorous removal of water from the samples is required) and qualitative at best, owing to the heat lability of the derivatives formed[3].

In this paper is reported a reliable, rapid high-performance liquid chromatographic (HPLC) method for the measurement of D-glucosone.

EXPERIMENTAL

High-performance liguid chromatography

A Spectra-Physics 8000 high-performance liquid chromatograph equipped with dual detectors —refractive index (RI) and ultraviolet (UV) at 192 nm— was used. Aqueous samples (10 μl) were injected onto a 30 cm $\times$ 4.6 mm I.D. Waters Assoc. (Milford, MA, U.S.A.) carbohydrate analysis column (10 μm). The mobile phase was 20% aqueous acetonitrile containing 0.003 *M* (final) potassium phosphate buffer (pH 6.0). Flow was set at 2.0 ml/min and column temperature was set at 25°C. Attenuation was 8 $\times$ on the RI detector and 0.25 a.u.f.s. on the UV detector.

Standards

The sugar-type compounds shown in Table I were used. All standards were prepared in aqueous solution. Concentrations were 30 mg/ml, unless otherwise noted.

RESULTS AND DISCUSSION

Separation of sugars on the carbohydrate analysis column using an aqueous acetonitrile mobile phase is a well-documented method[4]. Although D-glucosone also eluted under these standard conditions, loss of component was occasionally observed, especially when a new column was used. It is possible for D-glucosone, which has a

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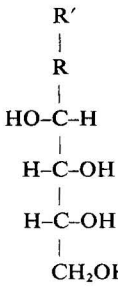
TABLE I

STRUCTURE AND SOURCE OF SUGAR-TYPE COMPOUNDS



No.	*Name*	*R*	*R'*	*Source*
I	D-Glucose	H–C–OH	CHO	Applied Science Corp., State College, PA, U.S.A.
II	D-Gluconic acid	H–C–OH	CO_2H	Applied Science Corp.
III	D-Fructose	C=O	CH_2OH	Applied Science Corp.
IV	D-Glucosone	C=O	CHO	Enzymatically synthesized from glucose[1]
V	D-2-Ketogluconic acid	C=O	CO_2H	Sigma, St. Louis, MO, U.S.A.

reactive aldehyde functional group, to form Schiff bases with the column packing resulting in irreversible adsorption[5]. Also, it is known that D-glucosone, which is chemically unstable, decomposes in the presence of traces of base[6]. To overcome these problems, the mobile phase was buffered on the acidic side (pH 6.0). Reliable measurements were then possible. Fig. 1 shows the measurement of D-glucosone using RI detection and UV absorption at 192 nm.

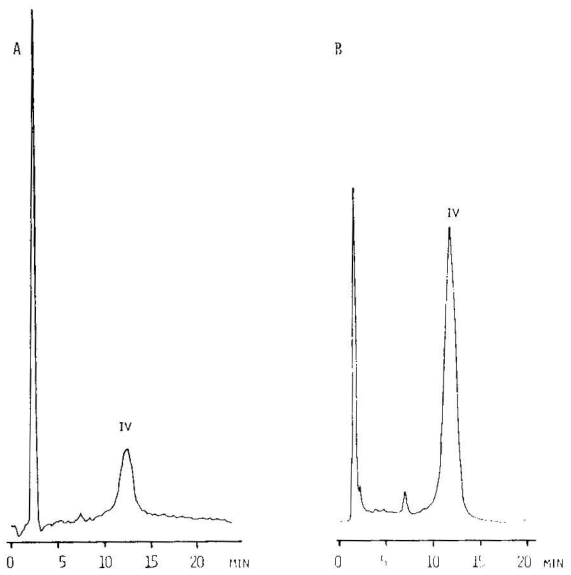


Fig. 1. Chromatograms of D-glucosone (IV) using RI detection (A) and UV detection at 192 nm (B).

The minimum detectable quantity of D-glucosone is 20 μg using RI detection and 0.1 μg using UV detection at 192 nm. The precision of this assay method is 10.1 % standard deviation and 7.3 % standard deviation for D-glucosone standards of 10 mg/ml and 30 mg/ml, respectively; assayed fourteen times over a 48-h time period, using peak areas obtained with the RI detector. A linear relationship is found between peak area and D-glucosone concentration in the range of 8 mg/ml through 100 mg/ml D-glucosone, using the RI detector.

The chromatographic conditions developed also permitted the measurement of the other sugars in the commercial process. Fig. 2 shows the separation of D-glucose and D-fructose in the presence of D-glucosone. To be able to measure simultaneously both D-glucose and D-glucosone is important. Low levels of D-glucose, were they to be carried over in the process, might interfere with the crystallization of D-fructose.

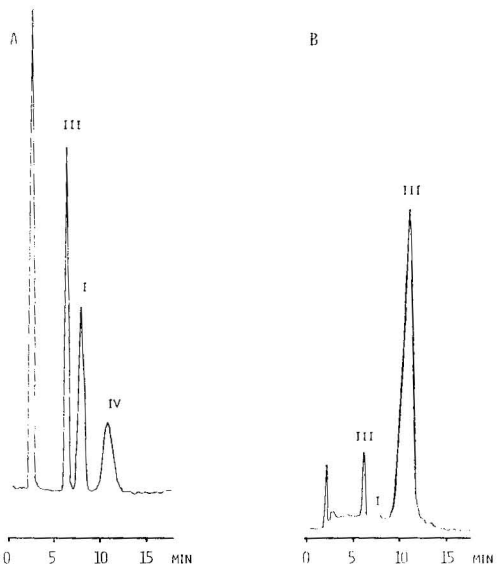
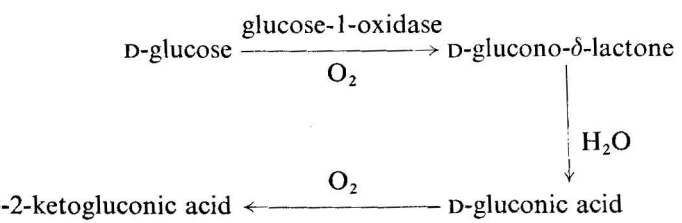


Fig. 2. Chromatograms of D-glucose (I), D-fructose (III) and D-glucosone (IV) using RI detection (A) and UV detection at 192 nm (B).

Since the first step of the process is an enzymatic step, a competing enzymatic reaction on D-glucose was possible. A potential competing enzyme would be glucose-1-oxidase:



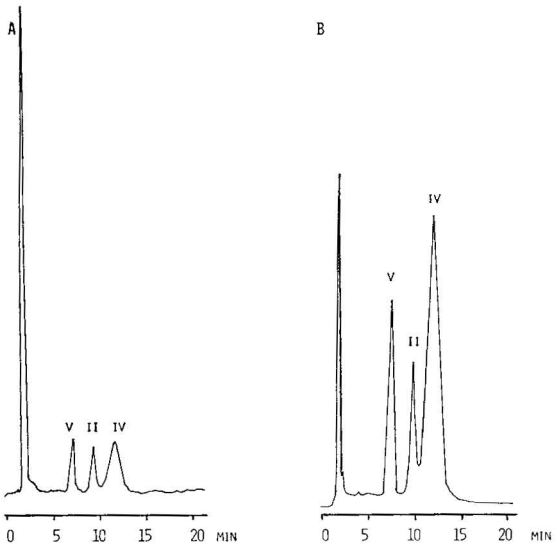


Fig. 3. Chromatograms of D-gluconic acid (II), D-glucosone (IV) and D-2-ketogluconic acid (V) using RI detection (A) and UV detection at 192 nm (B). Compounds II and V are each 10 mg/ml.

Fig. 3 shows the separation of D-gluconic acid and D-2-ketogluconic acid in the presence of D-glucosone. D-Glucono-δ-lactone eluted at 4 min under these conditions. The presence of acidic buffer in the mobile phase is important for these compounds. The absence of acidic buffer in the HPLC mobile phase caused the D-gluconic acid to elute with broad tailing and the D-2-ketogluconic acid to not elute at all.

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Note

Column and thin-layer chromatography of cholic, deoxycholic and chenodeoxycholic bile acids and their sodium salts

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Column and thin-layer chromatography (TLC) are convenient techniques for separating bile acids and their derivatives. The qualitative separation of certain free and conjugated bile acids was investigated by Hofmann[1] using TLC. A group separation of bile acids and salts by column chromatography has been examined by several authors[2–4]. Hamilton[5] reported the separation of bile acids by applying two-dimensional thin-layer and glass–paper chromatography. Sundaram *et al.*[6] improved the separation of chenodeoxycholic acid from deoxycholic acid and several publications have appeared[7–9] which deal with the conjugated and keto derivatives of bile acids. Thus, although the chromatographic analysis of bile acids and their derivatives has been well studied there are apparently no data on the qualitative and quantitative separation of alkali bile salts, taking into account impurities. In the present study we describe TLC and column chromatography methods to do this which use a new developing solvent system.

MATERIAL AND METHODS

Bile salts were sodium deoxycholate, sodium cholate (Calbiochem, San Diego, CA, U.S.A.), and sodium chenodeoxycholate (Weddell, London, Great Britain). Deoxycholic acid and chenodeoxycholic acid were obtained from Sigma (St. Louis, MO, U.S.A.) and cholic acid was from Fluka (Buchs, Switzerland), puriss grade. Silica gel sheets (TLC aluminium sheets (20 × 20 cm) pre-coated with silica gel 60; layer thickness 0.2 mm) were from E. Merck (Darmstadt, G.F.R.), and were cut into plates 15 cm × 5 cm. All solvents were laboratory grade. Ceric ammonium sulphate reagent was prepared according to Sundaram *et al.*[6].

Bile salts and bile acids were dissolved in methanol and were applied with a Hamilton microlitre syringe as a thin line at 1.5 cm from the bottom of the plate. Plates were developed at room temperature in a pre-saturated chamber, lined with filter paper. The solvent was allowed to rise 13–14 cm from the starting line. The plates were removed, dried at 110°C, and sprayed with ceric ammonium sulphate reagent. Plates were dried then, heated, for 15 min at 120–130°C to develop the colour. In a study examining the qualitative separation of sodium cholate from sodium deoxycholate, nine different solvent systems at different mixing ratios were tested (Table I).

TABLE I

SOLVENT SYSTEMS TESTED FOR THE SEPARATION OF SODIUM CHOLATE FROM SODIUM DEOXYCHOLATE

Solvent system	*Ratios of mixing*	*Solvent composition*	*Comments*	*Ref.*
S-I	5 25 70	Methanol Acetone Chloroform	No separation	10
S-II	1, 8 1, 92	Acetone Benzene	No separation	10
S-III	4 1 2	Acetone Benzene Glacial acetic acid	Poor separation	
S-IV	4.5 0.5 3	Methanol Benzene Glacial acetic acid	No separation	
S-V	10, 2, 16, 2 4, 1, 4, 1 9, 1, 8, 2	Glacial acetic acid Diisopropyl ether Isooctane	Better separation compared with solvent systems S-III	10
S-VI	4, 20, 4 3, 15, 3 1, 5, 1 1, 5, 1 2, 5, 4 1, 2.5, 3	Isoamyl acetate Diisopropyl ether Carbon tetrachloride Benzene *n*-Propanol Glacial acetic acid	Better separation	10
S-VII	10* 7 3 4	Isooctane Ethyl acetate Glacial acetic acid *n*-Butanol	Better separation than solvent systems S-III, S-V, S-VI	
S-VIII	3, 5 4, 10 1, 2	light petroleum (b.p. 40–60°C) Methanol Glacial acetic acid	No separation	
S-IX	21** 8 13.5 6 7	light petroleum (b.p. 40–60°C) *n*-Butanol Glacial acetic acid Ethyl acetate Benzene	Optimum separation	

* Developed by testing 12 different ratios.
** Developed by testing 100 different ratios.

For the column separation of bile salts, a column (25 cm × 4 cm I.D.) with glass wool at the bottom was prepared. A slurry of Kieselgel 60 (0.04–0.06 mm 230–400 mesh, ASTM, Merck) was poured into the column, containing 300 ml solvent system and was allowed to settle. The prepared column was washed with eluting solvent system. One gram samples of bile salts were applied and 50-ml portions of eluents were collected at a flow-rate of 1.2 ml/min.

RESULTS AND DISCUSSION

Thin-layer chromatography

Table I summarizes the results of nine solvent systems for the separation of sodium cholate from sodium deoxycholate. The solvent systems S-I and S-II gave no separation because of the low polarity of the mixture, whereas separation improved slightly when the polarity increased (S-III). Replacement of a solvent component by methanol did not increase separation (S-IV and S-VIII). However, with further increase in polarity, separation again improved (S-V, S-VI and S-VII). By further manipulation of the solvent composition, sodium cholate, sodium deoxycholate and sodium chenodeoxycholate were separated from each other and from impurities with solvent system, S-IX (light petroleum (b.p. 40–60°C)–benzene–glacial acetic acid–*n*-butanol–ethyl acetate, 21:8:13.5:6:7).

Commercial bile acids and their derivatives generally contain impurities (Table II). The quantities of impurities of bile acids and their sodium salts were investigated by TLC and were compared with the purity of sodium cholate which was accepted as a standard. Thus, Barry and Gray[11] reported that sodium cholate and sodium deoxycholate contained a slight amount of deoxycholate and chenodeoxycholate respectively. Fig. 1 illustrates the separation of impurities from bile acids and their sodium salts using solvent system S-IX, (Table I). The R_F values in Table III confirm that we can analyse qualitatively the compounds on a microscale with the solvent system.

Bile acids

Several TLC methods have been reported to separate bile acids[1,5,6,12]. In the

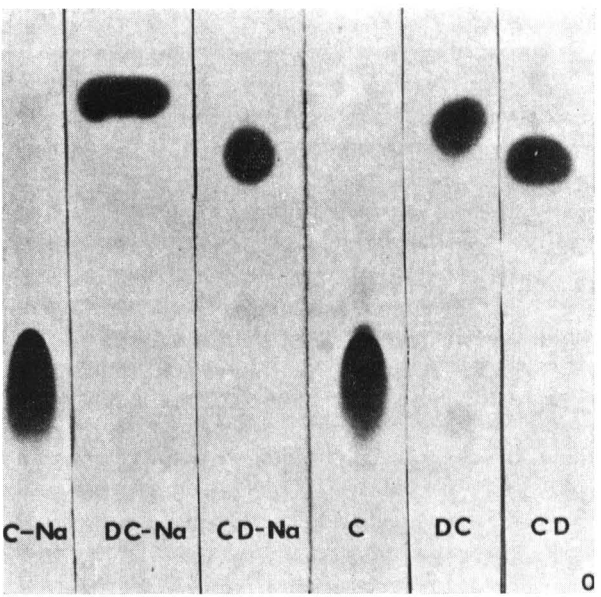

Fig. 1. Separation of bile salts and bile acids from impurities. C-Na = sodium cholate; DC-Na = sodium deoxycholate; CD-Na = sodium chenodeoxycholate; C = cholic acid; DC = deoxycholic acid; CD = chenodeoxycholic acid; O = origin.

TABLE II

GRADE AND PURITY OF COMMERCIAL BILE ACIDS AND THEIR SODIUM SALTS, AND THE ESTIMATED PERCENTAGE OF IMPURITIES ƆDUCED BY TLC

Ia = Impurity claimed by manufacturer; Ib = estimated impurity by TLC.

Bile acids	*Manufacturer*	*Ia (%)*	*Ib (%)*	*Sodium salts*	*Manufacturer*	*Ia (%)*	*Ib (%)*
Cholic	Fluka (Puriss, TLC)	<1	1–1.5	Cholate	Calbiochem (analytical grade, TLC)	3	3 (=Ia)
Deoxycholic	Sigma (grade II)	—	2–3	Deoxycholate	Calbiochem (analytical grade, TLC)	Trace spots	1
Chenodeoxycholic	Sigma	—	1–1.5	Chenodeoxycholate	Weddel (batch: MW 159)	—	2–3

TABLE III

R_F VALUES FOR THE BILE ACIDS, THEIR SODIUM SALTS AND IMPURITIES; (300 μg APPLIED)

I = Impurity.

Bile acids	$R_F \pm$ *S.D.* ($n = 6$)	*Sodium salts*	$R_F \pm$ *S.D.* ($n = 6$)
Cholic	0.38 ± 0.02	Cholate	0.51 ± 0.02
I_1	0.50	I	0.77
I_2	0.76		
Deoxycholic	0.73 ± 0.02	Deoxycholate	0.79 ± 0.02
I_1	0.30	I	0.42
I_2	0.87		
Chenodeoxycholic	0.70 ± 0.02	Chenodeoxycholate	0.70 ± 0.02
I	0.83	I	0.47

present study mixtures of the bile acids in the ratio 1:1:1 were run and the R_F values obtained were correlated with those given in the literature. Fig. 2 shows the results for mixtures of bile acids applied in total amounts of 50, 100, 200, 250, 300, and 400 μg. Table IV indicates that the separation of bile acids at 400 μg was as good as that obtained at 50 μg. Since the average R_F values of bile acids are essentially constant up to 400 μg, means of average R_F values (R_{F_m}) are also shown in Table IV together with standard deviations. The differences in R_{F_m} values for the separation of cholic acid from deoxycholic acid and chenodeoxycholic acid are slightly higher (0.45 and 0.39) than literature values (0.43 and 0.37)[1] and (0.42 and 0.34)[6]. The difference obtained for the separation of chenodeoxycholic acid from deoxycholic acid (0.06) was lower than 0.08 given by Sundaram *et al.*[6]. To check the efficiency of the solvent

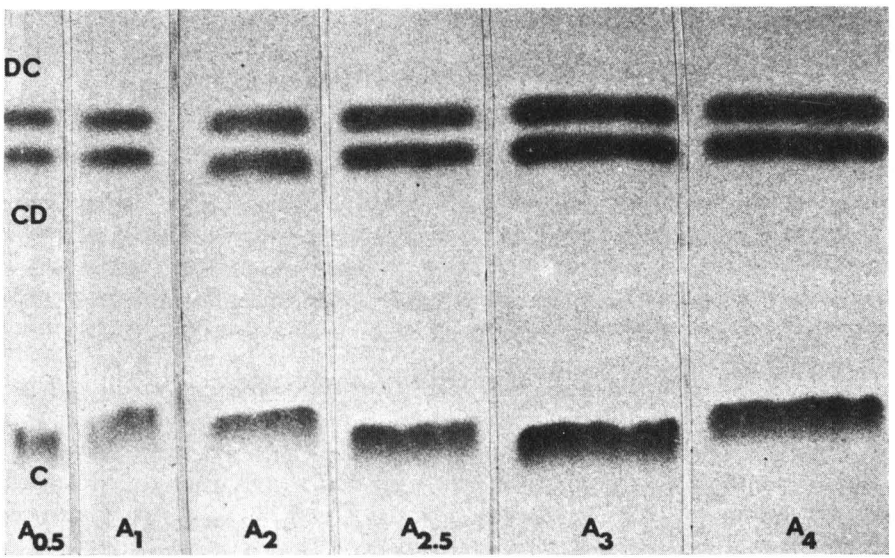

Fig. 2. Separation of bile acids from their 1:1:1 mixtures; C = cholic acid; CD = chenodeoxycholic acid; DC = deoxycholic acid. Applied quantity: $A_{0.5}$, 50 μg; A_1, 100 μg; A_2, 200 μg; $A_{2.5}$, 250 μg; A_3, 300 μg; A_4, 400 μg.

TABLE IV

R_F VALUES FOR THE BILE ACIDS OF VARIOUS APPLIED QUANTITIES

Bile acids	$R_F \pm S.D.$ $(n = 6)$						
	50 μg	*100 μg*	*200 μg*	*250 μg*	*300 μg*	*400 μg*	R_{Fm}
Deoxycholic	0.76 ± 0.03	0.76 ± 0.02	0.74 ± 0.02	0.76 ± 0.02	0.77 ± 0.02	0.77 ± 0.02	0.76 ± 0.01
Chenodeoxycholic	0.69 ± 0.02	0.70 ± 0.02	0.68 ± 0.02	0.70 ± 0.02	0.71 ± 0.01	0.71 ± 0.02	0.70 ± 0.01
Cholic	0.30 ± 0.02	0.30 ± 0.02	0.30 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.02	0.31 ± 0.01

TABLE V

R_F VALUES FOR THE BILE SALTS OF VARIOUS APPLIED QUANTITIES

Sodium salts	$R_F \pm S.D.$ $(n = 6)$						
	50 μg	*100 μg*	*200 μg*	*250 μg*	*300 μg*	*400 μg*	R_{Fm}
Deoxycholate	0.77 ± 0.03	0.75 ± 0.01	0.76 ± 0.03	0.76 ± 0.02	0.76 ± 0.02	0.76 ± 0.02	0.76 ± 0.01
Chenodeoxycholate	0.70 ± 0.02	0.68 ± 0.02	0.69 ± 0.02	0.69 ± 0.03	0.69 ± 0.01	0.69 ± 0.02	0.69 ± 0.01
Cholate	0.29 ± 0.02	0.30 ± 0.02	0.30 ± 0.01	0.31 ± 0.02	0.31 ± 0.02	0.31 ± 0.03	0.30 ± 0.01

system of these authors, mixtures of 250 μg of each acid (cholic, deoxycholic and chenodeoxycholic) were run with their solvent system (isooctane–ethyl acetate–*n*-butanol–acetic acid, 10:5:1.5:1.5). The average R_F values of six replicates for deoxycholic acid, chenodeoxycholic acid and cholic acid were 0.35 $\pm$ 0.03, 0.31 $\pm$ 0.03 and 0.12 $\pm$ 0.01 respectively. The difference between the R_F values for deoxycholic acid and chenodeoxycholic acid (0.04) is lower than 0.08 as given by Sundaram *et al.*[6], under identical conditions. The same mixture of bile acids was run with our solvent system. The average R_F values of six replicates for corresponding bile acids were 0.73 $\pm$ 0.01, 0.67 $\pm$ 0.02 and 0.29 $\pm$ 0.01. The results show that the differences between the R_F values for deoxycholic acid, chenodeoxycholic acid and cholic acid were greater for our solvent system and reproducibility was better compared with the system of Sundaram *et al.*[6].

Bile salts

Mixtures of sodium chenodeoxycholate, sodium cholate, and sodium deoxycholate (1:1:1) in amounts of 50, 100, 200, 250, 300 and 400 μg were run. Table V and Fig. 3 indicate that the bile salts separate as well from each other as do the bile acids. Again separation was good up to 400 μg, a higher limit than for the methyl esters reported in the literature[6]. The R_F values change little with applied quantity of mixture. The difference between R_{F_m} values for sodium cholate and the other salts (0.46 and 0.39 for sodium deoxycholate and chenodeoxycholate respectively) were greater than bile acid differences reported by Sundaram *et al.*[6]. However, the difference between sodium chenodeoxycholate and deoxycholate values was lower (0.07) than the value (0.08) given[6] for the corresponding bile acids.

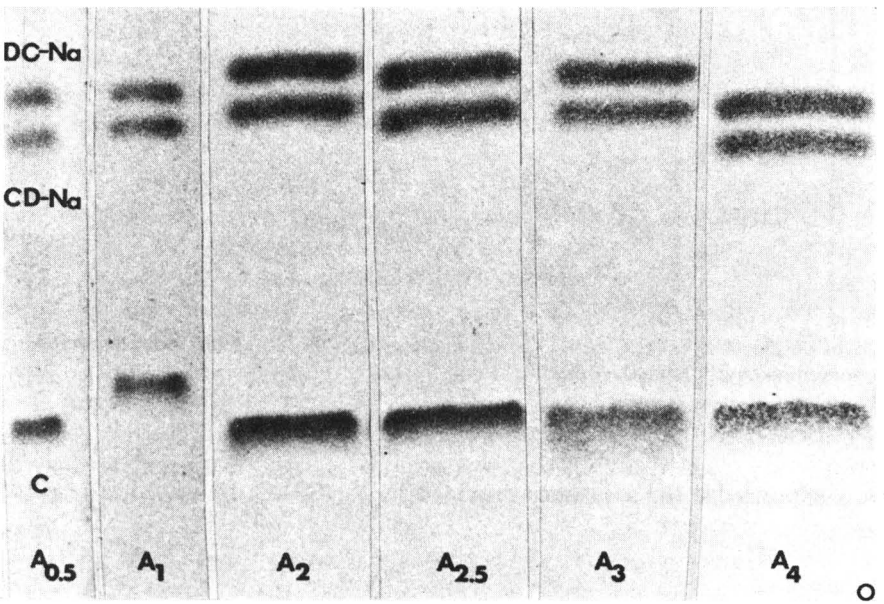

Fig. 3. Separation of bile salts from each other; C = sodium cholate; CD-Na = sodium chenodeoxycholate; DC-Na = sodium deoxycholate. Applied quantity: $A_{0.5}$, 50 μg; A_1, 100 μg; A_2, 200 μg; $A_{2.5}$, 250 μg; A_3, 300 μg; A_4, 400 μg; O = origin.

Column chromatography

The separation of bile salts from impurities by TLC using our solvent system appears to offer the possibility for preparative applications. Thus, purification of bile salts was studied by column chromatography using the same solvent system. Samples were applied to the column and the collected eluents were checked by TLC. Those portions which showed impurities were rejected. The remaining eluents were combined, dried and applied in amounts of 300 μg in methanol on TLC. Fig. 4 shows no detectable impurities of bile salts on TLC; the recovery was 70%. The R_F values for six replicates for the purified bile salts were sodium cholate (0.48 $\pm$ 0.01), sodium deoxycholate (0.83 $\pm$ 0.01) and sodium chenodeoxycholate (0.71 $\pm$ 0.01). Because of the rapid and efficient separation, column chromatography with our solvent system is a convenient technique for preparing pure samples of the bile salts.

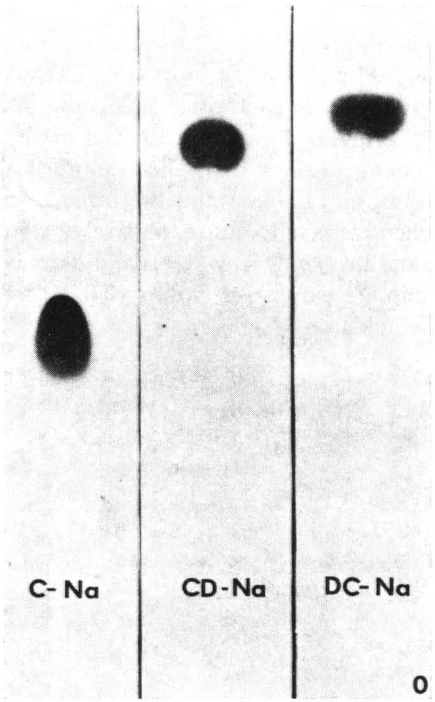

Fig. 4. TLC of purified bile salts; C-Na = sodium cholate; CD-Na = sodium chenodeoxycholate; DC-Na = sodium deoxycholate; O = origin.

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Book Review

Paper and thin layer chromatographic analysis of environmental toxicants, by M. E. Getz, Heyden & Son, London, Philadelphia, Rheine, 1980, X + 164 pp., price US$ 16.50, £ 7.30, DM 34.50, ISBN 0-85501-451-2.

Unless the reader has an opportunity to survey the contents of this book prior to purchase, he/she may be misled by the title of the book. The seven chapters cover Drugs and animal feed additives, Food additives, Air and water pollution, Pesticides, Cosmetics, Naturally occurring toxicants, and Chromatographic techniques and reagents. By definition, toxicant is a poison. Drugs and animal feed additives, food additives, and cosmetics are not toxicants *per se* unless, perhaps, they are used in an abusive manner. A better description, at least for the first two categories, should have included the term adjuvants. The Editor of this series classes the book as a monograph. A monograph is defined as a treatise on a particular subject; a highly detailed and thoroughly documented study written about a limited area of a subject or field of inquiry. This book is not a monograph. Rather, it is a brief compilation of abbreviated techniques on paper and thin-layer chromatography culled from the literature. The analyst would be required to refer to the original publication quoted as a reference for the necessary complete analytical procedures. The Editor does qualify his opening remark on the "monograph" by stating that "Techniques, ideas and applications are described in sufficient detail to enable those who are not specialists in a particular subject to appreciate the applicability of the subject matter to their own work. Bibliographies included in these monographs will enable the reader to pursue the subject to any desired depth". He further states that the volume would be of special interest to analysts in developing countries; such areas may not have ready access to the supplementary reference works required for completeness of analytical details.

The listing of chemicals in the various tables were unnecessary, because the enormous number of such chemicals precludes completeness. This page space could have been utilized to better advantage with tables of retention times (R_F values) and detection limits (lowest amount of chemical detectable). Detection limits, important criteria for the analyst, are not apparent anywhere in the text, even though the author states that paper and thin-layer chromatography can now be accomplished in a quantitative manner. The format of the R_F data is awkward for the reader. Tabulated data would have been preferable. Also, more chromatographic illustrations of each chemical group would have been most informative. No doubt, present-day economic restrictions because of increased printing costs limited the scope and size of this publication.

The bibliography section of each chapter suggests that a thorough search of the literature was made for the period when paper and thin-layer chromatography were predominant analytical techniques (prior to gas chromatography), and it is also apparent that the author made an effort to update the reference lists through the period 1977–1978.

Honolulu, HI (U.S.A.) A. BEVENUE

PUBLICATION SCHEDULE FOR 1980

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

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Chromatographic Reviews			184/1	184/2					184/3			184/4	
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