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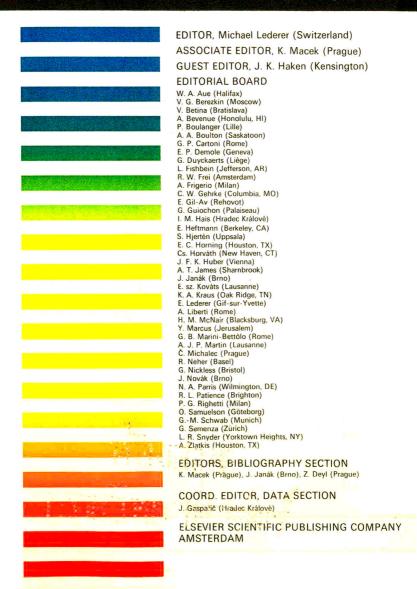
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JOURNAL OF

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INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS



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STUDY OF THE PERFORMANCES OF THIN-LAYER CHROMATOGRA-PHY

VII. SPOT CAPACITY IN TWO-DIMENSIONAL THIN-LAYER CHROMA-TOGRAPHY

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SUMMARY

In two-dimensional thin-layer chromatography the spot capacity is the number of spots, resolved with a resolution unity, that can be placed on the plate between the two solvent fronts and the parallels to these fronts through the center of the original sample spot. This is difficult to calculate because the plate height in thin-layer chromatography (TLC) is a complex function of the characteristics of the solvents and the plate, since during development in one direction the spots spread in both directions and since calculation of the density of the most dense spot packing requires topological information that is not available. Some simplifying assumptions are made and an iteration method is used.

The results show that it is very easy to achieve a spot capacity between 100 and 250, but difficult to reach 400 and nearly impossible to exceed 500, except in very favourable circumstances. As for one-dimensional TLC, the spot capacity in two-dimensional TLC increases with decreasing diffusion coefficients and with increasing plate quality (*i.e.*, packing homogeneity) and kinetic coefficients of the solvents. For a given solvent and development length there is an optimum particle size which provides the maximum spot capacity.

The development time for a capacity of 300 spots is around 30 min but it is very difficult to obtain accurate quantitative results if the analysis is fast.

INTRODUCTION

Thin-layer chromatography (TLC) can easily be carried out in two dimensions, successively. Only one sample spot is developed on a square (or rectangular) plate. The sample is placed at a corner of the plate, and the two developments are carried out successively, parallel to the two sides of the plate, using two different chromatographic systems, for example two different solvents. It is more difficult to spread the components of a mixture evenly over the entire plate than to spread them over the one dimension of standard TLC or column liquid chromatography; this requires far more ingenuity from the analyst in combining the different retention mechanisms.

Two-dimensional TLC was first reported by Consden *et al.*¹. They used a 45 \times 55 cm paper sheet to separate proteinic amino acids. The first development using collidine–water lasted 72 h. After drying, this was followed by a development using phenol–water in an atmosphere containing a small amount of ammonia, that lasted from 27 to 48 h. At least 15 of the 22 amino acids were separated². Detection was carried out using ninhydrin. The sensitivity was of the order of 1 μ g allowing the analysis of 200- μ g samples of protein hydrolyzates.

Later this technique was used by Munier and co-workers³ to separate a variety of acids important in biochemistry (malonic, lactic, citric, malic, tartric, etc.) and by Nordmann *et al.*^{4,5} to separate 21 drganic acids in urine. The spots on the chromatogram published differ widely in size, reflecting not only variations from spot to spot in both development directions, but also differences in concentration^{4,5}. It is well known that spot shapes drawn after visual inspection have a size depending markedly on the amount of the corresponding compound⁶. Nevertheless, taking the average surface area of a spot on the chromatogram (8 × 10⁻³ R_F^2) we derive a spot capacity of 126 which is remarkably large in view of the crude technique used.

Two-dimensional TLC has been used for a large number of difficult separations⁷. For example, excellent separations of amino acids have been reported by Von Arx and Neher⁸ and very impressive separations of carbohydrates by Lato and coworkers^{9–11}. This technique has had an important impact on the development of several important fields of biochemistry, such as the elucidation of the reduction cycle of carbon in photosynthesis and its connection to other metabolic pathways^{12,13} and the unravelling of other biochemical pathways¹⁴.

This method is also related to other techniques used in biochemical analysis. For example, the separation of oligonucleotides can be carried out by ionophoresis on a two-dimensional system using cellulose acetate in one dimension and DEAE-paper in the other¹⁵. Similarly, large numbers of proteins are separated by two-dimensional electrophoresis¹⁶.

Two basic techniques have been used. In the first the same chromatographic bed is developed successively with two different solvent mixtures along the two directions. In the second method a plate is coated with a strip of a sorbent along one edge and a large layer of a second sorbent, and two successive developments are carried out, with two different solvents. The preparation of such plates is difficult^{7,17}.

The main advantage of the technique is its high resolution power, already exemplified above, associated with the simplicity of TLC. The drawbacks are the necessity of selecting two different retention mechanisms, the possible interference between the solvent used for the first development and the second retention mechanisms.

nism and particularly the detection of the separated compounds for quantitative analysis.

Already TLC is plagued by the lack of a good measuring device. The human eye is a wonderful instrument to detect a pattern of spots but is unable to perform any quantitative measurement^{6,18}. A scanning photometer, although not very practical and rather slow¹⁹, can be used to scan a one-dimensional TLC chromatogram. To obtain quantitative results several minutes are required to scan a conventional TLC plate. It would be almost impossible to scan a complete plate for a two-dimensional chromatogram. This would require several hundred parallel scans and would take many hours, since we know from column chromatography that at least ten data points are required per standard deviation¹⁹. For the same reason, although seemingly attractive, the use of a Vidicon tube²⁰ raises a difficult problem of optical resolution. Equipment able to handle 10×10 cm plates with a spot capacity of 400 (spot diameter ca. 5 mm) should have a resolution of 0.13 mm, *i.e.*, 800 points should be distinguished along one side of the plate. This largely exceeds the specifications for the screen of commercial TV sets or video display monitors (512 \times 512 pixels).

Up to now the problem has been solved satisfactorily only for the analysis of radioactive samples^{16,21}, using photographic techniques and autoradiography.

The purpose of this work is to calculate the performance expected from twodimensional TLC and the range of spot capacity attainable in practice. The specifications for a detection system could then be derived.

THEORETICAL

The peak capacity in one-dimensional TLC can be calculated using an approach developed recently²². As both the spot diameter and the height equivalent to a theoretical plate (HETP) corresponding to each spot vary along the distance on the plate between the sample spot and the solvent front, an iteration method is used.

It is assumed that the distance between two successive spots which are separated with a resolution of unity is equal to the diameter of the first of these two spots. The migration distance, z_{p+1} , of the spot number p+1 is thus related to the migration distance of spot p and the width of that spot by

$$z_{p+1} = z_p + 4\sigma_p \tag{1}$$

where σ_p is the standard deviation of the concentration distribution of spot p along the development direction, assuming a Gaussian profile. The spot capacity, n, is such that:

$$\sum_{p=0}^{n} 4\sigma_{p} < L - z_{0} < \sum_{p=0}^{n+1} 4\sigma_{p}$$
 (2)

where L is the migration distance of the solvent front and z_0 is the distance between the solvent level in the tank and the original sample spot. The calculations are carried out using a HP 67 calculator. The retention ratio is:

$$R_F = z/L \tag{3}$$

In this calculation we neglect the variation of the density of the solvent near its front but assume a piston flow of the mobile phase. This is in part compensated by rounding off n to the lower integer, and assuming that the non-retained solute has a circular spot whereas it actually has a semi-circular or crescent-shaped one. Also the less strongly retained spots are also the longer ones in the direction of the development so that it is rare that the second spot has an R_F larger than 0.85-0.90 (ref. 22).

The spot diameter is obtained using the addition of variances

$$\sigma^2 = \sigma_i^2 + zH \tag{4}$$

where σ_i is the standard deviation of the sample spot deposited on the plate and H is the average HETP corresponding to the spot compound²³. H is obtained by integrating the Knox empirical equation for the reduced plate height²³

$$h = \frac{B}{v} + Av^{1/3} + Cv ag{5}$$

with

$$h = H/d_{\rm p} \text{ and } v = ud_{\rm p}/D_{\rm m}$$
 (6)

where $d_{\rm p}$ is the diameter of the particles used to make the chromatographic bed, u is the solvent velocity and $D_{\rm m}$ is the diffusion coefficient of the compound in the solvent. The integration is carried out to account for the variation of the solvent velocity during the development, since the movement of the solvent front obeys the quadratic law

$$L^2 = kt (7)$$

where t is the time, L the migration distance of the solvent above its level in the solvent tank and k the kinetic coefficient of the solvent:

$$k = \theta \, d_{\rm p} \tag{8}$$

 θ is a function of the nature of the solvent²⁴. Integration of eqn. 5 using eqns. 6–8 gives²³

$$H = b(L + z_0) + \frac{a}{L - z_0} (L^{2/3} - z_0^{2/3}) + \frac{c}{L - z_0} \ln \frac{L}{z_0}$$
 (9)

with

$$a = 3Ad_{\rm p}^{5/3}\theta^{1/3}/2(2D_{\rm m})^{1/3} \tag{10}$$

$$b = B/\theta d_{\rm p} \tag{11}$$

$$c = C\theta d_{\rm p}^3 / 2D_{\rm m} \tag{12}$$

while B is related to the diffusion coefficients by²³:

$$B = 2\left(\gamma_{\rm m}D_{\rm m} + \frac{1 - R_F}{R_F} \cdot \gamma_{\rm s}D_{\rm s}\right) \tag{13}$$

 γ is the tortuosity and D the diffusion coefficient, while the subscripts m and s refer to the mobile and stationary phases respectively. As a first approximation, $\gamma_{\rm m}D_{\rm m}$ and $\gamma_{\rm s}D_{\rm s}$ are similar and we assume them to be equal. Hence:

$$B = 2\gamma D/R_F \tag{14}$$

Combination of eqns. 3, 4, 9 and 14 gives

$$\sigma^2 = \sigma_i^2 + L \left[\frac{2\gamma D}{\theta d_p} \left(L + z_0 \right) + R_F H_0 \right]$$
 (15)

with:

$$H_0 = \frac{a}{L - z_0} \left(L^{2/3} - z_0^{2/3} \right) + \frac{c}{L - z_0} \ln \frac{L}{z_0}$$
 (16)

 σ_0 , σ_1 , ... σ_p are calculated using eqns. 1, 3, 15 and 16 and summed until n is obtained.

The calculation of the spot number in two-dimensional TLC is slightly more complicated, since all spots spread during the two successive developments, unequally in the direction of development and in the perpendicular direction. This is illustrated in Fig. 1. Let n_1 and n_2 be the spot capacities obtained in one-dimensional TLC along the two different development directions with a sample spot standard deviation of σ_i and σ_i be the spot capacity achieved in two-dimensional TLC. Obviously σ_i is smaller than the product σ_i for two reasons. First, when the second development starts the spots have a dimension (length along the second direction, *i.e.*, width perpendicular to the first direction) which is larger than σ_i . Accordingly the spot capacity for this second development is smaller than σ_i . The spot capacity in the second direction should be calculated for an original spot dimension σ_i , such that

$$\sigma_0^2 = \sigma_i^2 + 2\gamma Dt_1 = \sigma_i^2 + \frac{2\gamma D_1 L_1^2}{k_1} \tag{17}$$

where the subscript 1 refers to the first solvent. This gives n'_2 , the spot capacity along the second direction in two-dimensional TLC. Secondly, during the second development, the spots also spread laterally, so they must be separated with a resolution higher than unity at the beginning of this second development if they are to have a resolution of 1 at the end. Some of the resolution provided by the first development is lost during the second one.

Accordingly, in two-dimensional TLC the standard deviation to use in eqn. 1 to calculate n_1 is given by:

$$\sigma_p^2 = \sigma_i^2 + z_p H + \frac{2\gamma_2 D_2 L_2^2}{k_2} \tag{18}$$

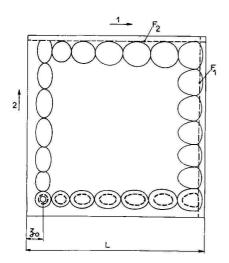


Fig. 1. Scheme of spot distribution on a two-dimensional TLC plate, after the two developments are completed. 1,2 = The two development directions; F_1 , F_2 = solvent fronts. In this case $n'_1 = n'_2 = 7$; n_1 is between 9 and 10. The spots corresponding to compounds which do not move during the second development ($R_{F,2} = 0$) spread to some extent (cf., dotted profiles).

Then the spot capacity in two-dimensional TLC is given by

$$^{2}n = n'_{1} n'_{2} \tag{19}$$

where n'_1 and n'_2 are calculated using eqns. 1–3, 9–12, 14–16 and 18. These calculations have been made for a number of combinations of plate and solvent characteristics to investigate the performances which are accessible.

These derivations assume that the thin-layer bed is homogeneous and isotropic, so that there is no coupling between the two developments. We have neglected the compression effect at the beginning of the second development; the solvent front reaches the lower side of the spots first and moves it towards the centre of the spots, so actually it reduces the effective spot width by a factor $(1 - R_F)$. This phenomenon was also neglected when it acts on the original sample. It may result, however, in a significant increase in the spot capacity.

We have considered it implicit that the spot capacity is equal to the product $n_1' \times n_2'$ and the spots are arranged in rows and columns after a regular square pattern, the numbers of spots n_1' and n_2' being calculated along the axes 1 and 2 through the centre of the sample spot. The spot capacities along these directions would be slightly smaller if calculated at the other end of the plate, along the solvent front F_1 for direction 2 and along solvent front F_2 for direction 1, since the corresponding spots have moved over a longer distance. The difference is not great in most cases however, as molecular diffusion tends to control spot broadening in TLC^{23} . This effect, which would result in a decrease in spot capacity, is approximatively compensated by the fact that the spots could be packed more densely than in a square-based tessellation: a regular hexagonal tessellation could accommodate $2/\sqrt{3}$ or 15% more spots.

Also neglected in eqns. 17 and 18 is the contribution to radial or lateral band

broadening due to the anastomosis of the flow stream pattern. It is at most equal to $0.15zd_n$ and thus negligible compared to $2\gamma Dt$.

Finally the limiting spot capacity, reached after an infinitely long development time in both directions, so that the sample spot size becomes negligible compared to the final spot size, is

$${}^{2}n_{\mathrm{T}} = \left[\frac{L}{4\sqrt{2\gamma\left(\frac{D_{1}}{k_{1}} + \frac{D_{2}}{k_{2}}\right)L^{2}}}\right]^{2} = \frac{d_{\mathrm{p}}}{32\gamma\left(\frac{D_{1}}{\theta_{1}} + \frac{D_{2}}{\theta_{2}}\right)}$$
(20)

as derived from eqns. 4, 7 and 8 of ref. 22.

RESULTS AND DISCUSSION

We have carried out calculations using the model developed above to assess the effects on spot capacity of the various characteristics of the chromatographic systems used, and of the parameters of the TLC bed.

We first studied the effect of the sample spot size and of the distance of this spot above the solvent level, then the most important parameters, the plate size (it is assumed to be square) and the average particle size. We assume that the TLC bed is thin enough so that the plate efficiency is not affected by vertical segregation of particles of different sizes during preparation of the bed. Then we calculated the effect of the quality of the TLC bed (parameters A and C of eqn. 5) and of the parameters of the chromatographic system: the diffusion coefficient of the solute and kinetic parameter of the solvent. The diffusion coefficient is assumed to be the same for all solutes. Another assumption (such as a relationship between D and R_F) would be equally arbitrary and would lead to extremely complicated calculations. Thus the reduced velocity is also taken to be the same for all solutes.

Throughout this work we have taken the bed tortuosity to be 0.7, a value often employed²². Except when the effects of these parameters is studied, the bed characteristics A and C are equal to 1 and 0.01 respectively, in agreement with experimental results^{24,25}.

Although it is quite reasonable to assume that the plate characteristics (A, C, γ, d_p) are the same in both directions, this is less acceptable for the solvent characteristics. The kinetic coefficient is quite different from one solvent to another and so is the diffusion coefficient. The latter can be approximated by the Wilke-Chang equation²⁶

$$D_{\rm m} = 7.4 \times 10^{-10} \sqrt{\varphi_1 M_1} \cdot \frac{T}{\eta_1 V_2^{0.6}}$$
 (21)

where M_1 and η_1 are the molecular weight and viscosity of the solvent, respectively. T the temperature (°K) and V_2 the molar volume of the solute. φ is an association constant (2.6 for water, 1.9 for methanol, 1.5 for ethanol, 1 for non-associated liquids). A correlation exists between θ and the diffusion coefficient of any given solute, at least in normal chromatography, as in this case the cosine of the wetting

angle is unity for all solvents: θ is inversely proportional to the solvent viscosity as is $D_{\rm m}$ and the surface tension increases gradually with the molecular weight, except for light, very polar solvents like acetonitrile and acetone. Accordingly, most of the calculations have been made using values of θ and $D_{\rm m}$ which are both smaller in one direction than in the other one. We have chosen for $D_{\rm 1}$ and $D_{\rm 2}$ values of 5×10^{-6} and 2×10^{-6} cm²/sec respectively, which are typical of medium size molecules constituting most of the complex mixtures of current interest, and for $\theta_{\rm 1}$ and $\theta_{\rm 2}$, values of 120 and 60 corresponding respectively to fast and rather slowly moving TLC solvents (cf. eqns. 7 and 8). In a separate section, the influence of these two parameters is studied and calculations are made using different combinations of values for $\theta_{\rm 1}$, $D_{\rm 1}$ and $\theta_{\rm 2}$, $D_{\rm 2}$.

As very little experimental work has yet been done in two-dimensional TLC, it is not useful at this stage to make a thorough investigation of the whole situation; it is sufficient to obtain enough data to give a flavour of the potential of the technique.

Theoretical limit of the performance

The theoretical spot capacity, achieved with either a sample spot diameter of zero or an infinitely long development time, unrealistic conditions in both cases, has been calculated for a variety of experimental conditions, using eqn. 20. The results are reported in Table I, together with the corresponding values for one-dimensional TLC. The spot capacity in two-dimensional TLC exceeds that in conventional TLC, using the same plate characteristics and solvent systems, by about one order of magnitude, although it is markedly smaller than the product of the spot capacities in both directions, as expected from the radial diffusion of the spots.

The theoretical performance cannot be reached, as usual in chromatography, but we can expect to be able to achieve rather easily half the theoretical limit, since it has already been demonstrated that the development time required for a similar effect in one-dimensional TLC is very reasonable²².

This means that, in spite of the limits of the TLC technique, two-dimensional TLC could be comparable to column liquid chromatography in terms of resolution power, provided two independent retention mechanisms can be found.

Influence of sample spot size

The results are given in Table II. The calculations have been made for square plates having sides from 1 to 5 cm. The sample spot is placed on the plate diagonal at

TABLE I
THEORETICAL LIMIT OF THE SPOT CAPACITY IN TWO-DIMENSIONAL TLC (cf., EQN. 20) v = 0.70: $n_x = \sqrt{\theta d/32 vD}$

y = 0.70, m	- V 00	p/327D					5250		-	
d_n	3	5	7	10	15	20	7	7	7	7
$D_1 \times 10^6$	5	5	5	5	- 5	5	1	2	10	10
θ_1	120	120	120	120	120	120	30	60	120	120
$D_2 \times 10^6$	2	2	2	2	2	2	1	1	5	10
θ_2	60	60	60	60	60	60	30	30	100	120
$n_{\mathrm{T,1}}$	18	23	27	32	40	46	30	30	19	19
$n_{\mathrm{T,2}}$	20	25	30	36	44	51	30	30	25	19
$^{2}n_{\mathrm{T}}$	178	297	416	595	892	1190	468	468	234	187

TABLE II

INFLUENCE OF THE SAMPLE SPOT SIZE ON THE SPOT CAPACITY

A=1; C=0.01; $\gamma=0.70$; $d_{\rm p}=5~\mu{\rm m}$; $D_1=5~\times~10^{-6}~{\rm cm}^2/{\rm sec}$; $D_2=2~\times~10^{-6}~{\rm cm}^2/{\rm sec}$; $\theta_1=120~{\rm cm/sec}$; $\theta_2=60~{\rm cm/sec}$. σ_i is the standard deviation of the sample spot, in cm. The theoretical limit, $^2n_{\rm r}$, is $297~({\rm cf}, {\rm Table~I})$.

L(cm)	$\sigma_i = 0$	0		$\sigma_i =$	0.02	1	$\sigma_i = 0.04$	0.04		$\sigma_i = 0.06$	90.0		$\sigma_i =$	$\sigma_i = 0.10$	i	$\sigma_i =$	0.20	
	n_1'	n_2'	^{2}n	$n_1^{'}$	n' ₂	^{2}n	n_1'	n_2'	u_z	n_1'	n_2'	2n	n' ₁	n' ₂	2 _m	n_1'	n_2'	2,11
*!	10	10	100	7	7	49	S	2	25		ŧ			J			1	
5 *	13	12	156	11	11	121	6	6	81	7	7	46	5	S	25		J	
5 *	15	15	225	15	15	225	14	4	196	12	12	144	10	10	100		į	
10	91	16	256	16	16	256	16	16	256	15	15	225	14	14	961	10	10	100
15	17	17	289	17	17	289	91	16	256	16	91	256	15	15	225	13	13	691

 $[\]star$ $z_0 = 0.2$ cm; otherwise $z_0 = 0.5$ cm.

TABLE III

ANALYSIS TIME (MIN) IN TWO-DIMENSIONAL TLC

 $t_A = t_1 + t_2$; $\theta_1 = 120 \text{ cm/sec}$; $\theta_2 = 60 \text{ cm/sec}$.

L(cm)	$d_{\rm p}=3~\mu m$	3 рт		$d_p = 5 \ \mu m$	5 µт		$d_p = 7 \ \mu m$	7 рт		$d_p = 10 \ \mu m$	ит о		$d_p =$	$d_p = 15 \ \mu m$		$d_p = 2$	20 µm	
	t,	[[t _A	1,	<i>t</i> ₂	l _A	t ₁	12	t _A	t_1	12	t _A	t ₁	12	t _A	1	t ₂	l'A
·	0.46	0.46 0.92	1.40	0.28	3 0.56	0.83	0.20	0.40	09.0			ji S						
7	8.1	3.70		Ξ			0.75			0.56			0.4	0.7	_			
4	7	15	22	4.4			3.2			2.2		9.9	1.5		4.5	3		
															ì	5 mi	п	
9	. 17	33	50	10	20	30	7		21	5			3.33	3 6.66	10	2.5	2	7.5
10	I n 46	93	139	28	55	83	20		09	14			6	19	28	7	14	21
15		208	312	62	125	187	44.6	68	134	31	62	94	21	42	63	15.6	31	47
20	185	370	555	111	222	333	79		238	55			37	74	111	27.8	55	83
25				173.6	347	521	124		372	87	U		58	911	174	43	87	130
30							179		536	125		1	83	167	250	62.5	125	187.5
40													148	296	444	Ξ	222	333
50																174	347	520
																200000000000000000000000000000000000000		

a distance z_0 from each side and development is carried out successively in both directions, until the solvent front reaches the opposite edge of the plate in both cases. Only 5- μ m particles are considered here, as it has already been shown that the effect of the sample spot size is most important on short plates made from small particles²².

As expected the spot capacity falls dramatically for sample spot sizes larger than 0.1–0.2 mm with the small plates. With larger plates it becomes easy to achieve half the theoretical spot capacity of the plate with acceptable sample size: with a sample spot diameter of 2 mm and a 5-cm plate it is still possible to resolve 144 spots, close to half the limit of 297 (Table I, column 2). The total development time is only 21 min, to which some time should be added to allow for an intermediate, drying step between the two developments.

It will be possible to achieve more than half the theoretical performance in most cases with quite reasonable specifications, except for small plates, which are very fast to develop but conversely require very small samples²⁷.

Plates larger than 15 cm have not been considered because of an excessive development time. Development times calculated for a number of combinations of plate size and average particle diameter are reported in Table III. The total analysis time is the sum of the two development times and the time necessary to dry the plate

TABLE IV INFLUENCE OF z_0 ON THE SPOT CAPACITY $A=1; C=0.01; \gamma=0.70; D_1=5\times 10^{-6} \text{ cm}^2/\text{sec}; D_2=2\times 10^{-6} \text{ cm}^2/\text{sec}; \theta_1=120 \text{ cm/sec}; \theta_2=60 \text{ cm/sec}.$

L(cm)	$d_p(\mu m)$	$z_0(cm)$	$\sigma_i(cm)$	n_1'	n_2'	² n	$^{2}n_{T}$
1	5	0.1	0	10	10	100	297
		0.2	0	10	10	100	
		0.5	0	7	7	49	
2	3	0.1	0	12	12	144	178
			0.04	9	9	81	
		0.2	0	12	12	144	
			0.04	8	8	64	
		0.5	0	10	10	100	
			0.04	7	7	49	
	5	0.1	0	13	13	169	297
			0.04	9	9	81	
		0.2	0	13	12	156	
			0.04	9	9	81	
		0.5	0	11	11	121	
			0.04	8	7	56	
	7	0.1	0	12	12	144	416
			0.04	9	9	81	
		0.2	0	12	12	144	
			0.04	9	8	72	
		0.5	0	11	11	121	
			0.04	7	7	49	
4	5	0.1	0	15	15	225	297
		0.2	0	15	15	225	
		0.5	0	15	14	210	

between the two developments. This drying should be made very carefully ¹⁸ as the reproducibility of the retention data during the second development is strongly influenced by the presence of minor amounts of the first solvent sorbed on the stationary phase. The last step cannot be undertaken in less than 10 min.

Calculations have been made for combinations of plate length and particle size which result in a total development time not exceeding 10 h. In spite of the work of the pioneers in this field¹⁻⁵, it seems that longer times are not realistic and we do not consider further conditions which require development times in excess of a few hours.

Influence of z_0

This influence is particularly significant on small plates, so it has been studied on plates having sides from 1 to 4 cm, made from 3–7 μ m particles. The data are reported in Table IV. They show that z_0 has little influence as long as it is less than 20% of the plate side and no influence at all if it is 10% or less. However, with a small plate it is not possible to achieve a large fraction of the theoretical limit. This is discussed in the next section.

In the following we have used values of $z_0 = 0.2$ cm for plates smaller than 5 cm square, and 0.5 cm for larger plates. This is reasonable and meeting these specifications does not seem to raise any significant experimental problem. It is worth noting also that retention data are reproducible only if the migration distance of the solvent front is large compared to z_0 , at least three times and preferably ten times larger¹⁸.

Influence of plate length and particle size

These are the most important characteristics of a plate, together with the homogeneity of the packing which is considered in the next section. Performances have been calculated for various combinations of plate length and particle size and the results are reported in Table V, together with the theoretical maximum spot capacity as calculated by eqn. 20. The original spot size used ($\sigma_i = 0.4$ mm), although quite realistic for most TLC applications, may appear somewhat large in view of the progress which may be expected in the near future. The data in Table II show that with such a spot size there is a marked decrease in the performances of short plates. To allow further comparison, other data are given in Table VI, calculated for a much smaller sample size, close to the technical minimum with present technology ($\sigma_i = 0.1$ mm). The results in Table VI agree with those in Table II showing that the sample spot size has a significant effect only for plates smaller than 5 cm. For 2-cm plates, for example, the improvement obtained with a four-fold decrease in sample spot size is very important.

For plates made from small particles it does not seem too difficult to reach a spot capacity close to the theoretical limit within an acceptable analysis time. Analysis times are given in Table III and calculations have been carried out only for combinations of L and d_p which lead to analysis times shorter than about 3 h, already a long time by present day standards. In 2 h and 20 min it is possible to achieve 98% of the maximum spot capacity using a 10 cm long plate made from 3- μ m particles if the sample spot standard deviation is 0.4 mm, while the same performance is achieved within 30 min with $\sigma_i = 0.1$ mm. In a similar time, only about half of the theoretical spot capacity is achieved with a 30 cm long plate made from 20- μ m particles.

TABLE V INFLUENCE OF PLATE DIMENSIONS AND PARTICLE SIZE ON SPOT CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; D_1 ; $C > 5$ cm.	0.01; 7	= 0.70;	200	× 10_6	cm²/sec;	$D_2 = 2$	= 5×10^{-6} cm ² /sec; $D_2 = 2 \times 10^{-6}$ cm ² /sec; $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec; $\sigma_i = 0.04$ cm; $z_0 = 0.2$ cm for $L < 5$ cm, 0.5 cm for	m²/sec;	$\theta_1 = 120$	cm/sec;	$\theta_2 = 6$	0 cm/sec;	$\sigma_i = 0.0$)4 cm; z ₍	, = 0.2 cr	n for L	< 5 cm, C	.5 cm for
L(cm)	$d_p =$	$d_p = 3 \ \mu m$		$d_p =$	$d_p = 5 \mu m$	İ	$d_p = 7 \mu m$	7 µm		$d_p =$	$d_p = 10 \ \mu m$	5	$d_p = 15 \ \mu m$	15 µm		$d_p = 20 \ \mu m$	ип 0.	
	$n_1^{'}$	n_2'	2 _n	n_1'	n_2'	^{2}n	$n_1^{'}$	n'z	2n	n' ₁	n'z	2 n	n_1'	n_2'	^{2}n	$n_1^{'}$	n_2'	2n
-	S	S	25	S	ν,	25												
2	∞	∞	4	6	6	81	6	8	72									
3	10	10	100	12	12	144	=	11	121	=	10	110	6	6	81			
4	12	12	144	14	14	196	14	14	196	14	14	196	13	12	156	1	=	121
5	12	12	144	15	15	225	16	16	256	16	16	256	15	15	225	13	13	169
7	13	13	169	16	16	256	17	17	588	18	18	324	18	17	306	16	16	256
10				16	16	256	19	19	361	70	20	400	21	20	420	19	19	361
15										21	21	441	22	22	484	22	21	462
20 30																25	25	625
$^2n_{\mathrm{T}}$		172			297			416			595			892			1190	

TABLE VI INFLUENCE OF PLATE DIMENSIONS AND PARTICLE SIZE ON SPOT CAPACITY As for Table V except $\sigma_i=0.01$ cm.

L(cm)	$d_p =$	3 μm		$d_p =$	5 μm		$d_p =$	7 μm		$d_p =$	10 μm	
	n_1'	n_2'	² n									
2	11	11	121	12	12	144	12	12	144	11	10	110
3	12	12	144	14	14	196	14	14	196	13	13	169
5	13	13	169	15	15	225	16	16	256	16	15	240
7	13	13	169	15	15	225	17	17	289	17	17	289
$^2n_{\mathrm{T}}$		172			297			416			595	

Although the theoretical spot capacity is much larger, development is much slower and the time required to reach 90% of the spot capacity would be prohibitively long.

As in conventional TLC, the spot capacity increases monotonously towards the theoretical limit (eqn. 20) with increasing development length, while at constant length there is an optimum particle size (cf., Fig. 2). For smaller particle sizes the spot capacity decreases with decreasing d_p because the development is too slow and diffusion becomes more and more important, while for larger particle sizes the spot capacity decreases with increasing particle size because of increasing flow velocity and band broadening due to packing heterogeneity. Nevertheless, the large spot capacities which can be achieved in rather moderate analysis times are striking. They are com-

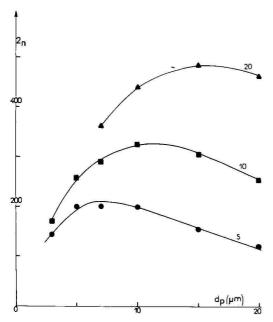


Fig. 2. Plot of the spot capacity in two-dimensional TLC versus the particle size for three different development lengths as indicated on the corresponding curves (L in cm). Conditions as in Table V.

TABLE VII
INFLUENCE OF THE DIFFUSION COEFFICIENT AND KINETIC PARAMETER ON THE SPOT
CAPACITY

$A = 1$; $C = 0.01$; $\gamma = 0.70$; $L = 10$	0 cm; $d_p = 10 \mu m$; $z_0 = 0.5 \text{ cm}$; $\sigma_i = 0.04 \text{ cm}$.
---	--

$\begin{array}{l}D_1\times 10^6\\(cm^2/sec)\end{array}$	θ* (cm/sec)	$\begin{array}{c} D_2 \times 10^6 \\ (cm^2/sec) \end{array}$	θ_2^* (cm/sec)	n_1'	n' ₂	² n	$^{2}n_{T}^{\star\star}$
2	60	2	60	19	19	361	669
2	120	2	60	19	20	380	892
2	120	2	120	19	19	361	1339
5	120	2	60	18	18	324	595
_	_	2	120	19	18	342	765
_	_	5	20	11	12	132	153
_	_	5	60	15	16	240	357
	_	5	100	17	17	289	487
_		5	120	18	18	324	535
_	_	5	140	18	18	324	576
_	_	7	60	14	15	210	281
_	_	7	120	17	17	289	446
_	_	10	60	13	13	169	214
_	_	10	120	15	16	240	357
10	60	10	60	11	11	121	133
10	120	10	60	12	12	144	178
10	120	10	120	14	14	196	267

^{*} Development time: 14 min for $\theta = 120$ cm/sec, 28 min for $\theta = 60$ cm/sec.

parable to or larger than the peak capacities which can be obtained with the best columns available in high-performance liquid chromatography (HPLC).

A peak capacity of 100 requires a 40,000-theoretical plate column, which is more than most HPLC columns can produce: it requires at least a 40 cm long column packed with 5-um particles and the analysis time at v = 3 would be 2 h 45 min for k' = 6.4. This is certainly possible to achieve with current technology, but it becomes increasingly difficult to do better, while spot capacities in the range 200-300 and more do not seem terribly difficult to achieve in TLC (Tables III, V and VI). Fairly large values of the sample spot standard deviation can be tolerated for practical applications, and dilution does not greatly exceed one order of magnitude, which still permits sensitive detection. A peak capacity of 300 requires a 360,000-plate column, which is more than half the world record²⁸ and more than almost anybody has yet been able to achieve. Nevertheless, data from Tables III and V show that it can be achieved in two-dimensional TLC in an hour or so. For example, a 10-cm square plate coated with a layer of $10-\mu m$ particles has a spot capacity of 324 with $\sigma_i = 0.4$ mm and its two developments take a total of 42 min. The ultimate performance achievable in two-dimensional TLC, in terms of spot capacity, is of the order of 500, which exceeds that which can be obtained in column chromatography with reasonable experimental conditions²⁹.

Finally it should be noted that the procedure of calculation resulting from the application of the law of variance addition ensures that the spot capacity is independent of the order in which the two developments are carried out. We also

^{**} From eqn. 20.

TABLE VIII
INFLUENCE OF PLATE CHARACTERISTICS ON SPOT CAPACITY

 $D_1 = 5 \times 10^{-6} \text{ cm}^2/\text{sec}; D_2 = 2 \times 10^{-6} \text{ cm}^2/\text{sec}; \theta_1 = 120 \text{ cm/sec}; \theta_2 = 60 \text{ cm/sec}; L = 10 \text{ cm}; d_p = 10 \text{ } \mu\text{m}; \sigma_i = 0.04 \text{ cm}; \gamma = 0.70; ^2n_T = 595.$

	The state of the s		1 1-10/02/1-10/03/15	
A	C	n' ₁	n_2'	² n
3	0.01	15	15	225
1	0.01	18	18	324
0.8	0.01	19	19	361
0.5	0.01	20	20	400
1	0.03	18	18	324
1	0.10	18	17	306
1	0.30	17	16	272

observe from Tables V and VI that with the solvent characteristics chosen (D_m, θ) the spot capacities in the two directions are almost always identical. This would not be true for more dissimilar solvents, but this is an improbable situation.

Influence of the solvent characteristics

There are two important parameters which depend on the solvent used: the diffusion coefficient, which for a given solute can vary by a factor of 2 to 5, and the kinetic coefficient which is usually between 60 and 120 and can vary between 20 and 140 at most²⁴. Calculations have been made using different set of values for both solvents and are reported in Table VII. We have chosen a plate with good potential performance for these calculations, a 10×10 cm square coated with 10- μ m particles.

The spot capacity which can be achieved in a reasonable time (total development time about 45 min) increases markedly with decreasing diffusion coefficient in and increasing velocity coefficient of the two solvents. TLC is not well suited to the analysis of low-molecular-weight compounds because the average reduced velocity during a development carried out under the usual conditions is too low and spot broadening by molecular diffusion is too important.

We observe also that the performances achieved with the plate considered are markedly lower than the theoretical performances and increase much more slowly. In fact it is extremely difficult to find conditions in which the spot capacity would reach 400 without drastic requirements, especially regarding analysis time.

Influence of the plate characteristics

Besides the plate dimensions and the particle size already discussed, other characteristics to be considered are the coefficients of the theoretical plate height equation (eqn. 5), the bed tortuosity, γ , the packing homogeneity coefficient, A, and the coefficient of resistance to mass transfer, C.

There is little one can do about γ . The axial diffusion term has not been studied intensively since the classical work by $Knox^{30}$. Recent data by Theumneum and Hawkes³¹ show that in gas chromatography it is not constant but increases slightly with increasing gas velocity. Whether the same is true in liquid chromatography and to what extent is still unknown. In all our calculations γ is taken as constant and equal to 0.7. Significant changes of γ , however, much larger than the range of variations

reported by Hawkes, would be required to affect markedly the spot capacity. For example, under the conditions given in Table VIII, for A=1 and C=0.01, $n_1'=16$ for $\gamma=0.50$, 18 for $\gamma=0.70$ and 20 for $\gamma=1.0$. In practice we can consider that γ is between 0.65 and 0.75 which leads to a value of n_1' of either 19 or 18, hardly a significant variation.

The influence of A and C has been studied and results are reported in Table VIII. The influence of A has been studied in the range from a value of 0.5 which corresponds to an extremely homogeneous bed to one of 3 which corresponds to a fairly poor bed. In column chromatography it is very difficult to achieve values of A less than 1, but making an homogeneous thin packing as in TLC seems an easier task and values of A smaller than 1 have been obtained for commercial plates²⁴. Reducing A seems to be the easiest way to improve the plate performance, since this does not change the analysis time nor does it require any adjustment of the chromatographic system properties. However, there is as yet little information on how to do it, and the improvement, although major when performances of plates of high and low packing

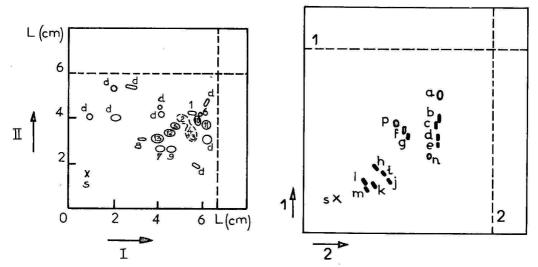


Fig. 3. Separation of azaarenes by two-dimensional TLC. Stationary phase: RP-18 (Merck). Development 1: ethanol–water (20:1). After drying the plate is briefly dipped in a solution of ethanol–water–ammonia +1 M Cu(NO₃)₂ (13:1:6) carefully avoiding wetting the strip where are the compounds separated by the first development. Development II: after drying: ethanol–water +1 M Cu(NO₃)₂ (15:2). Plate size: 10×10 cm. Spots: 1 = indenopyridine; 2 = benzo-5,6-quinoline; 3 = benzo-3,4-quinoline; 4 = benzo-7,8-quinoline; 5 = 7-azafluoranthene; 6 = 2-tolyl-3-methylquinoline; 7 = 9-methylbenzo-5,6-acridine; 8 = 5-ethyl-9-methylbenzo-1,2-actidine; 9 = 2,2'-biquinoline; 10 = acridine; 11 = phenazine; 12 = benz[a]acridine; 13 = 4-azapyrene; 12 = denz[a]acridine; 13 = 4-azapyrene; 13 = 5-azapyrene; 13 = 6-azapyrene; 13 = 8-azapyrene; 13 = 9-azapyrene; 13 = 9-azapyre

Fig. 4. Separation of a mixture of nucleic acid components by two-dimensional TLC. Stationary phase: silica gel $60F_{25}$, 5- μ m particles. Plate size: 10×10 cm. No activation prior to analysis. Development I: 1-butanol-acetic acid-water (12:3:5); drying for 5 min at 110° C and 2 h at ambient temperature. Development II: 1-propanol-ammonia-water (50:5:10). Spots: a = thymidine; b = adenosine; c = hypoxanthine; d = guanine; e = cytosine; f = xanthosine; g = guanosine; h = 5'-thymidine monophosphate; h = 5'-cytidine monophosphate; h = 5'-adenosine monophosphate; h = 5'-cytidine monophosphate; h = 5'-guanosine
qualities are compared, is not very important for values of A lower than 1. This is because during a TLC development the solvent velocity is so low most of the time that the contribution to spot broadening of the A term of the plate height equation is minor²³.

A marked reduction of this contribution is accordingly not very significant, even so the conditions selected for Table VII are such that the first term of the plate height equation (axial diffusion) is not as predominant as it is in many TLC analyses.

All this discussion applies as well to the influence of the parameter C. The third term of the plate height equation usually gives a very minor contribution to band broadening in TLC^{23} . A thirty-fold increase of C only reduces n_1' by 1, which is hardly significant. Any kind of packing material which gives fair results in column chromatography, as far as resistance to mass transfer is concerned, will be useful in TLC and will not contribute significantly to band broadening.

Comparison with experimental data

Few quantitative data are available in two-dimensional TLC. This technique is hardly amenable to scanning because of the difficulty in localizing the exact centre of a spot and the fact that a number of parallel profiles (about 40–50) should be obtained for each spot. We have attempted such scans on spots obtained on various plates, but it takes a very long time to scan a small part of a plate and it was not possible to achieve illustrative results; so we show two chromatograms in a conventional way, the spots being drawn as the contours of the luminous spots seen when the developed plate is placed under an UV lamp.

Fig. 3 shows the separation of thirteen different azaarenes and nine unidentified impurities, probably other azaarenes. The separation compares favourably to those obtained by Engel and Sawicki³². From the measurements of the spot dimensions in the x and y directions it appears that the spot capacities in these two directions are 12 and 15 respectively, hence the total spot capacity of the plate is 180. Theory predicts 19 for one single TLC development²², 14 for each development in two-dimensional TLC and a total of 196 (cf., Table V). The agreement is excellent. It will be noted, however, that the spot capacity is markedly larger in the direction of the second development. This results from the concentration effect at the beginning of the second development as the lower edge of the spots starts moving upward before the upper end. Account of this effect could be taken by multiplying the second term of the right-hand side of eqn. 17 by R_F .

A similar effect is observed in Fig. 4 which shows a separation of fifteen nucleic acid components. Although, as in the chromatogram of Fig. 3, the same adsorbent is used with two different chromatographic systems, the spots are much narrower in the second direction $(16.6 \times 10^{-3} R_F)$ instead of $31 \times 10^{-3} R_F$). Accordingly the spot capacities along the two directions are 60 and 31 respectively, with a total two-dimensional TLC capacity of 1860, whereas theory would predict about only 320, because of the low values of the diffusion coefficients. Part of the considerable difference probably results from the low sensitivity of the detection and the necessity to draw spot shapes in dim light. In such a case there can be little relationship between spot width and zone standard deviation⁶. Nevertheless, chromatograms such as this one attest to the power of the technique.

TABLE IX

Conditions for all systems: A = 1; C = 0.01; $\gamma = 0.70$; $D_{m,1} = 5 \times 10^{-6}$ cm²/sec.

COMPARISON BETWEEN ANALYSIS TIMES IN TLC, TWO-DIMENSIONAL TLC AND COLUMN CHROMATOGRAPHY

	-	1 10								
Spot or peak capacity required n	Condition	Conditions in TLC*		Conditions in two-dimer	Conditions in two-dimensional TLC**	*	Conditions in column cl	Conditions in column chromatography***	<i>hy</i> ***	
	L(cm)	$d_p(\mu m)$	t _A (min)	L(cm)	$L(cm) d_p(\mu m) t_A(min)$	t _A (min)	$L(c\dot{m})$	$L(cm)$ $d_p(\mu m)$ $\Delta P(atm)$ $t_A(min)$	AP(atm)	t _A (min)
10 15 20	s 2 0	3 5 5 7 10	2.5 5 17	Practically impos because it is too	Practically impossible because it is 100		Practically impo because it is too	Practically impossible because it is too		
	30	20		(m)			4.9	5	18	4.0
	Practical	ly impossible					13.6	٠	49	11.11
	because 1	it is <i>too</i>		3.5	5	10	54	5	195	45
	difficult			4	ŗ	2	122	5	439	100
	1			Ç. ,	,	71	73	3	1220	36
	1			9	10	15	217	5	780	178
	1			10	15	28	Practical	Practically impossible because	because	
200	1			22.5	20	105	it is too difficult	tifficult		

* $z_0 = 0.2$ cm for L < 5 cm; $\theta_1 = 120$ cm/sec; $\sigma_i = 0.5$ mm. Analysis time = development time. ** $\theta_1 = 120$ cm/sec; $\theta_2 = 60$ cm/sec; $D_2 = 2 \times 10^{-6}$ cm²/sec; $\sigma_i = 0.5$ mm. Analysis time = sum of the two development times. *** $v_0 = 2.8$; h = 1.94; $\eta = 0.6$ cP; $k_0 = 1 \times 10^{-3}$. Analysis time = elution time for k' = 6.4.

CONCLUSIONS

Whereas TLC offers a resolution power quite lower than column chromatography, with an analysis time which increases much faster than the necessary plate number, in contrast to what happens in column chromatography²¹, the situation in two-dimensional TLC is quite different (*cf.*, Table IX). The resolution power available is much larger than anything attainable in column chromatography and the analysis time remains quite reasonable, although again it increases rapidly with increasing spot capacity. This makes two-dimensional TLC very attractive in principle for the separation of complex mixtures, much more powerful, in theory at least, than column chromatography (*cf.*, Table IX).

However, two major practical problems remain to be solved, one of which seems to be much more difficult than the other one, as discussed in the Introduction.

First, whereas in TLC or column chromatography only one retention mechanism, or chromatographic system, has to be selected, in two-dimensional TLC we need two such mechanisms or systems which are compatible and which are independent or orthogonal, *i.e.*, there should be little correlation between the retention patterns in both systems, otherwise the spots tend to agglomerate along the bisector of the plate and the spot capacity is merely multiplied by $\sqrt[4]{2}$. True, neither system needs to separate all the constituents of the mixture, but the interferences must be different with the two systems. Thus the spots corresponding to the different components will be spread over the entire plate and advantage can be taken of the large spot capacity. Advances in the understanding of retention mechanisms and of the physico-chemical basis of selectivity in column chromatography could certainly be used to select such combinations of mechanisms as normal phase LC, reversed-phase LC, size exclusion LC, affinity chromatography, etc. Nevertheless two-dimensional TLC has been used with success in the past as explained in the Introduction and continues to be applied $^{1-5,7-18}$. There are thus many ways to solve this difficult problem.

However, data acquisition remains the real bottleneck of the technique. Neither spectrophotodensitometers, definitively too slow for this application, nor Vidicon cameras, which lack the optical resolution, offer even the hope of a satisfactory solution. Our calculations have shown that two-dimensional TLC offers spot capacities between 100 and 400 which are easy to achieve with current equipment. Only the use of diode arrays could be helpful in this situation, or advanced image analyzers³³.

Thus, our calculations demonstrated that whereas two-dimensional TLC offers an extremely high resolution power, it also presents a great challenge to the equipment designer and will certainly require a sophisticated and expensive system for data acquisition and handling.

It seems to us that, in the quest for an extremely high resolution power, a chromatographic system simpler than a multi-million-plate column^{28,29} but less crude than a TLC system¹ should be used. There seems to be a way to combine the resolution power of two-dimensional TLC and the flexibility and efficiency of column chromatography^{19,34,35}.

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ADSORPTION OF ACETONE AND BUTANE ON LIQUID-MODIFIED GRAPHITIZED CARBON BLACKS

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SUMMARY

Gas-liquid, gas-solid, and gas-liquid-solid equilibrium isotherms were determined for acetone and butane on graphitized carbon black (Carbopack C) and liquid-modified graphitized carbon black (Carbopack C plus 0.2% Carbowax 1500). The measurements were carried out over the range of temperatures from 30 to 75°C by mass spectrometric tracer pulse chromatography with stable isotopic solutes. Studies of the gas-liquid systems show that the bulk liquid undergoes a phase transition (wax \rightarrow liquid) at 40°C and that the equilibrium isotherms vary significantly with temperature for the wax form. The temperature of the phase transition is shifted to 50–55°C for the same liquid coated on Carbopack C as a thin film.

The adsorption isotherms of acetone on the coated and uncoated Carbopacks were interpreted by means of a model of simultaneous competitive and cooperative adsorption effects. The Carbowax-modified adsorbent generally adsorbed as much, if not more, acetone at any given pressure than the uncoated adsorbent. This effect was attributed to specific lateral interactions between the solute and liquid or wax modifier. The magnitude of the enhanced capacity was much greater than could be accounted for by bulk solubility of acetone in the Carbowax.

The effect of preadsorbed acetone on the adsorption and retention of other solutes was also investigated. The specific retention volumes of small samples of butane were measured as a function of the surface coverage by acetone or acetone plus Carbowax 1500. Small amounts of preadsorbed acetone had little or no effect on the retention volume of butane, and the retention volumes were greater on the uncoated adsorbent. However, at surface coverages close to a monolayer, the retention volume of butane decreased dramatically with increased acetone adsorption. Also, at higher surface coverages, the retention volumes were the same for the coated and uncoated adsorbent at any fixed amount of acetone adsorbed. These "interference plots" were also explained in terms of the model previously discussed.

INTRODUCTION

Graphitized carbon blacks (GCBs) are unique adsorbents which have been used extensively as trapping material for water and air analysis and as chromato-

graphic stationary phases for the separation of a wide variety of samples¹⁻³. These adsorbents have relatively homogeneous surfaces; however, many investigators have found that the chromatographic properties of these adsorbents could be improved by the addition of small amounts of a normal chromatographic liquid phase, presumably to block and deactivate high-energy, specific adsorption sites on the GCB surface^{2,4-6}.

Numerous investigations have been carried out to determine the exact effect of these high-molecular-weight, non-volatile liquids on the chromatographic properties of the adsorbents. The experiments usually involved the measurement of the isosteric heats of adsorption or some chromatographic retention parameter as a function of the amount of liquid phase coated on the adsorbent. Kiselev et al.7 studied polyethylene glycols of different molecular weights coated on GCB and found that the retention volumes of all of the solutes studied decreased dramatically at the point of formation of a monolayer of the liquid. Bruner and co-workers^{5,6} found that the isosteric heats of adsorption of alkanes and substituted benzenes increased slightly (1-3 kcal/mol) with surface coverage by high-molecular-weight hydrocarbons, such as squalane, up to the coverage corresponding to the formation of a monolayer. A sharp (4-6 kcal/mol) decrease in the heats was observed at the point of formation of a monolayer. These same general results were obtained^{4,5} for alkane solutes on GCB coated with a polar liquid, such as glycerol or Carbowax (PEG). The results for polar solutes (alcohols) were similar; however, the increase in the heats of adsorption at low surface coverage was greater (3-6 kcal/mol) and the decrease in the isosteric heats at coverages of a monolayer or greater was less dramatic (0-2 kcal/mol). It was also observed4 that much more of the liquid was required to form the initial monolayer than was required for the formation of any subsequent layer.

These observations have been discussed 4-8 in terms of a model incorporating both competitive and cooperative adsorption. This latter effect is enhanced adsorption caused by lateral interactions between the adsorbate and the liquid "modifier" on the adsorbent surface 8. All of the previously mentioned investigations were carried out with solutes at very low pressures $(P \rightarrow 0)$. Under other conditions, there are additional sorption mechanisms which may operate, especially at finite solute concentrations. These are (i) adsorption of the solute on the surface of the liquid, (ii) solution of the solute in the adsorbed liquid, (iii) adsorption of the solute on the liquid-modified surface of the GCB, and (iv) cooperative adsorption caused by solute—solute interactions in addition to solute—liquid phase interactions. Another factor which must be considered is the magnitude of the modifier—adsorbent interactions which control the type of adsorption (localized or mobile) of the polymeric liquid.

In addition, the properties of the adsorbed liquid and the liquid-modified surface may differ significantly from the properties of the bulk liquid and the uncoated solid surface. Kern et al.⁹ found that n-alkanes exhibited "prefreezing" (ordering) on the surface of graphite at temperatures above the normal freezing point of the bulk liquid, and several authors^{9,10} have reported that the vapor pressures of liquids are diminished near a solid surface. Serpinet¹² has shown that docosane exhibited unusual melting phenomena when coated on the surface of GCB. Shifted melting points and significant hysteresis effects were also observed for this system. This author¹² suggests that docosane does not exhibit normal solvent properties at the melting point of the bulk liquid if it is coated on GCB, even in thick multilayers.

In previous investigations^{13,14}, the interactions of solutes at finite concentrations on the surface of GCBs with different surface areas have been studied. The effect of a liquid modifier. Carbowax 1500 (CW-1500), on these interactions was also investigated for non-polar solutes. In general, it was found that the polar liquid modifier did, indeed, decrease the capacity of the adsorbent for alkane solutes and that the isotherms of the alkanes were linear and segmented for the Carbowax-modified adsorbents. Significant cooperative adsorption was observed between the non-polar adsorbates, but no cooperative adsorption effects were observed for the non-polar adsorbates with the polar liquid. The primary role of the liquid in these systems was to deactivate the surface and diminish the surface area available for adsorption of the alkanes.

The mechanisms of retention and adsorption for these alkane systems are fairly well understood. On the other hand, polar systems, especially with solutes at finite pressures, are not as well characterized or understood. In this study, an investigation of the interactions of polar solutes with other solutes and with adsorbed liquids on the surface of GCB was undertaken. Other objectives were to clarify the role of the liquid modifier in these adsorption systems and to test the significance of each of the possible mechanisms which may operate in these polar systems, especially at high solute concentrations and at temperatures close to the normal melting point of the liquid "modifier".

EXPERIMENTAL

The instrumentation and mass spectrometric tracer pulse techniques have been described elsewhere^{13–15}. The isotopic solutes used were [²H₆]acetone (99.8%) (Commissariat Pour l'Energie Atomique, France) and [²H₂]butane, which was synthesized from butan-2-one by condensation with tosylhydrazide to give the tosylhydrazone which was subsequently reduced with sodium cyanoborodeuteride.

Mixtures of helium and unlabeled acetone were used as the carrier gases. The analysis of these mixtures was carried out on a Hewlett-Packard 5840A gas chromatograph by comparison of the gas mixture with standard samples from a gas stream saturated with acetone at different temperatures. The GCBs were commercial chromatographic adsorbents (Carbopack C and Carbopack C plus 0.2 % CW-1500) (Supelco, Bellefonte, PA, U.S.A.).

The solubility studies were carried out with conventional packed columns. The solid support was Chromosorb P AW DMCS (Johns-Manville, Denver, CO, U.S.A.) coated with 21 % CW-1500. Acetone was injected as a vapor with an effective sample size of less than 5 μ g. The dead time of the column was determined from the retention time of the C_1 – C_4 alkanes or from the retention time of methane if the alkane peaks were unresolved.

RESULTS

Carbopack C with 0.2% Carbowax is a popular chromatographic adsorbent. Unfortunately the liquid modifier is not pure, but rather a mixture of equal amounts of CW-300 (PEG-300) and CW-1540 (PEG-1540). The material is a liquid at temperatures above 40°C and a wax at the lower temperatures studied. Most practical

TABLE I COMPARISON OF ISOTHERM AND ELUTION DATA FOR ACETONE IN CARBOWAX 1500

Temperature (°C)	Specific retention volume, V_g^0 (ml/g)	Calculated limiting slope of isotherm (mmol)
10	191	6.49
20	155	8.40
25	137	9.25
30	141	11.8
35	129	13.1
40	144	17.8
45	119	17.8
50	101	18.2
55	81.5	17.4
60	72.0	18.3
65	58.5	17.4
70	50.1	17.5
75	43.1	17.5
80	37.2	17.5
	ated limiting slope of isotherm range 40-80°C)	17.7
	ng slope of isotherm range 40–75°C)	17.9

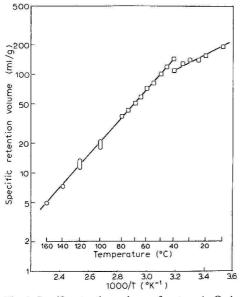


Fig. 1. Specific retention volume of acetone in Carbowax 1500. \bigcirc , Literature data¹⁷; \square , this work.

TABLE II
THE SOLUBILITY OF ACETONE IN CARBOWAX 1500

Relative pressure (P/P ⁰)	Acetone dissolved (mmol/g)	Relative pressure (P/P ⁰)	Acetone dissolved (mmol/g)
30°C		35°C	2 5000 00 00 00 00 00 00 00 00 00 00 00 0
0.023	0.044	0.006	0.012
0.033	0.065	0.030	0.072
0.092	0.187	0.061	0.151
0.134	0.291	0.110	0.289
0.193	0.456	0.159	0.471
0.283	0.750	0.222	0.703
0.369	1.106		
40°C		45°C	
0.023	0.065	0.032	0.103
0.066	0.223	0.080	0.279
0.115	0.413	0.138	0.486
0.166	0.600	0.199	0.764
0.231	0.929	0.268	1.139
60°C		75°C	
0.016	0.049	0.012	0.039
0.041	0.141	0.034	0.112
0.069	0.242	0.055	0.192
0.108	0.397	0.080	0.288
0.151	0.582		

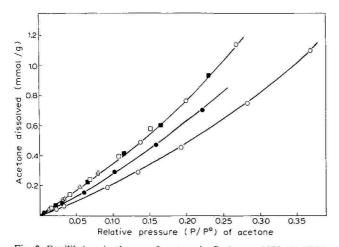


Fig. 2. Equilibrium isotherms of acetone in Carbowax 1500. \bigcirc , 30°C; \bullet , 35°C; \blacksquare , 40°C; \bigcirc , 45°C; \square , 60°C; \triangle , 75°C.

chromatographic applications of this adsorbent system require high temperatures, so the phase transition is not commonly a problem. However, sampling procedures are usually carried out at ambient temperatures, and the lower temperature regions are of more interest for a study of solute interactions on an adsorbent surface.

The low pressure $(P \to 0)$ solubility of acetone in bulk CW-1500 over a range of temperatures from 30 to 75°C was determined by normal elution chromatography with the liquid coated on a deactivated (DMCS-treated) support. These data are presented in Table I, and Fig. 1 is a plot of the data in the form of $\ln V_g^0$ vs. 1000/T. The break in Fig. 1 at 40°C is due to the phase transition. The plots are linear at both high and low temperatures; however, at temperatures close to 40°C a significant hystersis effect was observed. If the column was equilibrated at room temperature and then heated to 40°C, the measured retention volumes initially agreed with the extrapolated value for the wax. Over a period of hours, the retention volume eventually increased to the extrapolated value for the liquid and remained at that value. For this reason two data points are shown in Fig. 1 for 40°C.

McReynold's¹⁷ data at higher temperatures are also shown in Fig. 1. The agreement between the two data sets is very good, especially in view of the fact that the literature data is for a series of different molecular weight Carbowaxes. The heat of solution for acetone in liquid CW-1500 was determined from the slope of the plot and found to be -7.5 kcal/mol and the heat of "sorption" of acetone on the wax form of CW-1500 was only -2.7 kcal/mol.

The solubility isotherms were also determined over a range of temperatures and pressures by mass spectrometric tracer pulse chromatography (MSTPC)¹⁵. These data are given in Table II and Fig. 2. The plots of the amount of acetone dissolved as a function of the relative pressure (P/P^0) are congruent for the temperatures greater

TABLE III
THE SPECIFIC RETENTION VOLUMES OF INFINITE DILUTION SAMPLES OF BUTANE AND ACETONE ON GRAPHITIZED CARBON BLACK

Temperature	Specific retention volume (ml/m²)				
(°C)	Carbopack C		Carbopack C with 0.2% CW-1500		
	Butane	Acetone	Butane	Acetone	
10	3.08	_	1.63	_	
30	1.10	0.810	0.804	0.900	
35	_	_	0.674	0.785	
40	-	_	0.536	0.638	
40	0.83	_	0.600	_	
45	0.579	0.436	0.454	0.540	
50	_	_	0.395	0.475	
55	_	_	0.355	0.450	
60	0.360	0.265	0.297	0.376	
65	_	_	0.253	0.324	
70	0.28	-	0.220	0.279	
75	0.211	0.157	0.189	0.237	
80	-	_	0.158	0.194	
100	0.111	_	0.094	-	

than 40°C. This is indicative of a liquid polymeric system in which the athermal or configurational contribution to the activity coefficient is predominant. On the other hand, the isotherms at 30 and 35°C are more typical of solid adsorption isotherms. The limiting slopes of each of the isotherms agree well with the value calculated from the limiting elution data as shown in Table I. No hystersis effects were observed in the MSTPC experiments at any of the temperatures used. This set of solubility data was obtained in order to quantitatively evaluate the liquid solubility contribution to the retention (adsorption) mechanisms for the liquid-modified GCB adsorbents.

The limiting $(P \to 0)$ retention data for butane and acetone on Carbopack C and liquid-modified Carbopack C are given in Table III as specific retention volumes. These data were obtained by normal elution GC, however, the values are given in units of ml/m², rather that the normal units of ml/g. Fig. 3 is a plot of $\ln V_{\rm g}^0 vs. 1000/T$ for the acetone data. There is an obvious irregularity in the range from 50 to 55°C. This is indicative of the wax \to liquid transition, which has been shifted to higher temperatures by adsorption of the liquid on the GCB surface¹². This transition occurs at ca. 40°C for the bulk liquid, but is shifted to 50–55°C when the liquid is present as a monolayer on the adsorbent.

Fig. 3 also shows that there is a small contribution to the specific retention volume from the solubility of acetone in CW-1500, or more likely from some other mechanism which operates when the modifier is in the liquid form. The limiting heats of adsorption for these systems were $ca. -7.5 \, \text{kcal/mol}$ for acetone on both the GCB and liquid-modified GCB at temperatures above 40°C. The corresponding data for butane was $ca. -6.5 \, \text{kcal/mol}$.

Adsorption isotherms of acetone on the two adsorbents were determined at

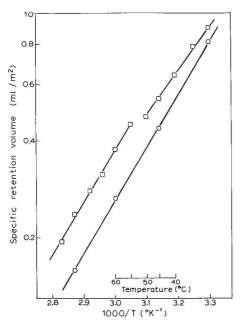


Fig. 3. Specific retention volumes of acetone and on Carbopack C (\bigcirc) and Carbopack C with 0.2% Carbowax 1500 (\square).

Temperature	Carbopack C				Carbopack C	Carbopack C with 0.2% Carbowax 1500	wax 1500		
<u>3</u>	Relative	Acetone	Specific rete	Specific retention volume (ml/m²)	Relative	Acetone	Specific rete	Specific retention volume (ml/m²,	
	pressure (P/P^0)	adsorbed (µmol/m²)	Acetone isotope	Butane	(P/P^0)	adsorbed $(\mu mol/m^2)$	Acetone isotope	Butane	Ĭ
75	0.008	0.13	0.205	0.245	0.0050	0.09	0.255	0.155	1
	0.033	0.50	0.188	0.204	0.020	0.36	0.218	0.154	
	0.045	0.79	0.216	0.213	0.029	0.57	0.243	0.161	
	0.053	16.0	0.209	0.197	0.042	0.90	0.260	0.156	
	690.0	1.25	0.222	0.190	0.054	1.15	0.261	0.141	
	980.0	1.72	0.239	0.185	0.072	1.57	0.268	0.131	
	0.100	1.75	0.214	0.184	0.091	1.96	0.263	0.120	
09	0.005	0.07	0.269	0.322	0.0065	0.15	0.445	0.336	
	0.021	0.34	0.313	0.329	0.029	0.72	0.476	0.342	
	0.042	0.73	0.328	0.344	0.045	1.21	0.517	0.333	
	0.047	0.91	0.377	0.348	0.063	1.75	0.530	0.264	
	0.068	1.26	0.380	0.267	980.0	2.13	0.475	0.185	
	0.00	1.49	0.418	0.281	0.116	2.41	0.393	0.125	
	0.099	2.05	0.408	0.217	0.131	2.67	0.392	0.115	
	0.124	2.30	0.368	0.162	0.154	2.74	0.340	0.089	
	0.155	2.56	0.324	0.123	0.163	2.91	0.341	0.087	

0.427 0.434 0.343 0.237 0.185 0.180 0.142 0.121 0.098	0.092 0.691 0.714 0.675 0.347 0.132 0.079
0.585 0.796 0.831 0.792 0.709 0.720 0.624 0.595	0.501 0.881 1.082 1.225 1.225 1.065 0.810
0.25 1.14 1.73 2.36 2.82 3.05 3.27 3.59 3.98	4.30 0.36 0.71 1.74 2.88 3.79 4.41 5.28
0.017	0.274
0.045	0.023
0.066	0.037
0.095	0.081
0.127	0.130
0.135	0.202
0.167	0.310
0.193	0.454
0.571 0.567 0.552 0.518 0.406 0.326 0.272 0.163	0.095 0.961 0.954 0.791 0.314 0.068 0.096
0.522	0.419
0.513	0.817
0.736	0.951
0.718	1.334
0.767	1.203
0.679	0.879
0.573	0.663
0.15	3.78
0.33	0.22
0.76	0.55
1.04	1.49
1.78	3.04
2.01	3.61
2.36	4.35
3.35	5.04
0.009	0.290
0.020	0.015
0.037	0.033
0.046	0.063
0.074	0.144
0.083	0.233
0.112	0.373
0.173	0.500

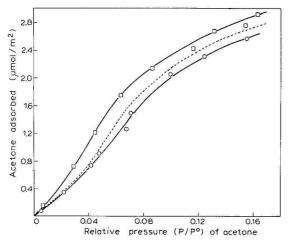


Fig. 4. Adsorption isotherms of acetone at 60°C. ○, Carbopack C; □, Carbopack C with 0.2% Carbowax 1500; ----, Carbowax 1500.

several temperatures using MSTPC and these data are given in Table IV. The first two columns for each adsorbent represent the adsorption isotherm for acetone, and this data is plotted in Figs. 4–6. The plot at 75°C is not shown; however, it has the same general form as the plot for 60°C. At these higher temperatures, the liquid-modified adsorbent adsorbed more acetone than the uncoated GCB at all pressures. On the other hand, there was little or no difference between the isotherms of the two sorbents at low temperatures and low pressures (Figs. 5 and 6, $P/P^0 \leq 0.1$). At higher pressures, the capacity of the liquid-modified GCB was again greater than that of the bare GCB.

This finite concentration data shows that several different adsorption mechanisms are operative in these systems. The relative contribution of each mechanism is a

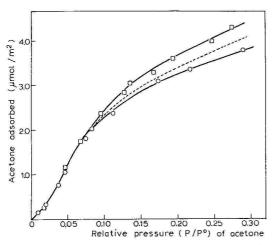


Fig. 5. Adsorption isotherms of acetone at 45°C. Legend same as Fig. 4.

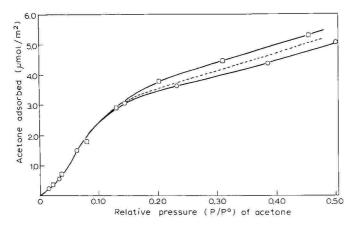


Fig. 6. Adsorption isotherms of acetone at 30°C. Legend same as Fig. 4.

function of temperature, pressure, surface condition (bare or liquid-modified), and the condition of the liquid or wax modifier.

DISCUSSION

In general, the results of this investigation agree with previous work. The liquid modifier (CW-1500) blocks some of the surface sites. This is indicated by the decreased adsorption of non-polar solutes, such as propane and butane, on the liquid-modified adsorbents¹⁴. The amount of surface blocked by the liquid cannot be determined exactly, but is *ca.* 1/3 to 1/2 of the total surface area. However, this blocking effect is only apparent for solutes which cannot specifically interact with the non-volatile liquid modifier.

Solutes such as acetone which can interact with the polar liquid modifier exhibit enhanced adsorption on the liquid-modified adsorbent in spite of the blocking effect of the liquid. This enhanced adsorption is due to lateral interactions on the adsorbent surface. The specific interactions are solute-modifier at low pressures and solute-solute at higher pressures. Cooperative adsorption effects are also observed for non-polar solutes for polar liquid-modifiers, but only at high pressures indicating only solute-solute interactions. Cooperative adsorption of non-polar solutes with non-polar liquids has been observed previously⁶ at low solute pressures.

The significance of bulk solubility effects in these systems is not clear because of the small amount of liquid (0.2%) and the monolayer character of the liquid film. The solubility of butane in CW-1500 was too low for an accurate assessment at the temperature used in this study; however, the solubility of acetone was significant and was dependent upon the physical state of the modifier as shown in Fig. 1.

The specific retention volumes of infinite dilution samples of butane and acetone on the GCB and liquid-modified GCB show that a phase transition occurs for the CW-1500 on the GCB but at elevated temperatures. In this case the transition occurs in the range 50–55°C, as indicated by both the acetone and butane data (Figs. 3 and 7). At higher temperatures, the heats of adsorption of acetone are equal for the coated and uncoated adsorbents; however, the retention is greater on the liquid-

modified adsorbent. The same is true for butane as a solute, except that the retention is greater on the uncoated adsorbent. For both solutes, the heats of adsorption at lower temperatures are significantly less than at high temperatures where CW-1500 is a liquid. This decrease in heats and retention for the wax phase could be caused by a change in the retention mechanism from solution to adsorption, as observed for the bulk liquid, or by a change in the magnitude of the cooperative and competitive adsorption processes on the liquid and wax coated adsorbents.

The modified adsorbents were studied at finite solute pressures in order to examine further the retention mechanisms. At temperatures above the transition temperatures for the CW-1500 on the GCB surface, the liquid-modified adsorbent consistently adsorbed more acetone at the same pressure than the uncoated GCB. This could possibly be due to the solubility of acetone in CW-1500. To determine the magnitude of this effect, the maximum possible solubility contribution to the sorption isotherms at each temperature was calculated from the data given in Table II and plotted in Figs. 4–6 as the dotted line. This represents the enhancement in the isotherm that would be expected if the CW-1500 did not block any of the surface and the CW-1500 had bulk solubility properties.

The increased capacity if the liquid-modified adsorbent is due to the cooperative (solute-liquid and solute-solute) adsorption effects which are significant for these systems when the modifier is a liquid. On the other hand, the wax-modified GCB (at temperatures less than 50°C) showed enhanced adsorption of acetone at very low pressures (Fig. 3) and high pressures (Figs. 5 and 6), but little or no effect at intermediate pressures. At intermediate pressures, i.e. $0 < P/P^0 \le 0.10$ –0.15, the amount of acetone adsorbed by the modified and uncoated GCBs are the same within the limits of the measurements. This observation cannot be explained by changes in the solubility of acetone in CW-1500. More likely, there is a significant difference in the relative magnitudes of the cooperative and competitive adsorption effects between the two modified surfaces (wax-modified and liquid-modified).

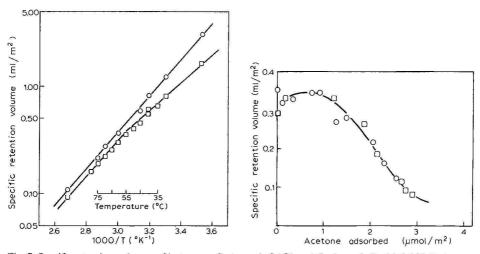


Fig. 7. Specific retention volumes of butane on Carbopack C (○) and Carbopack C with 0.2 % Carbowax 1500 (□).

Fig. 8. Specific retention volumes of butane on Carbopack C (○) and Carbopack C with 0.2 % Carbowax 1500 (□), as a function of amount of acetone adsorbed at 60°C.

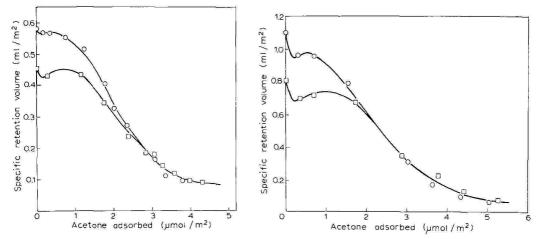


Fig. 9. Specific retention volumes of butane on Carbopack C \bigcirc and Carbopack C with 0.2% Carbowax 1500 \bigcirc as a function of amount of acetone adsorbed at 45°C.

Fig. 10. Specific retention volumes of butane on Carbopack C (○) and Carbopack C with 0.2 % Carbowax 1500 (□) as a function of amount of acetone adsorbed at 30°C.

Localized adsorption of the wax form, as opposed to delocalized (mobile) adsorption of the liquid form could account for the diminished magnitude of solute—modifier cooperative adsorption at the lower temperatures and pressures. Decreased mobility of the modifier on the surface would result in less accommodation of the solute molecules, diminished cooperative adsorption and enhanced competitive adsorption (blocking). At very low pressures of the solute, the mobility of the modifier is not critical so the usual cooperative adsorption effects are observed. At higher pressures, multilayer adsorption of the acetone is observed and the liquid simply provides a better surface for adsorption than the acetone itself in the form of multilayers on uncoated GCB. The same phenomenon is observed at high pressures for all of the temperatures investigated. That is, the physical state of the modifier (wax or liquid) (localized or delocalized adsorption) does not influence the adsorption of acetone after the formation of a monolayer.

This model was tested for additional solutes at infinite dilution, by measuring the effect of adsorbed acetone on the specific retention volumes of small samples of butane. This data is given in the fourth and last columns of Table IV for the two types of adsorbent. The results are also shown in Figs. 8-10. The curves all have the same general form as previously observed for butane and propane adsorption¹⁴. At low acetone pressures, the blocking effect of the CW-1500 is predominant. Increased amounts of acetone adsorbed on the surfaces result in decreased retention (adsorption) of the infinite dilution samples of butane. The sharp decrease in the retention volume with $1-3 \mu \text{mol/m}^2$ of acetone adsorbed corresponds to the formation of a monolayer of acetone or acetone plus CW-1500. Significantly, the presence of CW-1500 has little or no effect on the amount of acetone required to block out the butane samples. This indicates that the acetone, unlike butane, is not preferentially adsorbed on the solid (GCB) surface, but is adsorbed on regions of the surface "covered" by the CW-1500 polymer. That is, there exist regions on the surface that are inaccessible to butane because of competitive adsorption, but accessible to acetone due to cooperative adsorption effects.

CONCLUSIONS

CW-1500 present as a thin layer on the surface of GCB has significantly different physical properties, *e.g.* phase transition temperature and solubility, from the bulk liquid. This confirms the results of previous investigations of similar systems¹².

Cooperative adsorption due to solute–solute interactions was observed for both acetone and butane at finite concentrations on both adsorbents. However, solute–modifier interactions were observed only for solutes with polarity similar to that of the modifier. These cooperative adsorption effects differed in magnitude for the same system depending upon the mobility of the non-volatile modifier on the surface of the GCB.

Adsorption of a component from the carrier gas can significantly alter the adsorption properties of the system for other solutes. The exact effect of the adsorbed component depends upon the amount adsorbed and on the polarity and chemical characteristics of the adsorbed component and the other solutes. The influence of a non-volatile modifier is similar to that of a volatile component, except that the type of adsorption (localized–delocalized) of the non-volatile modifier may significantly alter the balance of the cooperative and competitive adsorption equilibria.

ACKNOWLEDGEMENTS

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ANALYSIS OF HALOPERIDOL TABLETS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY —AN INTER-LABORATORY STUDY

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SUMMARY

A high-performance liquid-chromatographic (HPLC) method for the determination of haloperidol in tablets was developed and evaluated by an inter-laboratory study. The spectrophotometric method of the British Pharmacopoeia 1973 was evaluated concurrently, and the accuracy and precision of the methods were compared. Two samples of a commercially available haloperidol tablet formulation were analysed by thirteen laboratories with satisfactory results for column performance and precision of assay. The total error standard deviations, S_D , for the HPLC method and the spectrophotometric method were 3.92 and 2.58%, respectively. The HPLC method is considered suitable for official testing purposes.

INTRODUCTION

In recent years, analysis of pharmaceuticals in dosage forms by chromatographic methods has become widespread. A number of high-performance liquid chromatographic (HPLC) procedures have been adopted as Pharmacopoeial referee methods and are used for the official testing of commercially available therapeutic goods. While HPLC methods have obvious attractions over many older pharmacopoeial procedures in terms of speed and selectivity, relatively little information has been made available on the precision and accuracy of chromatographic methods of pharmaceutical analysis under conditions of inter-laboratory usage. This paper describes an HPLC method for the major tranquiliser haloperidol {4-[4-(4-chlorophenyl)-4-hydroxypiperidino]-4'-fluorobutyrophenone} in tablets and an inter-laboratory study of its precision and accuracy as compared with a pharmacopoeial procedure based on spectrophotometry.

The previous Australian official method for the determination of haloperidol in tablets was that in the 1973 edition of the *British Pharmacopoeia* (B.P.)¹. This method involves direct extraction from the crushed tablet material, followed by measurement of the absorbance of the resulting solution at 245 nm. A difficulty arose in the use of this method for coloured haloperidol tablets because of interference from the dyestuffs. The experience of this laboratory was that, when the method was applied to coloured tablets, the results for haloperidol were up to 22% higher than the true

contents. It therefore seemed appropriate to use a chromatographic method as an alternative, and an HPLC separation proposed by an Australian manufacturer² was considered for further development.

EXPERIMENTAL.

Development of HPLC method

In the manufacturer's method, separation of haloperidol and the colouring material was achieved on a Waters μ Bondapak C₁₈ column, using methanol—water—glacial acetic acid (80:20:1) as the mobile phase. Further work with this system showed that the relationship between detector response (peak-height ratio) and concentration was non-linear. Linearity and peak shape improved when the amount of haloperidol injected was decreased, but to obtain an acceptable response—concentration relationship it was necessary to add potassium chloride to the mobile phase. A mobile phase consisting of methanol—0.01 M potassium chloride—glacial acetic acid (60:40:2) was found to be suitable.

Fig. 1 shows a chromatogram obtained using this mobile phase at a flow-rate of 1.5 ml/min, with a UV detector operating at 254 nm. The dyestuff was completely retained by the column and was therefore resolved from haloperidol and from 2-naphthol, which was selected as an internal standard.

Tablets were prepared for analysis by grinding them to an even, fine powder. An accurately weighed portion of sample powder, equivalent to 2.5 mg of haloperidol, was then vigorously shaken for 5 min in 50 ml of the mobile phase which contained 0.05 mg/ml of 2-naphthol. The resulting solution was filtered prior to injection.

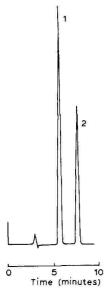


Fig. 1. Typical chromatogram from analysis of haloperidol tablets. Peaks: 1 = 2-naphthol; 2 = haloperidol.

Inter-laboratory trial of the method

In order to assess the suitability of the method for official testing of haloperidol tablets, an inter-laboratory trial was conducted, using thirteen participants. Each laboratory analysed two samples, consisting of tablets from separate batches of a commercially available 1.5-mg haloperidol formulation, by both the HPLC method and the spectrophotometric procedure of the B.P. 1973. In order to permit a valid comparison of the two methods, a sample formulation was selected which did not contain a dyestuff, so that interference in the B.P. assay was avoided.

The trial protocol specified use of a column packed with octadecylsilane-coated silica particles of mean diameter not more than 10 μ m. Column dimensions of 25 cm \times 2 mm I.D. and a nominal flow-rate of 1.0 ml/min were suggested.

Each laboratory received portions of the same haloperidol reference substance, which was checked for purity by HPLC before dispatch. Preliminary samples were not sent to the participating laboratories but criteria for resolution and reproducibility were to be met before participants proceeded to the analysis of the samples. Laboratories were asked to contact the National Biological Standards Laboratory if any difficulties were encountered or if any modifications to the method were desired.

A solution consisting of 0.1 mg/ml of haloperidol and 0.05 mg/ml of 2-naphthol in water-glacial acetic acid was used as a calibration solution. Prior to analysis of samples, six replicate injections of this solution were made and participating laboratories were asked to achieve a mean resolution factor of 3.0 with a coefficient of variation of less than 2.0%. The coefficient of variation of the peak-height ratios from the six chromatograms was also required to be less than 2.0%. A minimum height of 60% of full scale deflection was required for each peak. Some laboratories could not meet the requirement of not less than 3.0 for the resolution factor, R. However, it was considered that in view of current pharmacopoeial practice an R value greater than 2.0 was acceptable and the laboratories concerned were requested to proceed with analysis of the trial samples.

Laboratories analysed each sample once, using a mixture of powder from

TABLE I	
DETAILS OF COLUMN	PERFORMANCE

Laboratory No.	Resolution factor (R)	Coeff. of variation of peak-height ratios
1	3.77	1.71
2	4.28	2.63
3	3.59	0.35
4	4.85	1.26
5	2.09	0.26
6	2.76	0.33
7	2.65	0.40
8	2.47	0.71
9	2.71	0.02
10	3.26	0.01
11	3.00	0.35
12	3.39	0.82
13	6.50	2.4

TABLE II
ASSAY RESULTS

	Percent recove	ery by		
Laboratory No.	HPLC method	d	B.P. method	
140.	Sample A	Sample B	Sample A	Sample B
1	95.6	94.5	99.7	97.8
2	101.4	101.5	98.1	99.2
3	100.3	100.4	100.6	100.1
4	106.8	104.1	98.6	98.5
5	100.9	99.2	100.7	97.6
6	98.4	100.6	101.6	101.3
7 .	98.4	97.3	96.9	96.5
8	96.0	93.2	102.6	102.1
9	96.4	96.7	101.0	100.1
10	100.6	98.0	102.0	102.7
11	100.6	98.7	97.1	96.0
12	102.2	100.5	103.1	100.5
13	102.2	102.1	100.0	101.1
Mean	100.0	99.1	100.1	99.5
Standard deviation	3.2	3.2	2.0	2.1

twenty tablets in each case, and including 2-naphthol as an internal standard in the extracting solution of methanol—water—glacial acetic acid (80:20:2, v/v). Quantitation was achieved by comparison of the peak-height ratio with the mean peak-height ratio of the calibration solution.

RESULTS

Measurement of column performance

The values reported by the thirteen laboratories for the resolution factor (R) and coefficient of variation of peak-height ratio are shown in Table I. Laboratory 13 used a brand of column different from that used by all other laboratories and obtained a large value for R in addition to a reversal of elution order. This operator found that 2-naphthol was not practical as an internal standard and used an external-standard procedure.

Evaluation of the methods

Data from the analysis of the samples by the HPLC method are shown in Table II and Fig. 2. The mean results for content of haloperidol obtained for samples A and B were 100.0% and 99.2%, respectively. Laboratory 1 was the only participant to report the presence of decomposition products, which may have been partly responsible for the low assay values obtained by this operator. No attempt was made to compensate for the presence of these decomposition products in the computation of the haloperidol content.

Using the terminology of Youden and Steiner³ the total error, precision (repeatability) error and bias (reproducibility) error of a method can be measured by the standard deviations S_D , S_R and S_B obtained from the expressions:

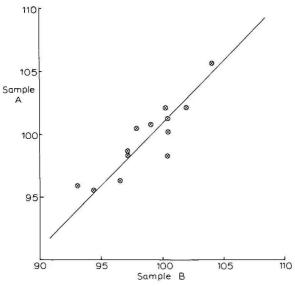


Fig. 2. Two-sample chart for the HPLC procedure.

$$S_D = \sqrt{\Sigma (T_i - T)^2 / 2(n - 1)}$$

$$S_R = \sqrt{\Sigma (D_i - D)^2 / 2(n - 1)}$$

$$S_B = \sqrt{(S_D^2 - S_R^2) / 2}$$

Where T_i refers to the sum and D_i to the difference of the results for content of each sample for n estimates (n = 13 in the trial reported here).

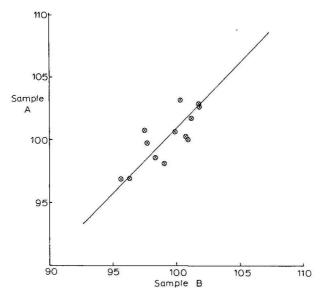


Fig. 3. Two-sample chart for the method of the B.P. 1973.

Laboratory	HPLC method	B.P. method	D = HPLC - BP
No.	A + B	A + B	
1	190.1	197.5	-7.4
2	202.9	197.4	5.5
2 3	200.7	200.7	-
4	210.9	197.1	13.8
4 5	200.1	198.3	1.8
6	199.0	202.9	-3.9
7 .	195.7	193.4	2.3
8	189.2	204.7	-15.5
8 9	193.1	201.1	-8.0
0	198.6	204.7	-6.1
11	199.3	193.1	6.2
12	202.6	203.6	-1.0
13	204.3	201.1	3.2
Mean	199.0	199.7	-0.7
Standard deviation	5.9	3.9	7.5

TABLE III
COMPARISON OF THE HPLC METHOD WITH THE B.P. METHOD, RELATIVE BIAS

For the HPLC method, the total error standard deviation S_D , is 4.20, the precision standard deviation, S_R , is 1.03 and the bias standard deviation, S_B , is 2.88. If the results from Laboratory 1 are rejected, because of the partial sample decomposition reported by that participant, the respective values for S_D , S_R and S_B are 3.92, 1.08 and 2.66.

The data obtained using the spectrophotometric method of the B.P. 1973 are shown in Table II and Fig. 3. The mean values obtained for samples A and B were 100.1% and 99.5%, respectively. The standard deviations S_D , S_R and S_B were found to be 2.76, 0.91 and 1.84.

Comparison of the methods

A comparison of the results obtained from the two procedures is shown in Table III. From the "difference" column it is apparent that the two test methods gave similar results, and use of a paired t test showed that no significant difference exists between the two methods with regard to the estimates of the mean contents of the two samples ($t_{12} = 0.30$). On the basis of the F test, at the 95% confidence level, there is no significant difference between the methods with regard to precision standard deviation (S_R), while bias standard deviation is significantly greater for the HPLC procedure. On the basis of these results, it is considered that the HPLC procedure is adequate for official testing of haloperidol tablets, with suitably low imprecision and bias, and with obvious selectivity advantages over the direct spectrophotometric method.

DISCUSSION

The most important point raised by participating laboratories concerned the difficulty in obtaining a suitable value for the resolution factor, R, and a range of

mobile phase compositions was used to achieve the required resolution. The composition of the mobile phase used by participants (methanol-electrolyte-acetic acid) ranged from 50:50:1 to 70:30:1. When the method is included in a standard, a range of solvent proportions will be specified with minimum column performance criteria. It is accepted that laboratories must be free to adjust mobile phase composition to achieve satisfactory resolution but it would seem necessary to set limits to this adjustment to avoid effectively different methods being used in a referee situation. A related problem is the task of appropriately specifying the type of column to be used in an official method. Possible approaches are to refer to commonly available commercial brands or to describe the column packing more closely, to take account of different methods of manufacture. This task is becoming increasingly difficult as the number of reversed-phase packings is rapidly proliferating. Majors⁴ has listed over 30 octadecyl-silane-bonded packings, all of which differ in percentage of phase loading, pore size and proportion of residual silanol groups.

Some laboratories neglected to use the electrolyte in the mobile phase as they considered that the peak shape obtained with methanol-water-acetic acid was symmetrical. Non-compliance with the trial protocol is always a potential problem with inter-laboratory trials, and also occurred in a previous study of an HPLC method conducted by this laboratory⁵. In the work described here, satisfactory results were obtained, but presumably over-all error in the HPLC method would have been less had all laboratories followed instructions more closely. A few participants were concerned that potassium chloride in the mobile phase could induce corrosion in the stainless steel of pumps and columns. This potential problem can be overcome by passifying the pump after use with 20–50 % nitric acid solution⁶. It was found at this laboratory, after the trial, that sodium sulphate solutions, which do not produce significant corrosion of stainless steel, are as effective as potassium chloride solutions in ensuring linearity of response, and the method will be modified accordingly when used for official testing.

One laboratory commented that the peak-height ratios from the calibration solution varied less than the ratios of the electronically integrated areas, the coefficients of variation being 2.5% for the area ratios and 0.26% for the peak-height ratios. Scott and Reese⁷ have pointed to the greater reliability of peak-height compared with peak-area measurement, and adoption of peak heights in a referee method also enables laboratories which do not have suitable integrators to carry out the official procedure.

The results of the inter-laboratory trial have shown that the HPLC method for haloperidol tablets compares favourably with the direct spectrophotometric procedure of the B.P. 1973 with regard to precision, but has greater systematic error. It may be possible to reduce the systematic error of the HPLC method by more closely specifying the procedure with regard to assurance of linear response of detector output and accurate temperature control of the column. Conditions of storage of the mobile phase might also be specified to ensure that evaporation of the volatile components does not occur. The HPLC method is, however, considered to be acceptable for the testing of haloperidol tablets, and is preferred to the spectrophotometric procedure for single-tablet analysis of low-dose (0.5 mg) formulations and for the assay of higher dose formulations containing dyestuffs.

During the course of the trial described in this paper, the method of the British

Pharmacopoeia for haloperidol tablets was modified to overcome the interference problems referred to above. The relevant monograph of the B.P. 1980 (see ref. 8) includes a spectrophotometric assay in which the powdered haloperidol tablets are successively triturated with portions of diethyl ether, which are then combined, and the drug substance is partitioned into dilute sulphuric acid. This relatively slow procedure successfully overcomes any interference from colouring materials, but in our hands gave low recoveries of drug substance and had lower precision than either the HPLC procedure or the method of the B.P. 1973. Analysis of sample B from the trial by this laboratory using the method of the B.P. 1980 gave a mean content of 94.8% with a standard deviation of 1.7. (n = 5) This compares with the inter-laboratory results for the B.P. 1973 method of a mean content of 99.5% with a precision standard deviation of 0.9, while the corresponding data for the HPLC method are 99.1% and 1.08. The HPLC method is considered to be a realistic alternative to pharmacopoeial methods in terms of speed, bias, precision and selectivity, and to be suitable for official testing purposes.

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SEPARATION OF STRUCTURALLY RELATED AROMATIC SULPHONIC ACIDS AND SULPHATES IN SYNTHESIS MIXTURES BY ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

Separation and determination of products formed by sulphonation of alkylphenols were accomplished by reversed-phase ion-pair liquid chromatography and UV detection. The chromatographic system consisted of aqueous eluents with methanol as organic modifier, tetraethylammonium as ion-pairing agent and LiChrosorb RP-8 as stationary phase. The presence of less than 0.1% of a compound could be determined with acceptable precision and accuracy.

A comparison was made between various chromatographic systems containing methanol or acetonitrile as organic modifiers and tetraethyl-, tetrapropyl- or tetrabutylammonium as ion-pairing agents. Separation factors were determined between compounds differing in the nature and positions of alkyl and polar substituents.

INTRODUCTION

The mechanism of sulphonation of phenolic compounds has been studied extensively¹⁻⁵, but a major problem has been the separation of the different reaction products prior to determination. Spectrophotometry¹, bromodesulphonation² and paper chromatography⁶ have been used along with gas-liquid chromatography⁷⁻⁹. The latter method could not be applied for the determination of more highly sulphonated products, *e.g.*, disulphonic acids, and also comprised a derivatization step thereby introducing the possibility for changes in the proportions between the different compounds in the reaction mixture and thereby jeopardizing the elucidation of the true reaction mechanism. However, during the last five years, high-performance liquid chromatography (HPLC) has been successfully applied for separation of sulphates and sulphonates using ion-exchange¹⁰, ion-pair normal-phase¹¹⁻¹³ and above all reversed-phase ion-pair modes¹⁴⁻²⁸.

The aim of this study was to develop LC systems for the separation and determination of the components of reaction mixtures resulting from the sulphonation of different alkylphenols, viz., 2-methyl-, 3-methyl-, 2-isopropyl-, 2-cyclohexyl- and 2-tert.-butylphenol, in order to elucidate the kinetics and reaction mechanism of the sulphonation³⁻⁵. The reaction products were mono- and disulphonic acids and phenyl

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hydrogen sulphates, the structures of which could be postulated from their retention behaviour.

EXPERIMENTAL

Chemicals and reagents

Methanol (p.a.; E. Merck, Darmstadt, G.F.R.) and acetonitrile (HPLC grade; Rathburn Chemicals, Walkerburn, Great Britain) were used without further purification. Tetraethylammonium (TEA), tetrapropylammonium (TPrA) and tetrabutylammonium (TBA) hydrogensulphate were obtained from the Department of Organic Chemistry, AB Hässle. Aqueous solutions of the ammonium compounds were neutralized to pH 7–10 with sodium hydroxide before use. All reagent and buffer solutions were prepared from analytical grade chemicals. All reference substances (Table I) were synthesized^{3–5} by Dr. Gert Strandlund, AB Hässle, and the purity checked by NMR and LC.

Liquid chromatographic system

The liquid chromatograph consisted of an LDC 711-47 LC-pump, an LDC SpectroMonitor III spectrophotometer and a Rheodyne sampling valve with a sample loop of 20 μ l. Chromatographic columns (150 \times 4.5 mm) were packed with LiChrosorb RP-8, 5 μ m (E. Merck), and operated at 1.0 ml/min. The performance of the columns was maintained over a long period if the top of the columns was exchanged every day. The eluents contained sodium phosphate buffer of pH 6.5. The total ionic strength was 0.20 which included added quaternary ammonium sodium sulphate (Table II). The retention time of an unretained solute, t_0 , was determined by injection of dichromate dissolved in the eluent without any alkylammonium present.

Analysis of reaction mixtures

Reaction mixtures from various sulphonations of monoalkylphenols^{3–5} were diluted 10–100 times in the mobile phase. The diluted samples (20 μ l) were injected onto the chromatographic column. The eluate was monitored by a UV-detector operated at 212 nm. Quantitations were based on peak height measurements and monitored reference samples. The minimum determinable concentration of a compound was less than 0.1% of the total sulphonate content.

RESULTS AND DISCUSSION

Retention principles

The retention of ionic solutes on a non-polar solid phase can be regulated by the kind and concentration of ionic or neutral modifier and ion-pairing agent (counter ion) present in the aqueous eluent. Ionic and neutral species will compete with the solute for the adsorption capacity of the solid phase. Methanol or acetonitrile was used as neutral modifier and different alkylammonium ions (Q^+) were tested as ion-pairing agents. The anionic solutes (sulphonates and sulphates) were either mono- or divalent anions (X^-, Y^{2-}) .

In a chromatographic system, the distribution process of a solute to an adsorbing surface can be illustrated by

$$Q_m^+ + X_m^- + A_s \rightleftharpoons QXA_s$$

Table I compounds studied and their capacity factors with 30 % methanol in phosphate buffer ph 6.5. As the eluent

Compound No.	Designation (R in R-Ph)	k'
2-Methylphenol seri	es	
1	1-SO ₃ H-2-OH-3-CH ₃	0.50
2	1-SO ₃ H-3-OH-4-CH ₃	-0.01
3	1-SO ₃ H-3-CH ₃ -4-OH	-0.43
4	1,3-di-SO ₃ H-4-OH-5-CH ₃	-0.55
5	1-OSO ₃ H-2-CH ₃	0.43
6	1-SO ₃ H-3-CH ₃ -4-OSO ₃ H	-0.94
7	1-OH-2-CH,	1.05
3-Methylphenol seri	3	1105
8	1-SO ₃ H-2-OH-4-CH ₃	0.26
9	1-SO ₃ H-2-OH-6-CH ₃	0.36
10	1-SO ₃ H-2-CH ₃ -4-OH	-0.57
11	1,3-di-SO ₃ H-4-OH-6-CH ₃	-0.45
12	1-OSO ₃ H-3-CH ₃	0.48
13	1-SO ₃ H-2-CH ₃ -4-OSO ₃ H	< -1
14	1-OH-3-CH ₃	1.03
2-Isopropylphenol s		1.03
15	1-OSO ₃ H-2-CH(CH ₃) ₂	1.14
16	1-SO ₃ H-2-OH-3-CH(CH ₃) ₂	1.32
17	1-SO ₃ H-3-CH(CH ₃) ₂ -4-OH	0.27
18	1-SO ₃ H-2-OH-5-CH(CH ₃) ₂	1.02
2-Cyclohexylphenol	5	1.02
19	1-OSO ₃ H-2-cyclohexyl	>2
20	1-SO ₃ H-2-OH-3-cyclohexyl	>2
21	1-SO ₃ H-3-cyclohexyl-4-OH	1.12
22	1-SO ₃ H-3-cyclohexyl-4-OSO ₃ H	0.30
23	1,3-di-SO ₃ H-4-OH-5-cyclohexyl	1.01
2-tertButylphenol		1.01
24	1-OSO ₃ H-2-C(CH ₃) ₃	1.45
25	1-SO ₃ H-2-OH-3-C(CH ₃) ₃	1.80
26	1-SO ₃ H-3-C(CH ₃) ₃ -4-OH	0.84
27	1-SO ₃ H-3-C(CH ₃) ₃ -4-OSO ₃ H	-0.19
28	1,3-di-SO ₃ H-4-OH-5-C(CH ₃) ₃	0.68
29	1-OSO ₃ H-2,4-di-C(CH ₃) ₃	>2
30	1-SO ₃ H-2-OH-3,5-di-C(CH ₃) ₃	>2
31	1-OSO ₃ H-4-C(CH ₃) ₃	1.55
32	1-SO ₃ H-2-OH-5-C(CH ₃) ₃	1.26
33	1-SO ₃ H-2-OSO ₃ H-5-C(CH ₃) ₃	0.62
34	1-OH-2-C(CH ₃) ₃	1.70
35	1-OH-4-C(CH ₃) ₃	1.70
36	The same of the sa	0.08
37	1-OSO ₃ H	-0.11
38	1-SO ₃ H-2-OH	
	1-SO ₃ H-4-OH	-0.77
39	1,3-SO ₃ H-4-OH	< -1
40	1-OH	0.66

where the subscripts m and s refer to the eluent and solid phase respectively and A is the number of available adsorption sites in moles per gram of solid phase. The equilibrium constant for the process is given by:

$$[QXA]_{s}/[Q^{+}]_{m}[X^{-}]_{m}[A]_{s} = K_{QX}$$
 (1)

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TABLE II
CHROMATOGRAPHIC SYSTEMS

Stationary phase: LiChrosorb RP-8, 5 μ m, 150 \times 4.5 mm. Eluents: phosphate buffer solutions pH 6.5 with listed modifiers and ion-pair reagents. The total ionic strength is 0.20 in all cases. Flow-rate: 1 ml/min. Detector wavelength: 212 nm.

Eluent No.	Organic solver	ıt	Quaternary ammonium ion	
1	Methanol	10%	.=	
2		20 %	-	
3		30%	_	
4		35%	_	
2' 3 4 5 6		55%	_	
6		10%	Tetraethylammonium	0.01 mol/l
7 8		10%		0.03 mol/l
8		10%		0.05 mol/l
9		35%		0.01 mol/l
10		55%		0.01 mol/1
11		10%	Tetrapropylammonium	0.01 mol/1
12		30%		0.01 mol/l
13		10%	Tetrabutylammonium	0.01 mol/l
14	Acetonitrile	10%	-	
15		20%	_	
16		10%	Tetrapropylammonium	0.01 mol/l
17		20%		0.01 mol/l

Anions from the buffer are similarly distributed to the solid phase as ion pairs which compete with QX for the available adsorption sites. The buffer and solute anions can also be adsorbed as the NaX ion pair (Na⁺ is the buffer cation).

The capacity ratio of the sulphonates and sulphates retained as ion pairs is defined as:

$$k_{\mathbf{X}}' = q \left([\mathbf{QXA}]_{\mathbf{s}} + [\mathbf{NaXA}]_{\mathbf{s}} \right) / [\mathbf{X}^{-}]_{\mathbf{m}}$$
(2)

This expression is valid provided that $[HX]_m$ can be disregarded, as is the case at pH 6.5. $(q = W_s/V_m)$ is the ratio of solid phase to eluent in the column.)

Regulation of retention

Reversed-phase LC using aqueous eluents was preferred since the sample from the reaction mixtures could be injected directly on to the chromatographic column. Various chromatographic systems were used with LiChrosorb RP-8 as the column packing and eluents containing different quaternary ammonium ions such as tetraethyl-, tetrapropyl- and tetrabutylammonium as ion-pairing agents (Table II). Methanol or acetonitrile as organic modifier in sodium phosphate buffer solutions of pH 6.5 constituted the eluent. By varying the type and concentration of the ion-pairing agent (counter ion, Q^+) the retention could be adapted to the separation problem. One example is shown in Fig. 1, where the change in $\log k'$, $\Delta \log k'$, is plotted for mobile phases containing an increasing concentration of TEA, 0.01, 0.03 and 0.05 mol/l, or 0.01 mol/l of TPrA or TBA. Three groups of compounds can be distinguished, phenols (compound 7), monovalent sulphonates or sulphates (1–3,5) and divalent

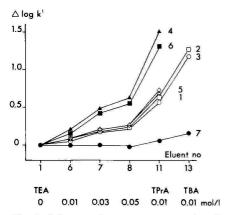


Fig. 1. Influence of quaternary ammonium ions in the eluent on $\log k'$ of some substituted 2-methylphenols. $\Delta \log k' = \log k'_{\text{quatern}} - \log k'_{\text{eluent 1}}$. Eluents 1, 6, 7, 8, 11 and 13 refer to Table II and compounds 1–7 to Table I.

anions (4,6). The divalent anions are more strongly influenced than the monovalent ones, while the phenol is only slightly affected. The effect of TEA (0.01 mol/l) is limited, resulting in an increase of $\log k'$ by about 0.1 (monovalent) and 0.2 \log units (divalent anion) as compared with 0.7 and 1.4 for TPrA and 1.2 and 2.1 for TBA (Table III). The increase in $\log k'$, calculated per additional methylene group in the counter ion, is as low as 0.13–0.15, going from TEA to TBA. By increasing the content of methanol from 10% to 30%, $\Delta \log k'$ between TPrA and TEA decreased from 0.69 to 0.22 for monovalent anions and from 1.40 to 0.47 for divalent ones. A decrease of the same magnitude was seen in acetonitrile, where a 10% increase in content lowered $\Delta \log k'$ from 0.55 to 0.40 (monovalent) and from 1.17 to 0.75 (divalent). Accordingly, a high selectivity is favoured by a low content of organic modifier.

TABLE III INCREASE IN LOG k' ($\Delta \log k'$) FOR MONOVALENT AND DIVALENT SULPHONIC ACIDS, OBTAINED WITH ELUENTS CONTAINING A QUATERNARY AMMONIUM ION (0.01 mol/l)

Eluent No.	Quaternary ammonium ion	$\Delta \log k'$				
100 10000	and organic solvent .	Monovalent acids	Divalent acids			
6 and 1	Tetraethylammonium	$0.08 \pm 0.02 (n = 15)$	$0.20 \pm 0.05 (n = 9)$			
ll and 1	Tetrapropylammonium 10% Methanol	$0.69 \pm 0.09 (n = 12)$	$1.40 \pm 0.22 (n = 6)$			
13 and 1	Tetrabutylammonium 10% Methanol	$1.22 \pm 0.04 (n = 4)$	(n = 1)			
16 and 14	Tetrapropylammonium 10% Acetonitrile	$0.55 \pm 0.07 (n = 12)$	$1.17 \pm 0.02 (n = 5)$			
12 and 13	Tetrapropylammonium 30% Methanol	$0.22 \pm 0.04 (n = 21)$	$0.47 \pm 0.06 (n = 8)$			
17 and 15	Tetrapropylammonium 20% Acetonitrile	$0.40 \pm 0.06 (n = 20)$	$0.75 \pm 0.05 (n = 6)$			

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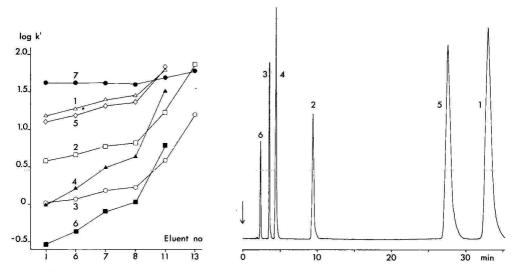


Fig. 2. Separation of some substituted 2-methylphenols using different quaternary ammonium ions in the eluent. Experimental conditions as in Fig. 1.

Fig. 3. Separation of substituted 2-methylphenols. Experimental conditions: eluent 6 (10% methanol + 0.01 mol/l TEA). Compounds 1–6 refer to Table I.

Plotting log k' of compounds 1–7 (Fig. 2) for the same eluents as in Fig. 1 shows that eluent 6 along with eluents 7 and 8 could be chosen for the separation (Fig. 3). Increasing the concentration of TEA (0.05 mol/l) or using TPrA or TBA (0.01 mol/l) would result in lower resolution.

Methanol was chosen as organic modifier in this study, its effect on the retention being compared with that of acetonitrile (Table IV). Log α (log $k_2' - \log k_1'$) was determined for compounds 1–7 in four eluents containing 10% of methanol or acetonitrile (eluents 1 and 14), 30% methanol or 20% acetonitrile (eluents 12 and 17, both of which also contained 0.01 mol/l TPrA). Comparing eluents 1 and 14 showed no difference in selectivity (log α) and the chromatographic performance was similar. Turning to eluents 12 and 17 (including TPrA) some pairs of compounds seemed to be better separated using methanol (e.g., compounds 1 and 5, 2 and 4, 3 and 6).

Substituent effects

Owing to the large number of eluents and compounds studied it was possible to elucidate the effect of different substituents. Table V shows the difference in $\log k'$ ($\log \alpha$) between unsubstituted phenols and substituted ones. The sulphonate group made the phenol more hydrophilic, the effect being more pronounced the larger the distance between the groups. In *ortho*-position (1,2), hydrogen-bonding has a strong influence, in *para*-position (1,4) the polar groups are completely separated, while in *meta*-position (1,3) a weak hydrogen-bonding seemed to occur. Phenols with two sulphonate groups (*ortho* and *para*) do not differ in $\log \alpha$ compared with the monosulphonate (*para*), the extra *ortho*-sulphonate thus not contributing to the polar character.

Table VI shows the separation factor (log α) for substituted 2-alkylphenols only differing in the size of the alkyl substituent. Four different eluents were ex-

TABLE IV SEPARATION FACTORS (log α) Obtained in the separation of substituted 2-methylphenols using either methanol or acetonitrile as the organic modifier

Compound	Eluent No.							777
No.	1 (10% CH	$_3OH)$	14(10% C	H ₃ CN)	12 (30% C	'H ₃ OH)*	17(20% C	H ₃ CN)*
	log k'	log a	log k'	log α	log k'	log a	log k'	log α
7	1.62		1.49		1.04		1.06	
		0.44		0.56		0.31		0.35
I	1.18		0.93		0.73		0.71	
		0.08		0.04		0.07		0.01
5	1.10		0.89		0.66		0.70	
		0.52		0.60		0.50		0.60
2	0.58		0.29		0.16		0.10	
		0.57		0.52		0.26		0.00
4	0.00**		-0.23		-0.10		0.10	
		0.01		0.00		0.19		0.11
3	0.01**		-0.23		-0.29		-0.21	
		0.53		0.53		0.25		0.02
6	-0.53		-0.76		-0.54		-0.23	

^{* 0.01} mol/l TPrA as counter ion.

amined, 1 and 3 with 10 and 30% methanol, 15 with 20% acetonitrile and 17 the corresponding eluent with 0.01 mol/l TPrA. Three different groups of compounds were studied, one with the sulphonate group *ortho* to the phenol, one with the sulphonate *para* to the phenol and one with a sulphate ester group replacing the phenol. The results were similar for the last two groups, *i.e.*, the increase in $\log k'$ calculated per carbon atom in the alkyl substituent (= $\log \alpha$ per carbon) was about 0.25–0.45, the larger figure being obtained with the eluent with a low content of organic modifier. The first group, with the sulphonate group *ortho* to the phenol, gave values of

TABLE V SUBSTITUENT EFFECT: SEPARATION FACTORS (log α) BETWEEN NON-SULPHONATED AND SULPHONATED ALKYLPHENOLS

 $\log \alpha = \log k'_{\text{alkylphenol}} - \log k'_{\text{substituted}}$. Eluents 1 and 3, 10 and 30% methanol. Eluents 14 and 15: 10 and 20% acctonitrile.

Substituent	log a	n			
	1	3	14	15	
1-SO ₃ -2-OH	0.60	0.61	0.78	0.82	5
1-SO ₃ H-3-OH	1.04	1.06	1.20	1.34	1
1-SO ₃ H-4-OH	1.60	1.45	1.65	1.70	4
1-OSO ₃ H	0.52	0.55	0.50	0.76	4
1-SO ₃ H-4-OSO ₃ H	2.01	2.04	2.06	2.43	2
1,3-di-SO ₃ H-4-OH	1.51	1,48	1.61	1.83	3

^{**} Retention order is reversed.

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TABLE VI SEPARATION FACTORS (log α) PER CARBON IN THE SUBSTITUENT FOR SUBSTITUTED ALKYLPHENOLS

Eluents: 1 (10% CH_3OH); 3 (30% CH_3OH); 15 (20% CH_3CN) and 17 (20% CH_3CN , 0.01 mol/l TPrA).

Compound	R	log a per	log α per carbon in eluent					
No.		1	3	15	1	7		
Substituents: 1	-SO ₃ H-2-OH-3-R							
37	Н							
1	CH_3	0.80	0.61	0.49	. (),48.		
16	$CH(CH_3)_2$	15 	0.48	0.42	().58		
25	$C(CH_3)_3$		0.48	0.43	().55		
20	Cyclohexyl	_	-	0.34	19	-		
Substituents: 1	-SO ₃ H-3-R-4-OH							
38	Н							
3	CH ₃	0.48	0.34	_	().21		
17	$CH(CH_3)_2$	0.46	0.35	0.28	(0.25		
26	$C(CH_3)_3$	0.51	0.40	0.33	().31		
21	Cyclohexyl	0.41	0.32	0.27	(0.25		
Substituents: 1	-OSO ₃ H-2-R							
36	H							
5	CH ₃	0.44	0.35	0.35	(0.23		
15	CH(CH ₃) ₂		0.35	0.33	0	0.29		
24	$C(CH_3)_3$	-	0.34	0.32	(0.29		
19	Cyclohexyl	-	-	0.31		-0.1		

TABLE VII

THE INFLUENCE ON $\log k'$ FROM ALKYL SUBSTITUTION IN DIFFERENT POSITIONS Experimental conditions: see Table II. $\log \alpha = \log k'_1 - \log k'_2$.

Substance No.	Substituents	log α in eluent		
8		I .	14	
7 14	1-OH-2-CH ₃ 1-OH-3-CH ₃	0.01	0.03	
1	1-SO ₃ H-2-OH-3-CH ₃ 1-SO ₃ H-2-OH-6-CH ₃	0.14	0.14	
9 3 10	1-SO ₃ H-3-CH ₃ -4-OH 1-SO ₃ H-2-CH ₃ -4-OH	0.21	0.07	
	1 30311 2 6113 4 011	log α in eluent		
		10	15	
34	1-OH-2-C(CH ₃) ₃	0.19	0.19	
35	1-OH-4-C(CH ₃) ₃	0.17	0.17	
25	1-SO ₃ H-2-OH-3-C(CH ₃) ₃	0.31	0.51	
32	$1-SO_3H-2-OH-5-C(CH_3)_3$	0.51		
24	1-OSO ₃ H-2-C(CH ₃) ₃	-0.06	-0.18	
31	1-OSO ₃ H-4-C(CH ₃) ₃	5.00		

log α per carbon of around 0.4–0.6. An explanation for this might be that the phenol function is directed away from the alkyl substituent towards the hydrogen-bonding sulphonate. Going vertically down the groups it can be concluded that the cyclohexyl substituent gives somewhat lower values of log α per carbon than the other substituents. The addition of a counter ion, TPrA, to the mobile phase had no effect on the separation factor.

Table VII shows the difference in separation factor ($\log \alpha$) for the compounds containing alkyl substituents in different positions with respect to the phenol group, viz. ortho, meta and para. No difference in $\log k'$ was obtained for the 2- and 3-methylphenols. A sulphonate group ortho or meta to the methyl group made a significant difference since the compounds with the methyl group meta to the sulphonate were more lipophilic than the ortho-methyl sulphonate. The methyl group is so small that the larger sulphonate group shields it from the interaction with the stationary phase. A corresponding behaviour was seen with the last three pairs of compounds in Table VII, where in two cases a bulky tert.-butyl group shields the smaller phenol group, thus giving the ortho-tert.-butyl compounds a more lipophilic character than the para analogues. The situation was slightly more complicated with a tert.-butyl and a sulphate ester ortho and para to each other (nos. 24 and 31).

Applications

The sulphonation of phenols can result in rather complex reactions as illus-

Fig. 4. Proposed reaction scheme for sulphonation of 2-tert.-butylphenol with chlorosulphonic acid (taken from ref. 5).

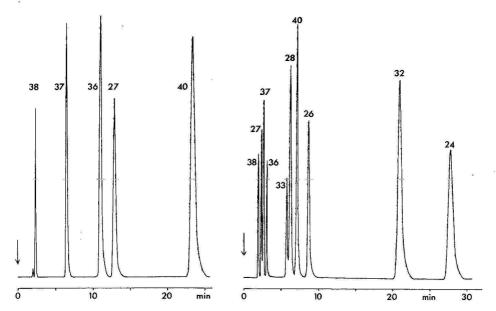


Fig. 5. Separation of substituted phenols and 2-tert.-butylphenols. Experimental conditions: eluent 6. Compounds as in Table I.

Fig. 6. Separation of substituted phenols and 2-tert.-butylphenols. Experimental conditions: eluent 9 (35% methanol + 0.01 mol/l TEA). Compounds as in Table I.

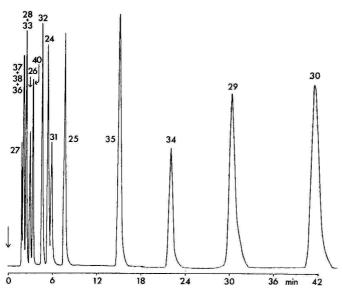


Fig. 7. Separation of substituted phenols and 2-tert.-butylphenols. Experimental conditions: eluent 10 (55% methanol + 0.01 mol/l TEA). Compounds as in Table I.

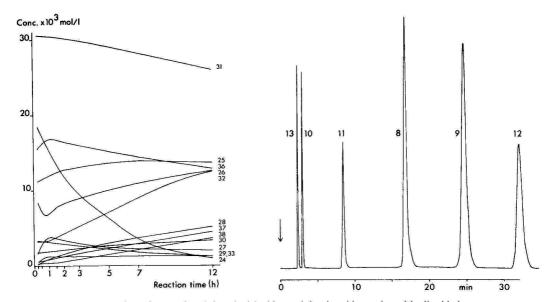


Fig. 8. Sulphonation of 2-tert.-butylphenol with chlorosulphonic acid, monitored by liquid chromatography using eluents 6, 9 and 10. Data taken from ref. 5.

Fig. 9. Separation of substituted 3-methylphenols. Experimental conditions: eluent 6. Compounds as in Table I.

trated in Fig. 4 for a proposed reaction scheme for the sulphonation of tert.butylphenol with chlorosulphonic acid. At least thirteen different sulphonic acids or sulphates were obtained. By applying ion-pair liquid chromatography it was possible to separate all of these compounds. Since the capacity factors of the different products varied by almost three orders of magnitude and no gradient elution was

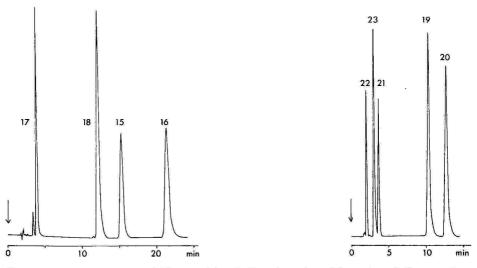


Fig. 10. Separation of substituted 2-isopropylphenols. Experimental conditions: eluent 9. Compounds as in Table I.

Fig. 11. Separation of substituted 2-cyclohexylphenols. Experimental conditions: eluent 10. Compounds as in Table I.

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available, it was necessary to use three different mobile phases, all containing 0.01 mol/1 TEA. For the more polar compounds (27, 36–38), 10% methanol was used (eluent 6, Fig. 5), for the medium polar compounds (26, 28, 33, 40), 35% methanol (eluent 9, Fig. 6) and for the most lipophilic products (24, 25, 29–32, 34, 35), 55% methanol (eluent 10, Fig. 7). The results (cf., ref. 5) from the liquid chromatographic measurements are shown in Fig. 8. It can be seen that sulphates are formed as intermediates since their concentrations decreased with time (compounds 24, 27, 31 and 36).

Examples are also given on the separation of 3-methylphenols (Fig. 9; cf., ref. 3), 2-isopropylphenols (Fig. 10; cf., ref. 4) and 2-cyclohexylphenols (Fig. 11; cf., ref. 4). In all cases methanol was used as the organic modifier (10, 35 and 55% respectively) and TEA as the counter ion (0.01 mol/l). As is seen in the figures, excellent separations were obtained in all cases.

ACKNOWLEDGEMENTS

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CHROM. 15,158

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF INORGANIC ANIONS USING Fe³⁺ AS A DETECTION REAGENT

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SUMMARY

Fe³⁺ was examined as a detection reagent in the analysis of inorganic anions by high-performance liquid chromatography. The chromatographic conditions were: the stainless-steel tube was packed with TSK-GEL IEX-520 QAE; 0.05 M sodium acetate buffer (pH 5.48) containing 0.05 M sodium nitrate was used as eluent; and 0.8 M perchloric acid containing 0.05 M iron(III) perchlorate was chosen as a complex-forming reagent. Under these conditions, chloride, sulphate and thiocyanate ion were determined in the range 2–500 nmol, and phosphate, nitrite and thiosulfate ion in the range 8–500 nmol.

INTRODUCTION

A few methods for the simultaneous determination of inorganic anions by high-performance liquid chromatography (HPLC) have been reported¹⁻⁵. Conductometry is generally used to detect the most ionic species by the method known as ion chromatography (IC)⁶. One of the disadvantages of this method is the lack of selectivity in the analysis of biological samples. UV detectors also lack selectivity, and cannot detect some major anions, such as chloride, phosphate and sulphate ions, because they have no absorption in UV region.

Recently, we reported the determination of free and bound sulphate⁷ and thiocyanate⁸ in human urine or serum by HPLC. The principle of this method was based on the formation of sulphate or thiocyanate complexes with Fe³⁺ (refs. 9 and 10), which was used as eluent and colour-developing reagent. It was also known that Fe³⁺ forms coloured complexes with chloride, phosphate and sulphate ions^{10,11}.

In this paper, we report the utility of Fe³⁺ as a detection reagent for the analysis of inorganic anions by HPLC, using a post-column derivatization method.

EXPERIMENTAL

Reagents

All chemicals used were of analytical grade. Water was redistilled after passage through anion-exchange resin.

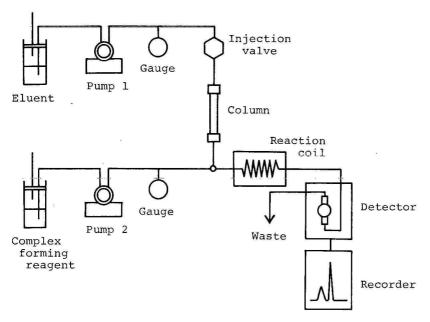


Fig. 1. Flow diagram of the chromatographic system.

Complex-forming reagent

Perchloric acid (0.8 M) containing 0.05 M iron(III) perchlorate was used.

Eluent

Sodium acetate buffer (0.05 M, pH 5.48) containing 0.05 M sodium nitrate was used.

Instruments

Pumps (Model PSU-2.5), a variable injection valve (Model VMD-350), pressure gauges (Model GN-100), a water-jacketed stainless-steel tube and a UV detector (Model D-340C) were obtained from Seishin Pharmaceutical (Tokyo, Japan). A recorder (Model ss-250F) was obtained from Sekonic (Tokyo, Japan). A water bath with a thermoregulator (Model BT-25) was obtained from Yamato Scientific (Tokyo, Japan). UV spectra were measured by a Hitachi 340 automatic recording spectrophotometer.

HPLC apparatus

Fig. 1 is a flow diagram of the chromatographic system. A stainless-steel tube (150 mm \times 4 mm I.D.) was slurry-packed with TSK-GEL IEX-520 QAE (silica type pellicular anion exchanger). Pump 1 was used to deliver an eluent at a flow-rate of 0.8 ml/min. The temperature of column and reaction coil was kept at 25°C. The complex-forming reagent was delivered by pump 2 at a flow-rate of 0.4 ml/min. The reagent from pump 2 was mixed with the effluent. The mixed solution was delivered to the reaction coil, and was then monitored with the UV detector at 340 nm. PTFE reaction coil was 2 m \times 0.25 mm I.D.

TABLE I COMPLEX FORMATION OF INORGANIC ANIONS WITH Fe³⁺

Reactions were carried out in 0.8 M· HClO₄ containing 0.05 M Fe(ClO₄)₃. UV spectra were measured within 5 min using a reagent blank as a reference. Detection limits were obtained by the system without a column illustrated in Fig. 1. Distilled water was used as a carrier solution from pump 1 keeping the flow-rate at 0.2 ml/min.

Anion	$\lambda_{max} \ (nm)$	Detection limit (nmol)	Anion	λ_{max} (nm)	Detection limit (nmol)
CrO ₄ ²⁻	305, 344	0.4	SO ₃ ² -	308	12.7
SCN-	310	1.3	PO_3^{3}	_	24.8
Fe(CN) ₆ ⁴⁻	305	1.6	$H_2PO_2^-$	1-0	28.8
Fe(CN)3-	305	1.9	IO_3^-	-	73.7
SO ₄ ² - Cl - P ₂ O ₇ ⁴ - I -	306	2.8.	CO_3^{2}	_	141.3
Cl-	335	4.8	Br -	No.	144.3
P2O2-	310	5.2	$B_4O_7^2$	_	206.4
I-	306, 350	5.6	BrO_3^-	_	931.7
P ₃ O ₁₀ ⁵⁻	310	6.4	CN-	<u></u>	392.2
$P_3O_{10}^{5-}$ S^{2-}	-	7.1	SiO ₃ ²	_	285.7
$S_2O_3^{2-}$	308	8.1	NO_3^-	<u>-</u>	-
NO ₂	372, 360	10.8	F- "	_	_
NO ₂ PO ₄ ³⁻	310	11.8	ClO ₃	_	-

RESULTS AND DISCUSSION

Complex formation between inorganic anions and Fe³⁺

Iron(III) perchlorate was used as a complex-forming reagent for each anion, and perchloric acid was chosen as a reaction medium, because of its lower complexing ability which would minimize ligand exchange during the analysis¹². Iron(III) perchlorate and perchloric acid concentrations were determined according to the conditions of sulphate ion analyses by Nakae *et al.*¹¹. Tested inorganic anions formed iron(III) complexes exhibiting an absorption at *ca.* 300 nm, except for nitrate, fluoride and chlorate ions. Twenty-six inorganic anions were examined, and their detection limits were measured (Table I).

Since considerable absorption by the reagent blank was observed at 300 nm⁹, the monitoring wavelength for these anions was set at 340 nm, where 60–70 % of the maximum absorbance was obtained. Fourteen inorganic anions from chromium(VI) oxide to sulphite ion shown in Table I exhibited strong absorption at 340 nm; the detection limits for these were 0.4–12 nmol by a peak height method, and for other inorganic anions were 73–930 nmol. These results suggest that Fe³⁺ is very useful as a reagent for the detection of certain anions such as phosphate, sulphate, chloride, nitrite, thiosulphate, iron(III) cyanide and iron(II) cyanide ions, which might be present in biological fluids, foods or environmental pollutants.

Separation of inorganic anions by HPLC

The separation of nine inorganic anions (thiocyanate, iron(III) cyanide, iron(II) cyanide, sulphate, chloride, iodide, thiosulphate, nitrite and phosphate) was examined using the chromatographic system illustrated in Fig. 1. Perchloric acid (0.8)

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M) containing 0.05 M iron(III) perchlorate was used as a complex-forming reagent. Sodium nitrate solution or sodium acetate buffer containing sodium nitrate, which exhibited little absorption at 340 nm, was chosen as eluent. The effect of sodium nitrate concentrations on the retention time and the separation of inorganic anions on a column of the pellicular anion exchanger, TSK-GEL IEX-520 QAE, were examined. Seven anions (thiocyanate, sulphate, chloride, iodide, thiosulphate, nitrite and phosphate) were not separated completely with 0.1 M sodium nitrate; however, these anions were separated completely within 30 min when the flow-rate was changed from 0.8 to 0.32 ml/min (Fig. 2).

Iron(III) cyanide and iron(II) cyanide ion were not eluted with sodium nitrate in the concentration range 0.1–0.3 M within 60 min because they were strongly absorbed on the resin. In order to keep the pH constant, sodium acetate buffer containing sodium nitrate was examined. When 0.05 M acetate buffer (pH 5.48) containing 0.05 M sodium nitrate was employed as eluent, complete separation of these anions was obtained within 20 min (Fig. 3).

Complex-forming conditions

The effect of different concentrations of perchloric acid and iron(III) perchlorate on complex formation was examined. The eluent and its flow-rate were kept

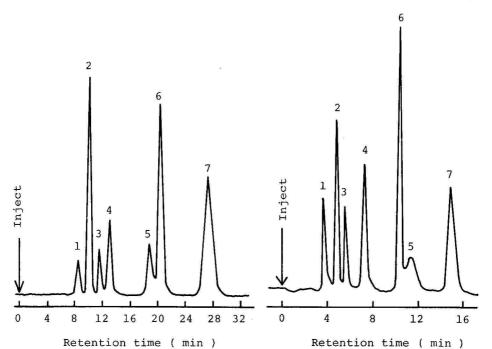


Fig. 2. Chromatogram of a standard mixture of inorganic anions. Eluent, 0.1 M NaNO₃; flow-rate, 0.32 ml/min; other conditions are described in the text. Peaks: $1 = PO_4^{3-}$; $2 = Cl^-$; $3 = NO_2^-$; $4 = SO_4^{2-}$; $5 = S_2O_3^{2-}$; $6 = I^-$; $7 = SCN^-$.

Fig. 3. Chromatogram of a standard mixture of inorganic anions. Eluent, 0.05 M acetate buffer (pH 5.48) containing 0.05 M NaNO₃; flow-rate, 0.8 ml/min; other conditions are described in the text. Peaks: $1 = PO_4^{3-}$; $2 = Cl^-$; $3 = NO_2^-$; $4 = SO_4^{2-}$; $5 = S_2O_3^{2-}$; $6 = I^-$; $7 = SCN^-$.

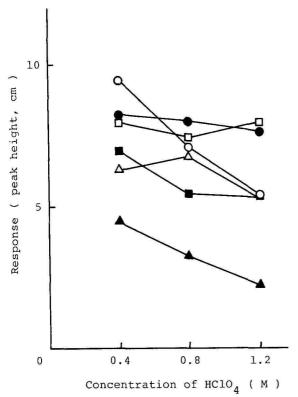


Fig. 4. Effect of concentration of $HClO_4$ on complex formation of inorganic anions in HPLC, using 0.05 M $Fe(ClO_4)_3$. Curves: $\bigcirc = SO_4^{2-}$; $\bullet = Cl^-$; $\square = SCN^-$; $\blacksquare = NO_2^-$; $\triangle = PO_3^{3-}$; $\triangle = S_2O_3^{3-}$.

constant at 0.05 M acetate buffer (pH 5.48) containing 0.05 M sodium nitrate and 0.8 ml/min, respectively. The flow-rate of the complex-forming reagent was kept constant at 0.4 ml/min. The effects of various concentrations of perchloric acid and iron(III) perchlorate on the response for inorganic anions are shown in Figs. 4 and 5.

The response for the six anions increased with decreasing concentration of perchloric acid (Fig. 4), but the response for the reagent blank also increased. On the other hand, the response for these anions increased with increasing concentration of iron(III) perchlorate (Fig. 5).

Considering the background absorption, 0.8 M perchloric acid and 0.05 M iron(III) perchlorate were used in this system.

Under the chromatographic conditions and using the post-column method, it seemed that the iron(III) complexes of these anions (chloride, sulphate, thiocyanate, phosphate, nitrite and thiosulphate) were stable during the time passing the flow cell, because these absorbances were unchanged for 5 min. On the other hand, the absorbance of iodide ion increased, because of oxidation by Fe³⁺.

Chloride, sulphate and thiocyanate ion were determined in the range 2–500 nmol, and phosphate, nitrite and thiosulphate ion in the range 8–500 nmol.

The precisions (coefficients of variation, n = 7) were 1.1, 0.8, 5.1, 3.8, 1.6 and

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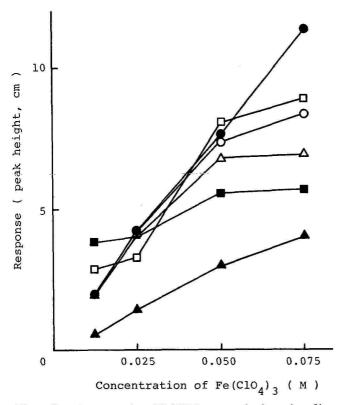


Fig. 5. Effect of concentration of Fe(ClO₄)₃ on complex formation of inorganic anions in HPLC, using 0.8 M HClO₄. Curves: $\bigcirc = SO_4^{2-}$; $\bullet = Cl^-$; $\square = SCN^-$; $\blacksquare = NO_2^-$; $\triangle = PO_4^{3-}$; $\triangle = S_2O_3^{2-}$.

1.2% for 50 nmol chloride, 25 nmol sulphate, 25 nmol thiocyanate, 100 nmol phosphate, 100 nmol nitrite and 100 nmol thiosulphate, respectively.

APPLICATION

Fig. 6 illustrates a chromatogram for the analysis of waste water. High levels of chloride and sulphate ions were detected in this sample.

A chromatogram for the analysis of human urine is shown in Fig. 7. The second, third and fourth peaks were attributable to phosphate, chloride and sulphate ion, respectively, and the trace amount of thiocyanate ion (1.4 nmol) was determined with only $10 \mu l$ urine sample.

CONCLUSION

Fe³⁺ was used as a detection reagent for the analysis of inorganic anions by HPLC. This method could determine major anions such as chloride, phosphate and sulphate, which have no absorption in UV region, and was selective in the analysis of biological samples. Thus it may be suggested that this method is useful for the simul-

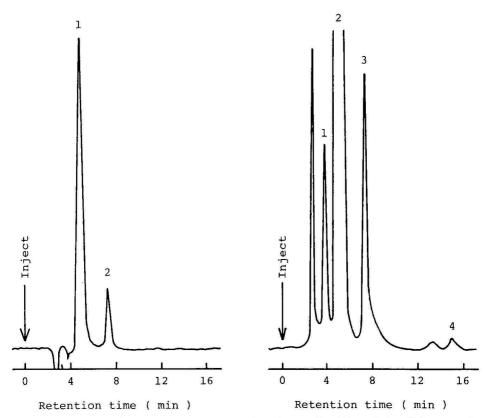


Fig. 6. Chromatogram of waste water. Sample size, $10 \,\mu$ l. Peaks: $1 = \text{Cl}^-(200 \,\text{nmol})$; $2 = \text{SO}_4^{2-}(15 \,\text{nmol})$.

Fig. 7. Chromatogram of human urine. Sample size, $10 \mu l$. Peaks: $1 = PO_4^{3-}$ (257 nmol); $2 = Cl^-$ (1800 nmol); $3 = SO_4^{2-}$ (100 nmol); $4 = SCN^-$ (1.4 nmol).

taneous determination of inorganic anions, such as phosphate, nitrite, chloride, sulphate, thiosulphate and thiocyanate, with sufficient sensitivity and simplicity.

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CHROM. 15,260

Note

Extra-column band spreading in high-performance liquid chromatography—mass spectrometry using a moving belt interface

Numerical evaluation of system variance

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The detrimental effects of extra-column band spreading in chromatographic separations have long been recognised and elegant theoretical treatments of the problem have appeared¹⁻⁴.

An increasing interest in microbore (and capillary) high-performance liquid chromatography (HPLC), techniques which impose great demands on chromatographic equipment, has to some extent rekindled awareness of these effects.

During our development of microbore technques for use with mass spectrometry (MS)⁵⁻⁷ we decided to investigate the band spreading effects of the mass spectrometer when used as HPLC detector. Two important spreading effects have been identified in HPLC detectors, viz. those due to, for example, flow effects, including dead volumes and cross sectional area changes, and those due to electronic time constants. It has been usual practice to evaluate these effects by considering the increase in variance (or second moment of mass) of a chromatographic band. The variances have useful properties including their additivity when the contributions are independent, i.e. if the system time-constant is independent of the dead volume effects then the two separately calculable variances can be added to give the overall system variance. Also the commonly used measure of column efficiency, the height equivalent to a theoretical plate, is itself a measure of the increase in second moment of mass of a chromatographic band as a function of the distance travelled down the column. Although the importance of variance in extra-column band spreading has been recognised, it is unfortunately not in general practical use by chromatographers, reference being made simply to a measured or estimated dead volume in most cases.

This paper describes some results obtained using a Finnigan 4000 mass spectrometer with a moving belt liquid chromatographic (LC) interface⁸, when used as a dectector for a high efficiency microbore HPLC system. In this system the column eluent is fed onto a moving belt which carries the solutes in solution under an infrared heater where the solvent is removed. Since in this technique there is no flow cell to be measured, the approach outlined here was adopted. Samples of the pesticide Lindane (γ -hexachlorocyclohexane) dissolved in methanol were injected into the LC-MS

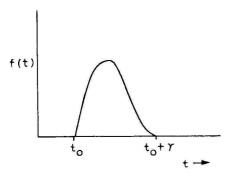


Fig. 1. Mass spectrometer output expressed as a function of time, f(t).

interface in a controlled manner using a micro-feeder. These input pulses were considered to approximate to square waves, the variance of which is given by¹:

$$\sigma_{\rm in}^2 = \frac{T_{\rm in}^2}{12} \tag{1}$$

where $\sigma_{\rm in}^2$ is the input variance, and $T_{\rm in}$ is the time over which the input takes place. Mass spectra were recorded using an Incos data system scanning the molecular ion region vary rapidly (0.1 sec per scan). The broadened output appeared to be a complex function together with a great deal of noise. It was not considered feasible to analyse the output algebraically and so the following calculations involving numerical integration based on Simpson's rule were used.

The ion current values produced by the mass spectrometer after subtraction of a baseline value is considered as a function of time f(t) (Fig. 1). We can then define the following:

$$A = \int_{t_0}^{t_0+r} f(t) dt = \text{area under curve}$$
 (2)

$$\bar{t} = \int_{t_0}^{t_0+Y} tf(t) dt/A$$
 (3)

$$\vec{t}^2 = \int_{t_0}^{t_0+r} t^2 f(t) dt/A$$
(4)

The variance is defined as the average of the squares minus the square of the average, *i.e.*

$$\sigma^2 = \overline{t^2} - (\overline{t})^2 \tag{5}$$

The output of the mass spectrometer is discrete, each scan being taken at a fixed time.

This type of data lends itself to analysis by numerical methods, and it was decided to

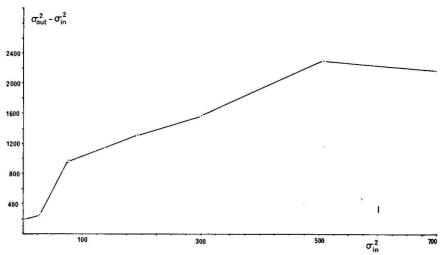


Fig. 2. Plot of output variance against input variance.

evaluate the above integrals by using Simpson's rule. Using this rule the integrals can be formulated as:

$$A = \frac{T}{3} \left\{ f_1 + f_N + \sum_{n=2}^{N-1} [3 + (-1)^n] f_n \right\}$$
 (6)

$$\overline{t} = \frac{T}{3} \left\{ tf_1 + tf_N + \sum_{n=2}^{N-1} \left[3 + (-1)^n \right] t^f n \right\}$$
 (7)

$$\overline{t^2} = \frac{T}{3} \left\{ t^2 f_1 + t^2 f_N + \sum_{n=2}^{N-1} \left[3 + (-1)^n \right] t^2 f_n \right\}$$
 (8)

The rule requires that the peak is split into an equal number of equally spaced strips of width T (the interval between scans), which requires an odd number of data points (or scans). A Fortran computer programme was then written to evaluate the summations and to compute the output variance σ_{out}^2 .

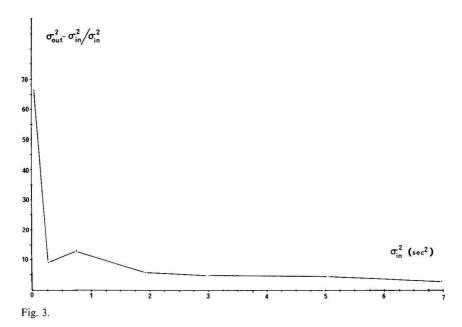
mations and to compute the output variance σ_{out}^2 .

Plots of $\Delta \sigma^2$ (= $\sigma_{\text{out}}^2 - \sigma_{\text{in}}^2$) against σ_{in}^2 , and of $\Delta \sigma^2/\sigma_{\text{in}}^2$ against σ_{in}^2 are shown in Figs. 2 and 3. Fig. 3 indicates that σ_{out}^2 is proportional to σ_{in}^2 at reasonable values of σ_{in}^2 , but an anomaly exists at vary low values of σ_{in}^2 . The volume standard deviation, σ_{v} and the time standard deviation, σ_{t} , of a chromatographic band can be calculated using the equations:

$$\sigma_{\rm v} = V_{\rm R}/N^{\frac{1}{2}}$$

and

$$\sigma_{t} = V_{R}/N^{\frac{1}{2}}Q$$



where V_R = retention volume, N = number of theoretical plates, and Q = volume flow-rate. For a typical microbore case using a 250 \times 0.5 mm I.D. column we may have:

$$N = 10,000;$$
 $V_0 = 30 \,\mu\text{l};$ $Q = 10 \,\mu\text{l min}^{-1}$

 V_R is replaced by V_0 , the column void volume, as this represents at the most difficult

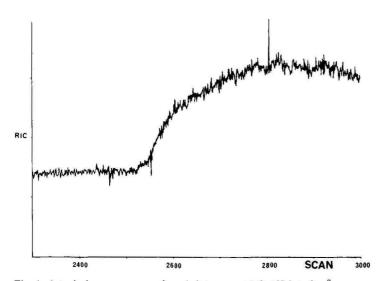


Fig. 4. A typical response curve for a belt transport LC-MS interface8.

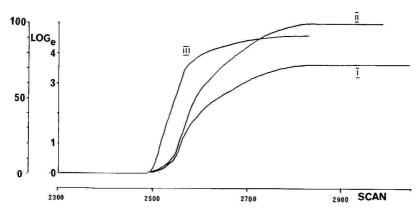


Fig. 5. Curve I: Smoothed response curve taken from Fig. 4; curve II: normalised response curve; curve III: logarithm curve, the slope of the straight line portion giving the instrument time constant.

peak to handle. The time based standard deviation and variance then compute to:

$$\sigma_t = 1.8 \text{ sec and } \sigma_t^2 = 3.24 \text{ sec}^2$$

By reference to Fig. 3 we see that this takes us well into the flat area of the plot. From this and a comparison of the mass spectrometer detector with a micro flow cell UV detector of dead volume $0.3 \mu l$, we conclude that the mass spectrometer is a suitable low effective dead volume detector for microbore HPLC.

We have also measured the time constant of the mass spectrometer detector. A single ion was monitored at maximum scan rate and a flow of sample was injected into the interface. A typical response is shown in Fig. 4. This response curve shows a high level of noise which makes further manipulation difficult. We decided to simply average the noise by drawing a line through the centre points of the curve to produce Fig. 5 (line I). After normalisation (line II) the logarithm was plotted (line III). The slope of the straight line portion of the logarithm curve gives the instrument time constant. An average of three values gave a commendably low 0.08 sec.

In conclusion this work shows the mass spectrometer to be a low time constant, low effective dead volume detector suitable for microbore HPLC. We also hope that this approach to the estimation of extra-column band broadening by consideration of system variance will promote further discussion of the problem among chromatographers.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatography of peptides obtained from elastin by alkaline hydrolysis

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Soluble peptides obtained by partial hydrolysis of cross-linked elastin were used as models for studies of structure and interactions of this protein^{1,2}. One of the chemical methods for the preparation of soluble elastin degradation products is alkaline hydrolysis in aqueous alcohol². The mixture of the soluble peptides obtained by this method was called kappa-elastin. It was suggested that ethanol and higher alcohols would facilitate the hydrolysis by deorganising the hydrophobic regions of elastin. It has been shown that, after hydrolysis for 18-72 h at room temperature³, non-coacervable, desmosin-containing, low-molecular-weight (molecular weight ≈ 10,000 daltons) peptides are formed. These peptides are remarkably resistent to further hydrolysis. The peptide mixture obtained contains several populations of homologous peptides, exhibiting different characteristic glycine-alanine ratios (1.2:1, 1:2, and 1:1.2, respectively). The ratio of these peptide populations changes as hydrolysis proceeds. Earlier studies indicated that the peptide mixture obtained by alkaline hydrolysis is heterogeneous. Some peptides were partially separated from this mixture by isoelectric focusing3. As the most important difference between the individual peptide populations is the ratio of hydrophobic amino-acid residues, methods involving hydrophobic interactions should be more effective for the separation of the different classes of these peptides than methods based on charge differences.

To obtain more insight into the aggregation properties and the separation of the different peptide populations in kappa-elastin, we investigated the behaviour of these peptides on high-performance liquid chromatographic (HPLC) columns used for the separation of proteins.

MATERIALS AND METHODS

Preparation of kappa-elastin

Elastin from Ligamentum nuchae, prepared by the Lansing procedure⁴, was used for the preparation of kappa-elastin². Hydrolysis in 80% aqueous ethanol con-

taining 1 N potassium hydroxide was performed for 24 h at 37°C, as described elsewhere³. The low-molecular-weight (10,000–16,000 daltons) peptide fraction was isolated by exclusion chromatography on a Sephadex G-100 column³.

Chromatography

The HPLC columns (7.5 cm \times 0.45 cm I.D.) used for this study were filled with chemically modified LiChrospher SI 100 (Merck, Darmstadt, G.F.R.) by grafting hydrophilic diol function by a procedure previously described⁵.

Solvent delivery was carried out by a Waters Model 6000 A pump, and the injector was a Waters U6K (Waters Assoc., Milford, MA, U.S.A.). Detection was performed with an Varichrom multiwave-length detector from Varian (Walnut Creek, CA, U.S.A.) at 220 nm.

The eluents were mixtures of ethanol and ammonium acetate $(10^{-2} M)$ in different proportions. The pH of the eluent was adjusted by adding acetic acid to the solution.

Experimental conditions

Hydrolysed elastin (5 mg) was dissolved in 5 ml of the eluting solution, and 5 μ l of this solution were injected onto the column.

We have studied the influence of pH, eluent composition and concentration of the sample on separation.

For the semi-preparative experiments, we used 30×0.45 cm I.D. columns and a sample concentration of 1 mg/ml. The eluent was ethanol- 10^{-2} M ammonium acetate (60:40, v/v).

RESULTS AND DISCUSSION

Column, 7.5 \times 0.45 cm I.D.; injected volume, 5 μ l.

The ammonium acetate solution, without the addition of alcohol, resolved the peptide mixture into two peaks at pH 7 and 8.5 (Table I). At pH 5 all the material is

TABLE I THE $_{\mbox{\scriptsize ph}}$ Dependence of the separation of the low-molecular-weight elastin peptide

pΗ	10 ⁻² M ammonium acetate		Ethanol -10^{-2} M ammonium acetate (60:40, v/v)		
	Elution time (min)	Ratio of peak areas*	Elution time (min)	Ratio of peak areas	
8.5	1.4	62	1.8	81	
	2	38	3.3	19	
7	1.4	58	1.8	82	
1	2	42	3.3	32	
	- ×	_	1.9	86	
3	2 .	100	3.3	14	

^{*} The distributions of the peak areas (absorbance at 220 nm) are expressed as the percentage of the sum.

TABLE II
DEPENDENCE OF THE ELUTION TIMES ON THE ETHANOL CONCENTRATION IN THE ELUENT

Injected volume, 5 μ l; concentration, 1 mg/ml; column, 7.5 × 0.45 cm I.D.; flow-rate: 0.5 ml/min; eluen	t,
ethanol -10^{-2} M ammonium acetate (v/v).	

Ethanol (%)	Retention time (min)	Proportion of the peak areas as a percentage of the total
0	1.4	62
	2	38
60	1.8	80
	3.2	20
70	1.8	82
	2.9	18
80	1.8	64
	2.7	36

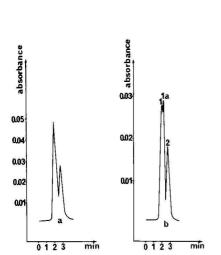
retained in the elution volume corresponding to peak 2 (Table I). The ethanol-aqueous solution mixture (60:40, v/v) resolves the peptides into two peaks, at all the pH values studied.

These findings may be attributed to hydrophobic interactions, similar to those participating in the coacervation phenomena. The pH optimum for the coacervation of soluble elastin peptides of higher molecular weight¹ is between pH 4.5 and 5. The low-molecular-weight kappa-elastin peptides do not form visible aggregates under the conditions of coacervation, but the formation of soluble aggregates for these sub-

TABLE III EFFECT OF THE CONCENTRATION OF THE INJECTED SAMPLE ON THE SEPARATION OF THE KAPPA-ELASTIN PEPTIDE

Column, 7.5 \times 0.45 cm I.D.; eluent, ethanol-10 ⁻² M ammonium	acetate	(60:40, v/v)
--	---------	--------------

Concentration (mg/ml)	Peak number	Retention time (min)	Ratio of peak area	Injected volume (µl)	
1	1	1.8	62	_	
ì	2	2.7	38	5	
(1/2)	Ī	1.8	66	10	
(1/2)	2	2.7	34	10	
(1/4)	1	1.8	64	20	
(1/4)	2	2.7	36	20	
(1/8)	1	1.8	53	20	
(1/8)	2	2.7	47	20	
(1/16)	Ĭ	1.8	55	40	
(1/16)	2	2.7	45	40	
(1/32)	ì	1.9	39		
(1/32)	1a	2.2	37	80	
(1/32)	2	2.7	24		
(1/64)	1	1.9	37		
(1/64)	la	2.2	37	80	
(1/64)	2	2.7	26		



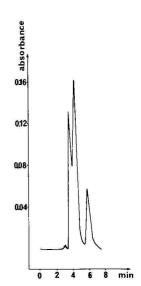


Fig. 1. Effect of the sample concentration on the elution diagram of the kappa-elastin peptides. Concentration of the samples (mg/ml): (a) 1; (b) 0.0156 (1/64). Experimental conditions: see Table III.

Fig. 2. Elution diagram of the kappa-elastin peptides from the 30-cm column (0.45 cm I.D.). Eluent, ethanol- 10^{-2} M ammonium acetate (60:40, v/v); injected volume, 5 μ l.

stances was demonstrated by electric birefringence⁶.

The possibility of the separation of the elastin fraction with the column decreases with increasing ethanol concentration in the eluent (Table II). The difference in the elution times of peaks 1 and 2 decreases from 1.4 to 0.9 min when the alcohol-buffer ratio is altered from 60:40 to 80:20. Alcohol concentrations higher than 70% (v/v) seem to increase the amount of material eluted in peak 2, but separation is less complete under these conditions.

Elution volumes remain constant with the dilution of the injected samples (Table III). A new peak (1a) appears between peaks 1 and 2, when highly diluted samples (from 1/32 of the initial concentration, 1 mg/ml) are injected, even on a short (7.5 cm) column (Fig. 1). It seems that high dilution of the injected sample increases the dissociation of the different interacting peptides and allows better separation.

With 30-cm columns with the same stationary phase and the ethanol-ammonium acetate (60:40) solution as the eluent, the elastin peptide mixture was separated in three fractions, even for samples of 1 mg/ml concentration (Fig. 2). Thus the preparative separation of the three fractions was achieved with this eluent system. Upon eluting this column with aqueous solution alone, or with a 90:10 ethanol-aqueous solution, only two peaks appear.

Amino-acid analysis of the separated peak materials (Table IV) indicates that the individual peaks represent different polypeptide populations. Alkaline degradation of fibrous elastin under the conditions used^{2,3} yields a large number of homologous peptides. Thus the three main peaks obtained cannot represent homogeneous substances, but it can be assumed that different polypeptide populations³ are enriched in each of the peaks. Peaks 1 and 2 contain alanine- and glycine-rich peptides, respectively. Such peptide populations were isolated also by isoelectric focusing from

TABLE IV

AMINO ACID COMPOSITION OF THE SEPARATED PEPTIDES

Values are residues per 100 residues.

Peak 1	Peak 1a	Peak 2	Amino acid
1.6	1.9	1	Нур
1.5	0.7	2.6	Asp
2.5	0.6	1.3	Trp
1.4	0.5	1.9	Ser
4.2	1.7	4.5	Gly
9.8	13.6	10.3	Pvo
21.3	27.9	26.28	Gly
39.6	19.7	32.05	Ala
10.1	25.8	10.26	Val
1.5	1.3	1.3	Ile
3.8	4.0	4.5	Leu
0.5	0.4	0.5	Tyr
2.5	1.4	3.2	Phe
0.2	0.3	0.6	Ids
0.3	0.4	0.6	Des
0.2	0.3	0.4	Lys
Trace	Trace	0.1	Arg

the low molecular weight kappa-elastin preparations³. Valine-rich peptides enriched in the peak 1a could not been isolated from those preparations in the isoelectric focusing systems already described³. Valine-rich peptides were isolated from proteolytic digests of tropoelastine, the precursor of the fibrous elastin^{7,8}. These peptides containing repetitive penta- or hexapeptides sequences are distant from the crosslinking regions, and the main structural element is the β -sheet conformation⁹. It has been suggested that this molecular arrangement is a requirement for the elastomeric properties of the elastic fibres¹⁰.

It appears that HPLC on diol-bonded silica supports is suitable for the separation, assay, and study of the aggregation of the main peptide population of alcoholsolubilized elastin peptides. As these different peptides are derived from different regions of the native elastin molecule^{3,8}, the study of their ratio may be useful in the comparison of elastin preparations originating from normal and pathological tissues.

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Note

Titration curves by combined isoelectric focusing—electrophoresis on a thin layer of agarose gel

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The possibility of obtaining protein titration curves by electrophoresis in a stationary pH gradient, stabilized by focused carrier ampholytes in a flat-bed polyacrylamide gel, was demonstrated by Righetti et al.¹ who exploited an original idea of Rosegren et al.². These authors showed that an analysis of the shape of the pH vs. mobility curves of a protein and of its genetic mutants makes it possible to determine which charged amino acid has been substituted. Krishnamoorthy et al.³ used this technique to study liganded states of proteins. They were able to isolate complexes of hemoglobin with different organic phosphates, measure their half-lives, the pH range of stability and the stoichiometry of the protein-ligand complexes.

Protein-protein interactions between cytochrome b_5 and hemoglobin on the one hand^{4,5} and cytochrome b_5 and cytochrome b_5 reductase⁶ on the other have also been studied by this technique, allowing the determination of the nature of the amino acids involved in these interactions. This technique is also useful to define a strategy for the purification of a specific protein, based on its charge properties. Furthermore, it constitutes one of the best criteria of charge homogeneity for a protein. Thus, this simple method has become a powerful tool in studying proteins.

However, polyacrylamide gel, due to molecular sieving, restricts the mobility of many large proteins and hence for them a mobility curve cannot be obtained. In this report we establish the conditions for obtaining mobility curves in large-pore-size agarose gel and discuss the difficulties encountered and the advantages of this medium over acrylamide gel for specific problems.

MATERIALS AND METHODS

Agarose IEF, Pharmalytes (pH range 3–10), gel bond plates and electrode strips (6 \times 10 mm) were obtained from Pharmacia (Uppsala, Sweden). Ampholine PAG plates, pH gradient 3.5–9.5 were from LKB (Stockholm, Sweden). Sorbitol and

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Coomassie Brillant Blue R250 were from E. Merck (Darmstadt, G.F.R.). All other reagents were of the best available grade.

Agarose gels (1.3 mm thick) were prepared in the following way. Agarose and sorbitol, 1% and 12% (w/v) final, respectively, were dissolved in 15 ml of double distilled water by heating under constant stirring in a water-bath at 95°C. The solution was then allowed to cool and Pharmalytes were added (6.3%, v/v) when the temperature reached 75°C. The mixture was layered on a gel bond plastic plate disposed with its hydrophilic side upwards on an horizontal support preheated at 60°C. The dimensions of the gel were limited by a plexiglass cast (120 × 120 mm). After gelification at room temperature, the gel was left in a humid atmosphere at 4°C overnight to increase its mechanical strength. Excess of liquid was absorbed from the gel with Whatman No. 3 MM filter-paper. Electrode strips were soaked in 1 M sodium hydroxide solution on the cathodic site and in 0.05 M sulphuric acid on the anodic side. The pH gradient was preformed by applying a power of 0.5 mW/mm³ with an LKB 2103 constant-voltage power supply for 90 min at 8°C. At this point a 10 cm long incision was made with a scalpel in the middle of the gel perpendicular to the electrode strips to be used as sample well.

Both anodic and cathodic regions were then removed by cutting the gel on the inner side of the electrode strips. Salt-free sample (7–10 μ l containing 100–200 μ g of protein) was applied in the well and electrophoresis was run perpendicularly to the preformed gradient using the same electrolytes. To insure a good penetration of the sample into the gel, the voltage was first maintained at 500 V for 3 min. Electropho-

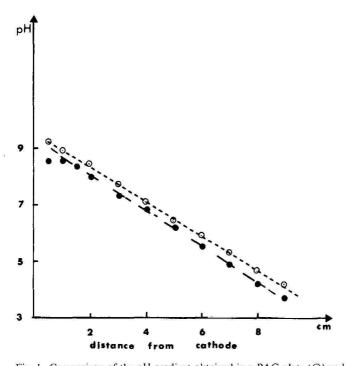
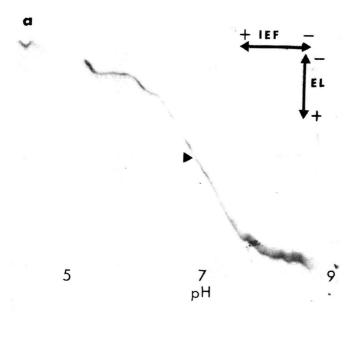


Fig. 1. Comparison of the pH gradient obtained in a PAG plate (○) and in agarose IEF gel (●).

resis was then pursued at 1000 V for 7 min. At the end of the electrophoresis, the pH gradient was measured as described previously⁷ and the gel was fixed and stained according to ref. 8.



b

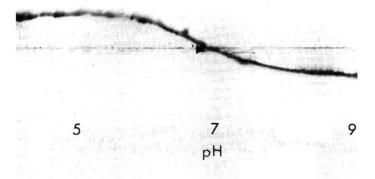


Fig. 2. (Continued on p. 76)

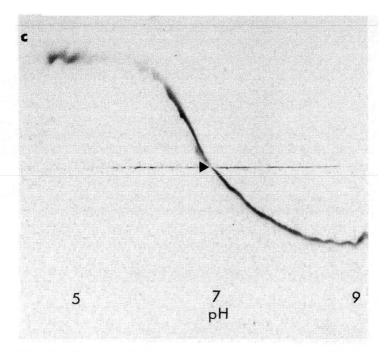


Fig. 2. Titration curves of total red blood cell lysates. The bidirectional arrows and the + and - symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (EL). The position at which the titration curve crosses the application well (indicated by an arrow) represents the zero-mobility point of the macromolecule, *i.e.*, its isoelectric point (pI). Gel fixing and staining are as described ¹¹. a, Agarose IEF gel. Conditions: IEF in the first dimension as described in the text; EL in the second dimension for 3 min at 500 V then for 7 min at 1000 V (constant voltage). b, PAG plate gel, pH gradient 3.5–9.5. Anode electrode solution: 1 M H₃PO₄. Cathode electrode solution: 1 M NaOH. Conditions: IEF in the first dimension at 7 W (constant power) for 90 min, 700 V at equilibrium; EL in the second dimension for 3 min at 250 V then 7 min at 400 V (constant voltage). Temperature = 4°C. c, PAG plate gel, pH gradient 3.5–9.5. Conditions as in Fig. 2b, except that EL was performed for 3 min at 250 V and then for 25 min at 400 V (constant voltage) to obtain a curve with a comparable mobility as in Fig. 2a.

RESULTS

As a first step the formation of the pH gradient in the agarose gel matrix was compared with that obtained in polyacrylamide gel. As shown in Fig. 1 a pH gradient can be generated along the gel. The shape of the gradient is similar and linear in both cases.

A total red blood cell lysate was used to show the feasibility of obtaining titration curves in agarose IEF gel. Fig. 2a shows the results obtained using the conditions described above. A sharp titration curve is generated which crosses the sample well at pH 6.95 corresponding to the pI of hemoglobin A. This result has been compared to those from a PAG plate LKB gel, pH gradient 3.5–9.5.

Fig. 2b shows the titration curves obtained after an electrophoresis of the same duration (10 min) as that used in agarose IEF gel, whereas in Fig. 2c a longer electrophoresis duration (28 min) had to be used to achieve a similar mobility as in agarose IEF gel.

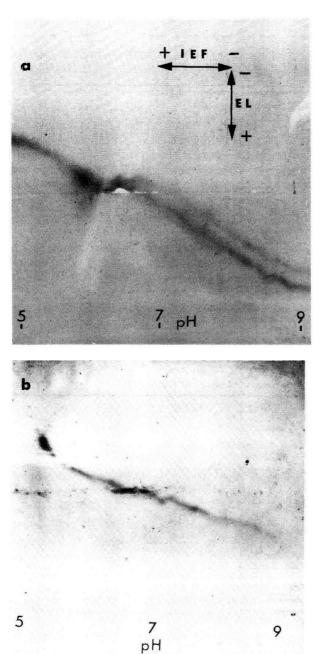


Fig. 3. Titration curves of a mutant of NADH cytochrome b_5 reductase (Diaphorase). The sample was the non-heme protein fraction purified from total red blood cell lysates according to ref. 12. Specific staining for NADH cytochrome b_5 reductase was performed according to the method of Kaplan and Beutler¹³. a, Agarose IEF gel, pH gradient 3–10. Electrical conditions as described in the text. b, PAG plate, pH gradient 3.5–9.5. Conditions as in Fig. 2b. A p*I* of 6.8 has been found in both experiments.

The method has been used to analyze genetically substituted proteins and the superiority of agarose IEF gel as compared to polyacrylamide gel has been demonstrated in specific problems. An example is provided by a mutant of cytochrome b_5 reductase (diaphorase)^{9,10}.

The same sample from a heterozygote patient was run on agarose IEF gel (pH gradient 3–10) and on a PAG plate gel (pH gradient 3.5–9.5). After specific staining the following patterns were obtained: on agarose IEF gel (Fig. 3a) a single band on the acidic side of the pI, and two bands on the basic side with different migrations; on PAG plates (Fig. 3b) a single band along the whole pH gradient.

DISCUSSION

A reliable interpretation of electrophoretic mobility curves first requires a stable linear gradient and an efficient migration which itself must take place in a relatively short time so as not to disrupt the gradient.

In polyacrylamide gels migrations of high-molecular-weight molecules is slowed down by a molecular sieving effect, due to the pore size of the gel, and to compensate for this a prolonged migration time is required which partially destabilizes the gradient. We therefore developed, as an alternative electrophoretic support, the use of agarose gel which has larger pores.

One problem is related to electro-osmosis¹⁴ of ordinary agarose which carries negative charges. This can be solved by using highly purified agarose IEF, where selective positive charges neutralize the existing negative charges¹⁵. The electro-osmotic effect was also minimized by the use of sorbitol which increases the viscosity of the gel, decreasing the osmotic flow as a secondary effect and by overnight storage of the gel at 4°C in a humid chamber which increases its mechanical strength. As seen in Fig. 1, these conditions provide a linear gradient.

The results shown in Fig. 2 clearly demonstrate that the resolution obtained in generating titration curves on agarose gel is as good as that achieved by the use of polyacrylamide as a support and in a shorter time of migration. It should be noted that the sample well is replaced by a slit made with a scalpel, thus reducing the volume of the sample; this results in a sharper and better resolved curve.

We found it essential that the electrophoresis be run initially at low voltage in order to allow the sample to penetrate into the gel. If a high voltage is applied from the beginning, the sample migrates at the surface of the gel, and is washed out during the staining procedure.

Fig. 3 gives an example of the usefulness of this technique in the study of a mutant of cytochrome b_5 reductase. When analyzed on a PAG plate, the mixture of the substituted enzyme and its wild type gives rise to a single visible band (Fig. 3b). On the agarose gel, the same sample is separated into two distinct bands at pH values above the pI (6.8), Fig. 3a. One explanation for the behaviour on agarose gel could be a more efficient penetration of the chromogenic agents into the gel. Other advantages of agarose IEF gel have also to be considered, e.g., the ease, rapidity and reliability of gel preparation, the aerobic polymerization, the rapidity of staining and destaining, the use of non-toxic chemicals, the absence of chemically reactive components in the gel and the unrestricted mobility for large proteins. All these advantages together make agarose IEF a suitable support for isoelectric focusing experiments.

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CHROM. 15,171

Note

Silica gel-impregnated paper chromatographic determination, by differential staining, of N-acyl lipids

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The increasing interest in N-acyl phosphatidyl ethanolamine (NAPE)¹ from various biological sources has required techniques which can suitably differentiate these substances from other N-acyl lipids and from amino lipids, notably phosphatidyl ethanolamine (PE). We report here our observations derived from chromatography on Whatman SG-81 silica gel-impregnated filter paper and modifications of the N-chlorination/o-tolidine–KI procedure representing, largely, the cumulative experience of others²⁻⁴. To facilitate this examination a convenient chlorination apparatus has been assembled from commonly available laboratory glassware.

EXPERIMENTAL

The chlorination apparatus

As shown in Fig. 1, this consists of a 500-ml, three-neck, round-bottomed flask with a 21-cm 45/50 standard-taper tube, inserted into the center neck, to contain the rolled chromatogram. This tube serves as the chlorination chamber and is capped by a PTFE plate through which extends a short stainless-steel rod. The 24/40 side-arm at the left is for introduction of the pulverized NaCl:KMnO₄ stoichiometric mixture (500 mg) and the separatory funnel in the right side-arm contains the 9 M sulfuric acid necessary for the chlorine generation. To ensure that the chlorine promptly reaches the top of the tube a brief (20 sec) gentle flow of nitrogen is introduced via a connection with the left side-arm. Evidence that chlorination has been adequate is provided by inspection of the inner end of the stainless-steel rod, which acts as a cold-finger; 10 min exposure to the chlorine appears to be sufficient.

Spot-tests

The actual spot-testing occurs after (1) aeration of the chlorinated chromatogram for 10 min, (2) three 20-sec washes in water, (3) blotting with paper towels and air drying (complete dryness is not required). Both sides of the chromatogram can be briefly sprayed with the o-tolidine–KI (2:1) mixture for maximal visualization of the

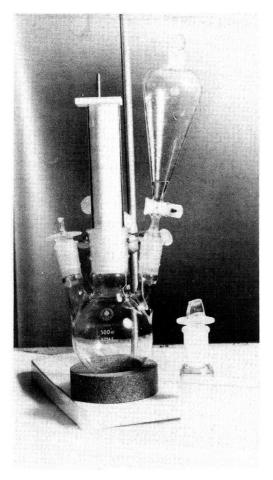


Fig. 1. Chlorination apparatus with rolled chromatogram in place.

N-acyl compounds. Alternatively, spraying can be done first with the ethanolic o-tolidine followed (5 min later) by the aqueous KI (or by the o-tolidine–KI mixture as discussed in Results. Because of the transience of the resulting blue spots the chromatograms are then photographed prior to any subsequent staining. Visualization of monoglyceride (MG) and ceramide monohexosides is achieved by the PAS reaction and the appropriate use of fluorescamine. Rhodamine 6G, OSPAS and the plasmal reaction, etc. have been described earlier⁵; together they aid in the full characterization of the chromatograms. Commercial preparations of o-tolidine are frequently too oxidized to use for the described purpose. However, filtrates prepared from hot saturated solutions of o-tolidine in 50% aqueous ethanol, which have been decolorized by Norite and kept reduced by added ascorbate, produce crystalline material of respectable purity from which the spray reagent is prepared fresh daily (1 mg/ml ethanol); the KI is 1 mg/ml water and is stable.

Chromatography

Extract samples of $10-30~\mu l$ are applied to $12\times19~cm$ sheets of Whatman SG-81 paper, previously washed with chloroform—methanol (2:1) and by acetone, and run in one of the following developing mixtures in accordance with the nature of the resolution required: chloroform—methanol-14 M ammonium hydroxide solution (85:15:1.5), (120:15:1.5) or (180:5:0.5). As indicated in the figures, empiricism remains desirable to achieve the desired optimal resolutions. Repeated use of the developing solvents produces subtle separation effects, as the solvent ratios change, which are not always useful.

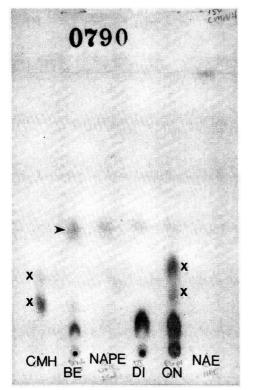
The above procedures have been applied to chromatograms prepared from chloroform–methanol (2:1) extracts from freeze-dried samples of (a) normal and infarcted cardiac muscle from dog, rabbit and man, (b) normal hearts of cat, sheep, cow, guinea-pig and monkey, (c) normal brain and optic nerve of the fish *Amia calva* and *Elops saurus*, (d) yolk (hen's egg), yolk-sac and stage-20 chick embryo, (e) NAPE-containing seeds of oat and pea. To assist in the interpretation of the above extracts, co-chromatography with various commercially available lipid standards, of high purity, was resorted to. The palmitoylethanolamide (NAE) was a synthetic product from Calbiochem-Behring (La Jolla, CA, U.S.A.); $10^{-3} \mu M$ was readily detectable. The concentration of the extracts was adjusted so that $10{\text -}30 \mu l$ would produce usable chromatograms, and generally represented $100{\text -}500$ mg, dry-weight, of tissue per millilitre of solvent. The standards were used at a concentration of 1 mg/ml.

RESULTS

o-Tolidine–KI was found to be a positive spot test for the entire NAE group so far encountered (e.g. NAE, NAPE and NA-lyso-PE), for all of the ceramide (Cer) derivatives (e.g. n- and h-Cer, CMH, CDH and sphingomyelin), for PE, lyso-PE and MMePE. Neither DiMePE nor PC (TriMePE) stained. The zwitterionic properties of PS probably explain its failure to stain. As expected, the *imine* produced by reaction of PE with acetone was positive but was easily chromatographically resolvable from NAPE. Bis-phosphatidic acids were negative (Figs. 2–4).

Of the above compounds the NAE group was the most refractory to *o*-tolidine staining in the absence of KI (Fig. 5). This behavior therefore provided a useful differential manipulation, particularly under conditions where the ceramides were not clearly resolved from NAE nor CMH from NA-lyso-PE. Cer/NAE resolutions were best in the 180:5:0.5 system, while the 120:15:1.5 system was most useful for extracts containing NAPE, NA-lyso-PE, CMH and PE. More polar NAE/Cer derivatives would require the most polar system.

It was consequently observed, in the DI series (six specimens of 17–24-h infarcts), that there was a wide quantitative variation in NAPE and NA-lyso-PE content (mainly as plasmalogen) with only one specimen containing detectable amounts of NAE. The PAS reaction revealed that, in this series, MG was also present and easily co-chromatographed with NAE (although separable when alerted to the problem). None of the plant seeds examined contained NAE, although NAPE and NA-lyso-PE was present. As with the other infarct (and normal) heart specimens Cer was also present; this increased the resolution problem re: NAE, MG and n- and h-Cer, although these, too, could be resolved and differentiated as indicated. None of the



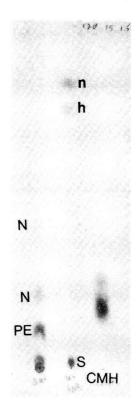


Fig. 2. *o*-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 *M* ammonium hydroxide solution. From left to rigth: ceramide monohexoside (CMH) with one major and one minor spot (×), black-eye pea, NAPE (silicic acid column isolate from dog-heart infarct), DI, *Elops* optic nerve (ON) and NAE standard. The tissue samples show varying levels of NAPE with pea DI ON; only in the pea (above PE) is an NA-lyso-PE detectable. NAE, right lane, is near the solvent front.

Fig. 3. *o*-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 *M* ammonium hydroxide solution (120:15:1.5). The samples are, left to right, oat, n- and h-Cer, CMH (two spots). No NAE is present.

other heart specimens contained detectable amounts of the NAE group of compounds. Our exploratory studies showed an exceedingly small amount of NAPE in the yok of hen's egg, while yolk-sac specimens contained somehwat more; none was detected in stage-20 embryos. Although the *o*-tolidine–KI reaction had the desired selectivity, the sensitivity (as a detector of NAPE) was less than that provided by rhodamine 6G and, for the alk-1-enyl species, by the plasmal HgCl₂–Schiff reaction (Fig. 6), neither of which stained the NAE. Olefinic NAE species, however, were appropriately positively spot-tested by the OSPAS reaction.

Other biphenyl diamines (e.g. benzidine and o-dianisidine) were also examined as visualizing reagents but were found to be no more sensitive than was o-tolidine and are not further described.

DISCUSSION

On the basis of gas—liquid chromatographic analysis of the amide-linked fatty acids of NAE, isolated from dog heart infarct by thin-layer chromatography, Epps *et al.*⁶ have calculated the NAE content and established the FA profile. The presence of



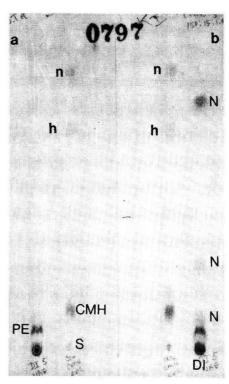


Fig. 4. o-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 M ammonium hydroxide solution (120:15:1.5). Left lane, oat; middle lane, CMH (two); right lane, NAE.

Fig. 5. (a) Sprayed only with ethanolic o-tolidine; left lane, DI with added NAE; right lane, Cer (normal and hydroxy standards), CMH and sphingomyelin (S). (b) Sprayed first with o-tolidine and then with KI (both sides); same samples as (a), but lanes reversed as shown. From top to bottom (N) refers to NAE, NAPE and NA-lyso-PE, respectively. The solvent (chloroform–methanol–14 M ammonium hydroxide solution) ratio is 150:15:1.5.

MG was detected and removed prior to the analysis. From our experience we would expect that any n-Cer present could have contributed to the data.

There have been relatively few reports dealing with the free Cer of tissues⁷. The technique described here may more readily permit the demonstration that the distribution of ceramides is more widespread than currently appreciated and could facilitate its more direct metabolic correlation with the commonly observed sphingomyelin and CMH.

The presence of both NAPE and NA-lyso-PE in various seeds certainly suggests the presence of a phospholipase A as well.

It seems quite remarkable that the two mammalian representatives (cat and dog) for which cardiac infarction results in the appearance of lipids of the NAE group are the same ones whose kidneys are characterized by normal histochemical distribution of neutral lipids in the kidney cortex^{8–11}. Does this represent a generalized phenomenon, for these two animals, in the way they handle their fatty acid metabolism?

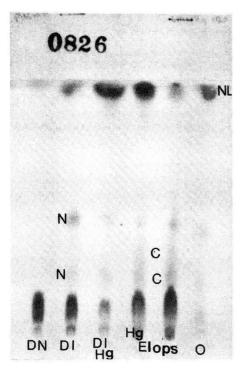


Fig. 6. Plasmal reaction followed by rhodamine 6G; chloroform methanol–14 *M* ammonium hydroxide solution (120:15:1.5). Left to right, normal dog heart (DN), dog-heart infarct (DI), DI with *in situ* application of aqueous HgCl₂ to release free aldehyde from the plasmalogen prior to the chromatographic run, *Elops* optic nerve with and without added HgCl₂, oat. The free aldehydes appear at the solvent front with other neutral lipids (NL). Two CMH spots (c) are in the *Elops* samples. The HgCl₂ produces Na-lyso-PE (the lower N spot) in both the DI and *Elops* samples.

ACKNOWLEDGEMENTS

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Note

Quantitative separation of B-6 vitamers in selected foods by a gas-liquid chromatographic system equipped with an electron-capture detector

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Gas chromatography (GC) is one of the most important and widely used separation techniques to date. Recent advances in the techniques of GC offer potential for the detection and quantitative determination of the various forms of vitamin B-6 in foods and similar materials with satisfactory specificity, sensitivity, convenience, and reduction in analysis time. Several investigators¹⁻³ have used trimethylsilylation for the analysis of the various B-6 vitamers. Imanari and Tamura⁴ examined the GC separation of the trifluoroacetyl derivatives of pyridoxol (also called pyridoxine, PN), pyridoxamine (PM), methyloxime of pyridoxal (PL), and pyriodoxic acid lactone. Sennello and Argoudelis⁵ have used N,O-bis-(trimethylsilyl)acetamide as a derivatizing reagent in the GC determination of vitamin B-6. GC separations of acetylated derivatives of PL, PN, and PM have been reported by Sheppard and Prosser⁶, Prosser et al.⁷, and Korytnyk⁸. The heptafluorobutyryl derivatives of PL, PN, and PM have also been separated with GC by Williams9. Patzer and Hilker¹⁰ have recently used a new reagent N-methyl-bis-trifluoroacetamide (MBTFA) for the formation of vitamin B-6 derivatives which offers the advantage of being a rapid, clean, and simple analytical procedure; the hydrochlorides of PL, PN, and PM were used; the detection minimum was at least 250 ng using a flame ionization detector (FID).

Preliminary studies in our laboratory indicated that enhanced sensitivity in the detection of B-6 vitamers in standard solutions was obtained when the electron-capture detector (ECD) was utilized as compared to the FID. We utilized MBTFA as a derivatizing reagent and a GC system equipped with a ⁶³Ni ECD (GC-ECD) for the separation and quantitation of PL, PN, and PM. Preliminary studies demonstrating the application of this technique to the separation and quantitation of naturally occurring PL, PN, and PM in selected foods are also described.

EXPERIMENTAL

Hydrochloride forms of PL, PN, and PM (Sigma, St. Louis, MO, U.S.A.) were used as standards. MBTFA, obtained from Pierce (Rockford, IL, U.S.A.), was used as the derivatizing reagent. MBTFA was used to trifluoroacylate primary and secondary amines, hydroxyl, and thio groups under mild, non-acidic conditions¹¹. Pesticide grade absolute ethanol and ethyl acetate were glass-distilled before use.

A stock solution containing a mixture of the three B-6 vitamers (1000 ng/ μ l each of PL, PN, and PM) were prepared in deionized–distilled water and protected from light. Trifluoroacetylation of the B-6 forms was carried out using a modification of the method of Patzer and Hilker¹⁰. A 50- μ l volume of the aqueous mixture of the three B-6 vitamers was introduced into each of two 1 ml reactivials and dried under a gentle stream of nitrogen at 65°C with the use of a No. 18800 Reactitherm heating module equipped with a No. 18804 Reacti-block (Pierce).

Absolute ethanol, 50 μ l, was added to each vial to convert PL to its hemiacetal in order to distinguish it from PN after derivation³. The vials were covered with PTFE, silicone discs and sealed with open top screw caps. The contents of the vials were refluxed at 85°C for 30 min, cooled to room temperature, and then the ethanol was evaporated under nitrogen at 65°C. MBTFA, 50 μ l, was added to the contents of each vial; refluxing with closed tops was carried out at 130°C for 20 min. The contents of the vials were allowed to cool to room temperature and 450 μ l of ethyl acetate were added to bring about a 1:10 dilution of the derivatized mixture so that a concentration of 100 ng/ μ l of each B-6 vitamer was obtained. The contents were mixed using a vortex for 0.5 min to ensure homogeneity. The derivatized B-6 compounds were further diluted with ethyl acetate to obtain concentrations of 0.01 to 100.0 ng/ μ l of each of the B-6 vitamers. Volumes of 1.0 μ l were then injected directly into the gas chromatograph and a calibration curve prepared. All injections were done in duplicate.

Analyses of food extracts

Brand names of selected foods were purchased at a local grocery store; these included Rainbo enriched white bread, Carnation instant non-fat dry milk, and Green Giant sweet peas. Aqueous slurries of the foods (1:2; solid—water) were prepared by homogenization.

The B-6 vitamers in the homogenates were solubilized and released by acid hydrolysis followed by enzymatic treatment using alpha-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), and papain (EC 3.4.22.2); all enzymes were obtained from Sigma Chem. Co., St. Louis, MO. To 50 ml (bread or milk) or 25 ml (peas) slurry, 30 ml 0.2 M hydrochloric acid were added; samples were placed in a boiling water bath for 1 h with constant stirring using a magnetic stirring bar. Flasks were then cooled to room temperature and 2 ml alpha-amylase solution (3 g in 2.5 M sodium acetate), 2 ml pepsin solution (3 g in 2.5 M sodium acetate), and 1 ml 1% papain solution were added; then samples were incubated at 37°C for 16 h in a shaker water bath. Samples were then filtered through No. 1 filter paper using a Buchner funnel. The filtrate was then passed through an ion-exchange column (AC-50W-X8, 100-200 mesh, Bio-Rad Labs., Richmond, CA, U.S.A.) in order to further remove contaminants.

Aliquots (150 μ l) of the food extracts were derivatized under the same conditions as for the B-6 standards. Calibration curves with external standards were prepared to accompany each set of chromatographic determinations. This allowed the GC analyst to compensate for sensitivity changes that occurred during normal GC operations.

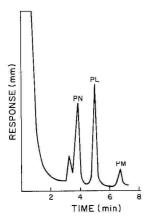
GC operating conditions

GC was carried out using a MT-220 (Microtek Instruments, Baton Rouge, LA, U.S.A.) gas-liquid chromatograph fitted with a ⁶³Ni ECD. The detector voltage was set at 10² and the sensitivity at 1/32. Several different columns and operating conditions including that of temperature were tried and those described below were found to be the best for the quantitative separation of the B-6 vitamers. The column was 1.54 m × 2 mm I.D., glass, packed with 10% SP-2100 on Supelcoport 80–100 mesh (Supelco, Bellefonte, PA, U.S.A.). The column temperature was maintained at 125°C, the injection port was operated at 205°C, and the detector temperature was set at 350°C. The carrier gas was nitrogen with a regulator pressure of 40 p.s.i.g. and a flow-rate of 30 ml/min.

RESULTS AND DISCUSSION

A representative chromatogram of B-6 standards separated by GC-ECD is shown in Fig. 1. The separation of all three B-6 compounds was completed in less than 8 min. Trifluoroacylation of PL and PM gives rise to single peaks; whereas, trifluoroacylation of PN gives rise to two peaks —a major peak with a retention time of approximately 3.8 min and a minor peak with a retention time of approximately 3.2 min which sometimes appeared as a shoulder to the major peak. Korytnyk³ had reported that trimethylsilylation of PN generally yielded 2 peaks. The variation in areas of the two peaks was dependent on the time the vitamer was exposed to the trimethylsilylation mixture. In the present study, variation in areas of the two peaks resulting from formation of the MBTFA derivative of PN was observed. The peak with the longer retention time was always the predominant peak. The calibration curve for PN was plotted using the sum of the peak heights. This gave a satisfactory linear plot.

The log response vs. log ng standard calibration curves for PN and PL were linear between 0.01 and 0.5 ng; whereas the curve for PM was linear between 0.01 and 5.0 ng. The minimum detectable quantity for all three vitamers was 0.01 ng. Several factors affect the operation of the gas chromatograph such as purity and dryness of the carrier gas, sensitivity of the ECD, temperature, column bleed, column conditioning times, and electrical noises also often contribute to variability in the signal output in GC-ECD techniques.



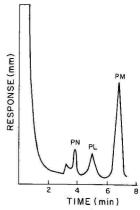


Fig. 1. Separation of B-6 standards by GC–ECD. Conditions: Microtek MT-220; column, $1.54 \,\mathrm{m} \times 2 \,\mathrm{mm}$ I.D., glass, packed with $10 \,\%$ SP-2100 on Supelcoport 80–100 mesh; column, $125 \,^{\circ}$ C, injection port, $205 \,^{\circ}$ C, detector, $350 \,^{\circ}$ C; carrier gas, nitrogen at 30 ml/min; 63 Ni ECD.

Fig. 2. Separation of B-6 vitamers in milk by GC-ECD. Conditions as for Fig. 1.

GC-ECD analyses of food extracts

A representative chromatographic pattern of separated B-6 vitamers in derivatized extracts of milk is shown in Fig. 2. Similar patterns were obtained for extracts of bread and peas. Identification of the B-6 vitamers was accomplished by matching peak patterns and retention times of the B-6 vitamers from the foods with those of pure standards chromatographed in the same set of determinations. In general, good separations with little interference were obtained.

The B-6 vitamer content of these selected foods as measured by GC-ECD techniques is shown in Table I. Published values for GC analyses of B-6 vitamer content were not available for comparison. The values which we obtained using GC-ECD methods are higher than reported values as ascertained by microbiological assay¹².

TABLE I B-6 VITAMER CONTENT OF SELECTED FOODS AS MEASURED BY GC–ECD $\overline{X} \pm \text{S.D.}$ for duplicate analyses of two separate extractions.

Food	mg/100 g wet wt.				
	PM	PL	PN	Total B-6	
Enriched bread	0.41 ± 0.11	0.34 ± 0.07	1.04 ± 0.24	1.79 ± 0.41	
Non-fat dry milk	0.95 ± 0.08	0.41 ± 0.20	4.58 ± 0.64	5.94 ± 0.91	
Sweet peas	0.99 ± 0.32	0.39 ± 0.01	5.33 ± 1.32	6.71 ± 1.00	

The short separation time of the MBTFA derivatives of PL, PN, and PM as well as the exceptional sensitivity of the ECD suggest the potential use of this GC–ECD method for the detection and quantitation of B-6 vitamers in food. Additional precision could be obtained by use of an internal standard such as deoxypyridoxine. Further studies should involve the use of mass spectrometry for additional confirmation of peak identities.

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CHROM. 15,197

Note

Fused-silica capillary gas chromatographic separation of alditol acetates of neutral and amino sugars

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In a previous paper¹ we demonstrated that fused-silica wall-coated open tubular (WCOT) columns provide a convenient and efficient tool for the analysis of alditol acetates of neutral monosaccharides, which are the most commonly used derivatives for analyses of polysaccharides and glycoconjugates². Those coated with polar liquid phases such as FFAP and PEG 20M gave excellent separations of either alditol acetates or partially methylated substances under isothermal operations.

In carbohydrate chemistry, fused-silica WCOT columns have recently been employed in separations of partially methylated alditol acetates derived from the extracellular polysaccharide of the bacterium *Rhizobium japonicum*³ and in analysis of the methanolysate of lipopolysaccharides⁴ on a column with methylsilicone (SE-30) stationary phase.

Here we extend the use of such columns to the analysis of amino sugars as alditol acetates. Separation of the alditol acetates of glucosamine and galacosamine by gas-liquid chromatography (GLC) was first reported on a packed column with 1% ECNSS-M on Gas-Chrom A⁵, and was followed by some improvements in packed column GLC⁶⁻⁸. Recently, Doctor and co-workers⁹ reported that a glass capillary SCOT column coated with chiral polysiloxane liquid phase is able to separate the alditol acetates of glucosamine, galactosamine and mannosamine and thirteen neutral monosaccharides.

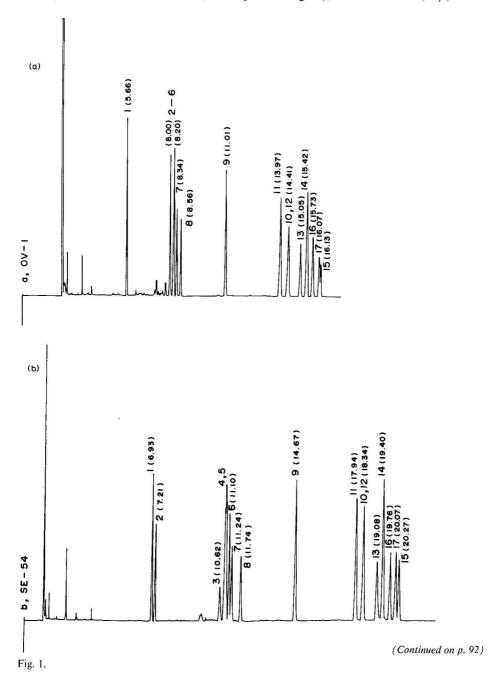
In the present study, the separation of alditol acetates of neutral and amino sugars has been examined on fused-silica WCOT columns with non-polar (silicone OV-1), slightly polar (silicone SE-54) and polar stationary phases (Carbowax 20M). Glucosamine, galacosamine and mannosamine were readily separated as alditol acetates by any column employed, whereas the Carbowax 20M column was superior for the separation of the neutral alditol axetates.

EXPERIMENTAL

GLC was carried out with a Hewlett-Packard 5880A instrument equipped with

a flame ionization detector at linear carrier gas (helium of hydrogen) velocities of 39–53 cm/sec. A sample solution (1 % w/v) in methylene chloride (0.2 μ l) was applied to a column in split mode (splitting ratio 100/1).

The following fused-silica WCOT columns (Hewlett-Packard, Avondale, PA, U.S.A.) were used: silicone OV-1 (dimethylsilicone gum), 50 m \times 0.2 mm, $D_{\rm f}$ (thick-



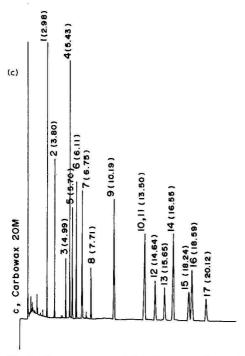


Fig. 1. Chromatograms of alditol acetates of neutral sugars on fused-silica WCOT columns using helium as the carrier gas: a, OV-1 (50 m \times 0.2 mm, $D_{\rm f}=0.17~\mu{\rm m}$), linear velocity (\bar{u}) 39 cm/sec, temperature 170–190°C at 1°C/min; b, SE-54 (25 m \times 0.3 mm, $D_{\rm f}=0.52~\mu{\rm m}$, $\bar{u}=40~{\rm cm/sec}$, temperature 140–200°C at 2°C/min; c, Carbowax 20M (25 m \times 0.2 mm), $\bar{u}=50~{\rm cm/sec}$, temperature 20°C. Peaks correspond to acetates of: 1 = D-digitoxitol; 2 = 2-deoxy-D-ribitol; 3 = L-rhamnitol; 4 = D-fucitol; 5 = 6-deoxy-D-glucitol; 6 = D-ribitol; 7 = L-arabinitol; 8 = D-xylitol; 9 = 2-deoxy-D-galactitol; 10 = D-allitol; 11 = 3-O-methyl-D-glucitol; 12 = 4-O-methyl-D-glucitol; 13 = D-allritol; 14 = D-mannitol; 15 = L-glucitol; 16 = D-galactitol; 17 = L-iditol. The retention times (min) are also shown.

ness of liquid phase film) = 0.17 μ m; silicone SE-54 (1 % vinyl, 5 % phenyl), 25 m × 0.3 mm, $D_f = 0.52 \ \mu$ m; Carbowax 20M, 25 or 12 m × 0.2 mm.

Alditol acetates were prepared as described previously¹ from corresponding neutral and amino sugars.

RESULTS AND DISCUSSION

Separations of alditol acetates of neutral and amino sugars are compared on three different types of columns in Figs. 1 and 2, respectively.

Hexitol acetates are well separated on each of the present columns; it may be noticed that glucitol acetate emerges after galactitol acetate on the OV-1 or SE-54 column, but the elution order is the opposite on the Carbowax 20M column. Resolution of acetates of pentitols and deoxyhexitols was improved on the SE-54 column compared to the OV-1 column, and complete separation was achieved on the Carbowax 20M column as on the FFAP column described previously¹.

Amino sugars are completely separated as alditol acetates in 8-12 min (Fig. 2);

the shorter Carbowax 20M column (12 m) was used, since it was required to operate near the maximum operating temperature for the separation of these compounds. The elution order of galactosaminitol and mannosaminitol acetates on the Carbowax 20M column is opposite to that on the OV-1 or SE-54 column.

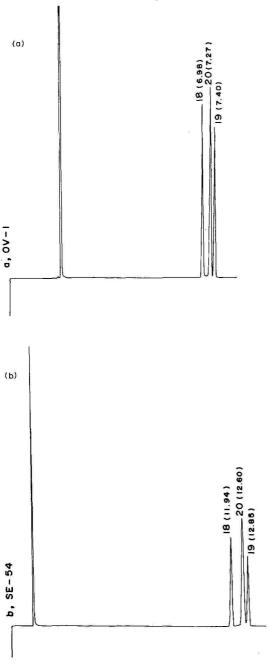


Fig. 2. (Continued on p. 94)

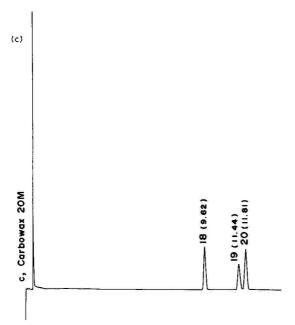


Fig. 2. Chromatograms of alditol acetates of amino sugars on fused-silica WCOT columns using helium as the carrier gas: a, OV-1, $\bar{u}=45$ cm/sec, temperature 210°C; b, SE-54, $\bar{u}=35$ cm/sec, temperature 210°C; c, Carbowax 20M (12 m × 0.2 mm), $\bar{u}=53$ cm/sec, temperature 220°C; characteristics of the OV-1 and SE-54 columns as in Fig. 1. Peaks correspond to acetates of: 18= D-glucosaminitol; 19= D-galactosaminitol; 20= D-mannosaminitol.

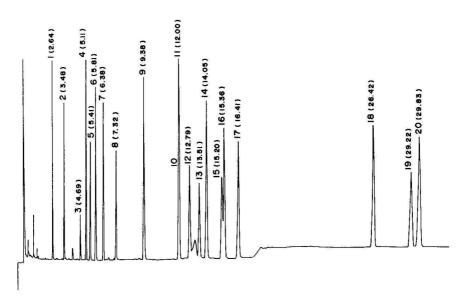


Fig. 3. Chromatogram of alditol acetates on the Carbowax 20M ($12 \text{ m} \times 0.2 \text{ mm}$) fused-silica WCOT column using hydrogen as the carrier gas. Temperature, $185-200^{\circ}\text{C}$ at 1°C/min , maintained at 200°C for 2 min and increased to 220°C at 20°C/min . Peak identities as in Figs. 1 and 2.

Simultaneous separation of alditol acetates of both neutral and amino sugars was tried on the 12-m Carbowax 20M column, giving the chromatogram of Fig. 3. Although the resolution of the acetates of glucitol and galactitol was not satisfactory, almost all monosaccharides could be rapidly separated as alditol acetates by this GLC system.

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CHROM. 15,275

Note

Gas chromatography of homologous esters

XVIII*. Polychlorinated propionate and butyrate esters

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A recent paper¹ has considered the retention behaviour of the methyl and chloromethyl esters of isomeric monochloro esters of C_2 – C_{18} *n*-carboxylic acids²⁻⁴ and of the corresponding monochloro esters of the isomeric C_5 aliphatic acids⁵.

The effect on retention of the position of the chlorine substituent and of branching in the acid chain shown in these reports was compared with the results from other studies on aliphatic esters containing a second and variable structural parameters.

The present work extends the earlier study using recently available data^{6,7} on the mono- and dichloro esters of n-butyric acid and on all the chlorinated esters of propionic acid^{8,9} such that the effect on retention of chlorine substituents in all of the positions in the chain is apparent.

EXPERIMENTAL

A Varian Model 2400 gas chromatograph with flame ionisation detector was used for the analyses. Glass capillary columns coated with 3% Carbowax 20M were used with temperature programming from 50°C at 6°C/min. The retention data were recorded as uncorrected retention times.

RESULTS AND DISCUSSION

The retention data for the methyl esters of mono- and dichlorobutyric acids are shown in Table I while the retention behaviour relative to the position of substitution is shown in Fig. 1.

The monochloro esters follow the trend previously observed for longer chain esters, where the retention increased with the distance of the chlorine atom from the carbonyl group, the ω - or terminally substituted compound having the highest retention due to minimisation of acceptor–donor effects.

Of the dichloro esters the 2,2-homologue has, predictably, the lowest retention time while the 3,3- and 4,4-homologues show progressively higher retention times, the

^{*} Part XVII: G. Crank and J. K. Haken, J. Chromatogr., 245 (1982) 346-349.

TABLE I
RETENTION TIMES OF MONO- AND DICHLOROBUTYRATE ESTERS

Compound	Retention time (sec)
Methyl butyrate	141
Methyl 2-chlorobutyrate	223
Methyl 3-chlorobutyrate	248
Methyl 2,2-dichlorobutyrate	282
Methyl 4-chlorobutyrate	340
Methyl 3,3-dichlorobutyrate	340
Methyl erythro-2,3-dichlorobutyrate	374
Methyl threo-2,3-dichlorobutyrate	484
Methyl 4,4-dichlorobutyrate	513
Methyl 2,4-dichlorobutyrate	561
Methyl 3,4-dichlorobutyrate	595

compounds following the pattern of the 2-, 3- and 4-chloro esters. The retention time of the 2,3-dichloro isomer is higher than that of the 3,3-isomer, that of the 2,4-isomer is similarly higher and the 3,4-dichloro ester shows the highest retention time. The polar effect of two chlorine substituents is maximised when the two atoms are not attached to the same carbon atom, which is simply due to the bulkiness of the substituents.

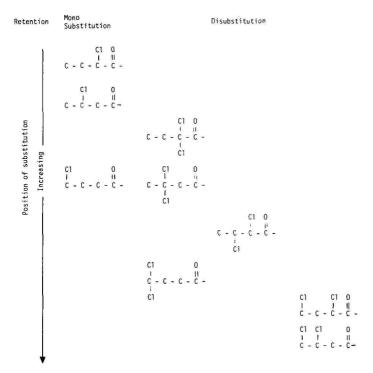


Fig. 1. Structure and retention behaviour of mono- and dichlorobutyrates.

TABLE II
RETENTION TIMES OF METHYL CHLOROPROPIONATES

Methyl	Retention time	
chloropropionates	(sec)	
2-Chloropropionate	195	
2,2-Dichloropropionate	218	
3-Chloropropionate	269	
3,3-Dichloropropionate	348	
2,3-Dichloropropionate	407	
3,3,3-Trichloropropionate	448	
2,2,3-Trichloropropionate	488	
2,3,3-Trichloropropionate	533	
2,3,3,3-Tetrachloropropionate	594	
2,2,3,3-Tetrachloropropionate	648	
Pentachloropropionate	749	

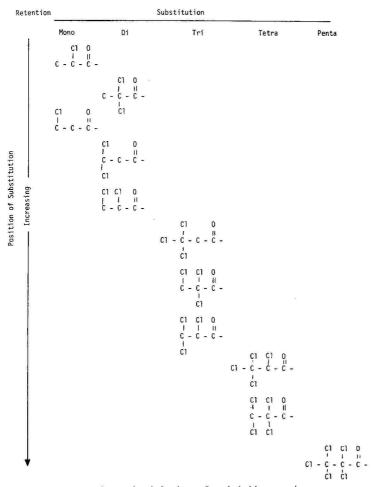


Fig. 2. Structure and retention behaviour of methyl chloropropionates.

The effect of further substitution may be observed from the data of the eleven methyl chloropropionates (Table II) while the retention behaviour relative to the position of the substituents is shown in Fig. 2. The increase in retention in the monoand dichloropropionates followed the same pattern as for the corresponding butyrate esters.

As expected, the trichloropropionates have increased retention times and the 3,3,3-trichloro ester, due to attachment of three substituent groups, exhibits a lower retention than the 2,2,3-trichloro ester which, in turn, has a lower retention than the 2,3,3-isomer with terminal di-substitution.

The same pattern is observed for the two tetra-substituted isomers, where terminal di-substitution produces greater retention than terminal tri-substitution. The pentachloropropionate, with an additional chlorine substituent, shows the greatest retention of the series.

The two ester series generally follow the same retention pattern with substitution, and it is possible, by observing the following simple rules, to predict the elution behaviour of longer chain polychlorinated esters.

- (1) The retention of an ester with single chlorine substitution increases as the distance from the carbonyl increases and retention is maximised with substitution in the terminal (ω) position.
- (2) ω,ω di-substitution produces lower retention than $\omega,\omega-2$ and $\omega,\omega-1$ di-substitution.
- (3) With trichloro esters retention is maximised with ω -di-substitution. The retention data, if available as retention indices, would allow the relative contributions of each substituent to be shown as retention increments as have been used with many series of saturated and unsaturated aliphatic esters.

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CHROM. 15,198

Note

Separation of prostaglandins and thromboxane $\,B_2$ by high-resolution gas chromatography coupled to mass spectrometry or electron-capture detection

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(Received June 10th, 1982)

In recent years much attention has been focused on the identification and measurement of prostaglandins (PGs)¹. It is generally recognized that mass spectrometry (MS) is the most specific and reliable method so far available for qualitative and quantitative analysis of PGs, especially when coupled to high-resolution gas chromatography (HRGC)^{2,3}. The sensitive electron-capture detector (ECD) can also be coupled to HRGC, yielding fairly specific methods of analysis⁴. The use of HRGC is mandatory when the stable cyclooxygenase metabolites of arachidonic acid [PGF_{2 α}, PGE₂, PGD₂, 6-keto-PGF_{1 α}, thromboxane B_2 (TXB₂)] have to be analyzed as a group, since these compounds cannot be separated by conventional packed columns.

This paper deals with the development of the simultaneous detection of PGs and TXB₂ by HRGC-MS and HRGC-ECD as alternative and integrated methods to be used when different degrees of specificity are required. A derivatization procedure suitable for the gas-phase analysis of all the compounds to be analyzed and for both the detectors to be used (MS and ECD), and a simple procedure for preparing high-resolution glass capillary columns tailored for PG analysis, were necessary.

Quantitative analysis and biological applications will be discussed elsewhere.

EXPERIMENTAL

Standards

 $PGF_{2\alpha}$, PGE_2 , PGD_2 , 6-keto- $PGF_{1\alpha}$, TXB_2 and 2a,2b-dihomo- $PGF_{2\alpha}$ were a generous gift from Dr. John Pike of the Upjohn Company, Kalamazoo, MI, U.S.A.

Derivatization

The pentafluorobenzyl ester trimethylsilyl ether (PFB-TMS) derivatives of $PGF_{2\alpha}$ and 2a,2b-dihomo- $PGF_{2\alpha}$ and the pentafluorobenzyl ester methyloxime trimethylsilyl ether (PFB-MO-TMS) derivatives of PGE_2 , PGD_2 , 6-keto- $PGF_{1\alpha}$ and TXB_2 were prepared as previously described⁵.

Mass spectrometry

An LKB 2091-051 gas chromatograph-mass spectrometer equipped with an

LKB 2130 computer system for data acquisition and calculation was used in the electron impact mode. The gas chromatograph was a DANI 3800.

The instrument was used in the selected ion monitoring (SIM) mode and was tuned on the following ions: m/z 301 for TXB₂, 461 for PGE₂, 544 for PGD₂ and 6-keto-PGF_{1 α}, 589 for PGF_{2 α} and 527 for 2a,2b-dihomo-PGF_{2 α} which was used as internal standard for quantitative work. The instrumental conditions were as follows: ion source temperature, 250°C; electron energy, 22.5 eV; trap current, 100 μ A; accelerating voltage, 3.5 kV; source slit width, 0.1 mm; collector slit width, 0.3 mm; resolution, 650. The mass spectra (recorded at 2.33 kV, 22.5 eV and resolution 900) are shown in Figs. 1–4, the salient fragments are assigned in Table I and the structures of the derivatives are shown in Fig. 5.

Electron-capture detection

A low dead-volume ⁶³Ni detector (DANI ECD 36/3), especially designed for connection to capillary columns, was used on a DANI 3900 gas chromatograph.

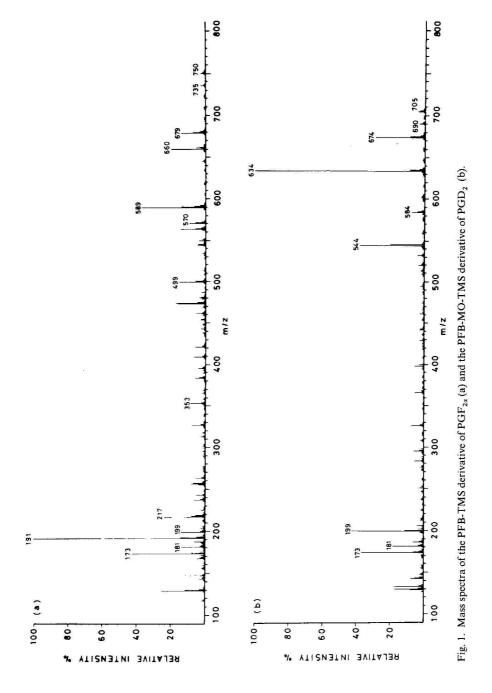
High-resolution gas chromatography

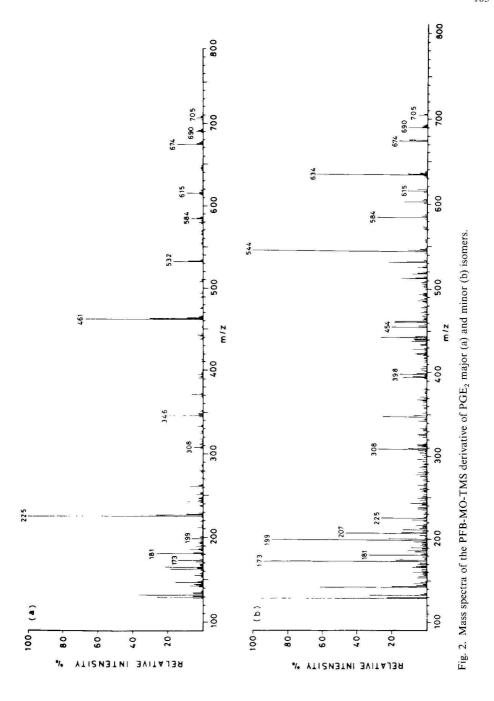
Support-coated open tubular (SCOT) capillary columns were prepared by a procedure developed in our laboratory as a modification of the method described by German and Horning⁶. Glass capillary columns (0.9 mm O.D., 0.3 mm I.D.) were drawn using a Shimadzu GDM 1 drawing machine. A 150-cm Pyrex glass tube (8 mm I.D., 3 mm I.D.) yields a coil about 90 m long, with a diameter of 12 cm. In this study 30-m columns were employed. The glass column was rinsed with a small amount of acetone. A chloroform plug was then introduced under air pressure (1 atm) in order to wet the column wall; this was followed by a plug (30% of the column internal volume) of the support suspension consisting of 2% LiChrosorb, 0.25% OV-101 and 0.25% OV-17 in chloroform-carbon tetrachloride-methanol (50:49:1 v/v). This suspension was forced through the column at a rate of about 1.5 cm/sec. The column was dried under an air stream for 3 h at room temperature, then coated with additional liquid phase by the same technique used for the support coating. A plug (30% of column internal volume) of a chloroform solution containing 1% of OV-101-OV-17 (8:2 v/v) was moved through the column at a rate of about 3 cm/sec. The column was then dried under an air flow for 12 h at room temperature. In the conditioning step the column was heated by increasing the temperature from 50°C to 260°C at a rate of 0.5°C/min under a helium flow (1 atm head pressure).

RESULTS AND DISCUSSION

Derivatization

Of the many different derivatives described in the literature for GC analysis of prostaglandins, the PFB-MO-TMS derivatives were chosen since they yielded satisfactory results in terms of GC properties, ECD and MS response and stability. These derivatives show single, well shaped peaks for each compound, except for PGE₂-PFB-MO-TMS, whose *syn-anti* isomers can be seen as well separated peaks. The PFB-MO-TMS derivatives, which are reportedly excellent for ECD analysis⁴, also gave satisfactory results when analysed by MS. The mass spectra of the derivatives, shown in Figs. 1–4, give significant intense ions in the high mass range, so that





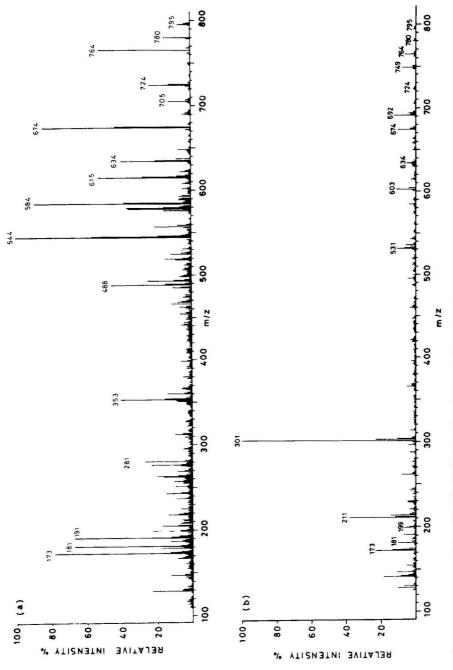
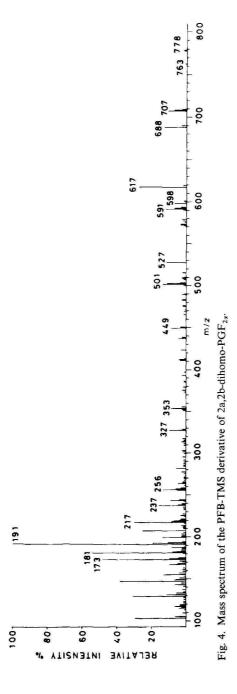


Fig. 3. Mass spectra of the PFB-MO-TMS derivative of 6-keto-PGF₁₄ (a) and TXB₂ (b).



PARTIAL MASS SPECTRAL DATA OF PROSTAGLANDINS AND THROMBOXANE B₂ AS PFB-TMS AND PFB-MO-TMS DERIVATIVES TABLE I

Fragment	$PGF_{2\alpha}$	ы	PGE_2 isomer	PGE_2 minor isomer	PGE_2 isomer	PGE_2 major isomer	PGD_2		TXB_2		6-keto	6-keto-PGF _{1α}	2a,2b-di	2a,2b-dihomo-PGF _{2a}
	z/m	%	z/w	%	z/w	%	z/w	%	z/w	%	z/w	%	z/w	%
[M] ⁺	750	7	705	4	705	3	705	4	795	<u>^</u>	795	5	778	7
$(M - 15)^{+*}$	735	7	069	11	069	4	069	3	780	_	780	10	763	-
$[M - 31]^{+**}$			674	15	674	15	674	29	764	9	764	44		
$[M-71]^{+***}$	629	14	634	63	634	7	634	100	724	_	724	20	707	Ξ
$[M - 90]^{+}$ §	099	16	615	=	615	6	615	<u>_</u>	705	$\overline{\lor}$	705	∞	889	14
$[M - (31 \div 90)]^{+}$			584	29	584	9	584	7	674	10	674	52		
$[M - (71 \div 90)]^{+}$	589	33	544	100			544	38	634	7	634	24	617	28
$[M - (2 \times 90)]^{+}$	570	10	525	4					615	c	615	22	865	œ
$[M - (71 + 173)]^{+ \$\$}$					461	<i>L</i> 9								
$[M - (71 + 2 \times 90)]^{+}$	499	12	454	20			454	-	544	7	544	100	527	12
$[M - (173 - 307)]^{+ \S \$ \$}$	an-				225	100								
									301	100				

^{*} Loss of CH₃ group. ** Loss of CH₃O. *** Loss of C₅H₁₁. § Loss of TMS-OH.

^{§§} Mass of 173 is equivalent to CH₃ON = C-CH₂-CH-OTMS¬+·· §§§ Mass of 307 is equivalent to top chain. † Equivalent to TMSO † = CH-CH = CH-CHOTMS-C₅H₁₁.

Fig. 5. Structures of PFB-MO-TMS and PFB-TMS derivatives of prostaglandins and TXB2.

specific and sensitive responses are obtained when the SIM technique is used.

A typical SIM analysis of authentic PGs and TXB is shown in Fig. 6.

Finally, as previously pointed out for 6-keto-PGF_{1 α}⁵, these derivatives are stable for months when kept in bis(trimethylsilyl)trifluoroacetamide (BSTFA) solution.

Capillary column properties

Columns were characterized with respect to the isothermal separation of tetracosane at 180°C, revealing a typical theoretical plate efficiency of about 1900 plates per metre. A TZ parameter of 7.3 was obtained by injecting tetradecane and pentadecane. No significant drop in column efficiency was noted after months of daily use.

These columns proved particularly suitable for prostaglandin analysis, since complete separation of all the major metabolites of arachidonic acid via the cyclo-oxygenase pathway can be obtained in a relatively short time (Fig. 7). The composition of the stationary phase mixture used for column coating seems to be critical for the separation of all the compounds, and columns prepared with less polar phase mixtures did not completely separate $PGF_{2\alpha}$ -PFB-TMS from the PGE_2 -PFB-MO-TMS minor isomer.

Interesting features of the method described are the constant and reproducible chromatographic properties of columns and the short time required for column preparation in that no time-consuming deactivation steps are required.

Electron-capture detection

As we previously demonstrated for 6-keto-PGF_{1x}, HRGC-ECD can be suc-

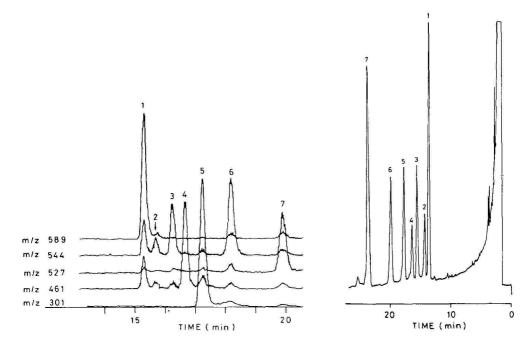


Fig. 6. Selected ion monitoring of (1) PGF_{2a}, (2) PGE₂ minor isomer, (3) PGD₂, (4) PGE₂ major isomer, (5) TXB₂, (6) 6-keto-PGF_{1a}, (7) 2a,2b-dihomo-PGF_{1a}. Column: 30 m OV-101–OV-17 (8:2), 220°C isothermal. Carrier gas: helium, 25 cm/sec.

Fig. 7. HRGC-ECD separation. Compounds and conditions as in Fig. 6.

cessfully used for PG analysis. A typical ECD chromatogram of authentic PGs is shown in Fig. 7 HRGC-ECD is a simple, sensitive and fairly specific alternative method for PG determination in selected experimental models previously characterized by mass spectrometry. If numerous samples have to be analyzed, the combined use of HRGC-MS and HRGC-ECD may be convenient using the first technique mainly for identification work and the second for routine quantitation.

Studies in progress on the applicability of this technique to complex biological matrices show that a critical aspect of HRGC-ECD is that it requires greater purification of biological samples than HRGC-MS; this question will be discussed in detail in a subsequent paper.

ACKNOWLEDGEMENTS

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CHROM. 15,167

Note

Rapid high-performance liquid chromatographic method for determining trace levels of fluometuron in soil

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Crop production systems often involve rotations of species from year to year, replanting to a different crop, or double-cropping within the same year. Fluometuron [1,1-dimethyl-3- $(\alpha,\alpha,\alpha$ -trifluoro-m-tolyl)urea], commonly used as a pre-emergence herbicide for cotton, can be injurious to soybeans used as a replacement crop when cotton stands fail. A rapid method for analyzing residual concentrations of fluometuron and a knowledge of the threshold level for crop damage would give the producer an estimate of the influence which this herbicide could have in cotton fields replanted to soybeans or other sensitive crops.

There are several analytical methods for fluometuron from various sources^{1–8}. Guth and Voss¹ reported a colorimetric procedure for fluometuron from soil; however, it is for the unchanged urea and the corresponding hydrolysis product. Analysis of the unchanged urea requires thin-layer chromatography to separate it from metabolites. A reversed-phase high-performance liquid chromatographic (HPLC) method is available for the separation of carbamates and ureas from each other and from some of their metabolites².

Many of the procedures available for analyzing fluometuron cannot be applied easily to a large number of samples per day because they involve extensive clean-up, large pieces of glassware, or Soxhlet extraction. The objective of this study was to develop a method for the rapid analysis of fluometuron residues in a large number of soil samples.

EXPERIMENTAL

Chemicals

Fluometuron (99.2%) was obtained from Ciba-Geigy (Greensboro, NC, U.S.A.). Working solutions of 0.3, 3.0, and 15 ppm were prepared by making appropriate dilutions of a 100-ppm stock solution in ethanol with deionized water. Propachlor (2-chloro-N-isopropylacetanilide) was obtained from Monsanto (St. Louis, MO, U.S.A.) and was used as a 50-ppm solution in 5% methanol in deionized water. The 20% saturated ammonium chloride was prepared by diluting saturated ammonium chloride (1:5). Diethyl ether was reagent grade and methanol and acetonitrile were HPLC grade.

Apparatus

The extraction bottles were 175 ml square, linear polyethylene Nalgene® with polypropylene caps. The samples were filtered through 0.22- μ m aqueous 13-mm diameter Millipore filters in a Swinney adapter fitted to a 10-ml syringe. The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system, Model 710A WISP, Data Module, Model 440 UV detector fixed at 254 nm, and a Radial Compression Module with a 5-mm I.D. Radial-Pak 10- μ m C₈ cartridge. A wrist-action shaker operating at 3 to 4 shakes per sec and a N-Evap (Organomation Assoc., Northborough, MA, U.S.A.) were also used.

Sample preparation

The samples were fortified with fluometuron in a manner that put them through a wet–dry cycle to simulate the "aging process" which occurs under field conditions. Dry soil (30 g) was placed in a polyethylene bag, and the desired volume of fluometuron solution was applied in drops evenly over the soil. Additional water was added in the same manner to make a total of 4 ml of water added to the soil. The bags were closed, shaken by hand for *ca.* 10 sec at a rate of 2 to 3 shakes per sec, and then opened and allowed to dry. Each sample was extracted and analyzed within 4 days of fortification.

Extraction

The soil was placed in a 175-ml plastic bottle. Then 25 ml of 20% saturated aqueous ammonium chloride and 50 ml of ether were added in sequence. The cap was screwed on securely, and the bottle was shaken for 30 min on a wrist-action shaker. The bottle was removed, and the soil was allowed to settle into the aqueous layer. The ether layer was transferred to a 20×2.5 cm I.D. test tube by means of a disposable Pasteur pipet. (When 30 samples were to be run, the second fifteen were shaking while the ether layers were being removed from the first fifteen). The volume of ether was reduced to ca. 10 ml on a 35°C N-Evap under a stream of dry nitrogen. The sample was extracted with two additional 50-ml portions of ether in the same manner with the ether portions being combined and reduced in volume. After the third extraction the ether was completely removed by evaporation. An internal standard of 2 ml of 50ppm propachlor in 5% methanol in water was added, followed by 1 ml of methanol. The sides of the test tube were washed down with the solution by means of a Pasteur pipet, and the tube was placed in a warm water bath for 5 min with occasional swirling. The sample was filtered into a sample vial through a 0.22-μm Millipore filter in a Swinney adapter and analyzed by HPLC.

Chromatography

The injection volume was 40 μ l. The sample was eluted with a mixture of acetonitrile—water (30:70) at a flow-rate of 2 ml/min. The retention times were 9.3 min for fluometuron and 12.0 min for propachlor.

RESULTS AND DISCUSSION

The results are given in Table I. Typical chromatograms of blanks and soil samples fortified at 0.02 and 0.1-ppm fluometuron in soil are shown in Fig. 1. The

TABLE I
RECOVERY OF FLUOMETURON FROM LORING AND CROWLEY SILT LOAMS

Soil	ppm in soil	Recovery \pm S.D. $(\%)^*$
Crowley	0.00	nd**
	0.02	88 <u>+</u> 4
	0.10	91 ± 3
	0.50	91 ± 1
	1.00	91 ± 7
Loring	0.00	nd
_	0.02	114 ± 13
	0.10	91 ± 2
	0.50	91 ± 5
	1.00	94 + 2

^{*} Seven replicate samples were analyzed at each concentration for each soil type.

limit of detection was 0.02 ppm for both soil types. The 91% recovery was the same for both soils at concentrations of 0.1–1.0 ppm in soil. The only difference in the results for the two soil types was at the lowest concentration of fluometuron (0.02 ppm in soil). The higher percentage recovery and larger standard deviation for the Loring soil at this level indicated higher and more varied background interferences. However, there was a difference in the results for the blank soil and the soil that was fortified at the 0.02-ppm level.

The method has limits of detection comparable to or better than most fluometuron techniques in the literature; it is simple and adaptable to running a large number of samples per day. One person can quickly learn to perform analyses of fifteen samples per day and after becoming more familiar with the procedure can analyze as many as 30 samples per day. This ability to analyze soil samples rapidly is important to farmers using rotational or double-cropping systems or those who wish to replant during the same season.

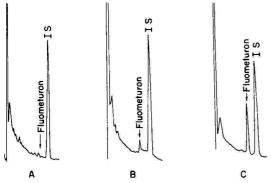


Fig. 1. Chromatograms of soil samples fortified with fluometuron. A, blank; B, 0.02 ppm in soil; C, 0.1 ppm in soil. The retention times for fluometuron and the internal standard (IS) are 9.3 and 12.0 min, respectively.

^{**} Not detected.

ACKNOWLEDGEMENTS

We thank Dave Graves and Joe Scott for their help in the laboratory during the development of the method and Martha Davis for her help in preparing the manuscript.

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CHROM. 15,206

Note

Separation of mycosporine-like amino acids in marine organisms using reversed-phase high-performance liquid chromatography

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Mycosporine-like amino acids are water-soluble nitrogenous substances with strong absorption maxima in the range 310–360 nm¹. As shown in Fig. 1, nine mycosporine-like amino acids, mycosporine-Gly (3) $(\lambda_{max}310 \text{ nm})^2$, palythine (4) $(\lambda_{max}320 \text{ nm})^{3-5}$, shinorine (5) $(\lambda_{max}334 \text{ nm})^{6,7}$, porphyra-334 (6) $(\lambda_{max}334 \text{ nm})^{7,8}$, asterina-330 (7) $(\lambda_{max}330 \text{ nm})^9$, palythinol (8) $(\lambda_{max}332 \text{ nm})^{10}$, palythenic acid (9 and 10) $(\lambda_{max}337 \text{ nm})^{11}$ and palythene (11) $(\lambda_{max}360 \text{ nm})^{10,12}$, and two related compounds (1¹³ and 2¹⁴) have been isolated from several marine animals (starfish, zoanthid, mussel and cod eggs) and plants (red algae). However, their rôles and biogenesis *in vivo* remain unknown. From their structural similarity and the variety of origins, the series of compounds are supposed to be related to one another, probably originating from shikimic acid, and to be distributed widely among numerous marine organisms. Preliminary results of our survey on the distribution of mycosporine-like amino acids in

Fig. 1. Structures of mycosporine-like amino acids 3-11 and the related substances 1 and 2 isolated from marine organisms.

marine organisms have shown that they are almost ubiquitous among algae and invertebrates, suggesting that they have important rôles in biological systems. Our interest in mycosporine-like amino acids is focused on their compositions in marine organisms and the structures of their biogenetically related metabolites. In the present work, we describe a method for separation of mycosporine-like amino acids 3–10 and for rapid determination of their compositions in marine organisms by using reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals

MCI gel CHP-20 (porous styrene polymer, 75–150 μ m; Mitsubishi), charcoal (activated, chromatographic grade; Wako), acetic acid and ethanol (Wako) and glass-distilled water were used.

Samples

Mycosporine-like amino acids 3-6, 9 and 10 were isolated from the ascidian *Halocynthia roretzi*, and 7 was isolated from the starfish *Asterina pectinifera* as previously reported^{9,11}. Substance 8 was kindly donated by Dr. Takano¹⁰.

The seaweeds Geldium amansii and Codium fragile were collected at Misaki in Kanagawa Prefecture in April, the seaweed Padina crassa and the sea sponge Haly-chondira japonica at Okinawa Islands in May, the zoanthid Palythoa tuberculosa at Ishigaki Island in May, and the mussel Mythilus edulis, the starfish Asterina pectinifera and the ascidian Halocynthia roretzi at Asamushi in Aomori Prefecture in November. The antarctic krill Euphasia sperba was obtained as frozen material. Marine organisms were stored at $-20^{\circ}\mathrm{C}$ until they were used.

Chromatographic apparatus and conditions

The HPLC apparatus consisted of an ALTEX pump Model 100A, a Rheodyne injector Model 7125 equipped with a 20- μ l loop, a JASCO spectrophotometer Model UVIDEC-100III and a System Instruments Intelligent Integrator Model 7000A. The prepacked columns used were MCI Hypersil ODS HY-5U (5 μ m, Mitsubishi), ALTEX Ultrasphere ODS (5 μ m) and Develosil ODS-3 (3 μ m, Nomura), 25 cm \times 4.6 mm I.D., and were eluted isocratically with dilute acetic acid. The mobile phase was filtered and degassed by using a Nucleopore polycarbonate membrane with a pore diameter of 0.2 μ m. Separations were carried out at room temperature (ca. 20°C) or at a temperature controlled by a constant-temperature water-bath Thermo Elites Model BH-41 equipped with Neocool Dip Model BD-11 (Yamato). Absorbance was detected at 330 nm.

Preparation of samples for HPLC analysis

Samples for HPLC analysis were prepared as follows. The marine organism (ca. 20 g) was homogenized and extracted three times with 70 % ethanol (40 ml). The extract was evaporated to dryness in vacuo. The residue was dissolved in 20 ml of water and the UV spectrum of the solution was measured after dilution in water to a convenient concentration. An aliquot (0.5–10 ml) of the solution, whose optical density was ca. 1 at around 330 nm when it was diluted in water to 40 ml, was applied on

TABLE I
RETENTION TIMES OF MYCOSPORINE-LIKE AMINO ACIDS ON THREE ODS COLUMNS AT ROOM TEMPERATURE

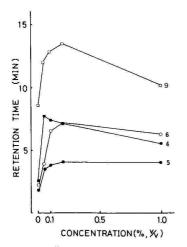
D1 0 1	01			5,000		0	1.0 ml/min.	
Ellient, II I	-/	acenc	acia	ın	Waler	HOW-rate	1 D mi/min	

Column	Retention	time (min)		
	5	6	4	9
Hypersil	3.81	6.51	7.44	13.00
Ultrasphere	3.85	8.62	8.84	20.19
Develosil	4.39	9.04	2.39	10.26

a column of CHP-20 (5 cm \times 7 mm I.D.), which was then eluted with water (15 ml). The eluate was applied on a column of charcoal (3.5 \times 1.5 cm I.D.), which was eluted with water (20 ml) and then with 50% ethanol, monitoring the UV absorption. The 50% ethanol fractions showing UV absorption in the range 310–340 nm were collected (50–100 ml) and evaporated to dryness *in vacuo*. The residue was dissolved in 500 μ l of water and aliquots of the solution were used for HPLC analysis.

RESULTS AND DISCUSSION

Mycosporine-like amino acids 3–10 were eluted from a charcoal column by addition of 50% ethanol to water, whereas substances 1, 2 and 11 could not be recovered from the column even by use of higher contents of ethanol. On the other hand, only substance 11 was absorbed on a column of CHP-20 and was eluted from the column by addition of 10% ethanol to water. For the HPLC analysis of mycosporine-like amino acids, crude extracts of marine organisms were purified by a charcoal



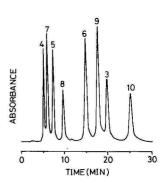
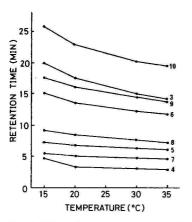


Fig. 2. Effect of the concentration of acetic acid in the mobile phase on retention times of mycosporine-like amino acids 4-6 and 9. Column: Hypersil ODS (25 cm × 4.6 mm I.D.). Flow-rate: 1 ml/min. Room temperature.

Fig. 3. Separation of mycosporine-like amino acids 3–10 on Develosil ODS column (25 cm × 4.6 mm I.D.). Mobile phase: 0.02 % acetic acid in water; flow-rate, 1.0 ml/min. Temperature: 15°C.



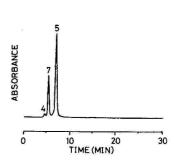


Fig. 4. Effect of temperature on retention times of mycosporine-like amino acids 3–10. Other conditions as in Fig. 3.

Fig. 5. Chromatogram of mycosporine-like amino acids of the red alga *Geldium amansii*. Peak numbers and chromatographic conditions as in Fig. 3.

column following a CHP-20 column. A mixture of components purified from each extract was dissolved in water and aliquots $(1-20 \mu l)$ of the solution were injected for HPLC analysis.

The separations of mycosporine-like amino acids were carried out on reversed-phase columns using isocratic elution with dilute acetic acid. The retention times of amino acids 4–6 and 9 were obtained on three different columns under identical solvent and flow conditions at room temperature (Table I). The retention time of 4 on Develosil ODS was much shorter than those on the other two columns. The difference may result from different amounts of free silanol groups. However, the substances 5, 6 and 9 were eluted in the same order from the three columns, according to their hydrophobic properties.

On Hypersil ODS, maximum retentions of 5, 6 and 9 were obtained at 0.2% acetic acid, whereas that of 4 was achieved at 0.05% acetic acid (Fig. 2). The difference is due to the presence of two carboxyl groups in substances 5, 6 and 9 but only one in 4.

Complete separation of substances 3-10 was achieved with Develosil ODS

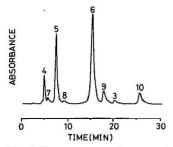


Fig. 6 Chromatogram of mycosporine-like amino acids of the antarctic krill Euphasia sperha. Peak numbers and chromatographic conditions as in Fig. 3.

TABLE II
COMPOSITIONS OF MYCOSPORINE-LIKE AMINO ACIDS IN MARINE ORGANISMS

	Relative	e molar rat	io	- Marie Programme - Shaff Commander of the Commander of t				
	3	4	7	8	5	6	9	10
Alga					300	180 10035		
Rhodophyceae								
Geldium amansii	-	1.0	9.5	-	23	-	_	-
Chlorophyceae								
Codium fragile	_	1.0	0.1	-	0.1	1.3	_	-
Phaeophyceae								
Padina crassa	_	1.0	0.9	1.9	15	9.4		_
Invertebrate								
Porifera								
Halychondria japonica	_	1.0	0.1	< 0.1	0.2	0.1	< 0.1	·
Coelenterate								
Palythoa tuberculosa	0.07	1.0	_	0.03	_		_	
Arthropoda								
Euphasia speba	-	1.0	0.1	< 0.1	1.9	3.8	0.4	0.6
Mollusca								
Mythilus edulis	0.2	1.0	0.1		2.4	0.9	0.2	1 2000 1
Echinodermata								
Asterina pectinifera	0.2	1.0	0.2	< 0.1	< 0.1	())	_	-
Protochordata								
Halocynthia roretzi	6.8	1.0	< 0.1	< 0.1	0.6	0.9	0.3	< 0.1

using 0.02% acetic acid as mobile phase at 15°C (Fig. 3). As shown in Fig. 4, their retentions depended on temperature to different extents, and baseline separation of each peak was obtained at 15°C. Under these conditions, substances 3–10 were separately eluted in order of their hydrophobic properties, from 4 of the lowest hydrophobicity to 10 of the highest, within 30 min.

HPLC analyses of mycosporine-like amino acids in about 40 marine organisms were carried out under the optimum conditions described above and good separations were obtained in all cases. In Figs. 5 and 6 are displayed chromatographic profiles of the red alga *Geldium amansii* and the antarctic krill *Euphasia sperba*, respectively. Each peak was identified by comparing the retention time with that of an authentic sample and the amount of each substance was determined on the basis of the peak area obtained by an integrator. Some of the results are summarized in Table II. The data show that substance 4 is contained in all the organisms examined and occurs as the main component in several organisms.

The wide distribution of these mycosporine-like amino acids could be explained in terms of the food chain. The differences in their compositions may result from differences in the biological systems (cf., metabolism) of the marine organisms.

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CHROM. 15,194

Note

Separation of amino acids by charge-transfer interaction chromatography in aqueous systems

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Most biosubstances have electron-donating groups such as an amino group or a heterocyclic group containing a nitrogen atom¹. Thus, it is expected that the use of a polymeric adsorbent containing an electron acceptor which specifically interacts with the electron donating groups will enable the separation of the biosubstances. Such methods using the so-called charge-transfer interaction between electron donor and acceptor have mainly been investigated in organic media²⁻⁴. Porath and co-workers⁵⁻⁹ studied charge-transfer interaction chromatography in aqueous media. However, the contribution of the charge-transfer interaction between substrates and adsorbents has not been clearly established.

In this article, chromatography of amino acids in aqueous media using a polymeric adsorbent containing the dinitrophenyl group, which is a strong electron acceptor, has been studied, and the interactions between the adsorbent and amino acids are discussed with attention centered on the charge-transfer interaction.

EXPERIMENTAL

Syntheses of polymeric adsorbents

The polymeric adsorbents were synthesized according to the following scheme:

The degrees of substitution of Sephadex (Pharmacia; cross-linked dextran) by dinitrophenyl (DNP) and phenyl (P) groups were 140 and 170 μ mol per gram of dry adsorbents respectively, as determined by elemental analysis. Unsubstituted Sephadex was used as a control adsorbent.

Chromatography of amino acids

The polymeric adsorbents were packed into a glass column (20×0.5 cm I.D.) by the slurry method, the temperature being kept constant by a thermostat. The column was equilibrated with an eluent, the total bed volume being 4.0 ml in each case. A 0.5-ml volume of sample solution was introduced into the column and eluted at a rate of 4.0 ml/h. Three amino acids, tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe), were used as substrates. The concentrations of amino acids in the eluate were determined spectrophotometrically at 280 nm (Trp), 276 nm (Tyr) and 259 nm (Phe).

RESULTS AND DISCUSSION

Fig. 1 shows the elution patterns of Trp on these polymeric adsorbents at 10° C. The elution volumes, $V_{\rm E}$, of Trp on DNP-Sephadex, P-Sephadex and Sephadex were 7.9, 7.5 and 6.5 ml, respectively. In general, an elution parameter, $K_{\rm d}$, is defined by following equations

$$V_{E} = V_{0} + K_{d}V_{I} V_{T} = V_{0} + V_{I} + V_{G}$$

where $V_0 = {\rm void}$ volume, $V_{\rm I} = {\rm internal}$ volume, $V_{\rm T} = {\rm total}$ bed volume and $V_{\rm G} = {\rm gel}$ volume^{10,11}. When the retention of a substrate is only caused by molecular sieving, $0 < K_{\rm d} \le 1$. From Fig. 1, however, the values of $K_{\rm d}$ were obviously larger than 1.0 for all the adsorbents used, because the value of $V_{\rm T}$ was 4.0 ml. Therefore, it is considered that the retention of Trp may be caused by other interactions in addition to molecular sieving, and the intensity of the interactions between the adsorbents and Trp decreases in the order DNP-Sephadex > P-Sephadex > Sephadex.

In order to examine the influence of temperature on the retention of Trp, the chromatography was carried out at different temperatures between 10°C and 70°C. Fig. 2 shows the relation between the value of $V_{\rm E}/V_{\rm T}$ and the elution temperature. The value of $V_{\rm E}/V_{\rm T}$ is used instead of $K_{\rm d}$ as a measure of the retentive power of adsorbents

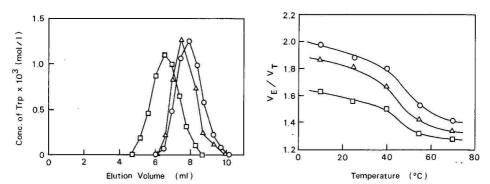


Fig. 1. Chromatography of tryptophan on DNP-Sephadex (\bigcirc), P-Sephadex (\triangle) and Sephadex (\square) at 10°C in 0.07 M phosphate buffer at pH 7.0.

Fig. 2. Temperature dependence of the values of $V_{\rm E}/V_{\rm T}$ for tryptophan on DNP-Sephadex (\bigcirc), P-Sephadex (\triangle) and Sephadex (\square) in 0.07 M phosphate buffer at pH 7.0.

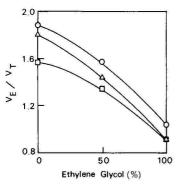


Fig. 3. Relation between the values of $V_{\rm E}/V_{\rm T}$ for tryptophan and ethylene glycol content of the eluent at 10°C on DNP-Sephadex (\bigcirc), P-Sephadex (\triangle) and Sephadex (\square).

since the relation between $V_{\rm E}/V_{\rm T}$ is $K_{\rm d}$ is linear, and the larger this value is the stronger is the retentive power⁹. It was found that the values of $V_{\rm E}/V_{\rm T}$ increased in the order Sephadex < P-Sephadex < DNP-Sephadex in the temperature range 10–70°C, and they gradually decreased with increasing temperature.

When column chromatography using hydrophilic gels is carried out, normal retention forces such as hydrogen bonding and van der Waals forces are weakened by a rise in temperature, owing to a decrease in enthalpy¹². In the present case, the value of $V_{\rm E}/V_{\rm T}$ decreased drastically at about 50°C. According to Némethy¹³, hydrophobic interactions become weaker at temperatures greater than 58°C because the ordered structure of water is broken down. Therefore, it is considered that the drastic change was caused by a decrease of the hydrophobic interaction between Trp and the adsorbents, *i.e.*, hydrophobic interactions are probably involved in the retention of Trp.

The decrease in the values of $V_{\rm E}/V_{\rm T}$ for Trp when an ethylene glycol (EG) was added to the eluent is indicated in Fig. 3. Since an EG disrupts the ordered structure of water, this again suggests that the decrease in $V_{\rm E}/V_{\rm T}$ is due to a decrease in the hydrophobic interaction between Trp and the adsorbents. The difference between the values of $V_{\rm E}/V_{\rm T}$ on DNP-Sephadex and on P-Sephadex was nearly the same with or without EG, and in the case of 100% EG —where there is no hydrophobic interaction—the value of $V_{\rm E}/V_{\rm T}$ on P-Sephadex is the same as that on Sephadex; the value of $V_{\rm E}/V_{\rm T}$ on DNP-Sephadex is large. From these results, the retentive power of P-Sephadex, which is larger than that of Sephadex in aqueous media, seems mainly due to the hydrophobic interaction between the phenyl groups and Trp. Moreover, it is suggested that the retentive power of DNP-Sephadex for Trp is enhanced compared with that of P-Sephadex by the introduction of the nitro groups.

In order to investigate the contribution of electrostatic interactions, the chromatography on DNP-Sephadex was carried out at 10° C and various pH values. The results are shown in Fig. 4. The values of $V_{\rm E}/V_{\rm T}$ were constant in the range pH 2.5–7.5, but decreased at pH > 7.5 or < 2.5 where Trp was negatively or positively charged, respectively. Thus, if the elution is carried out at a pH near the isoelectric point of Trp (5.89), the influence of electrostatic interactions is negligible.

Fig. 5 shows the elution pattern of an artificial mixture of Trp, Tyr and Phe on DNP-Sephadex. The elution volume increased in the order Phe < Tyr < Trp. The

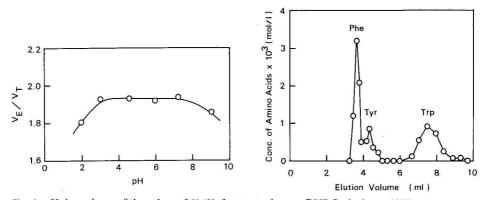


Fig. 4. pH dependence of the values of $V_{\rm E}/V_{\rm T}$ for tryptophan on DNP-Sephadex at 10°C. Fig. 5. Chromatography of an artificial mixture of tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) on DNP-Sephadex at 10°C in 0.07 M phosphate buffer at pH 7.0.

electrostatic interaction between DNP-Sephadex and the amino acids was negligible, because these amino acids are not charged (pH 7.0).

The values of hydrophobicity¹⁴, energy of the highest occupied molecular orbital (HOMO), which is a measure of the electron-donating ability¹⁵, and $V_{\rm E}/V_{\rm T}$ obtained from Fig. 5 are listed in Table I. The order of the hydrophobicity is Tyr < Phe < Trp, so that the order of the values of $V_{\rm E}/V_{\rm T}$, Phe < Tyr < Trp cannot be explained only by the hydrophobic interaction. Therefore, the introduction of nitro groups into the adsorbent considerably affects the separation of amino acids. On the other hand, the order of the electron-donating ability of these amino acids —the lower the energy of the HOMO is the stronger is the donating ability—agreed with the order of $V_{\rm E}/V_{\rm T}$. Moreover, the dinitrophenyl group introduced into DNP-Sephadex is a very strong electron acceptor. From these results, it is suggested that the effect of the nitro groups observed in the case of DNP-Sephadex is based on the charge-transfer interaction.

TABLE I
PHYSICAL PROPERTIES OF AROMATIC AMINO ACIDS

	Phenylalanine	Tyrosine	Tryptophan
Hydrophobicity ¹⁴ (cal/mol)	2500	2300	3400
Energy of HOMO ¹⁵	0.908	0.792	0.534
$V_{\rm E}/V_{\rm T}^{\star}$	0.91	1.07	1.91

^{*} Calculated from the result in Fig. 5.

In conclusion, the charge-transfer interaction and the hydrophobic interaction play an important rôle in the chromatography of amino acids on polymeric adsorbents containing the dinitrophenyl group as a ligand.

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We thank Dr. Naoki Negishi for valuable discussions.

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Note

Detection of N-acetyl amino acids on paper and sugars on thin-layer chromatograms by a thermal-ultraviolet method

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The detection of N-acetyl amino acids is not easily accomplished by the nin-hydrin procedure¹ due to the presence of a blocked amino group. Also, other techniques specific for certain acetyl amino acids such as bromophenol in ethanol and potassium permanganate proved to be unsatisfactory². However, more elaborate procedures such as synthesis "in vitro" of radiolabelled N-acetyl amino acids³ have been used as standards in paper chromatography⁴.

A thermal-UV procedure for the detection of a large variety of organic compounds after paper chromatography has recently been reported⁵. The application of this method to the detection of N-acetyl amino acids is described in this paper. We also report the application of the thermal–UV procedure after thin-layer chromatography (TLC) with high recovery of the sample.

MATERIALS AND METHODS

N-Acetyl amino acids were detected either after ascending paper chromatography or high-voltage electrophoresis. The former method was performed with Whatman No. 1 paper using the following solvent systems: (A) pyridine-1-butanol-acetic acid-water (15:10:3:12)⁶; (B) 1-propanol-12% ammonium hydroxide (3:1); (C) 1-propanol-methyl ethyl ketone-25% formic acid (15:3:2)⁷.

High-voltage electrophoresis was carried out in pyridine-acetic acid-water (1:10:189), pH 3.5, at 35 V/cm for 90 and 20 min for N-acetyl amino acids and phospho amino acids respectively.

TLC was carried out using the following solvent systems: (D) acetone-

benzene-35% ammonium hydroxide-water (200:50:1.35:1) and (E) 1-butanol-pyridine-water (6:4:3).

After each run the paper or the plate was dried and developed according to Alperin *et al.*⁵; in the latter case an oven was used instead of a domestic iron.

[U-14C]Glucose (250 Ci/mol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All N-acetyl amino acids, phospho amino acids and amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Silica gel glass plates were Kieselgel 60 from E. Merck (Darmstadt, G.F.R.) and silica gel coated sheet from Eastman-Kodak (Rochester, NY, U.S.A.). Glass microfibre filters were Whatman GF/C. Toluene–PPO (2,5'-diphenyloxazole) was used for radioactivity measurements in a Beckman Model 8100 liquid scintillation spectrometer. All other procedures were as previously described⁵.

RESULTS AND DISCUSSION

Detection of N-acetyl amino acids on paper chromatograms

About 1 μ mol of N-acetyl derivatives of lysine, valine, glutamic acid, alanine and methionine was spotted on paper and chromatographed using solvent system A during 30 h as described in Materials and methods. After the run, the paper was dried and developed by the thermal–UV method. All the N-acetyl amino acids gave a detectable fluorescent spot as shown in Fig. 1. A sensitivity test was performed using N-acetylserine. It was found that up to 0.25 μ mol/cm² could be detected after chro-

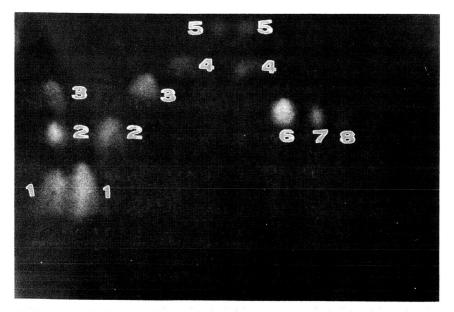


Fig. 1. Detection by the thermal–UV method of N-acetyl amino acids after paper chromatography using solvent system A. The spots correspond to about 1 μ mol/cm² of the following compounds: 1 = N-acetylvaline; 2 = N-acetylylysine; 3 = N-acetylglutamic acid; 4 = N-acetylglutanie; 5 = N-acetylmethionine. 6, 7 and 8 represent 1, 0.5 and 0.25 μ mol/cm² of N-acetylserine respectively. Spots 1 and 5 migrated 11 and 22 cm from the origin respectively.

TABLE I $R_{\rm F} \, {\rm AND} \, R_{\rm NAS} \, {\rm VALUES} \, {\rm OF} \, {\rm N-ACETYL} \, {\rm AMINO} \, {\rm ACIDS}, \\ {\rm PHOSPHO} \, {\rm AMINO} \, {\rm ACIDS} \, {\rm AND} \, {\rm AMINO} \, {\rm ACIDS} \, {\rm IN} \\ {\rm PAPER} \, {\rm CHROMATOGRAPHY} \, {\rm AND} \, {\rm ELECTROPHORESIS}$

Compound	R_F^{\star}	R_{NAS} **	Compound	R_F^*	$R_{NAS}^{\star\star}$
N-Acetylserine	0.63	1.0	Serine	0.39	-
N-Acetylalanine	0.92	_	Alanine	0.47	_
N-Acetylglutamic acid	0.76	_	Glutamic acid	0.37	0.39
N-Acetylaspartic acid	0.65	=	Aspartic acid	0.26	0.72
N-Acetyllysine	0.61	0.21	Lysine	0.33	-
N-Acetylmethionine	0.83	0.86	Methionine	0.68	1998
N-Acetylglycine	0.72	0.95	N-Acetylhistidine	_	0.03
N-Acetylleucine	-	0.61	N-Acetylphenylalanine	_	0.73
N-Acetylproline	0.82	1.13	N-Acetyltyrosine		0.69
N-Acetylvaline	0.47	0.73	Phosphotyrosine	_	1.37
O-Phosphothreonine	_	1.68	Threonine	0.48	1 1
O-Phosphoserine	0.18	1.80	Serine	0.39	-

^{*} Paper chromatography in solvent system A.

matography (Fig. 1). In addition, other N-substituted amino acids and phospho amino acids were detected by this method (Table I). Detection was also accomplished after high-voltage paper electrophoresis as described⁸.

As mentioned before, the presence of blocked amino groups does not allow the ninhydrin reaction. As far as we know, at present, no other satisfactory chemical methods are available to detect N-acetyl amino acids on paper chromatograms. The use of radiolabelled acetyl amino acids as standards in paper chromatography is an elaborate and expensive procedure⁴. The thermal–UV method overcomes these difficulties, being an extremely simple technique.

To evaluate the recovery of the compound after heating, the following experiment was performed. About 1 μ mol of N-acetylserine was spotted and chromatographed using solvent system B. After the run, the paper was dried and heated until the fluorescent spot had developed. Subsequently, the paper was run in the second dimension using solvent system C. In this case N-acetylserine and serine were used as standards. After chromatography the paper was developed again by heating and it was observed that the sample migrated as authentic N-acetylserine whereas no spot was visualized migrating at the position of serine. In addition the fluorescent spot detected in the first dimension remained at its original position (not shown).

Detection of sugars on thin-layer chromatograms

The present method was also applied for the detection of compounds after TLC. About 20,000 cpm of [14 C]glucose were spotted together with 1 μ mol of the same unlabelled compound on a silica gel coated sheet (Eastman-Kodak). Chromatography was carried out using solvent system E. After the run, the chromatogram was heated for 3 min at 135°C in an oven until a yellow fluorescent spot was observed under UV light. Subsequently, the layer was run in the second dimension-using the

^{**} Paper electrophoresis as described in Materials and Methods. About 0.5 μ mol/cm² of each substance has been used.

same solvent system. After the run, the plate was dried and heated as above. A new yellow spot was observed which was cut out and processed for liquid scintillation counting. It is noteworthy that the R_F of the sample was the same in both runs and the recovery of the original radioactivity was about 87%.

This method has recently been applied for the detection of methyl glucosides after TLC on silica gel glass coated plates⁹ (Kieselgel 60, Merck). Two methyl glucosides (2, 3, 4, 6-tetramethylglucose and 2, 3, 4-trimethylglucose) were chromatographed using solvent system D. After the run, the plate was heated for 10 min at 140°C in an oven and two fluorescent yellow spots were observed. The eluted samples revealed similar chromatographic behaviour to the authentic standard compounds when run under the same conditions as before. Thus, in this case the advantages of TLC together with the thermal–UV detection method allow the possibility of further analysis of the recovered compounds.

The fluorescence phenomena

It has been observed that after heating the paper at constant temperature (150°C) for 1–3 min only UV fluorescent spots appeared. If the heating was continued for 3–6 min the fluorescence decreased and dark visible spots could be seen. After further heating the fluorescence disappeared and maximal contrast in the visible spots was achieved. At present, we are not able to determine whether cancellation or quenching occurred during heating. Similar results were obtained using higher temperatures. As mentioned before, fluorescent spots cannot be removed from the paper matrix using water, organic solvents or several chromatographic solvent systems⁵.

With several compounds initial heating to develop fluorescence revealed a light blue colour under UV light (366 nm). This phenomenon appeared to be independent of the nature of the compound tested. However, the appearance of fluorescence takes place near the decomposition temperature of any given substance. The same light blue fluorescence occurred when the paper matrix without sample was heated at approximately its decomposition temperature (260–270°C). In contrast, when the substance was spotted on silica gel (Kieselgel 60) or glass microfibre filters and heated, the colours of the fluorescende generated depended only on the nature of the substance used. These results may suggest an interaction between the substance and the paper matrix, possibly due to a differential absorption of heat. Thus, a higher temperature is produced on the area where the compound is present when the paper is heated, and results in the appearance of the characteristic light blue fluorescence of the paper.

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Note

Separation of iodinated compounds of L-tyrosyl-L-tyrosine from iodothyronines by reversed-phase high-performance liquid chromatography

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The dipeptide 3,5,3',5'-tetraiodo-L-tyrosyl-L-tyrosine (I₂Tyr-I₂Tyr) has been extracted from trypsic digests of bovine thyroglobulin and investigations conducted in our laboratory have shown that this sequence forms part of the primary structure of thyroglobulin¹. "In vitro" experiments demonstrated that I₂Tyr-I₂Tyr led to synthesis of the iodothyronines by a mechanism involving a cyclic agent without breaking the peptide bond^{2,3}. This suggested that tyrosyltyrosine sequences in thyroglobulin might be hormonosynthesis sites "in vivo". To check this hypothesis we studied the "in vitro" enzymatic iodination of synthetic peptides which include the tyrosyltyrosine sequence. The iodination led to a mixture of iodotyrosines, iodinated derivatives of tyrosyltyrosine and iodothyronines. Previously we described a procedure allowing the complete separation of these compounds by column chromatography on Bio-Gel P-2⁴. However, this technique is limited in application due to the 24 h needed for a single analysis.

Recent developments in chromatography have yielded highly efficient reversed-phase columns employing ion-pair partition. In 1978 Hearn $et~al.^5$ described a procedure for the analysis of thyroidal iodoamino acids by hydrophilic ion-pair reversed-phase high-performance liquid chromatography (RP-HPLC). This method permits the rapid separation of a mixture of iodinated compounds, by use of a chemically bonded C_{18} hydrophobic support as the stationary phase and water-organic solvent mixtures containing phosphoric acid or other ion-pairing reagents as the mobile phase. Burman $et~al.^6$ measured serum thyronines by column chromatography on μ Bondapak C_{18} with a linear gradient of 25 to 90% acetonitrile in 0.025 M sodium acetate buffer, pH 4.

Neither method allowed a convenient separation in our particular case, but using a similar approach, we have developed a method for the rapid chromatographic analysis of the iodinated compounds of L-tyrosyl-L-tyrosine by reversed-phase partition HPLC.

EXPERIMENTAL

Apparatus

An Altex (Chromatem, Touzart et Matignon, France) HPLC system equipped with two C380 solvent delivery pumps and a 420 Altex solvent programmer was coupled to a UV absorbance detector operated at a wavelength of 254 nm and/or to a Berthold LB 5026 radioactivity detector and to a double-channel chart recorder.

Reagents

All solvents were AnalaR grade. Methanol supplied by E. Merck (Darmstadt, G.F.R.) was further bidistilled. Potassium dihydrogen phosphate and orthophosphoric acid were supplied by Riedel de Haen (Hannover, G.F.R.). The iodoamino acids, monoiodotyrosine (ITyr), diiodotyrosine (I_2 Tyr), triiodothyronine (I_3) and thyroxine (I_4), were obtained from Sigma.

Iodinated derivatives of L-tyrosyl-L-tyrosine (Tyr-Tyr) were synthesized in the laboratory by coupling with dicyclohexylcarbodiimide (DCC) the N-carboxybenzoxy (Cbzo) derivatives of L-tyrosine (Tyr), 3-iodo-L-tyrosine (ITyr) and 3,5-diido-L-tyrosine (I_2 Tyr) with their methyl ester analogues. Eight compounds were obtained: ITyr-Tyr, Tyr-ITyr, Tyr-ITyr, Tyr-ITyr, ITyr-ITyr, ITyr-ITyr and I_2 Tyr-ITyr.

 $^{125}\text{IT}_3$ and $^{125}\text{IT}_4$ were obtained from NEN; their specific activity was 100-150 $\mu\text{Ci}/\mu\text{g}$. $^{125}\text{IT}\text{yr}$ and $^{125}\text{I}_2\text{Tyr}$ were synthesized and labelled with ^{125}I by the Chloramine T method⁹; their specific activity was $100~\mu\text{Ci}/\mu\text{g}$. Iodinated derivatives of L-tyrosyl-L-tyrosine were labelled by isotopic exchange with unlabelled compounds; their specific activity was $10-20~\mu\text{Ci}/\mu\text{g}$.

Procedure

A 30 \times 0.47 cm I.D. column was packed with 10- μ m LiChrosorb RP-18 (Merck). The mobile phase used successively consisted of four buffers:

buffer 1: 5% to 80% methanol gradient in 0.1 M KH₂PO₄ containing 0.1% H₃PO₄; buffer 2: 30% methanol in 0.02 M KH₂PO₄ + 0.1% H₃PO₄; buffer 3: 50% methanol in 0.02 M KH₂PO₄ + 0.1% H₃PO₄; buffer 4: 20% to 40% methanol gradient in 0.02 M KH₂PO₄ + 0.1% H₃PO₄ for 8 min, 40% to 50% for 8 min and 50% to 70% for 8 min.

A flow-rate of 2 ml/min was maintained at a pressure of 800–1000 p.s.i. All separations were performed at ambient temperatures. The sample injections were made with Hamilton syringes $(0.10 \,\mu\text{l})$ or $0.50 \,\mu\text{l})$ by a Rheodyne injector with a $100 - \mu\text{l}$ loop. Samples of the iodinated compounds were diluted in the first buffer system. The concentrations varied between 20 and 50 μ g per $10 \,\mu\text{l}$. The radioactivity of each compound was $0.05 \,\mu\text{Ci}$ (maximum sensitivity of the detector $0.005 \,\mu\text{Ci}$). Simultaneously, the variations of optical density at 254 nm and the radioactivity in the eluate were measured.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a mixture containing ITyr, I_2 Tyr, iodinated derivatives of Tyr-Tyr, I_3 and I_4 , labelled with I_2 I. In this preliminary experiment,

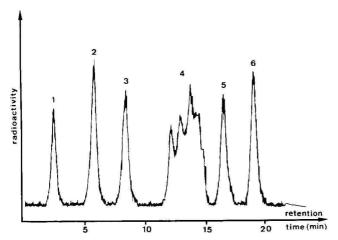


Fig. 1. Separation of a standard solution of iodide (1), ITyr (2), I_2 Tyr (3), iodinated derivatives of Tyr-Tyr (4), I_3 (5) and I_4 (6). Mobile phase: 5% to 80% methanol gradient in 0.1 M KH $_2$ PO $_4$ containing 0.1% H $_3$ PO $_4$ for 20 min. Flow-rate: 2.0 ml/min on a column (30 \times 0.47 cm I.D.) of LiChrosorb RP-18.

elution was performed as described by Hearn $et~al.^5$ using a 20-min linear gradient of 5% to 80% methanol in 0.1 M KH₂PO₄, containing 0.1% H₃PO₄ as ion-pairing reagent. A good separation of ITyr, I₂Tyr, T₃ and T₄ could be achieved but this elution system did not resolve adequately the mixture of iodinated derivatives of Tyr-Tyr. With a concentration in methanol higher than 60% in 0.1 M KH₂PO₄, we noted the appearance of crystals which disturbed the elution. The crystals did not appear when the concentration of KH₂PO₄ was below 0.02 M whatever the concentration of methanol. Under these conditions, ITyr is eluted at 25% methanol, I₂Tyr at 40%, iodinated derivatives of Tyr-Tyr between 45 and 55% and T₃ and T₄ at 60 and 70% respectively.

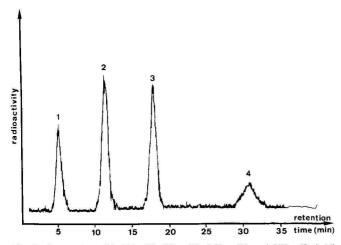
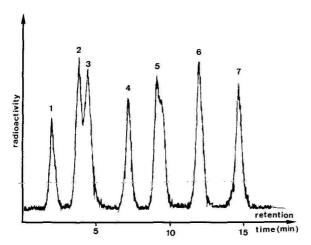


Fig. 2. Separation of iodide (1), ITyr (2), I_2 Tyr (3) and I(Tyr-Tyr) (4) by isocratic elution with 30% methanol in 0.02 M KH₂PO₄ containing 0.1% H₃PO₄. Other conditions as in Fig. 1.



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Fig. 3. Separation of iodide (1), ITyr (2), I_2 Tyr (3), I(Tyr-Tyr) (4), I_2 (Tyr-Tyr) (5), I_3 (Tyr-Tyr) (6) and I_2 Tyr- I_2 Tyr (7) by isocratic elution with 50% methanol in 0.02 M KH₂PO₄ containing 0.1% H₂PO₄. Other conditions as in Fig. 1.

The elution of iodinated compounds with three different concentrations of methanol was then examined. Fig. 2 shows an isocratic elution with buffer 2. ITyr is well separated from I_2 Tyr, but only monoiodinated derivatives are eluted and with poor peak shapes due to the long retention time of 30 min. This eluent does not allow the separation of the more highly iodinated derivatives of Tyr-Tyr.

Fig. 3 shows an isocratic elution with buffer 3. Under these conditions, ITyr and I_2 Tyr are poorly separated but a good separation of four peaks of the iodinated derivatives I(Tyr-Tyr), $I_2(Tyr-Tyr)$, $I_3(Tyr-Tyr)$ and I_2 Tyr- I_2 Tyr can be obtained. Iodothyronines are not eluted. From these chromatograms we can conclude that iodin-

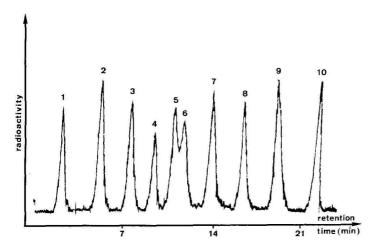


Fig. 4. Separation of iodide (1), ITyr (2), I_2 Tyr (3), I(Tyr-Tyr) (4), ITyr-ITyr (5), I_2 (Tyr-Tyr) (6), I_3 (Tyr-Tyr) (7), I_2 Tyr- I_2 Tyr (8), I_3 (9) and I_4 (10). Gradient elution in 0.02 I_4 KH $_2$ PO $_4$ containing 0.1 I_4 H $_3$ PO $_4$: 20–40 I_4 methanol for 8 min, 40–50 I_4 for 8 min and 50–70 I_4 for 8 min. Other conditions as in Fig. 1.

TABLE I
RETENTION TIMES OF IODOTYROSINES, IODOTYROSYLTYROSINES AND IODOTHYRONINES IN METHANOL MOBILE PHASES

Compound	Retention time (min)	% Methanol in 0.1 M $KH_2PO_4 + 0.1\% H_3PO_4$
Iodide	2.4	25
ITyr	6.3	36
I ₂ Tyr	9.7	42
I(Tyr-Tyr)	12,1	45
ITyr-ITyr	14.5	48
I ₂ (Tyr-Tyr)	15.2	49
I ₃ (Tyr-Tyr)	16.6	52
I ₂ Tyr-I ₂ Tyr	18.6	56
T_3	20.5	62
T_3 T_4	23.2	68

ated derivatives of Tyr-Tyr have a polarity in between that of iodotyrosines and iodothyronines. In order to obtain an optimal separation of these compounds, we chose a 20–40% methanol gradient in 0.02 M KH₂PO₄ + 0.1% H₃PO₄ for 8 min, then 40–50% methanol for 8 min and 50–70% methanol for 8 min. A good separation (Fig. 4) was obtained of: I⁻, ITyr, I₂Tyr, I(Tyr-Tyr), ITyr-ITyr, I₂Tyr-Tyr + Tyr-I₂Tyr, I₃(Tyr-Tyr), I₂Tyr-I₂Tyr, T₃ and T₄. The elution times and methanol concentrations are listed in Table I.

With this procedure, we are able to separate the iodinated derivatives of L-tyrosyl-L-tyrosine from iodotyrosines and iodothyronines in 24 min. This method should be suitable for quantifying the reaction products of "in vitro" iodination of synthetic peptides in order to elucidate the biosynthesis mechanism of iodothyronines applicable to thyroglobulin.

Application of this procedure associated with a derivatization method such as dansylation^{6,10} will be of considerable value for the identification and the dosage of putative derivatives of L-tyrosyl-L-tyrosine in human blood.

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Note

Ion-suppression reversed-phase liquid chromatographic determination of acetate in brine

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With the chemical industry ever increasingly going to closed-loop plants, recirculation of aqueous streams, including brine, is practiced. Build-up of organic impurities such as acetate in recycle streams, frequently must be closely monitored. There is a lack of methodology for the measurement of acetate, as acetic acid, in such a matrix. The determination of acetic acid in rainwater was accomplished by gas chromatography¹ and by isotachophoresis² in silage. Other methods employ steam distillation followed by titration³ and column chromatography with titration⁴. Ion chromatography⁵ can also be applied; however, the high salt concentration limits sensitivity. Richards⁶ chromatographed acetate and other weak organic acids using an eluent of dilute sulfuric acid and an ion-exchange column. However, the system required long analysis time and is complicated by the soft resin settling in the column. Therefore, a rapid and specific method requiring no sample treatment was needed.

EXPERIMENTAL

Acids were obtained from either Eastman Organic Chemicals or J. T. Baker, and used without further purification. Whenever ACS reagent requirements were applicable, compounds of that quality were employed.

Equipment

The liquid chromatograph consisted of an LDC UV III Monitor (1203) with a 214-nm source; a Waters Assoc. M-45 pump; a Rheodyne 7120 injection valve with 20- μ l loop; a Sargent-Welch SRG recorder; a Systems I (Spectra-Physics) computing integrator; and a Whatman Partisil 5 ODS-3 RAC 10 cm \times 9.4 mm I.D. column (minimum 90,000 plates per meter) protected with a 5 cm \times 2.1 mm I.D. precolumn containing Waters Assoc. pellicular μ Bondapak $C_{1.8}$ /Corasil.

Calibration solution

A 20% (w/w) aqueous solution of a sodium chloride brine was prepared. With a 100- μ l syringe, 100 μ l of glacial acetic acid (density 1.049) was added to 105 g of the brine solution. This gave a standard solution containing 1000 ppm acetic acid (983 ppm as acetate ion).

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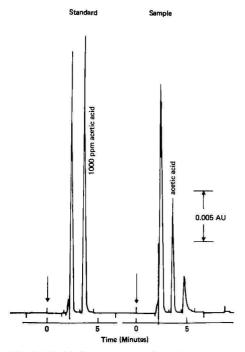


Fig. 1. Liquid chromatogram of sample and standard for the determination of acetate in brine according to the liquid chromatographic conditions described in the text.

Chromatographic conditions

The mobile phase was 0.01~N aqueous sulfuric acid prepared with water from a Milli-Q water purification system. The flow-rate was 2~ml/min (400 p.s.i.g.); injection volume, $20~\mu l$; detection wavelength, 214~nm; and attenuation, 0.064~a.u.f.s. for typical analyses, and 0.008~to obtain high sensitivity.

Procedure

Without any sample pretreatment, 20 μ l of the brine is injected and chromatographed as described above.

RESULTS AND DISCUSSION

Fig. 1 illustrates the measurement of acetate, as acetic acid, in a commercial 20% sodium chloride brine. The sodium chloride emerges at the column void volume.

This analysis has been carried out over an eight-month period using the same column. Minimal column degradation has been observed as indicated by less than a 10% decrease in peak height. Eluent was pumped continuously five days a week, 24 hours a day, on recycle. Fresh eluent was prepared monthly.

Because of the high polarity of the acetic acid molecule, it is important that the columns have an efficiency of > 90,000 plates/meter. Also, adsorption effects must be at a minimum; therefore, column packings having significant amounts of free hydroxyl sites cannot be tolerated.

TABLE I RETENTION TIMES AND SENSITIVITIES FOR VARIOUS WEAK ACIDS BY ION-SUPPRESSION REVERSED-PHASE LIQUID CHROMATOGRAPHY

	t_R (min)	Sensitivity (ng)*
Void volume	2.0	
Oxalic acid	S.F.**	
Lactic acid	2.6	0.2
Glycolic acid	2.6	0.1
Formic acid	2.8	0.2
Pyruvic acid	3.1	0.02
Malonic acid	3.3	0.04
Acetic acid	3.6	0.1
Monochloroacetic acid	4.8	0.5
Dichloroacetic acid	4.9	0.1
Maleic acid	5.0	0.006
Fumaric acid	5.9	0.004
Acrylic acid	6.1	0.06
Propionic acid	6.8	0.4
3-Chloropropionic acid	8.5	0.2
Trichloroacetic acid	8.9***	0.1
2-Chloropropionic acid	10.9***	1.0
2,3-Dichloropropionic acid	11.6***	0.5
2,2-Dichloropropionic acid	12.4***	0.5
Methacrylic acid	18.5	0.04

^{*} Amount in a 20-µl injection and 3 times signal-to-noise ratio.

In Table I are the retention times and sensitivities for a number of weak acids. Fig. 2 illustrates the separation of a select number of these acids.

Retention times for the more strongly retained acids can be shortened by the addition of 5% acetonitrile to the mobile phase.

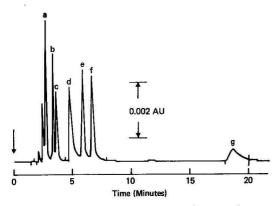


Fig. 2. Liquid chromatogram of weak acids according to conditions described in the text. Sample: $20 \mu l$ of a solution containing (a) 200 ppm glycolic acid, (b) 100 ppm malonic acid, (c) 190 ppm acetic acid, (d) 85 ppm dichloroacetic acid, (e) 1 ppm fumaric acid, (h) 320 ppm propionic acid and (g) 1 ppm methacrylic acid.

^{**} Excessive tailing.

^{***} Moderate tailing.

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TABLE II
PRECISION OF ACETATE IN BRINE MEASUREMENT

	Amount acetate (ppm)
Day I	417
•	416
	419
	416
	416
Day 2	414
•	416
	413
	416
	414
Mean	415.7
Standard deviation	1.7
Coefficient of variation	$\pm 0.4\%$

Lactic and glycolic acids, because of their proximity to the solvent front, are not measurable in 20% brine. For this same reason and because of excessive tailing, oxalic acid cannot be measured under these conditions.

Acids other than acetic have not been specifically measured in the presence of brine. However, it should be entirely possible to make such measurements.

Acetic acid was found to be linear from 10 to 5000 ppm in both area and peak height.

The column efficiency for acetic acid was studied as a function of eluent pH. With the 0.01 N sulfuric acid eluent (pH 2.2), the column gave 5048 theoretical plates; for a buffer of pH 3.5, it was 4986; and at pH 5.15, it fell to 822. As expected with an eluent buffer of pH 7.1, the acetic acid was not retained and came off at the column void volume.

The precision of the analysis was determined by measuring the acetate concentration five times on each of two consecutive days. Results are given in Table II.

This method can also be applied to calcium chloride brines. However, bromide containing brines cannot be analyzed for acetate because bromide is a strong ultraviolet absorber, and does not sufficiently clear the column before emergence of the acetate.

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CHROM, 15,220

Note

High-performance liquid chromatography of 12-dodecanelactam and its cyclic oligomers present in polyamide 12

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The equilibrium product of polymerization of lactams contains, in addition to the unreacted monomer, also linear oligomers and, in particular, cyclic oligomers:

Most attention has been paid to the analysis of the cyclic oligomers formed in polymerization of ε -caprolactam $(x=5)^1$. There are few data on the cyclic oligomers of other lactams. Zahn and Gleitsmann² were the first to report the cyclic dimer (II) and trimer (III) of 12-dodecanelactam (I). Mori *et al.*³ described the determination of cyclic oligomers of lactam I by gas chromatography after their previous reduction with LiAlH₄ in tetrahydrofuran (THF) solution. In industrial samples of polyamide 12, the contents of compounds I, II and III were 0.33, 0.94 and 0.25% (w/w), while the total amount of compounds extractable with ethanol was 1.70%. Feldmann and Feinauer⁴ found, in polyamide 12 prepared at 260, 270 and 280°C, 0.83 \pm 0.25% (w/w) of dimer II and 0.3 \pm 0.18% (w/w) of trimer III in good agreement with values calculated from cyclization constants.

We have worked out a simple method for direct determination of cyclic oligomers of ε-caprolactam by means of high-performance liquid chromatography (HPLC)¹. This method is applied here for the determination of oligomers of lactam I.

EXPERIMENTAL

Reagents and chemicals

The lactam I was crystallized three times from benzene and twice from acetone, dried at 50°C (2 kPa) for 50 h and then at 20°C (0.2 kPa) for 50 h. The equilibrium polyamide 12 was prepared by the polymerization for 600 h at 260°C of lactam I initiated with 2 mol. % of 6-aminocaproic acid in a sealed evacuated glass ampoule, according to ref. 5. The polymer was grated to shavings of thickness about 0.1 mm

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and extracted in a 125-fold (w/w) amount of methanol under reflux for 1 h. The completion of extraction was confirmed by repeated extraction. The extract was directly injected into a chromatographic column.

Equipment

The mixture of oligomers was separated in a Merck chromatographic column packed with LiChrosorb RP-18, using aqueous acetic acid (5 mM)-methanol (20:80, v/v) as eluent and a flow-rate of 0.7 ml/min. The injected volume was 10 μ l. The Spectra-Physics SP 8000 liquid chromatograph was equipped with a SP 8400 UV-VIS variable-wavelength detector. The separation was monitored at 210 nm.

RESULTS AND DISCUSSION

As in the case of ε -caprolactam and its oligomers¹, a very good separation of the lactam I and its cyclic oligomers up to the hexamer was attained, as seen in Fig. 1. Individual peaks were identified by comparison with pure oligomers obtained by preparative gel chromatography on a column packed with the gel LH-20, using methanol as the eluent. The gel chromatographic separation of individual oligomers of lactam I may be assumed to proceed in order of their molecular weights.

The molar absorption coefficients of the individual cyclic oligomers at 210 nm are presented in Table I. The quantitative evaluation was carried out analogously to that of the oligomers of ε -caprolactam by Mori and Takeuchi⁶. The areas of individual peaks were multiplied by correlation factors obtained from the ratios of the molar absorption coefficient of the monomer. The resulting value, when divided by the total area, is proportional to the weight per cent of the given oligomer in the mixture. The weight per cents of the individual cyclic oligomers in the equilibrium polyamide 12 are given in Table I.

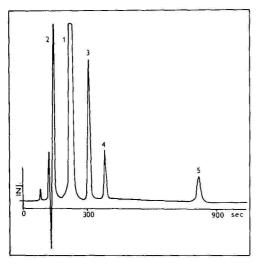


Fig. 1. HPLC separation of 12-dodecanelactam (I) and its cyclic oligomers. Peaks: 1 = I; 2 = dimer III; 3 = trimer III; 4 = tetramer IV; 5 = pentamer V.

NOTES NOTES

TABLE I
CYCLIC OLIGOMERS OF 12-DODECANELACTAM

A = Molar absorption coefficient; k = correlation factor; p = content (%, w/w) in the equilibrium polyamide 12.

Oligomer	A	k	p
I	1497	1.0	0.41
II	1847	0.81	1.25
III	2812	0.53	0.31
IV	2946	0.50	0.12
v	2970	0.50	0.10

The described HPLC method was also used for the determination of cyclic oligomers of lactam I during its polymerization and also during its copolymerization with ε-caprolactam, where the presence of a codimer and of cotrimers was shown. The latter result will be published in a subsequent paper.

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CHROM. 15,228

Note

Preparative high-performance liquid chromatography of adamantane-2,4-diols

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A mixture of three stereoisomeric adamantane-2,4-diols, *i.e.*, adamantane-2a,4a-diol (II), adamantane-2e,4a-diol (III) and adamantane-2e,4e-diol (IV), was prepared previously¹ by reduction of adamantane-2,4-dione (I) with LiAlH₄. This note describes the separation of these three stereoisomers by preparative high-performance liquid chromatography (HPLC).

EXPERIMENTAL

The separation was performed on Chromatospac Prep 100 preparative chromatograph (Jobin Yvon, Longjumeau, France). A 100-g amount of octadecyl silica of irregular shape (particle size $10-20~\mu m$) was packed into a column of 40 mm I.D.; the height of the bed was 170 mm. The silica was obtained by the method described previously². The chemically bonded phase was prepared by the method of Halász and Sebastian³ as modified by Hemetsberger *et al.*⁴, using octadecyltrichlorosilane as a reagent and toluene with pyridine as the reaction medium. No further "capping" has been done.

Methanol-water (50:50, w/w) was used as the mobile phase; it was degassed by connecting its reservoir to a vacuum for about 15 min. The flow-rate of the mobile phase was 14 ml/min at a pressure of 800 kPa. A slurry of stationary phase and methanol was used for packing of the column. The sample (0.3 g in 2 ml of methanol) was introduced directly into the column by an injection syringe.

Detection was effected with a refractive index (RI) detector (Varian, Palo Alto, CA, U.S.A.).

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HPLC analysis of fractions obtained by preparative separation was carried out on a Varian 8500 instrument equipped with an RI detector. Column: Micropak CH-10, 250 \times 2 mm I.D., packed with octadecyl silica, particle size 10 μ m (Varian). The flow-rate of the mobile phase, methanol-water (20:80, w/w), was 10 ml/h.

RESULTS AND DISCUSSION

A preparative chromatogram of the separated stereoisomeric adamantane-2,4-diols, together with analytical chromatograms of fractions obtained, is presented in Fig. 1. The chromatogram in Fig. 2 shows the analytical separation of all three stereoisomers. The conditions for the preparative separation were chosen in accordance with the analytical results published previously⁵.

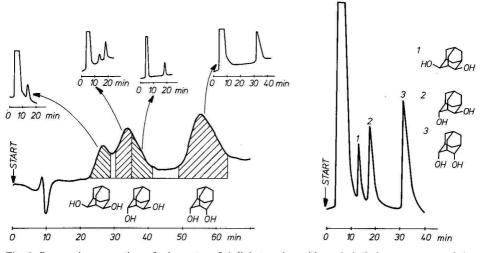


Fig. 1. Preparative separation of adamantane-2,4-diols together with analytical chromatograms of the separated fractions.

Fig. 2. Analytical HPLC separation of stereoisomeric adamantane-2,4-diols.

Owing to the small particle size and high viscosity of the mobile phase used, it was not possible to pack as long a column as when a mobile phase of lower viscosity was used². The purity of all separated isomers was higher than 97%. The yield of the second eluted peak (*i.e.*, 2e,4a-diol) was lower. It had to be collected after the maximum of the peak.

When comparing the described method of separation of these compounds with classical column chromatography on silica, it is evident that this method is quicker and more efficient.

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CHROM. 15,242

Note

High-performance liquid chromatography of free and bound phenolic acids in the egg-plant (Solanum melongena L.)

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It is well known that polyphenols are important in the physiology of the growth and development of plants¹. Since phenolic substituents usually improve the solubility characteristics of compounds and can interact with specific receptor groups by hydrogen bonding and/or by more stable covalent bonds, phenols are expected to influence a broad range of biological phenomena¹⁻⁸.

During a programme of plant breeding to obtain cultivars of the egg-plant for processing, the rôle of phenolic compounds on "browning" of vegetable tissues was considered. Most papers published on the high-performance liquid chromatography (HPLC) separations of polyphenolic compounds have dealt with a limited number of substances^{9–13}. Vande Casteele *et al.*¹⁴ studied the retention times of some 140 flavonoids and separated complex mixtures of about 40 substances.

The purpose of the present study was to develop a method for extending techniques previously applied to benzoic and cinnamic acid derivatives to more complex flavonoids extracted from plant tissues. The method, consisting of a combination of isocratic and linear gradient elution and a concave gradient elution, was applied to simple phenolic acids in the egg-plant.

EXPERIMENTAL

Chromatography

A Perkin-Elmer Series 2 liquid chromatograph, equipped with a spectrophotometric detector LC-55 and a Sigma 10B chromatography data station, was used. The column was a stainless-steel tube (30 cm \times 4 mm I.D.) packed with $\mu \rm Bondapak~C_{18}$ (Waters Assoc., Milford, MA, U.S.A.), having an average particle size of 10 $\mu \rm m$. A short stainless-steel precolumn, packed with $\mu \rm Bondapak~C_{18}$ –Corasil (37–50 $\mu \rm m$), was used. The UV detector was set at 280 nm and 325 nm.

Two solvents were used: A, methanol; B, acetic acid-water (5:95 v/v). The elution profile of the linear gradient (Programme I) was: 0-25 min, 15-40 % A; 25-30 min, 40 % A (isocratic); 30-45 min, 40-63 % A; 45-47 min, 63 % A (isocratic); 47-51 min, 63-99 % A. The concave gradient (Programme II) was: initial conditions 10 % A; programme time 44 min; final conditions 99 % A. The flow-rate was 2 ml/min and the column pressure was 2000-2200 p.s.i.

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TABLE I RETENTION TIMES (t_R) OF BENZOIC AND CINNAMIC ACID DERIVATIVES AND FLAVONOIDS

I = I	Linear	gradient;	II =	concave	gradient.
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Compound	und $t_R^I(min)$ $t_R^{II}(min)$ Compound		$t_R^I(min)$	$t_R^{II}(min)$	
Phloroglucinol	2.18	2.21	Sinapic acid	15.60	21.85
Gallic acid	2.48	2.52	Luteolin-7-glucoside	21.87	28.48
Protocatechuic acid	3.71	3.82	Quercetin-3-glucoside	23.35	29.20
Catechol	4.01	4.13	+ Rutin		
(+)-Catechin	4.73	5.76	Cinnamic acid	25.08	29.65
p-Hydroxybenzoic acid	5.81	6.30	Myricetin	25.90	30.63
Chlorogenic acid	6.53	7.68	Quercitrin	26.96	31.27
Esculetin	6.94	7.80	Morin + Quercetin	28.27	32.02
Vanillic acid	7.29	8.76	Naringenin	30.85	33.45
Caffeic acid	7.79	9.36	Fisetin	33.68	35.00
Syringic acid	8.83	11.20	Hesperetin	34.49	35.40
Cynarin	9.00	13.34	Luteolin	37.16	37.26
p-Coumaric acid	12.24	15.46	Kaempferol	40,27	39.60
Dihydroquercetin	12.82	16.72	Apigenin	41.79	41.01
Ferulic acid	14.33	19.25	Galangin	43.00	42.50

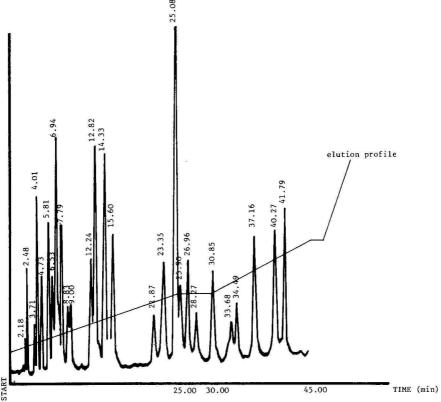


Fig. 1. The retention times of phenolic compounds eluted using the linear gradient on a μ Bondapak C_{18} column (30 cm \times 4 mm I.D.). A list of the compounds separated is given in Table I. For the elution system see Experimental.

Samples

Standards of benzoic and cinnamic acid derivatives and flavonoids were dissolved in methanol. Peeled fruits of the egg-plant were extracted with methanolethanol (1:1) by the procedure developed by the Laboratory of Plant Biochemistry of Ghent^{16,17}. The alcoholic extract, after concentration, was partitioned between 1-butanol and 6% Na₂CO₃. The aqueous layer was acidified to pH 3.5 and re-extracted with diethyl ether giving fraction A. The acidic aqueous layer was made alkaline with concentrated NaOH until 2 M, then refluxed, acidified and extracted with diethyl ether to give fraction B. The 1-butanol layer was refluxed in 2 M NaOH and the aqueous layer then acidified to pH 3.5 and re-extracted with diethyl ether to give fraction C. The residue insoluble in methanol-ethanol, after alkaline hydrolysis, acidification and ether extraction, gave fraction D. The four fractions obtained were: A, free phenolics; B, carbonate-soluble, alkali-labile bound phenolics; C, carbonate-insoluble, alkali-labile bound phenolics.

The ether fractions of free and bound phenolics were dried in vaci o at 30°C and the residue was dissolved in methanol. The injected volume of standard solutions and vegetable extracts was 10 μ l.

RESULTS AND DISCUSSION

Table I shows the retention times of the phenolic compounds eluted according to two elution profiles. The values are averages from six runs, the error being about

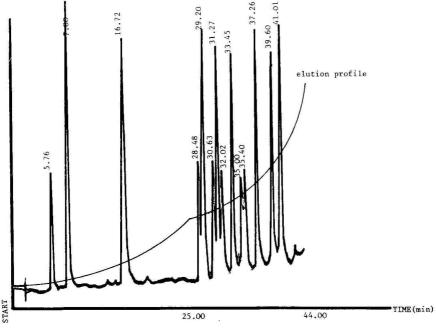


Fig. 2. The retention times of flavonoids eluted using the concave gradient on a μ Bondapak C_{18} column (30 cm \times 4 mm I.D.). Other details as in Fig. 1.

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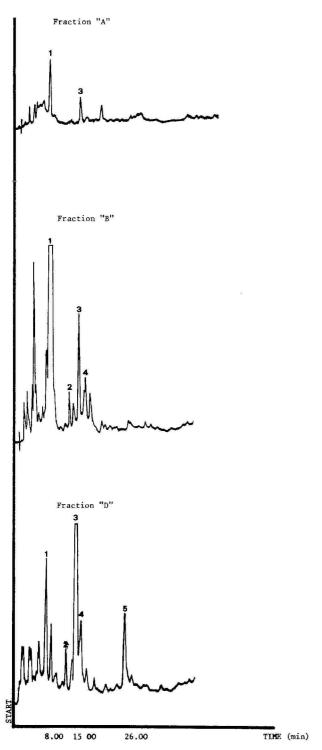


Fig. 3. The separation of simple phenols in vegetable extracts of the egg-plant using the linear gradient. Peaks: 1 = Caffeic acid; 2 = p-coumaric acid; 3 = ferulic acid; 4 = sinapic acid; 5 = cinnamic acid. For the elution system see Experimental.

TABLE II
SIMPLE PHENOLS IN VEGETABLE EXTRACTS OF THE EGG-PLANT

Cultivar	Caffeic acid	p-Coumaric acid	Ferulic acid	Sinapic acid	Cinnamic acid
Fraction .	4				
L39/78	+		+		
L17/79	Ť		+		
L.V.					
Fraction 1	В				
L39/78	+	+	+	+	+
L17/79	+	+	+	+	
L.V.	+	+	+	+	
Fraction .	D				
L39/78	+	+	+	+	
L17/79	+	+	+	+	+
L.V.	+	+	+	+	

2%. Fig. 1 shows the separation, using the linear gradient, in a single run of 30 substances: only quercetin-3-glucoside and rutin at 23.35 min and morin and quercetin at 28.27 min are not separated. Fig. 2 shows the elution of a set of flavonoids using the concave gradient. The two elution programmes are nearly equivalent, but permit more information to be obtained from the two different t_R values. Fig. 3 shows the separation of simple phenols from vegetable extracts of egg-plant fruits. A list of the compounds identified, according to their two different t_R values, is given in Table II. The UV spectra of the peaks identified corresponded to those of standard compounds. In addition to the components reported, a number of minor constituents in fractions B and D have not as yet been identified.

The highest content of simple phenols was found in the fractions B and D, while in fraction C they were absent. The content of free phenolics (fraction A) was very low and among them were identified caffeic and ferulic acids, the only ones existing in all fractions. Analysis carried out on cultivars of egg-plant with different "browning" characteristics showed differences mainly in the content of caffeic and ferulic acids, the most abundant components.

These results are in agreement with the theory that cinnamic acid and its derivatives, widely distributed in vascular plants, generally are found as esters rather than as free acids^{2–5,8,16,18}. In addition, large amounts of cinnamic acid derivatives, particularly p-coumaric acid and ferulic acid, are found after alkaline hydrolysis of the insoluble residue remaining after alcoholic extraction.

In the fraction D the content of ferulic acid was very high. El-Basyouni et al.³ suggested an alcohol insoluble enzyme ester of hydroxycinnamic acids as the active intermediate of lignin biosynthesis. Other possible interactions between alcohol insoluble phenolic acids and proteins are hydrogen bonding and irreversible oxidation followed by covalent condensation. Ferulic acid may also be bound by means of an amide linkage⁸. Fraction B contains phenolic acids esterified with the hydroxyls of compounds such as glucose or quinic acid. Among these phenolics, caffeic acid was particularly abundant and their rôle in plants is related to the biosynthesis of flavonoids and coumarins.

ACKNOWLEDGEMENT

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CHROM. 15,219

Note

Separation of the enantiomers of (\pm)-norephedrine by rotation locular counter-current chromatography

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In a recent paper, Prelog et al.¹ reported the separation of enantiomers by partition between two liquid phases. Racemic mixtures of salts of α -aminoalcohols, such as norephedrine, with lipophilic anions, such as hexafluorophosphate ion, could be separated by partition between the aqueous phase and the lipophilic phase (1,2-dichloroethane) containing an ester of tartaric acid (di-5-nonyl tartrate). The separation was achieved by flash partition chromatography², the stationary phase being the aqueous phase on a Kieselguhr support. In the present note we report a similar separation of the enantiomers of norephedrine by a support-free liquid–liquid technique using rotation locular counter-current chromatography (RLCC)^{3,4}.

RLCC is an adaptation of a technique based on a principle originally proposed by Signer et al.⁵ and modified by Winistorfer and Kováts⁶. A column is constructed by placing in a glass tube multiple centrally perforated partitions which divide the tube into compartiments called loculi. It is filled with the stationary phase which can be either the lower or the upper one, depending on the separation problem. The column is then inclined from the horizontal position at an angle of 25–40° and the mobile phase is continuously introduced, from the bottom if it is the upper phase or from the top if it is the lower phase. The mobile phase displaces the stationary phase in each loculus to the level of the hole leading to the next one; it is collected at the outlet of the column. In practice several columns, which are interconnected with fine PTFE tubings, are mounted on a rotating shaft. The rotation promotes the partition of substrate between two phases and prevents the formation of emulsions.

EXPERIMENTAL

In our work a RLCC instrument (Tokyo Rikakikai Co., Tokyo, Japan) was used which consisted of 16 columns (45 cm \times 11 mm I.D.) divided by centrally perforated PTFE disks into 37 loculi each. The flow-rate was 17–20 ml/h, the rotation speed was 60–70 rpm and the slope 40°. Two experiments were carried out at 2–3°C and 5–8°C, respectively.

The stationary phase was a $0.5\ M$ solution of sodium hexafluorophosphate (71.5 g) in water (850 ml) to which hydrochloric acid was added to pH 4. The mobile phase was a $0.3\ M$ solution of (R,R)-di-5-nonyl tartrate in 1,2-dichloroethane. A solution of 200 mg of racemic norephedrine hydrochloride and 360 mg sodium hexafluorophosphate in 2 ml water was injected into the inlet of the apparatus and eluted in the descending mode with the lipophilic phase, which was analyzed at the outlet by determining the UV absorption spectrum. The eluate containing norephedrine was divided into four fractions, which were treated separately with $0.25\ M$ sodium hydroxide, followed by extraction with $0.1\ M$ hydrochloric acid. The aqueous extracts were evaporated to dryness and each residue analyzed by determining the amount of norephedrine by UV absorption on a UVIKON 810 spectrometer and its optical purity by circular dichroism on a Jobin-Yvon III Dichrograph.

RESULTS AND DISCUSSION

The results of the first separation carried out at 5–8°C are summarized in Table I. The mobile phase front was observed at 456 ml, the two maxima corresponding to the 1S- and 1R-enantiomers respectively were at 1310 and 1730 ml.

TABLE I	
RLCC SEPARATION OF (±)-NOREPHEDRINE A	AT 5-8°C

Eluate volume (ml)	Norephedrine hydrochloride					
	mg	Enantiomeric excess (%)	1 <i>S</i> (%)	1 <i>R</i> (%)		
821-1310	52	87	93	7		
1311-1520	38	32	66	34		
1521-1730	38	86	7	93		
1731-2010	37	97	1	99		

The results of the second experiment, which was carried out at 2–3°C, are given in Table II.

Although no baseline separation has been achieved, these results show that practically pure enantiomers can be obtained by the RLCC technique; a complete resolution could be achieved using an apparatus with more loculi.

TABLE II RLCC SEPARATION OF (\pm)-NOREPHEDRINE AT 2–3°C

Eluate volume (ml)	Norephedrine hydrochloride					
	mg	Enantiomeric excess (%)	1S (%)	1 <i>R</i> (%)		
671-992	53	90	95	5		
993-1146	46	45	72	28		
1147-1314	41	76	12	88		
1315-1650	39	99	0.5	99.5		

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Note

Isotachophoretic assay of aminoglycosides and lincomycins in pharmaceuticals

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Aminoglycoside antibiotics are used in the treatment of severe infections of men and animals¹. They are indicated when antibiotics with inferior toxic potential are contraindicated and when the organisms are susceptible to the aminoglycosides concerned. Lincomycin and clindamycin, which are pyranosides, are indicated, for example, for the therapy of infections induced by penicillin-, oxacillin- and cephalosporin-resistant staphylococci².

The quantitation of the active substances of tobramycin sulphate, sisomicin sulphate, clindamycin hydrochloride, lincomycin hydrochloride as well as spectinomycin dihydrochloride by chromatographic techniques is still laborious and time-consuming^{3–5}. Microbiological techniques⁶ also do not meet the requirements of a rapid, precise and economical method for the quantitation of the aminoglycosides and lincomycins discussed herein. However, the present results show that analytical isotachophoresis can be successfully used for the determination of these active substances. This technique does not require more than 10 min for a complete assay.

MATERIALS AND METHODS

The pharmaceuticals were obtained from commercial sources. Spectinomycin dihydrochloride, clindamycin hydrochloride and lincomycin hydrochloride served as reference substances, e.g., for the construction of the calibration graphs (Fig. 1). The structural formulae of these compounds as well as those of the antibiotics sisomicin and tobramycin are listed in Table I. All reagents were prepared with double distilled water. They were purchased as analytical grade chemicals. Hydroxypropylmethylcellulose (HPMC 15000) was obtained from Dow Chemical (Stade-Brunshausen, G.F.R.), 4-amino-butyric acid from Serva (Heidelberg, G.F.R.), glycylglycine and potassium acetate from E. Merck (Darmstadt, G.F.R.) and β -alanine from Sigma (München, G.F.R.).

For isotachophoresis, a number of suitable aqueous electrolyte systems is available. A system of 0.020 mol/l potassium acetate plus 0.3% HPMC 15000 (to avoid electroendosmosis^{7,8}), pH 4.95, proved to be excellent for the qualitative and quantitative determination of the aminoglycosides and lincomycins. A mixture of 20 mmol/l 4-aminobutyric acid/acetic acid, pH 4.72, or 0.020 mol/l glycylglycine or 0.020 mol/l β -alanine, was selected as terminator. HPMC 15000 was purified by means of dialysis⁹.

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Determinations were performed with the "Tachophor" (LKB, Bromma, Sweden), Type 2127, at a constant current of 200 μ A and a constant temperature of 5°C. The length of the capillary was 23 cm. The measurement range of the recorder (LKB 2210) was 100 mV and the chart speed was 6 cm/min. Aqueous solutions of the antibiotics were injected with a 10- μ l Hamilton microsyringe, the volumes injected being 3–5 μ l. During the separation (cationic) by a discontinuous electrolyte, the cations migrate according to their net mobilities between the leading and terminating electrolyte¹⁰. The compounds discussed were investigated in the form of their sulphates or hydrochlorides, which are their common application forms. As these antibiotics show very little UV-absorption, they are identified by their differing electrical conductivities. The concentration of the standard solutions was chosen such that the concentration of the active substances based on the corresponding salts amounted to 1 mg/ml.

RESULTS AND DISCUSSION

The results confirm the possibility of using analytical isotachophoresis for the determination of aminoglycoside antibiotics and lincomycins, under the conditions mentioned. While, on the one hand, the lack of distinct UV absorption renders difficult the identification by means of high-performance liquid chromatography and no conditions are known under which the unaltered molecules can be exactly determined by gas chromatography, the conductivity detector proves to be excellent for the determination of these compounds.

The isotachopherograms show that the separation of the active substances in the pharmaceuticals is practicable without the application of other techniques of analysis, such as thin-layer, ion-exchange or column chromatography. However, the simultaneous determination of not only lincomycin hydrochloride and clindamycin hydrochloride but also of tobramycin sulphate and sisomicin sulphate implies some problems. The mobility of the cations depends on charge, viscosity, molecular size and shape, solvation, dielectric constant and temperature. The mixtures concerned contain weak organic bases differing only slightly in size (Table I) and in pK value

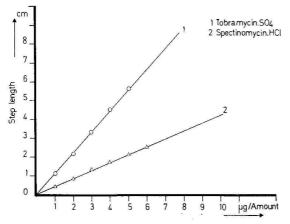


Fig. 1. Calibration graphs for quantitation of tobramycin sulphate and spectinomycin dihydrochloride.

TABLE I
ANTIBIOTICS STUDIED

Structural formulae (base)	Antibiotics	Anion
H_3C-NH	Spectinomycin	Cl-
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	$R^2 = H$ Lincomycin	Cl ⁻ Cl ⁻
H ₂ N-CH ₂ H ₃ C HO OH NH ₂ N	Sisomicin	SO ₄ ²⁻
H ₂ N — CH ₂ HO HO OH NH ₂ N N	Tobramycin	SO ₄ ²⁻

and, consequently, also in their effective mobilities. Nevertheless, since pharmaceuticals generally contain only one of these active compounds there is no practical difficulty. The isotachopherogram in Fig. 2 illustrates a separation of tobramycin/sisomicin, spectinomycin and lincomycin/clindamycin based on their different molecular structures¹¹.

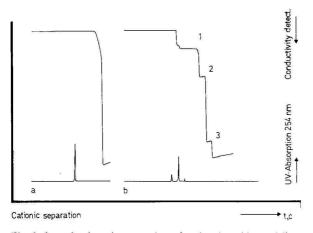


Fig. 2. Isotachophoretic separation of aminoglycosides and lincomycins (b) (1 = tobramycin sulphate; 2 = spectinomycin dihydrochloride; 3 = clindamycin hydrochloride) and UV and conductivity diagram of electrolyte system (a).

If the compounds discussed herein have to be determined during an in-process control, this is likely to be accomplished by means of spacer-ions¹². Although the salts of the antibiotics do not show any UV signals at 254 nm, the limits of the zones, in the case of spectinomycin and lincomycin/clindamycin, are still recognizable as

TABLE II
RESULTS OF THE QUANTITATION OF ACTIVE SUBSTANCES IN SOME PHARMA-CEUTICALS

Cps	= 0	Capsul	le: t	bl =	tablet.

Pharma- ceutical	Active substance	Quantity declared	Quantity found	Content related to quantity declared $\binom{9}{0}$
A	Clindamycin- hydrochloride	85.2 mg/cps.	90.3 mg/cps.	106.0
В	Lincomycin- hydrochloride	567.8 mg/cps.	620.0 mg/cps.	109.2
С	Lincomycin- hydrochloride	567.8 mg/cps.	555.3 mg/cps.	97.8
D	Lincomycin- hydrochloride	681.3 mg/2 ml	624.2 mg/2 ml	91.6
Ę,	Lincomycin- hydrochloride	113.4 mg/1 ml	123.0 mg/1 ml	108.5
F	Lincomycin- hydrochloride	226.8 mg/tbl.	240 mg/tbl.	105.8
G	Spectinomy- cin dihydro- chloride	3 g/package	3.01 g/package	100.3
Н	Spectinomy- cin dihydro- chloride	3.0 g/package	3.07 g/package	102.3

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there are always so-called spacers because of traces of natural UV-absorbing contaminant. These impurities are present even in analytical grade agents, such as glycyclglycine, β -alanine and 4-aminobutyric acid (Fig. 2).

For the quantitation of the antibiotics, a direct comparison of the length of the zones of a sample and those of a standard solution is used. Fig. 1 shows that there is a direct proportionality between the concentrations and the length, the coefficient of correlation being r=1.000. The lowest amount which can be quantitated is 1.6 nmol, under the conditions given. The reproducibility of the method was examined by replicate analyses and the coefficient of variation was found to be 2%. As shown in Table II, the pharmaceuticals contain a surplus of active substances —this is obviously to ensure a sufficient antibiotic content until the expiry date.

We hope that this publication will stimulate further research into more simple and precise assays of pharmaceuticals by means of isotachophoresis.

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Book Review

Organic trace analysis by liquid chromatography, by J. F. Lawrence, Academic Press, New York, 1981, XII + 288 pp., price US\$ 34.00, ISBN 0-12-439150-8.

The main requirements for the analysis of organic compounds in the ppm to ppb range by high-performance liquid chromatography (HPLC) are efficient isolation (clean-up) procedures and sensitive detectors. Whereas isolation methods are as varied as the analytes and matrices containing them, the choice of suitable detectors is necessarily limited in HPLC.

Steering a safe course between the vast literature on the analysis of traces of innumerable compounds and the meager possibilities of increasing their detectability, Lawrence has produced a very readable small book on HPLC. Actually, most of the book could be used as a general text for beginners in HPLC. There are some very lucid chapters on basic hardware (pumps, injectors, columns and detectors) with excellent schematic drawings, useful specifications and lists of suppliers.

Because he was forced to condense much information on chromatographic theory, Lawrence had to oversimplify and omit much of what can be found in other textbooks on HPLC, but what is presented is clear and coherent. The "meat" of this book, the applications of HPLC to various substances, occupies only half of it and is of necessity incomplete. There are chapters on derivatization and clean-up methods and some examples of analytical schemes. I have found only the subject index and the bibliography, which contains several errors, to be inadequate.

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Liquid Column Chromatography

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Erratum

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Page B219, heading "32b. Anions" should read "32b. Pharmacokinetic studies".

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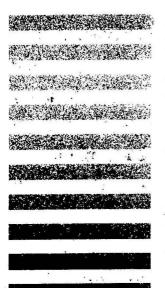
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