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LIQUID CHROMATOGRAPHY OF ADAMANTANES ON CARBON ADSORBENTS

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SUMMARY

The retention behaviour of various adamantanes has been investigated on carbon adsorbents with methanol–water and acetonitrile–water mobile phases. The relationships between the molecular connectivity and the rate of change of the capacity ratio with the mobile phase composition, and between the surface area of contact between the adsorbent and the molecular structure, indicate that the retention mechanism is better explained in terms of adsorption than in terms of hydrophobicity. Although methanol generally yields greater selectivities than acetonitrile for a given water content, acetonitrile gives much shorter analysis times for an equivalent selectivity. The influence of various substituents of the adamantanes on the contact surface area has been investigated, and the results explained on the basis of a change in the adsorption energy of one or several adsorption positions.

INTRODUCTION

Adamantane derivatives are very important compounds encountered in pharmacy, biochemistry, medicine, chemistry of plastics, synthesis of special lubricants, etc. The chromatographic behaviour of these compounds has only recently been systematically studied[1–3]. It has been shown that in normal-phase chromatography[1,2] there are two contributions to the adsorption energy, related respectively to the type of functional groups and to the arrangement of these groups. Normal-phase chromatography gives good separations of adamantane derivatives having the same number of polar functional groups. However, it is difficult to use this technique for solutes with different numbers of polar groups since the differences in retention times are too large and it is not possible to use gradient elution with this group of compounds because of the necessity for a refractive index detector.

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Reversed-phase chromatography with octadecyl-modified silica gel has proved to be very successful for such compounds[3]. However, this type of stationary phase is sometimes unable to resolve solutes of similar structures. Therefore, it is attractive to use carbon adsorbents as it is often possible to obtain very different selectivities with these materials than with the usual reversed-phase packings[4].

The purpose of this work was to investigate the retention of adamantane derivatives on carbon adsorbents. The retention mechanism was studied as well as the effects of the mobile phase composition and of various substituents.

EXPERIMENTAL

A Model 6000A pumping system (Waters Assoc., Milford, MA, U.S.A.) was employed. Sample injection was performed with a Rheodyne 7020 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) and solutes were detected with a Waters Model R 401 differential refractometer.

The carbon adsorbents were prepared as described previously[5]. The basic carbon materials were Black Pearls L and Sterling FT-FF (Cabot, Boston, MA, U.S.A.). Particle hardening was carried out by pyrolysis of benzene at 850°C. The particle size used was in the range 20–30 μm. The columns were packed at 200 bar using suspensions of carbon particles in acetonitrile–dibromomethane mixtures.

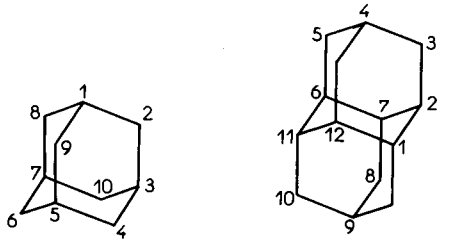
The mobile phases were prepared by pipetting appropriate volumes of methanol and acetonitrile (pro-analysi grade; E. Merck, Darmstadt, G.F.R.) or water (twice distilled). The compositions are given in v/v. Experiments were performed at room temperature (19–20°C).

The structures of the adamantane and diamantane molecules are shown in Fig. 1. All standards of adamantane derivatives were provided by the Laboratory of Synthetic Fuels, Institute of Chemical Technology, Prague.

RESULTS AND DISCUSSION

Retention mechanism

The retention mechanism on carbon surfaces is often pure adsorption[6], and the chromatographic behaviour of systems based on carbon materials can generally be explained using Snyder's approach developed for normal-phase adsorption chromatography[7]. It is clear, however, that the solvophobic effect associated with the use of aqueous mobile phases cannot always be neglected. This is particularly true for com-



ADAMANTANE DIAMANTANE

Fig. 1. Structures of adamantane and diamantane.

pounds of large molecular size (large molar volume) which leads to a greater solvophobic contribution, and spherical shape (small surface area of contact with a flat surface) which reduces the adsorption contribution. In such cases, the retention mechanism may become mixed. This situation may occur with the adamantane derivatives.

The study of possible relationships or correlations between retention, *i.e.*, capacity ratio, k', and the molecular connectivity or the contact surface area can give information on the retention mechanism and helps in determining which one of the two contributions (hydrophobic and adsorption) is predominant.

The molecular connectivity, χ, which is easy to calculate, gives a good estimate of the surface area of the cavity created in the solvent to accommodate the solute[8]. When the retention mechanism is governed by the solvophobic effect, the rate of variation of log k' with the mobile phase composition (volume fraction) is linearly related to the molecular connectivity. This assumes that log k' is also linearly related to the solvent composition. As will be seen below, this assumption does not hold exactly for all the adamantanes eluted on carbon materials; the curvature of the plots is small, however. It was thus assumed that over a limited range of solvent composition the plots log k' *vs.* water content were linear. The slopes of these straight lines were determined and then plotted *versus* the molecular connectivity for 27 adamantane and diamantane derivatives, Fig. 2 (methanol–water mixtures). The values of χ for the adamantanes are given in Table I. The results clearly indicate that retention cannot simply be explained in terms of hydrophobicity as the correlation is, at best, very weak.

It is more difficult to evaluate the contact surface area, CSA, between the adsorbent and the adsorbate molecule as this requires knowledge of the position of the molecule on the surface of the adsorbent. However, in some cases it is quite easy to estimate the CSA, *e.g.* when the adsorbate molecule is flat (*i.e.*, benzene) or when it

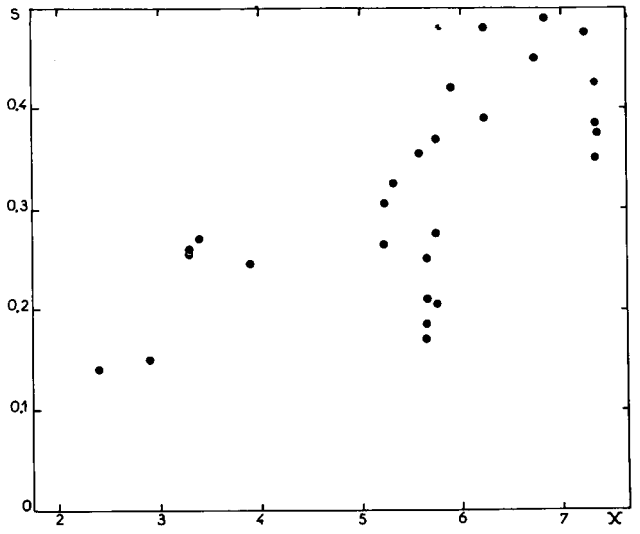


Fig. 2. Relationship between the molecular connectivity, χ, and the slope, S, of the log k' *vs.* solvent composition plots (methanol–water mixtures).

TABLE I

MOLECULAR CONNECTIVITY, χ, AND CONTACT SURFACE AREA, CSA, OF THE ADAMANTANE DERIVATIVES STUDIED

CSA = 1.00 for benzene

Solute	χ	*CSA**	*CSA***
Adamantane	4.900	–	1.00
Diamantane	6.899	–	1.48
Adamantan-1-ol	5.235	0.83	0.82
Adamantan-2-ol	5.326	1.01	1.04
Adamantanone	5.235	0.76	0.78
3-Methyladamantan-1-ol	5.571	1.05	1.03
3,5-Dimethyladamantan-1-ol	5.907	1.27	1.25
3,5,7-Trimethyladamantan-1-ol	6.243	1.50	1.46
3-Ethyl-5-methyladamantan-1-ol	6.468	1.44	1.40
3-Ethyl-5,7-dimethyladamantan-1-ol	6.596	1.66	1.61
2-Methyladamantan-1-ol	5.747	0.92	0.93
1-Methyladamantan-2-ol	5.747	1.09	1.09
2-Methyladamantan-2-ol	5.747	–	1.09
2-Ethyladamantan-2-ol	6.220	1.30	1.28
2-Propyladamantan-2-ol	6.720	1.48	1.48
2-Butyladamantan-2-ol	7.220	1.70	1.67
2-Isobutyladamantan-2-ol	7.078	1.63	1.62
Adamantane-1,4-diol	5.662	0.58	–
Adamantane-2,4-diol	5.662	0.78	–
Adamantane-2,6-diol	5.662	0.63	–
1-Hydroxyadamantan-4-one	5.662	0.58	–
2-Hydroxyadamantan-4-one	5.754	0.74	–
Diamantan-1-ol	7.256	1.27	1.24
Diamantan-3-ol	7.326	1.34	1.30
Diamantan-4-ol	7.235	1.19	1.11
Diamantanone	7.326	0.98	1.00
Cyclohexane	3.000	0.97	–
Cyclohexanol	3.394	0.81	–
Hexane	2.914	1.150	–
Decane	4.914	1.953	–

* Calculated from retention data in methanol–water mixtures.
** Calculated from retention data in acetonitrile–water mixtures.

is highly symmetrical (*i.e.*, carbon tetrachloride). It is clear from the structure of the adamantane molecule that there are four stable adsorption positions on the carbon surface, each of which corresponds to the contact of a cyclohexane ring with the adsorbent.

This indicates that, if the main contribution to the retention is adsorption, the rate of change of log k' with the mobile phase composition should be similar for adamantane and benzene and identical for adamantane and cyclohexane. This is indeed observed with methanol–water and acetonitrile–water mixtures.

The values of CSA for the adamantane derivatives investigated are given in Table I. The reference compound is benzene (CSA = 1.00). It can be seen that the

average relative CSA for adamantane is equal to that of benzene. It has already been shown that the CSA of cyclohexane is very close to that of benzene[6].

The values of CSA for the other compounds will be discussed in detail below. However, the results obtained with benzene, adamantane and methyladamantanol suggest that the retention mechanism is mainly adsorption.

Rôle of the water content of the mobile phase

As previously reported[4], the eluotropic strength of methanol–water and acetonitrile–water mixtures is approximately linearly related to the volume composition on carbon adsorbents, the linearity being greater with methanol than with acetonitrile. This results in almost linear plots of log k' *vs.* composition of the eluent. A significant curvature of these plots would indicate that the system is not governed by a pure adsorption process. It must be noted, however, that a non-linear behaviour can sometimes be observed with pure adsorption systems. This happens when the contact surface area between the solute molecule and the adsorbent changes with the solvent composition. In the case of carbon adsorbents, this should presumably occur with highly polar compounds containing conjugated benzene rings, but does not seem very likely with the adamantane derivatives.

Non-linear plots are observed for some solutes. As expected, the curvature of

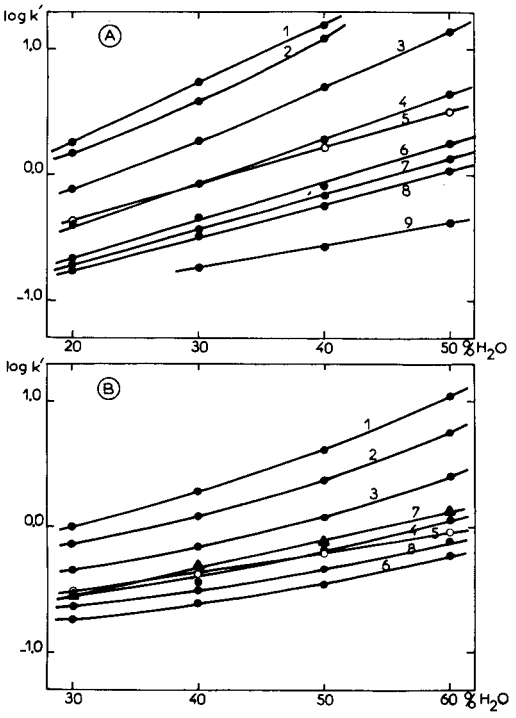


Fig. 3. Variation of log k' with the mobile phase water content (%, v/v). A, Methanol–water mixtures; B, acetonitrile–water mixtures. Solutes: 1 = 2-*n*-butyladamantan-2-ol; 2 = 3,5,7-trimethyladamantan-1-ol; 3 = 3,5-dimethyladamantan-1-ol; 4 = 3-methyladamantan-1-ol; 5 = 2-methyladamantan-1-ol; 6 = adamantan-1-ol; 7 = benzene; 8 = adamantanone; 9 = 1-hydroxyadamantan-4-one.

the plots is greater for acetonitrile than for methanol where the plots are quasi-linear. Some results are given in Fig. 3. In the case of acetonitrile the shape of the plots indicates that with increasing water content the solutes are more strongly retained than expected assuming a pure adsorption mechanism. It must be noted that the curvature of the plots becomes significant only at high water contents ($>50\%$). Because acetonitrile has a higher eluotropic strength than methanol, it has to be mixed with larger amounts of water to provide similar k' values. This partly explains why greater curvatures are observed for acetonitrile than for methanol. The curvature is probably the result of an increasing hydrophobic contribution to the retention.

The examination of the curves log k' *vs.* mobile phase composition reveals that inversions in the elution order occur for a few compounds, *e.g.*, 2-methyl- and 3-methyladamantan-1-ol in methanol–water and acetonitrile–water mixtures. The 3-methyl isomer is the more strongly retained at high water contents. This can simply be explained by the fact that the CSA for the 3-methyl isomer is larger than for the other one (see below). At low water contents, 2-methyladamantan-1-ol is eluted last (although close to the other isomer) because it interacts less with the solvent. With increasing water content, however, the contribution due to the solvent strength is larger for 3-methyladamantan-1-ol, the k' of which increases more rapidly than that of 2-methyladamantan-1-ol. This results in an inversion in the elution order.

Rôle of the organic modifier

Changing the organic solvent can result in a change in both the absolute and relative retentions. As far as the absolute retention is concerned, acetonitrile has a stronger eluting power than methanol: for a given water content the k' values are smaller with acetonitrile than with methanol. The change in absolute retention can be accompanied by a change in selectivity if the solutes studied have different CSA values. This happens with the adamantanes. It is difficult to determine *a priori* what is the best organic modifier for a given separation as this depends on the compounds investigated. Moreover, it also depends on what the analyst is interested in: maximum selectivity, minimum time of analysis, etc.

This is illustrated with a few examples showing some of the separation problems that can be encountered with the adamantanes:

(i) separation of positional isomers containing
— polar substituents (adamantan-1-ol and -2-ol)
— non-polar substituents (3-methyl- and 2-methyladamantan-1-ol)
— non-polar and polar substituents (2-methyladamantan-1-ol and 1-methyladamantan-2-ol)

(ii) separation of compounds with different degrees of substitution (non-polar substituents: 3-methyl- and 3,5-dimethyladamantan-1-ol)

These examples do not represent all the possible separation problems, but the conclusions that will be drawn give a good impression of the differences between the behaviours of acetonitrile and methanol.

The variations of the selectivity, α, with the water content of the eluent are indicated in Table II for two pairs of solutes in methanol–water and acetonitrile–water mixtures. Similar results are obtained for other pairs.

Two observations can be made from Table II. First, α increases with increasing water content. This stems from the fact that the solutes in each pair have different

TABLE II

INFLUENCE OF THE WATER CONTENT ON THE SELECTIVITY, α

A = 1-Methyladamantan-2-ol and 2-ethyladamantan-1-ol; B = 3-methyl- and 2-methyladamantan-1-ol.

Pair of solutes	*Modifier*	*Water content (%)*		
		30	*40*	*50*
A	Methanol	0.99	1.16	1.40
	Acetonitrile	0.93	–	1.05
B	Methanol	1.30	1.47	1.90
	Acetonitrile	1.26	1.33	1.46

CSA values, the most strongly retained solute having the largest CSA. Secondly, for a given water content, the selectivity is larger with methanol–water mixtures than with acetonitrile–water ones. This suggests that methanol is a better modifier if high selectivities are expected, regardless of the time of analysis.

It is also interesting to compare the two modifiers in terms of the time necessary to obtain a given selectivity. The corresponding data are reported in Table III. The time of analysis is accounted for by $1 + k'$, k' being the capacity ratio of the most strongly retained solute in each pair. The results indicate that acetonitrile is generally more "efficient" than methanol, on a time basis. This is particularly true when high α values are required (although, for a given water content, methanol yields higher selectivities). In some cases (low α values or compounds with very different CSA values) methanol becomes more efficient than acetonitrile. This corresponds, however, to low k' values (< 1) for which both modifiers are equivalent.

From these examples, and from other data not discussed here, it can be concluded that although methanol can potentially give larger selectivities than acetonitrile, it is generally better to use acetonitrile because, for a given selectivity, it requires shorter analysis times.

TABLE III

INFLUENCE OF THE ORGANIC SOLVENT ON THE ANALYSIS TIME FOR A GIVEN SELECTIVITY

A = 3-Methyl- and 2-methyladamantan-1-ol; B = adamantan-2-ol and -1-ol; C = 1-methyladamantan-2-ol and 2-methyladamantan-1-ol; D = 3,5-dimethyl- and 3-methyladamantan-1-ol.

Pair solutes	*Methanol*			*Acetonitrile*		
	α	$1 + k'$	*Water content (%)*	α	$1 + k'$	*Water content (%)*
A	1.40	5.48	50	1.40*	3.30*	70*
B	1.47	3.42	40	1.46	1.89	50
C	1.43	3.55	50	1.42	1.85	60
D	1.84	1.70	20	1.89	2.21	50

* Extrapolation.

Rôle of the solute structure

This has been investigated by studying the change in retention with the solvent composition. The relevant parameter is the contact surface area, as previously mentioned. CSA values (relative to benzene) are given in Table I. They have been calculated from the retention data obtained with acetonitrile–water and methanol–water mixtures. The CSA value for a given solute is the value of the slope of the plot of log k' *vs.* composition for that compound, divided by the corresponding slope for benzene. For these calculations it was assumed that the plots were linear (see above). The results in Table I show an excellent agreement between the values obtained in the two solvent systems. This does not mean, however, that methanol and acetonitrile are equivalent, as it has already been shown.

The examination of CSA values gives information on the rôle of the adamantane skeleton. The relative CSA values of benzene, adamantane and cyclohexane have been discussed above.

As for diamantane, it is clear from the structure of this molecule that its CSA is larger than that of adamantane, but not twice as large. The experiments give a value of 1.48, in good agreement with that (1.41) derived from a geometrical projection of the molecule onto a flat surface. On the other hand, it is remarkable that diamantane is much more strongly retained than adamantane [the relative retention is about 9 in acetonitrile–water (60:40)]. This is the result of the greater hydrophobic effect in the case of diamantane, in addition to the contribution of the extra four carbon atoms to the adsorption energy on the carbon surface.

The effect of a substituent on the adamantane molecule on the dependence of the retention on the mobile phase composition is related to the position and the nature of this group. Before discussing the effect on the CSA, it must be recalled that, because of its shape, adamantane has four stable and equivalent positions on the carbon surface. Depending on the location of the substituent, different adsorption positions will be affected. Moreover, according to the chemical nature of the substituent, the value of the contact surface area of a given adsorption can be increased or decreased. For instance, a methyl group increases the contact area whereas a polar group (such as–OH) decreases it because the molecule is partly shifted from the carbon surface in order to maximize specific polar interactions with the mobile phase. The situation is in fact more complex in the case of polar substituents because two opposite effects may occur. Indeed, the polar group can either come close to the surface of the adsorbent if its energy of adsorption is large enough, but it can also be pushed away from the surface if its interactions with the polar mobile phase are larger than its interactions with the adsorbent. This can result in a decrease or an increase of the CSA, the actual situation depending on the polar group and on the mobile phase composition.

The effect of non-polar substituents was examined with two groups of solutes: (i) polymethyladamantan-1-ols and (ii) 2-*n*-alkyladamantan-2-ols. The substitution of a tertiary carbon by a methyl group increases the CSA by 0.22 units, independently of the organic modifier. This appears in the series adamantan-1-ol, 3-methyl-, 3,5-dimethyl- and 3,5,7-trimethyladamantan-1-ol, and 3-methyl-, 5-methyl- and 3-ethyl-5,7-dimethyladamantan-1-ol. If the methyl substitution is made on a secondary carbon, the effect on the CSA is smaller: an increase of about 0.10 units in the series adamantan-1-ol, 2-methyladamantan-1-ol. The smaller effect in this case can be ex-

plained in terms of modified adsorption positions. In tertiary substitution, three adsorption positions are modified (and the corresponding CSA values are increased), whereas only two positions are modified for secondary substitutions.

As far as alkyladamantanes are concerned, the addition of a CH_2 group increases the CSA by about 0.20 units, slightly less than that for a methyl substitution on the basic skeleton. This is in agreement with other results obtained with carbon materials[6], showing that the effect on retention of the substitution of a CH_3 group on a ring is slightly larger than that of the addition of a CH_2 group to a chain. As can be seen in Table I, the CSA increase due to a CH_2 group calculated from the retention data of hexane and decane is also very close to 0.20 units (0.194). As is well known for both adsorption and hydrophobic effects, branching decreases the effect of the alkyl chain: isobutyladamantan-2-ol is less strongly retained than *n*-butyladamantan-2-ol and its retention is less dependent on the mobile phase composition.

From the previous results it follows that a linear plot of log k' *vs.* carbon atom number, n_C, should be obtained in the different series discussed, as in a given series the adsorption energy is proportional to the contact surface area. Such linear plots are shown in Fig. 4.

In the case of polar substituents, the effect is more complex because, as previously mentioned, the mobile phase might play a rôle. Moreover, the intensity of solute–solvent interactions is also related to the position of the polar group in the solute molecule (charge density and steric hindrance of the group). It is thus difficult to predict the effect of a given substituent on the CSA.

The comparison of the CSA values of adamantane, adamantan-1-ol and -2-ol indicates that the addition of an OH group on a tertiary carbon decreases the CSA by

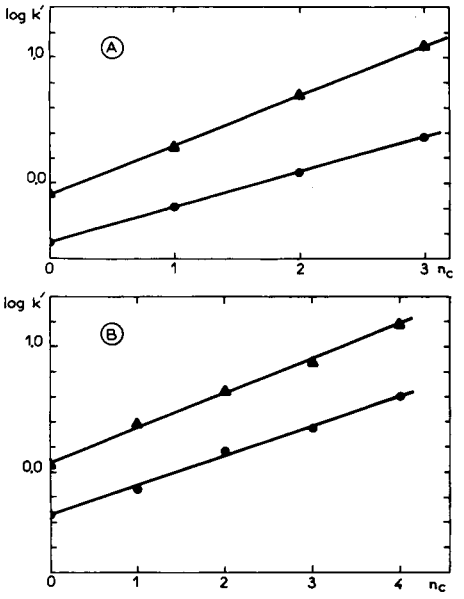


Fig. 4. Variation of log k' with the number of carbon atoms, n_C. A, Polytertiary methyladamantan-1-ols; B, 2-*n*-alkyladamantan-2-ols. ▲, Methanol–water (60:40); ●, acetonitrile–water (50:50).

0.18 units, but no significant effect if it is added at a secondary carbon. The result is similar to that obtained with a methyl group; the greater effect of tertiary substitution and the smaller CSA value for tertiary OH-substituted adamantanes can be explained by a smaller contact surface area for three adsorption positions. It must be noted that the CSA value of adamantan-1-ol is very similar to that of cyclohexanol (0.82 and 0.81 respectively). The negligible effect of a secondary OH group on the CSA is probably due to the compensation between adsorption of the OH group and interaction with the mobile phase (the charge density on the oxygen atom is larger in adamantan-1-ol than in -2-ol). The comparison between the values obtained for adamantanone 1-hydroxy- and 2-hydroxyadamantan-4-one indicates that the effect of a tertiary OH group is to decrease the CSA by 0.12 units and that of a secondary OH group to decrease it by 0.03 units. These values are very close to those obtained with the adamantanols.

The data obtained with adamantanediols show that, as it could be predicted, increasing the number of polar groups significantly decreases the CSA (*i.e.*, the CSA value for adamantane-1,4-diol is only about 50% of that of adamantane). The effect of the position of the groups is difficult to explain as the different groups may interact with each other. It can be noted, however, that the difference between the CSA values of adamantan-2-ol and -1-ol (0.18 units) is similar to that between those of adamantane-2,4-diol and -1,4-diol (0.20 units). The CSA value of adamantane-2,6-diol is close to that of adamantane-1,4-diol although there is no tertiary OH group in the first molecule. This result can perhaps be explained by the fact that, for these two compounds, all the four adsorption positions are affected instead of only three for adamantane-2,4-diol.

The contribution of a keto group can be calculated from the data for the adamantanone derivatives. The comparison between adamantane and adamantanone gives a decrease in CSA of 0.23 units; the corresponding decrease from adamantan-1-ol to 1-hydroxyadamantan-4-one and from adamantan-2-ol to 2-hydroxyadamantan-4-one is in both cases 0.25 units. Although there is a good agreement between these values, the result is nevertheless surprising as experiments with different types of solutes have indicated that: (i) the adsorption of a keto group is stronger than that of an OH group on carbon surfaces; and (ii) the keto group is generally less polar (and less hydrophobic) than the OH group[6]. We have no explanation for this behaviour.

As far as diamantanols are concerned, it is more difficult to interpret the results as the geometry of the molecule is not as simple as that of the adamantanols. If it can be assumed that the molecule has six stable adsorption positions, three are affected by the presence of a substituent on a tertiary carbon, independently of the position of this carbon. Only two positions are affected in the case of a secondary carbon. This is in agreement with experimental results (diamantan-3-ol has the largest CSA).

The tertiary carbon atoms are not equivalent and can be classified in two groups: the central carbons (1,2,6,7,11 and 12) and the extremity carbons (4,9) (*cf.*, Fig. 1). It is observed that the effect of substitution is larger for the extremity carbons: diamantan-4-ol has a smaller CSA than diamantan-1-ol. The difference between the effects associated with the two groups of tertiary carbons is rather large. The average decrease in CSA is 0.24 units for a central tertiary OH group, 0.36 units for an extremity tertiary OH group and 0.14 units for a secondary OH group.

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NEGATIVE IONS OF DICARBOXYLIC ACIDS OBSERVED BY PLASMA CHROMATOGRAPHY AND ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRY

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SUMMARY

The negative ions observed by plasma chromatography (PIC) and atmospheric pressure ionization mass spectrometry (APIMS) were compared for maleic acid, fumaric acid, and the isomeric phthalic acids. Although the product ionic species observed by PIC and APIMS are expected to be the same, based on similar ionization mechanisms and conditions, there are some exceptions. All three isomers of phthalic acid show the identical ionic species of $(M - 18)^-$ and $(M + O)^-$ in APIMS, while phthalic acid and isophthalic acid show a single ionic species with different ion mobilities in PIC. Maleic acid and fumaric acid display the same patterns of negative product ions in either PIC or APIMS. It may be concluded that if the ion survival time τ of product ion from a compound is longer than the time of PIC detection, *i.e.* $\tau \geqslant 10^{-2}$ sec, then the ion can be observed by both techniques; if $\tau \leqslant 10^{-5}$ sec then the ion can be observed only by APIMS.

INTRODUCTION

Plasma chromatography (PIC)[1] and atmospheric pressure ionization mass spectrometry (APIMS)[2] utilize a Ni-63 β-ray emitter as an ionization source at atmospheric pressure. These two techniques are virtually identical with respect to the ionization mechanism for organic compounds in the vapour phase, producting both positive and negative ions. The negative ion modes of PIC and APIMS are essentially those of an electron-capture detector (ECD) in which negative ions as well as electron standing current can be simultaneously measured.

The ECD is one of the most sensitive and selective devices available for detection in gas chromatography[3]. This detector has been widely used for the analysis of trace compounds in biomedical samples and for environmental pollutants, in spite of its anomalous responses and linearity limits[4]. Wentworth *et al.* have reported electron-capture mechanisms, electron-capture coefficients, and relative electron affinities for a

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wide range of organic compounds[5,6]. Karasek and Kane reported the oxygen effect on ECD response using the negative ion mode of PIC[7]. The mobility data of negative ions formed in PIC can be used to distinguish isomeric phthalic acids[8]. A summary of ECD response mechanisms for polynuclear aromatic compounds was reported using the negative ion mode of APIMS[9]. Most organic compounds, except some halogenated compounds, usually show less sensitive responses in the negative ion mode compared to the positive ion mode in PIC and APIMS. This lower sensitivity has been an obstacle to its use as an analytical method. However, it has been found that minor impurities, such as oxygen, in the nitrogen carrier gas appear to be involved in electron–molecule reactions displaying strong negative ion response. *p*-Chloronitrobenzene is one such example, exhibiting strong negative phenoxide ion $(M + O - Cl)^-$ with the same level as that of positive product ion in intensity[10]. This result suggests that intentional doping of electrophilic gas molecules, such as NO, O_2, SO_2 or N_2O into nitrogen carrier gas could enhance the response for organic compounds. Recently an enhancement of ECD sensitivity to non-electron-attaching compounds by addition of N_2O to the carrier gas has been reported[11].

Grimsrud and Miller reported an oxygen effect in ECD response enhancement of two or three orders of magnitude for halogenated hydrocarbons[12]. Research on the response enhancement by intentionally doping oxygen or other gases into nitrogen carrier gas has been expanded to a wide range of organic and environmentally interesting compounds. Some results for polynuclear aromatic compounds and observation on the dependence of ion signals on electrostatic fields applied to the ionization cell, as a new insight into the role of ions in influencing the measured ECD current, have been reported[13]. Meanwhile, very sensitive responses were reported with a d.c. mode of ECD using various mixtures of gases[14,15].

For these reasons it is meaningful to compare the negative product ions formed by both techniques of PIC and APIMS when adopting similar experimental conditions. It is currently thought that the resultant ions observed by both techniques are the same. We report here some comparisons for isomeric phthalic acids, and maleic and fumaric acids in forming their negative ions by these two techniques.

EXPERIMENTAL

All of the PIC data used in this study were obtained using the Beta VI plasma chromatograph. Fig. 1 shows the schematic diagram of the PIC tube. The details of the instrument and its operation have been described previously[1]. Experimental conditions for this study were: PIC tube, 150°C; inlet temperature, 150°C; carrier gas flow-rate 100 ml/min; drift gas flow-rate 400 ml/min; electric field gradient, 250 V/cm; injection and scan gate width, 0.2 msec; time base, 20 msec; recorded scan time, 2 min; ambient pressure, 724–746 torr. The carrier and drift gases were nitrogen (Linde high purity 99.996%) passed through individual stainless steel traps of 2.25-l capacity with Linde Molecular Sieve 13X to remove impurities.

The API mass spectrometer used in this study has been described in detail previously[13]. The schematic diagram of this instrument is also shown in Fig. 1. A sample can be introduced to the API source either via the injection port of a Autoprep Aerograph Model A-700 gas chromatograph using a stainless steel column (1.5 ft. × $\frac{1}{8}$ in. O.D.) packed with 3% OV-17 directly via a direct injection port connected the

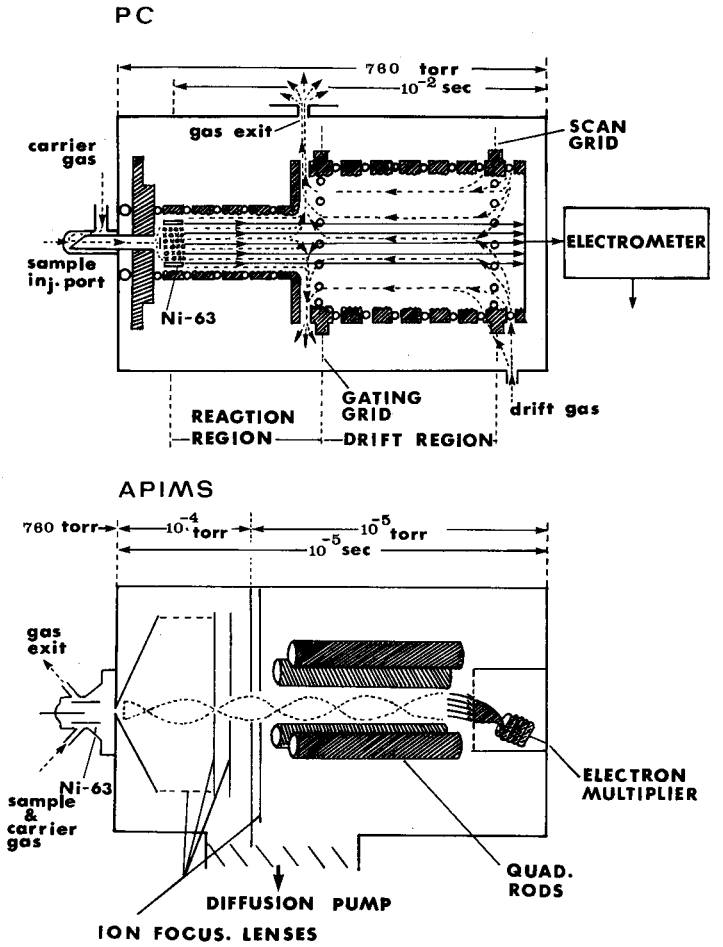

Fig. 1. A schematic diagram for PIC tube (top) and for APIMS (bottom).

API source. Alternate sample injection can also be made into a 3.7-l dilutor located between the carrier gas cylinder and the API source. Experimental conditions of the APIMS were: nitrogen carrier gas (Matheson, ultra high purity) flow-rate, 50 ml/min; injection port temperature, 140°C; API source temperature, 150°C. The electron standing current was measured with a pulse mode (pulse period 300 μsec, pulse width 2 μsec), and the individual m/z ion measurement was made by monitoring one single ion by tuning at specific m/z ion during the course of an experiment with pulse mode off.

Samples were prepared as 10^{-4}–10^{-5} g/ml solutions in ethanol and introduced directly to the PIC or APIMS system using a 1-μl syringe Alternatively, for direct injection for PIC, sample solution (1.0 μl solution) was dispensed onto a clean platinum wire, and the solvent was allowed to evaporate before introducing it into the PIC inlet system.

The phthalic and isophthalic acids were from J. T. Baker (Phillipsburg, NJ, U.S.A.). The terephthalic acid was reagent grade from BDH (Poole, Great Britain).

The maleic acid was reagent grade from Eastern Organic (Hauppauge, NY, U.S.A.). The fumaric acid was reagent grade from Aldrich (Milwaukee, WI, U.S.A.). The ethanol used as solvent was absolute (100%) ethanol for spectroscopic use from J. T. Baker.

RESULTS AND DISCUSSION

APIMS and PIC

Either a Ni-63 or a corona discharge ionization source is used as a β-ray source in both APIMS and PIC at atmospheric pressure. Schematic diagrams of both techniques are shown in Fig. 1. Sample vapour introduced to the ionization source via a GC column or a direct inlet system undergoes ion–molecule reactions with the reactant ions of $(H_2O)_nH^+$, $(H_2O)_nNO^+$, and $(H_2O)_nNH_4^+$ when nitrogen is used as a carrier gas. In the negative ion mode, sample molecules undergo electron–molecule reactions producing negative ions via associative electron capture, dissociative electron capture, or additive rearrangement reaction with negatively charged ions from trace molecules in the carrier gas. In APIMS the product ions are drawn through the same aperture via adiabatic expansion into the mass analyzer, while in PIC the product ions move through a nitrogen drift gas stream in the counter-current direction under the influence of an electric field. Positive and negative polarizing voltages are applied to observe positive and negative ions. The API sources in both techniques are always under an electric field influence; an API source is under a 107–250 V/cm field gradient in PIC. In APIMS, the field gradient is 15–25 V/cm in the d.c. model and in a 1–2 μsec pulse period of the pulse mode. The details of d.c. and pulse mode effects to the negative ions formed with polynuclear aromatic compounds in an ECD adopted APIMS have been previously reported[13]. The negative ionic species from polynuclear aromatic compounds such as anthracene, 1,2-benzanthracene, pyrene, and tetracene appear to be M^-, $(M + O - 1)^-$, and $(M + O_2 - 2)^-$ when a pulse mode is adopted in APIMS[16]. The formation mechanisms of these ions have previously been reported[9,10]. If a d.c. mode is adopted in APIMS, then neither product ion nor an ECD standing current decrease appears unless the sample size reaches the level of saturation for these polynuclear aromatics[12]. However, neither an ECD standing current decrease nor a product ion is observed in the negative ion mode of PIC, even with fairly large sample size. Presumably, the d.c. mode of the negative APIMS roughly corresponds to the API source environment of PIC except for the field gradient difference. These results indicate that electrons barely undergo electron–molecule reaction with sample species under constant electric field. Further, we could predict the following two reasons which might explain the absence of response.

(i) Electrons are drawn so rapidly to the electrode extended into the center of the ionization source due to the attraction force of the applied voltage in APIMS, and toward the ion collector due to the repulsive force in PIC, that electron-capturing sample species seldom have a chance to capture electrons or to react with them. The small portion of the ions formed by electron capture, assuming some electron-capture reaction still occurs under these conditions, might undergo recombination reactions between negative ions and positive product ions or charge neutralization reactions between negative ions and positive reactant ions, which presumably exist as space charge not only inside the wall of the ionization source cell but also along the inner wall of at least the first guard ring of the reaction region. If these assumptions are correct, the recombination or charge neutralization effect must be more profound in

PIC than in APIMS, since no such effect can be expected in the mass analyzer used in APIMS. In a PIC–MS system, the collected total ion current appears to be only 10% of the total ion current obtainable from the source[17]. Another possible path for the removal of the electron from the small portion of the negative ions formed under the conditions mentioned above is an electron decapturing process in the equilibrium state achieved between an electron and a molecular ion produced by an associative electron capture: Suppose a sample molecule "M" captures an electron via reaction 1

$$M + e \rightleftharpoons M^- \tag{1}$$

to form a negative molecular ion, M^-. This system can be considered an equilibrium mixture that depends upon the concentration of the sample, if the electron concentration is almost constant before the system reaches a saturation condition. Electron affinities (EA) of most organic compounds are reported to be below 1.0 eV and the EA of halide ions and of some nitrocompounds lies in the range 2.0–4.0 eV[6]. The negative ions formed via electron capture may be stabilized by a collision with neutral molecules or the captured electrons will be ejected if the energy level of the charged particles is quenched abruptly via a collision with neutral molecules. The reversible reaction

$$M^- \rightleftharpoons M + e \tag{2}$$

will occur. This reversible reaction can cause the decapturing of electrons from negative ions through several millions of collisions per second. The free electrons ejected thus might be detected by the PIC detedtor. This effect would be advantageous for the compounds of bulky structure such as polynuclear aromatic hydrocarbons (PAHs) having larger collisional cross-sections than those of relatively small ions such as the halide ions, Cl^-, I^-, and Br^-.

(ii) Another alternate view which might be responsible for the absence of response is the strength of electron energy in the source. The energy of electrons under PIC conditions appears to be $\sim$ 0.33 V cm^{-1} $torr^{-1}$, which corresponds to 0.58 eV at 165°C. The energy under APIMS conditions is 0.115 V cm^{-1} $torr^{-1}$ or 0.015 eV. The difference in the electron energy level between these two techniques might be responsible for the difference in ECD response for the aromatic compounds and for the lack of response from PAHs, since the EA of these compounds varies. The electron–molecule reaction is a function of the electron energy, and the electron energy is at or near the thermal energy level when no electric field is applied. However, the electron energy is considerably higher if an electric field is present[18,19]. Associative electron-capture responses were reported to be observed at 0.03 eV for both *o*-nitrotoluene and *m*-nitrotoluene[20]. The EA of naphthalene, anthracene, pyrene, benz[*a*]anthracene, and azulene were reported by Wentworth[5] to be 0.152, 0.552, 0.579, 0.696 and 0.587 eV, respectively.

The ECD conditions in APIMS used in this investigation are similar to those of the ECD used widely in ECD–gas chromatography. By applying 35 V to the ionization source of APIMS, the electron energy with a pulse mode operation is 0.115 V cm^{-1} $torr^{-1}$ or 0.015 eV, which corresponds roughly to one tenth of the reported values of electron affinities. The EA reported by Wentworth seem to be too high to be

supported by the data of ECD response obtained by ECD adopted negative APIMS[13]. These affinities appear to be close to the energy level of electrons in the PIC ionization source. However, no electron-capture response is obtained in PIC from PAHs. This discrepancy means that the reported EA values for these particular compounds might not be correct.

The ECD standing current, which is higher with the d.c. mode than with the pulse mode, and the absence of response with such structurally bulky compounds as polynuclear aromatics support view (i), because the ions formed from these compounds are expected to have slower ion mobilities. If view (ii) is responsible for the absence of response, the electron energy in PIC must be too strong to be captured by polynuclear aromatics.

Once view (i) is assumed correct, the charge neutralization reactions between positive reactant ions and the sluggish negative ions are expected to occur very rapidly based on Lovelock's model[3].

Under these circumstances, the neutralization reaction between positive and negative ions will occur and can be expressed as follows:

$$(H_2O)_nH^+ \text{ (or } MH^+) + M^- + N_2 \rightleftharpoons MH \text{ (or } M_2H) + nH_2O + N_2 \qquad (3)$$

The three-body recombination represented by eqn. 3 is the most important mechanism occurring in the range of a few torr to atmospheric pressure[21]. If the number of neutralization or recombination events is R, then eqn. 4 can be used to express the neutralization or recombination events as a unit of the number of events per second:

$$R = \alpha n^+ n^- \qquad (4)$$

where α is the neutralization or recombination coefficient, and n^+ and n^- are the number of positive and negative ions, respectively. Thomson[21] set up a model criterion for condition of recombination events between two oppositely charged particles and defined the coefficient α as follows: recombination will occur if the kinetic energy of the charged particles is at the same level as the average of the thermal energy. According to the premises suggested by Thomson, an ion pair will combine if its total relative energy ever becomes negative. De-excitation by collision with a third body is necessary to restore the kinetic energy to the average thermal value within a critical distance "r" of the other ion for recombination. The critical distance is determined by the relation of $3/2\,kT = e^2/r$ (or $r = 2e^2/3kT$), where k is the Boltzman constant, e is the electronic charge (e.s.u.), and T is the Kelvin temperature. Further, Thomson took into account collision probabilities between positive and negative ions, which are a function of the mean free paths of both positive and negative ions, and finalized the neutralization or recombination coefficient α for the pressure range from a few torr to 1 atm:

$$\alpha = \left(\frac{32\,(2\pi e^6)^{\frac{1}{2}}}{27\,(M_r)^{\frac{1}{2}}}\right)\left(\frac{1}{(kT)^{5/2}}\right)\left(\frac{1}{\lambda^+} + \frac{1}{\lambda^-}\right) \qquad (5)$$

where M_r is the reduced mass between ion and neutral gas, and λ^+ and λ^- are the mean free paths for positive and negative ions, respectively. At constant temperature,

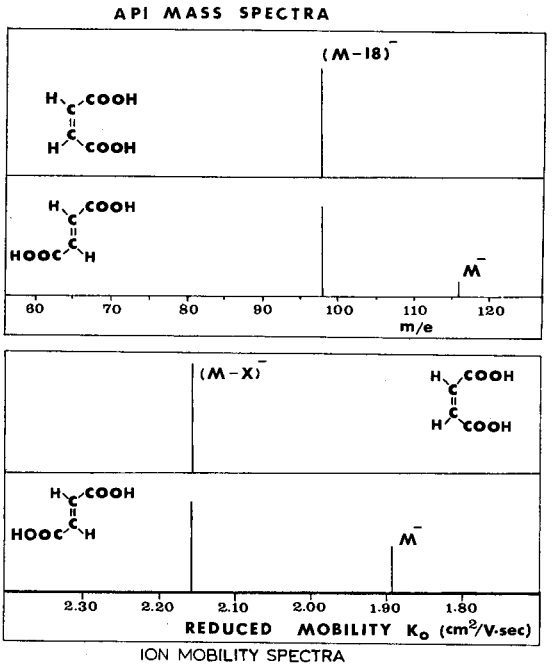

Fig. 2. Negative APIMS data for maleic and fumaric acid (top), and negative PIC ion mobility data (bottom). See the text for discussion.

α increases linearly with pressure up to 1 atm and becomes pressure independent at high pressure (see Fig. 12-4-1 in ref. 21). If the temperature is fixed, α is a function of the mean free path $\lambda^{\pm}$, which depends upon pressure. This means that a thousand-fold more recombination events will occur at atmospheric pressure than will occur at a pressure of 1 torr, and 10^7 times more than at 10^{-4} torr, which corresponds to the pressure of the ion focusing section in APIMS. It is apparent that recombination reactions between positive and negative ions cause shorter ion survival time and lower density of ions for the product ions formed in the API source. It takes $\sim 10^{-5}$ sec for the ions to reach the detector via a quadrupole mass filter in APIMS and $\sim 10^{-2}$ sec in PIC, depending on the ionic size and mass. Therefore the thousand-fold longer detection time in PIC could be responsible for the further recombination and neutralization of charges. This may mean that the charge neutralization probability is 10^7 times higher in PIC than in APIMS. Accordingly it is probable that the ions which have survival times shorter than 10^{-2} sec would not be detected in PIC, while such ions could still be detected in APIMS. If the absence of ECD response from PAHs were caused by the reason based on view (ii), a sensitive response from PAHs has to be obtained by applying a low ion polarizing voltage to the ionization source. Under these circumstances, the negative product ions of isomeric phthalic acids, maleic acid and fumaric acid which are geometric isomers were observed by both APIMS and PIC.

Ion mobility spectra of dicarboxylic acids

It is well known that phthalic acid loses one molecule of H_2O from the two

neighboring carboxylic groups to form phthalic anhydride, when the compound is heated above 150°C[22]. This type of dehydration is not believed to occur for isophthalic acid, terephthalic acid or fumaric acid, when we consider the position of the two carboxylic groups of these dicarboxylic acids and the impossible rotation mode between the two C=C carbons of fumaric acid. This means that thermal dehydration is responsible partly for the dehydrated ion $(M - 18)^-$ observed from phthalic acid and maleic acid. Fig. 2 shows the APIMS negative product ion spectra (top) and PIC ion mobility spectra (bottom) for maleic acid and its geometric isomer, fumaric acid. As one can see, the patterns of the APIMS and PIC ion mobility spectra for maleic acid and fumaric acid are identical: *i.e.* one single prominent ion $(M - 18)^-$ for maleic acid and two ion peaks, $(M - 18)^-$ and M^-, for fumaric acid. Obviously, $(M - 18)^-$ of the APIMS from maleic acid is the negative maleic anhydride ion with *m/e* 98. The $(M - 18)^-$ ion from fumaric acid is also considered to be $(M - H_2O)^-$, although the course of elimination of H_2O between the OH of the carboxyl groups and the hydrogen attached to the C=C group has not been reported. Presently, it is not clear whether one single ion of the PIC ion mobility spectra labelled as $(M - X)^-$ from maleic acid corresponds to the $(M - H_2O)^-$ of the APIMS spectra. The data of the positive ion mobility spectra of maleic and fumaric acids are available in ref. 22 as shown in Table I. No investigation has been made of the course of H_2O elimination and the accurate ionic mass formed from fumaric acid. The point we would like to make clear though is that these two geometric isomers display exactly the same pattern of spectra in both APIMS and PIC.

TABLE I

APIMS AND PIC DATA FOR MALEIC AND FUMARIC ACIDS

Compounds	*m/z*	*Ion*	*Abundance (%)*		*Reduced mobilities**
			APIMS	*PIC*	K_0 *(cm²/V sec)*
Maleic acid	98	$(M - 18)^-$	100.0	100.0	2.15
	99	$(M - Y)H^+$	–	100.0	1.99
	117	MH^+	–	20.0	1.89
Fumaric acid	98	$(M - X)^-$	100.0	100.0	2.15
	116	M^-	20.8	50.0	1.89
	99	$(M - Y)H^+$	–	100.0	1.99
	117	MH^+	–	60.0	1.89

* Calculated from $K_0 = \frac{L}{td \cdot E} \frac{273}{T} \cdot \frac{P}{760}$, where L = drift length (cm), td = drift time (sec), E = electric field gradient of drift (V), T = temp. (°K), and P = tube pressure (torr) (see ref. 22).

Fig. 3 shows a comparison of the APIMS and ion mobility spectra for isomeric phthalic acids. Unlike the spectra of maleic and fumaric acids, responses from these isomers are identical for the three isomers, while one single ion mobility peak is observed from phthalic acid and isophthalic acid with a mobility difference: from terephthalic acid, no negative ion is observed in PIC[8]; however, a prominent $(M - 18)^-$ with a weak $(M + 16)^-$ ion was observed in APIMS. No argument is

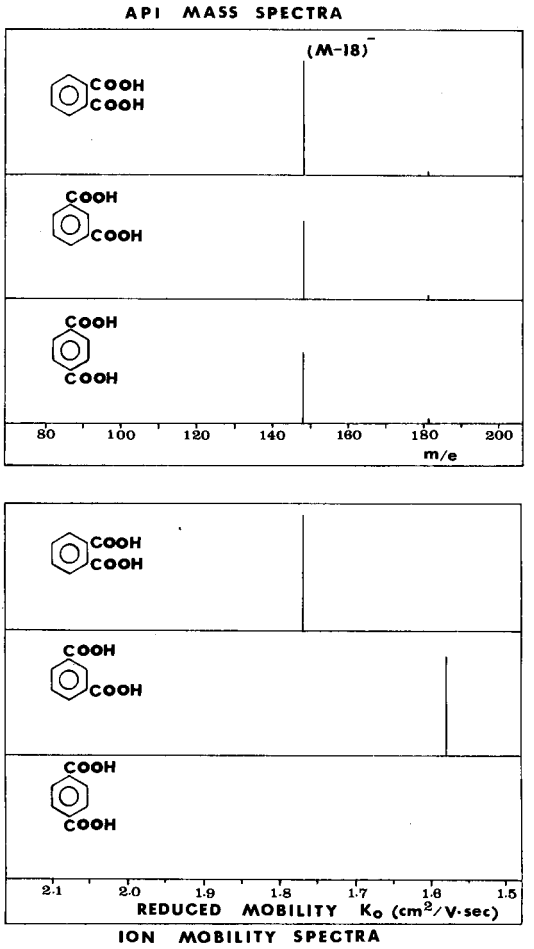

Fig. 3. Negative APIMS data (top) and PIC ion mobility data (bottom) for isomeric phthalic acids. See the text for discussion.

necessary for the $(M - H_2O)^-$ ion formation from phthalic acid; however, the observation of the identical pattern of ions from iso- and terephthalic acids forces us to conclude that H_2O elimination reactions may also occur in the two isomers by some elimination path under the conditions employed in this work. It is well known that a conversion of these three isomers into another is impossible under the conditions employed in this work, considering the synthesis routes of the three isomers.

Similar comments on the mechanism of dehydration of these compounds could be made for maleic acid and fumaric acid. What we are interested in is that the negative ion mobility data of isomeric phthalic acids obtained by PIC can be used for the identification of these three isomers[8], while APIMS data of the negative ions do not distinguish these isomers. The resultant ionic structures of the three dehydrated isomeric phthalic acids may be attributed to ion mobility differences in spite of identical ionic masses. Although Hagen[24] reported that terephthalic acid (*para* form) has a smaller collisional cross-section than that of isophthalic acid (*meta* form), it seems to

be reasonable to predict that the $(M - H_2O)^-$ ions formed from terephthalic acid apparently have a large ionic size because of the *para* position of the two carboxylic groups. As a result of random motions of the ion, the negative ion of the dehydrated terephthalic acid could be expected to have the largest collisional cross-section, which causes the highest chance of recombination with positive ions. This may mean that the $(M - H_2O)^-$ ion from terephthalic acid could be neutralized completely during the drift time of 10^{-2} sec. Indeed, the weakest intensity of this ion is observed in APIMS data as shown in Table II. The additional ionic mass and ionic radii of the $(M + 16)^-$ ion, which can be interpreted as the $(M + O)^-$ ion for phthalic acid and isophthalic acid, will also certainly be a factor for these ion mobilities, if these two different ions drift together in PIC. However, this effect should not be great when we consider the observed relative intensities between these two ions, which is less than 10%. Details of negative ions from isomeric phthalic acids are presented in Table II.

TABLE II

APIMS AND PIC DATA FOR ISOMERIC PHTHALIC ACIDS

Compound	*m/z*	*Ion*	*Abundance**		*Reduced mobility* K_0 *(cm²/V sec)*
			APIMS	*PIC*	
Phthalic acid	148	$(M - 18)^-$	100.0 (2.5K)	100.0	1.77 (ref. 8)
	166	M^-	1.7		
	182	$(M + 16)^-$	1.7		
Isophthalic acid	148	$(M - 18)^-$	100.0 (1.7K)	100.0	1.58 (ref. 8)
	166	M^-	5.3		
	182	$(M + 16)^-$	8.8		
Terephthalic acid	148	$(M - 18)^-$	100.0 (1.6K)	None	
	166	M^-	6.4		
	182	$(M + 16)^-$	6.4		

* The values in parentheses for $(M - 18)^-$ ions denote the ion intensities observed by APIMS with 10^{-9} g sample size for the three isomers.

To confirm the absence of response from terephthalic acids in PIC, careful measurements have been repeated several times with time bases of 20 msec and 50 msec, respectively, to search further for any possible heavily clustered ion (ion with slower ion mobility due to its bulky structure). However, no such ion was observed. These results again force us to predict that the ion survival time of the $(M - H_2O)^-$ ion from terephthalic acid must be shorter than 10^{-2} sec. In other words, the ion survival time, τ, of the $(M - H_2O)^-$ ion from terephthalic acid could be between 10^{-5} and 10^{-2} sec; these are the detection time limits for APIMS and PIC, respectively. This may mean that the ionic species observed by PIC reveal the truly stabilized ionic species at atmospheric pressure.

Thus we may conclude that the most probable reasons for the absence of response of polynuclear aromatics could be the low electron density at the source because of the existence of an electric field, as well as view of (ii) related to the energy of the electrons in the ionization source.

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LIQUID CHROMATOGRAPHY STUDY OF BROMINATED ANILINES AND INVESTIGATION OF PRODUCT FORMATION IN THE BROMINATION REACTION

I. ANILINES WITHOUT RING SUBSTITUENTS OR WITH ALKYL GROUPS IN THE *ortho*- AND *para*-POSITIONS

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SUMMARY

Straight- and reversed-phase liquid chromatography (LC) have been used to study product formation in the quantitative coulometric bromination of various anilines. The coulometric method yields, up to the end-point, predictable unambiguous products by exchange of hydrogen for bromine in free *ortho*- and *para*-positions. After the end-point, oxidation products may be formed from primary anilines and N-alkyl groups splitt off from secondary and tertiary anilines.

A detailed study of retention behaviour of some 70 bromoanilines in straight- and reversed-phase LC has been made. An increase in retention generally took place on the introduction of bromine into the aniline nucleus, except for the formation of *o*-bromoanilines in straight-phase LC, where retention decreases. Retention behaviour of bromoanilines in straight-phase LC is discussed in terms of base strength, steric hindrance around the nitrogen atom and solvent effects. A semi-linear relationship was found to exist between capacity factors of brominated and non-brominated anilines in both the LC systems.

INTRODUCTION

In a previous paper[1] it was shown that alkylanilines can be quantitatively brominated by a coulometric technique, based on reaction with anodically generated bromine. The advantage of the method, compared with other methods based mainly on volumetric bromination[2], is that the reaction can be controlled by means of the titration medium and by using an optimal generating current. The method can therefore be applied to a large number of anilines, even those that are usually sensitive to side-reactions such as oxidation.

The aims of this investigation were to study, by liquid chromatography (LC), product formation at various stages during the coulometric bromination, and to

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examine the retention behaviour of brominated alkylanilines in different LC systems. The choice of anilines was restricted to compounds that either have no ring substituents or contain alkyl groups in the *ortho*- and/or *para*-positions, *i.e.* 2- and 4-monoalkylanilines and 2,4- and 2,6-dialkylanilines. Primary, secondary and tertiary anilines are included. In Part II[3] *meta*-alkyl-substituted alkylanilines are considered.

EXPERIMENTAL

Apparatus

Coulometric titration. The apparatus and procedure for the coulometric bromination have been described in detail elsewhere[1,4].

Liquid chromatography. The LC pump used was a Varian Model 4100 (Varian, Palo Alto, CA, U.S.A.) and the detector was a Laboratory Data Control Model 1285 UV monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) used at 280 nm. Sample application was accomplished by a valve injector (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl loop.

Columns. The bonded-phase packing materials were the commercially available Nucleosil C_{18} (5 μm) and Nucleosil CN (5 μm) (Macherey, Nagel & Co., Düren, G.F.R.), packed by the upward-slurry packing technique[5,6]. All columns consisted of precision-bore stainless-steel tubing (200 mm × 4.4 mm I.D.) and were used at room temperature.

For accurate work it is necessary to reactivate the columns, especially the nitrile column, regularly. This was made according to recommended procedures. The stability of the columns was tested daily using a mixture of phenol, 2,6-dimethylphenol and 4-*tert.* butylphenol for the reversed-phases and a mixture of diphenylamine, triphenylamine and N-methyl-2-methylaniline for the nitrile phase.

Chemicals

Isooctane (certified ACS grade, Fisher Scientific, Fairlawn, NJ, U.S.A.), methanol (analytical-reagent grade, May & Baker, Dagenham, Great Britain), 2-propanol (*pro analysi* grade, E. Merck, Darmstadt, G.F.R.), sodium dihydrogen phosphate, $NaH_2PO_4,2H_2O$ (99%, BDH, Poole, Great Britain), disodium hydrogen phosphate, $Na_2HPO_4,2H_2O$ (according to Sörensen, E. Merck) and orthophosphoric acid (*pro analysi* grade, E. Merck) were used for preparing the LC eluents.

Acetic acid (*pro analysi* grade, E. Merck) and sodium bromide (99%, BDH) were used for the preparation of the titration media.

The anilines were of the best grade commercially available. Some of them were further purified by distillation or recrystallization. N,N-Dimethyl-2,6-dimethylaniline, N,N-diethyl-2-ethylaniline and N-ethyl-2-ethylaniline were prepared at this laboratory.

Mobile phases

Methanol–aqueous buffer was used for reversed-phase LC on the C_{18} phase, and isooctane, containing 0.2% (v/v) 2-propanol, was applied for straight-phase LC on the nitrile phase.

Methanol–aqueous buffer (70:30, v/v, pH 7.0). This buffer was made from 15 ml of 0.025 *M* Na_2HPO_4, 250 ml of 0.025 *M* NaH_2PO_4, and 618.33 ml of methanol;

the pH was adjusted to 7.0 with small amounts of orthophosphoric acid and dilute sodium hydroxide (1 *M*).

Methanol–aqueous buffer (80:20, v/v, pH 7.0). This buffer was made from 15 ml of 0.05 *M* Na_2HPO_4, 250 ml of 0.05 *M* NaH_2PO_4 and 1060 ml of methanol; the pH was adjusted as above.

Procedure

Coulometric titration. Anilines, *ca.* 20 μequiv. in 20 ml of titration medium, were coulometrically brominated as described by Truedsson and Smith[1]. All titrations were performed in medium III-1 (acetic acid–water, 60:40, v/v), bromide concentration 0.1 *M*.

Chromatography. For the reversed-phase studies, 20-μl aliquots containing *ca.* 5 nmol were removed at different stages of the titration directly from the titration vessel by means of a micro-syringe, after stopping the generating current. The samples were then injected directly on to the column. In the case of the nitrile phase studies, an extraction procedure was included in order to remove the acetic acid and water.

Extraction procedure. An aliquot (4 ml) of the titration mixture was transferred to a 50-ml separating funnel, and 5 ml of water and 5 ml of isooctane were added. After shaking, sodium hydroxide (10 *M*, 4–5 ml) was added in order to make the water phase alkaline, and the mixture was shaken again. The isooctane phase was removed and dried over sodium sulphate, and the solvent was partly evaporated with a gentle flow of nitrogen to a volume of 1–2 ml. From this solution, 20 μl were injected on to the nitrile phase column.

Capacity factor, k′. The capacity factor given is a mean value from at least three injections with a relative standard deviation of *ca.* 3%. Retention times of unretained solutes were determined by injecting *n*-hexane on the nitrile phase and aqueous sodium nitrate solution (0.05%) on the reversed phases. The capacity factor k' was calculated from the following formula

$$k' = \frac{t_R - t_0}{t_0} \tag{1}$$

where t_R is the retention time of sample and t_0 the retention time of unretained solute.

Separation factor, α. The separation factor for a pair of substances A and B is given by ratio of their capacity factors

$$\alpha_{A,B} = \frac{k'_A}{k'_B} \tag{2}$$

RESULTS AND DISCUSSION

Choice of liquid chromatographic systems

In a previous investigation[7] it was shown that valuable information about the structure of different kinds of aniline can be gained by a combination of straight- and reversed-phase LC.

For the study of brominated anilines in this work the same kinds of chromato-

graphic systems were chosen, *viz.* one straight-phase system on nitrile phase with isooctane, containing 0.2% (v/v) 2-propanol, as eluent and one reversed-phase octadecylsilane (C_{18}) system with methanol–aqueous buffer as eluent. Two eluents were used, one with methanol–aqueous buffer (70:30, v/v) and one with the proportions 80:20 (v/v). In both cases the eluents were buffered at pH 7.0 in order to improve peak symmetry and reduce the tendency to formation of double peaks.

It appeared, on varying the methanol content in the eluent, that the retention of brominated anilines on the C_{18} phase decreased when the percentage of methanol increased. In order to obtain appropriate elution times and good resolution, the composition methanol–aqueous buffer (70:30, v/v) was chosen for brominated primary anilines and the composition 80:20 (v/v) for brominated secondary and tertiary anilines. However, for comparison some primary anilines were also run with the latter eluent. On the nitrile phase, retention of brominated anilines is very much dependent on the content of 2-propanol in the isooctane eluent. On that account, the eluent must be carefully prepared and it is also essential to use dry solvents.

Introduction of sample. On the reversed-phase column, direct injection of aliquots taken from the titration vessel was carried out without any disturbances of the chromatogram or impairment in column performance. On the straight-phase system, however, an extraction procedure had to be carried out before injection, in order to remove acetic acid and water as previously described.

Product formation in coulometric bromination

In the coulometric bromination method for the titration of anilines described by the present authors[1], the reaction is carried out in a water–acetic acid medium and the reactivity is controlled by varying the water content and the bromide ion concentration and by the addition of pyridine. The reaction is believed to involve substitution with bromine at free *ortho*- and *para*-positions. For anilines with several such positions, the titration can be carried out either to the fully brominated stage or to a stage corresponding to the introduction of a smaller number of bromine atoms than the number of available free *ortho*- and *para*-positions. For a certain aniline, the outcome of a titration is dependent both on the structure and on the bromination-promoting properties of the titration medium.

In the present investigation, the aim was to study product formation during

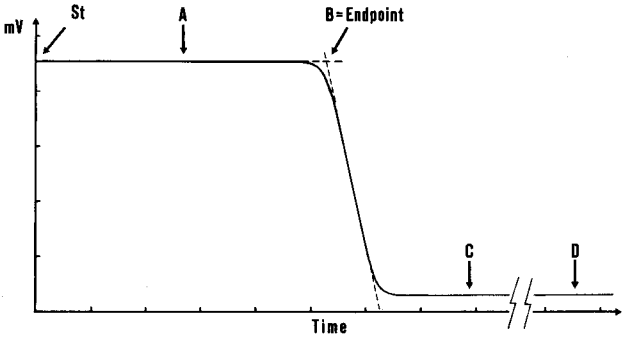

Fig. 1. Titration curve for coulometric bromination of alkylanilines. St, A, B, C and D indicate points at which samples were removed from the titration vessel.

titration in the pyridine-free medium III-1, containing acetic acid and water in the proportions 60:40 (v/v) and with a bromide concentration of 0.1 *M*. For this purpose samples were removed from the titration vessel at various stages of the titration and injected on to the LC columns either directly (C_{18} phase) or after extraction (nitrile phase). Fig. 1, which gives a typical titration curve, illustrates the procedure. Samples were removed at points indicated by A, B, C and D, and analyzed. The resulting chromatograms obtained for 2-methylaniline can be seen in Fig. 2. At point A, halfway to the end-point, the main bromination products formed are 4-bromo-2-methylaniline (c) and 4,6-dibromo-2-methylaniline (e), while very little of the 6-bromo derivative (d) appears. At the end-point (B) only the dibrominated product (e) is present. Analogous results were obtained for other 2-alkylanilines when titrated in medium III-1.

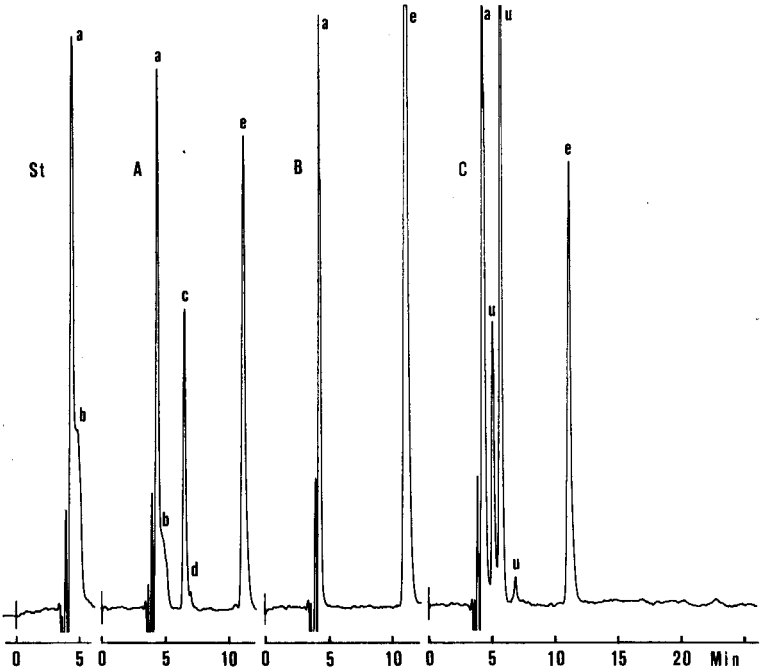

Fig. 2. Chromatograms of the product mixture after bromination of 2-methylaniline. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}; wavelength, 280 nm; volume injected, 20 μl. Peaks: a = acetic acid; b = 2-methylaniline; c = 4-bromo-2-methylaniline; d = 6-bromo-2-methylaniline; e = 4,6-dibromo-2-methylaniline; u = unknown compound. St, A, B and C refer to Fig. 1.

The results of the monobromination of different kinds of aniline up to point A on the titration curve can be gathered from Table I, which gives the k' values on the C_{18} phase of the various bromoanilines formed. As can be seen, all tested primary anilines with a free *ortho*-position yield an *ortho*-bromo derivative, while secondary and tertiary anilines furnish this derivative only when the *para*-position is occupied by an alkyl group. The increased steric hindrance at the *ortho*-positions of secondary and tertiary anilines obviously precludes the introduction of a bromine atom, unless the *para*-position is occupied.

Diphenylamine constitutes an exception from other secondary anilines in that

an *ortho*-bromo derivative is formed on bromination in medium III-1. Samples taken during the bromination show that the order of formation of bromo derivatives is 2- and 4-monobromo-, 2,4′- and 4,4′-dibromo-, 2,4,4′-tribromo- and 2,2′,4,4′-tetrabromodiphenylamine.

Triphenylamine, however, does not yield any *ortho*-bromo derivative. In this case the order of formation of bromo derivatives is 4-monobromo-, 4,4′-dibromo- and 4,4′,4″-tribromotriphenylamine.

Effect of over-bromination. All evidence points to the fact that coulometric bromination of anilines up to the end-point in the proper titration medium proceeds with the formation of bromoanilines owing to exchange of hydrogen for bromine at free *ortho*- and *para*-positions. However, it is well known that, in the analysis of anilines by volumetric bromination, some compounds are able to consume more bromine than the stoichiometric amount corresponding to the free *ortho*- and *para*-positions. Examples of side-reactions that have been suggested or proved to take place are replacement of *ortho*- and *para*-situated groups with bromine and various oxidation reactions[8–11].

The reason for the good quantitative results obtained on coulometric bromination of anilines, in comparison with results from volumetric bromination, is undoubtedly the careful choice of proper titration media and the fact that an excess of bromine is never allowed to build up before the end-point. Nevertheless, it was considered to be of interest also to examine the behaviour of the anilines in question after the end-point. For this purpose an excess of bromine was generated which depended on the kind of aniline. Thus, for primary anilines, where all vacant *ortho*- and *para*-positions are substituted with bromine at the end-point, an excess of *ca.* 50% of bromine was generated (point C in Fig. 1). Secondary and tertiary anilines, which are not fully brominated in medium III-1, were exposed to an excess of bromine corresponding to full bromination (point D in Fig. 1).

Among primary anilines, *ortho*-alkyl-substituted compounds seem to be particularly prone to react further on over-bromination. Thus, 2,6-dimethylaniline formed an over-bromination product which was investigated by means of gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy and shown to be 4-amino-4′-bromo-3,5,2′,6′-tetramethyldiphenylamine (b in Fig. 3). It is accordingly an oxidation product from 4-bromo-2,6-dimethylaniline, which latter compound is present at the end-point. This oxidation product is formed soon after the end-point; in fact, a small amount is present even before. We have also found it to arise on volumetric over-bromination and similar structures have been isolated on anodic oxidation of *p*-chloroaniline[12].

Further generation of bromine in the reaction mixture of 2,6-dimethylaniline yielded another product which, on the nitrile phase, was eluted nearer the front than the diphenylamine above (a in Fig. 3). It was tentatively identified as 4,4′-dibromo-2,6,2′,6′-tetramethylhydrazobenzene, a type of compound which has been reported to be formed on anodic oxidation of anilines[12]. As shown by the small peaks (u in Fig. 3), other over-bromination products are formed, the nature of which, however, is as yet unknown.

On over-bromination of 2-methylaniline, some early peaks of unknown origin appear at separation on the C_{18} phase (u in Fig. 2C). They are formed from 2-methyl-4,6-dibromoaniline (e) and spectral evidence indicates that their structure is quin-

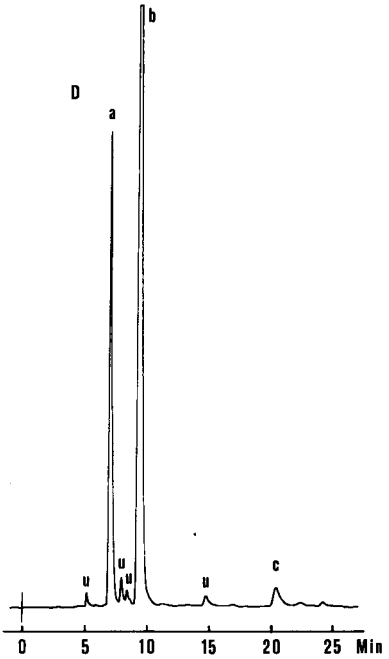

Fig. 3. Chromatogram of the product mixture after over-bromination of 2,6-dimethylaniline. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}; wavelength, 280 nm; volume injected, 20 µl. Peaks: a = 4,4′-dibromo-2,6,2′,6′-tetramethylhydrazobenzene; b = 4-amino-4′-bromo-3,5,2′,6′-tetramethyldiphenylamine; c = 4-bromo-2,6-dimethylaniline; u = unknown compound. D refers to Fig. 1.

oidic. These peaks are late on the nitrile phase in contrast to the substituted diphenylamine and hydrazobenzene, previously discussed in connection with over-bromination of 2,6-dimethylaniline (a and b in Fig. 3).

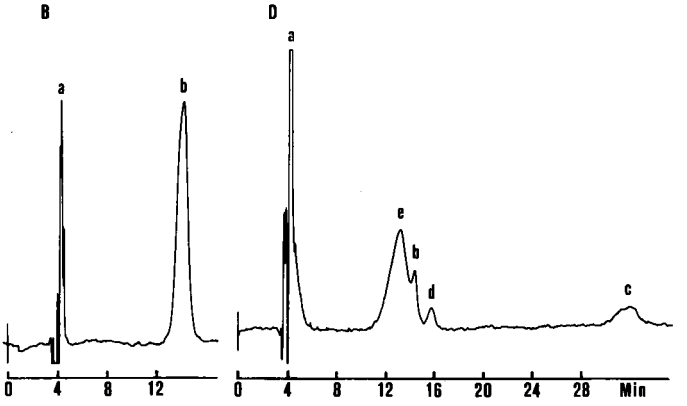

Fig. 4. Chromatogram of the product mixture after bromination of N,N-dimethyl-2-methylaniline. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}; wavelength, 280 nm; volume injected, 20 µl. Peaks: a = acetic acid; b = N,N-dimethyl-2-methyl-4-bromoaniline; c = N,N-dimethyl-2-methyl-4,6-dibromoaniline; d = N-methyl-2-methyl-4,6-dibromoaniline; e = 2-methyl-4,6-dibromoaniline. B and D refer to Fig. 1.

TABLE I

CAPACITY FACTORS AND SEPARATION FACTORS FOR *ortho*- AND *para*-ALKYL-SUBSTITUTED ANILINES AND THEIR BROMINATION PRODUCTS IN THE REVERSED-PHASE LC SYSTEM

Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20 and 70:30*, v/v, pH 7.0)

No.	*Aniline (substituent)*	*Capacity factor, k'*						*Separation factor, α*					
		Non-brominated	*Monobrominated*		*Dibrominated*		*Tri-brominated*	*Mono-/non-*		*Di-/mono-ortho-*		*Di-/mono-para*	*Tri-/di-*
			Ortho	*Para*	*Di-ortho*	*Ortho-para*		*Ortho*	*Para*	*Di-ortho*	*Ortho-para*	*Ortho-para*	
1	None*	0.55	1.33	1.16		3.33	8.87	2.42	2.11		2.50	2.87	2.66
1	None	0.41					3.48						
2	2-Methyl*	0.85	2.20	1.82		5.68		2.59	2.14		2.58	3.12	
2	2-Methyl	0.55	1.10	0.96		2.35		2.00	1.75		2.14	2.45	
3	2-Ethyl*	1.26	3.14	2.54		7.87		2.49	2.02		2.51	3.10	
4	2-Isopropyl*	1.69	4.20	3.33		10.2		2.49	1.97		2.43	3.06	
5	4-Methyl*	0.86	2.01		5.36			2.34		2.67			
6	4-Ethyl*	1.26	3.04		8.25			2.41		2.71			
7	4-Isopropyl*	1.80	4.08		10.8			2.27		2.65			
8	4-*n*-Butyl*	3.29	7.84		21.5			2.38		2.74			
9	2,4-Dimethyl*	1.25	3.36					2.69					
10	2-Methyl-4-butyl*	5.02	12.7					2.53					
11	2,6-Dimethyl*	1.31		2.89					2.21				
11	2,6-Dimethyl	0.78		1.39					1.78				
12	N-Methyl	0.68		1.25		3.04	5.14		1.84			2.43	1.69
13	N-Ethyl	0.87		1.57		4.39	7.18		1.80			2.80	1.64
14	N-Propyl	1.23		2.26		6.46	11.3		1.84			2.86	1.75

15	N-*n*-Butyl	1.69		2.79		8.87	15.3		1.65			3.18	1.72
16	N-Phenyl	1.52	3.20	2.79	4.89**	4.71***	8.75[§]	2.11	1.84				
17	N-Benzyl	1.50		2.61		6.55	12.3		1.74			2.51	1.88
18	N-Methyl-2-methyl	0.97		1.83		3.80			1.89			2.08	
19	N-Ethyl-2-methyl	1.34		2.48		5.30			1.85			2.14	
20	N-Ethyl-2-ethyl	1.86		3.23		6.90			1.74			2.14	
21	N-Methyl-4-methyl	0.90	2.17		2.95			2.41		1.36			
22	N-Ethyl-4-methyl	1.15	3.26		4.20			2.83		1.29			
23	N,N-Dimethyl	1.35		2.45		3.64	5.02		1.81			1.49	1.38
24	N,N-Diethyl	2.32		4.02		7.06			1.73			1.76	
25	N,N-Diphenyl	7.40		13.0		21.4***	31.9[§§]		1.76			1.65	1.49
26	N,N-Dimethyl-2-methyl	1.76		3.20		8.91			1.82			2.78	
27	N,N-Diethyl-2-methyl	3.45		6.40		16.2			1.86			2.53	
28	N,N-Diethyl-2-ethyl	5.21		8.94		21.8			1.72			2.44	
29	N,N-Dimethyl-4-methyl	1.80	2.32		7.37			1.29		3.18			
30	N,N-Diethyl-4-methyl	2.95	4.61		13.5			1.56		2.93			
31	N,N-Dimethyl-2,6-dimethyl	4.20		7.84					1.87				

* 70:30 eluent.
** 2,4′-Dibrominated.
*** 4,4′-Dibrominated.
[§] 2,4,4′-Tribrominated; 2,4,2′,4′-tetrabrominated; $k' = 2.03$.
[§§] 4,4′,4″-Tribrominated.

para-Alkyl-substituted primary anilines show a far better stability against excess of bromine than *ortho*-substituted. The 4-alkylanilines, for example, gave essentially the same chromatogram at 50% over-bromination as at the end-point.

Secondary and tertiary anilines of most of the kinds studied in this work are not fully brominated at the end-point in medium III-1, but still contain vacant *ortho*-positions. The result of over-bromination is that these free positions are substituted with bromine, producing the fully brominated aniline. However, this reaction cannot be utilized for quantitative purposes. The fully brominated secondary and tertiary anilines are very sensitive to further excess of bromine and react with loss of N-alkyl groups to the corresponding primary and secondary anilines (Fig. 4). An exception from this reaction order was constituted by N,N-diethylaniline. This compound is present as the 4-bromo derivative at the end-point and, on additional generation of bromine, one of the N-ethyl groups is split off, before further bromination takes place.

Retention behaviour of brominated anilines

Reversed-phase chromatography. The retention of solutes in reversed-phase chromatography is determined primarily by dispersion forces between the solute and the stationary phase and by the solubility in the mobile phase[13–15]. The size and shape

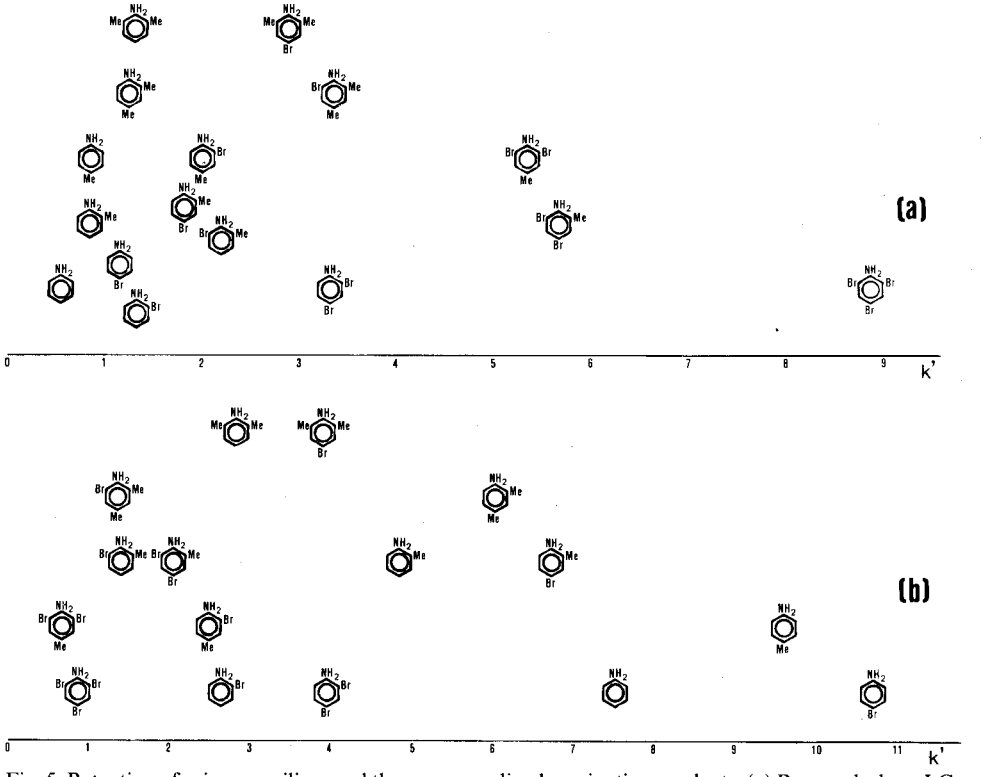

Fig. 5. Retention of primary anilines and the corresponding bromination products. (a) Reversed-phase LC. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. (b) Straight-phase LC. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane; 45 ml h^{-1}. Me = Methyl.

of the molecule is of importance for the magnitude of the dispersion forces. Thus, it has been shown that the retention of primary, secondary and tertiary anilines in reversed-phase chromatography is mainly determined by the total alkyl carbon number, representing the sum of the nuclear and N-substituted alkyl groups[7].

The introduction of bromine into the aniline nucleus gives a considerable increase in retention in reversed-phase chromatography (Table I and Figs. 5a and 6a). The great molar volume of the bromine atom is considered to be of special importance for this change in retention, decreasing the solubility of the bromoanilines in the eluent[16].

There exists a semi-linear relationship between k' values of the original anilines on one hand, and k' values of the corresponding mono-, di- and tribrominated derivatives, respectively, on the other hand, as demonstrated by Fig. 7.

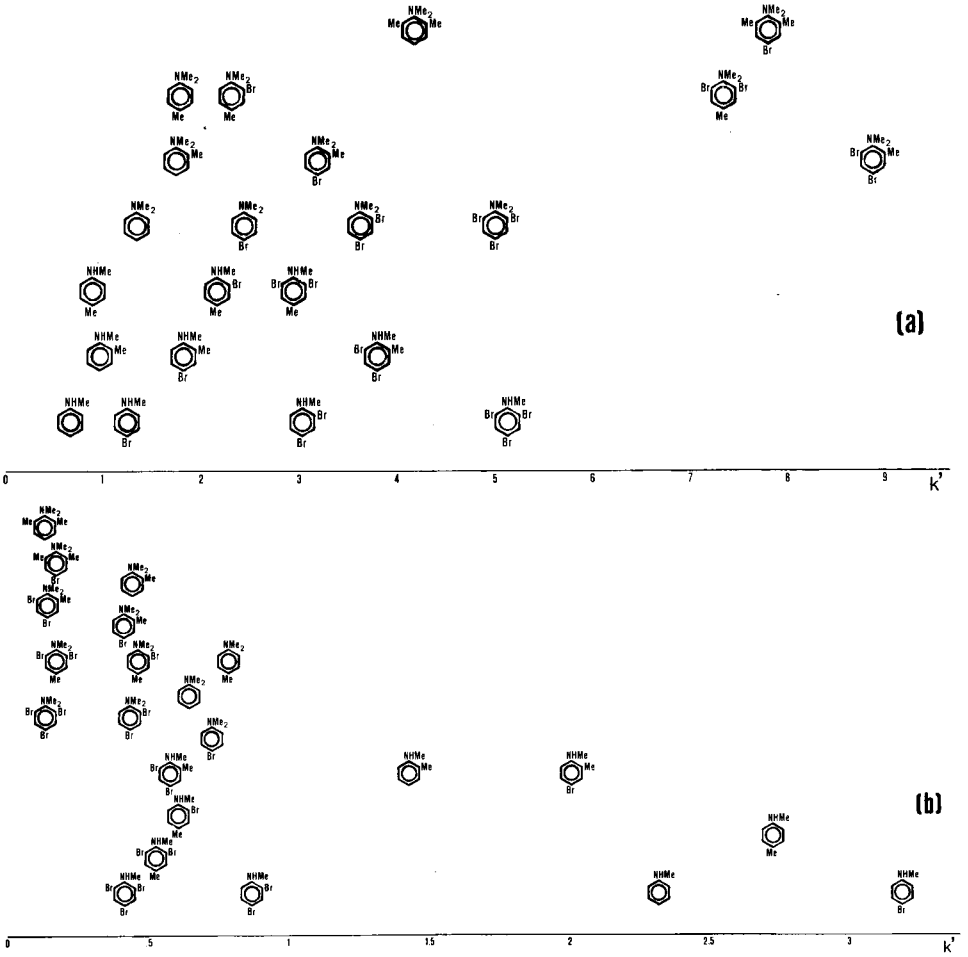

Fig. 6. Retention of secondary and tertiary anilines and the corresponding bromination products. (a) Reversed-phase LC. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. (b) Straight-phase LC. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}.

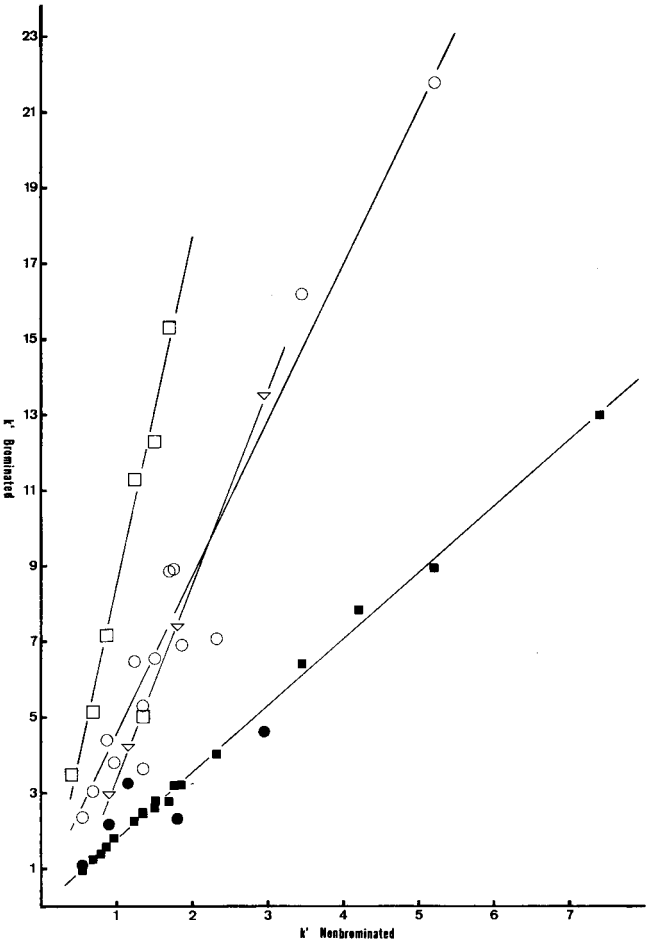

Fig. 7. Relationship between capacity factor, k', for alkylanilines and the corresponding bromination products in reversed-phase LC. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. ■ = Mono-*para*-brominated; ● = mono-*ortho*-brominated; ○ = *ortho-para*-brominated; ▽ = di-*ortho*-brominated; □ = tribrominated.

(a) Primary anilines. When bromine is substituted into aniline itself, or into a 2- or 4-alkylaniline, retention on the C_{18} phase increases with the number of bromine atoms entering. This is evident from the values of the separation factors, α, given in Table I. Among monobrominated anilines, the *para*-bromo-isomer is eluted before the corresponding *ortho*-bromo-isomer. Because *o*-bromoaniline, for example, is a weaker base than *p*-bromoaniline, it seems that the elution order is governed by the base strength, the least basic amine being eluted last. It is of interest to note that the same rule applies to *o*- and *p*-bromophenol on the C_{18} phase[17]. In this case *p*-bromophenol, which is the strongest acid, is eluted last. These facts are in accordance with the hydrophobic theory put forward by Horváth *et al.*[18] for the interaction between solutes and hydrocarbonaceous bonded stationary phases, using mixtures of water and organic solvents as mobile phases.

The retention effect of introducing bromine into a primary aniline is demonstrated in Fig. 8a and b, where the separation factors, α, for the monobrominated aniline and the aniline, respective the di- and mono-brominated anilines are plotted against carbon number. As can be seen, the increase in retention on mono-*ortho*-bromination is greater than on mono-*para*-bromination (Fig. 8a).

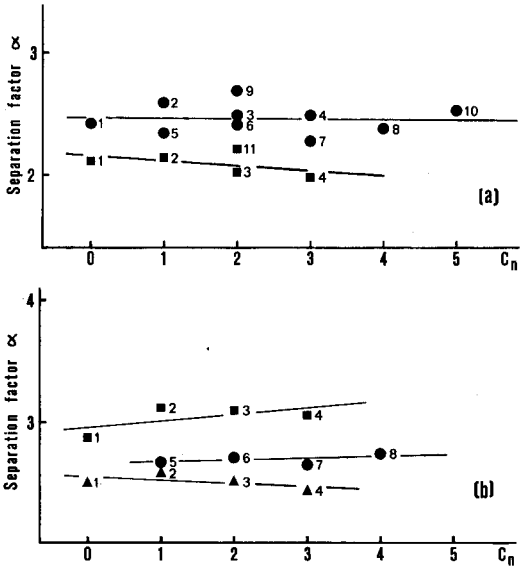

Fig. 8. Relationship between the separation factor, α, for monobrominated to non-brominated and for dibrominated to monobrominated primary alkylanilines, and alkyl carbon number. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (70:30, v/v, pH 7.0), 45 ml h^{-1}. (a) Mono-/non-brominated primary alkylaniline: ■, *para*-/non-brominated; ●, *ortho*-/non-brominated. (b) Di-/monobrominated primary alkylaniline: ▲, *ortho-para*-/*ortho*-brominated; ●, di-*ortho*-/*ortho*-brominated; ■, *ortho-para*-/*para*-brominated. The numbers refer to Table I.

On further bromination of the originally formed *o*-bromoanilines, either *o,p*- or *o,o*-dibromoanilines are formed. As shown by Fig. 8b, the separation factors are slightly higher for the *o,o*-dibromoanilines, which is in accordance with the results from the monobromination. Dibromination of the *p*-bromoanilines yields only one product *viz. p,o*-dibromoanilines. In this case the separation factor for the di- and monobromination products is distinctly higher than for the above-mentioned series of dibrominated anilines.

The difference between, and relative constancy of, the separation factors for compounds formed on mono- and dibromination of primary anilines, makes it possible to predict the order of elution of bromination products formed on coulometric bromination of this kind of anilines, thereby facilitating the interpretation of reversed-phase chromatograms. The linear relationship between k' values of brominated and non-brominated primary anilines is also of value for this purpose.

The elution order on the C_{18} phase of aniline and some primary methylanilines and their bromination products is demonstrated in Fig. 5a, which also gives a further illustration of the retention rules previously discussed.

(b) Secondary and tertiary anilines. As for primary anilines, there exists a semi-linear relationship between k' values of non-brominated anilines and k' values of mono-, di- and tribrominated analogues, respectively (Fig. 7). However, it is obvious that certain compounds show a considerable deviation from a linear relationship.

The plot of separation factors against carbon numbers in Fig. 9a and b gives information about the retention change caused by the introduction of one, two and three bromine atoms, respectively, into a secondary aniline. As previously established for primary anilines, the increase in retention on mono-*ortho*-bromination is greater than that on mono-*para*-bromination (Fig. 9a). On further bromination of *o*-bromoanilines, *o*,*o*-dibromoanilines are formed. The increase in retention on this bromination is smaller than when a *p*-bromoaniline is brominated to a *p*,*o*-dibromoaniline, just as for the corresponding primary anilines. For the secondary *p*,*o*-dibromoanilines two different series can be discerned, *viz.* compounds with or without an *ortho*-situated alkyl group. The separation factors for the former series are lower and do not change with the carbon number. For the latter series the separation factor increases linearly with the carbon number, *i.e.* with the size of the N-alkyl group, when the first *ortho*-situated bromine atom is introduced, but remain constant

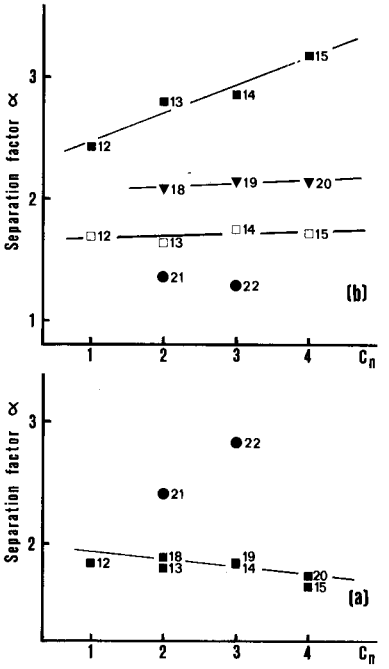

Fig. 9. Relationship between the separation factor, α, for monobrominated to non-brominated, dibrominated to monobrominated and tribrominated to dibrominated secondary alkylanilines, and alkyl carbon number. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. (a) Mono-/non-brominated secondary alkylaniline: ■, *para*-/non-brominated; ●, *ortho*-/non-brominated. (b) Di-/monobrominated and tri-/*ortho*-*para*-brominated secondary alkylanilines: ●, di-*ortho*-/*ortho*-brominated; ▼, *ortho*-*para*-/*para*-brominated (*ortho*-alkyl-substituted); ■, *ortho*-*para*-/*para*-brominated (non-*ortho*-alkyl-substituted); □, tri-/*ortho*-*para*-brominated. The numbers refer to Table I.

when, on tribromination, the second *ortho*-standing bromine atom is substituted (Fig. 9b).

For brominated tertiary anilines the retention pattern on the C_{18} phase is somewhat different from that of primary and secondary anilines. Thus, for the latter two groups the greatest change in retention, on monobromination, occurred on formation of *o*-bromoanilines from anilines, and the smallest change, on dibromination, occurred on formation of *o,o*-dibromoanilines from *o*-bromoanilines. For tertiary anilines the reverse is true as shown by Fig. 10a and b. There is also a reversal of the separation factors for the two series formed on *ortho*-bromination of *p*-bromoanilines.

It has been stated by Locke[15] that for compounds of similar type, selectivity in reversed-phase chromatography is primarily determined by the eluent and that elution order is in the inverse order of solute solubilities in the moving phase. However, it is uncertain to what extent the actual anilines should be regarded as being of "similar type", and it is more likely that the disparity in screening and steric hindrance between different *ortho*-brominated anilines affects the dispersion forces acting upon the molecule as well as its hydrophobicity.

The elution order on the C_{18} phase of some secondary and tertiary anilines and their bromination products is demonstrated in Fig. 6a, which also gives a further illustration of the retention rules discussed above.

Straight-phase chromatography. In a previous work[7], it was shown that the

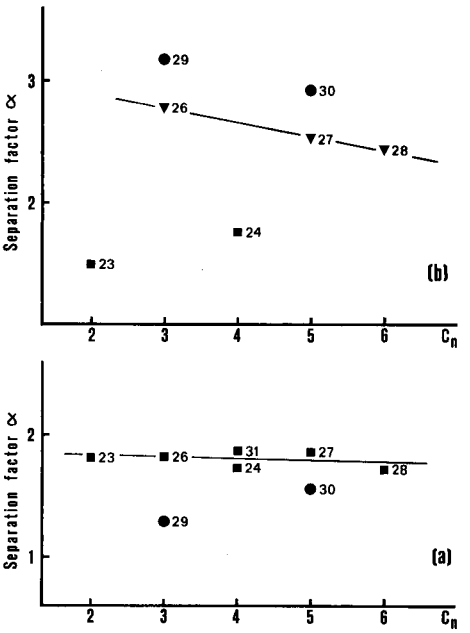

Fig. 10. Relationship between the separation factor, α, for monobrominated to non-brominated and dibrominated to monobrominated tertiary alkylanilines, and alkyl carbon number. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. (a) Mono-/non-brominated tertiary alkylaniline; ●, *ortho*-/non-brominated; ■, *para*-non/brominated. (b) Di-/monobrominated tertiary alkylaniline: ■, *ortho-para*-/*para*-brominated (non-*ortho*-alkyl-substituted); ▼, *ortho-para*-/*para*-brominated (*ortho*-alkyl-substituted); ●, di-*ortho*-/*ortho*-brominated. The numbers refer to Table I.

TABLE II

CAPACITY FACTORS AND SEPARATION FACTORS FOR *ortho-* AND *para*-ALKYL-SUBSTITUTED ANILINES AND THEIR BROMINATION PRODUCTS IN THE STRAIGHT-PHASE LC SYSTEM

Column, Nucleosil CN; eluent: 0.2% (v/v) 2-propanol in isooctane

No.	*Aniline (substituent)*	*Capacity factor, k'*						*Separation factor, α*					
		Non-brominated	*Monobrominated*		*Dibrominated*		*Tri-brominated*	*Mono-/non-*		*Di-/mono-ortho-*		*Di-/mono-para-*	*Tri-/di-*
			Ortho	*Para*	*Di-ortho*	*Ortho-para*		*Ortho*	*Para*	*Di-ortho*	*Ortho-para*	*Ortho-para*	
1	None	7.53	2.65	10.7		3.96	0.87	0.35	1.42		1.49	0.37	0.22
2	2-Methyl	4.84	1.41	6.74		2.06		0.29	1.39		1.46	0.31	
3	2-Ethyl	3.65	1.13	5.20		1.57		0.31	1.42		1.39	0.30	
4	2-Isopropyl	3.14	0.93	4.45		1.28		0.30	1.42		1.38	0.29	
5	4-Methyl	9.62	2.50		0.67			0.26		0.27			
6	4-Ethyl	8.72	2.26		0.60			0.26		0.27			
7	4-Isopropyl	8.87	2.16		0.57			0.24		0.26			
8	4-*n*-Butyl	8.72	2.11		0.54			0.24		0.26			
9	2,4-Dimethyl	6.04	1.36					0.23					
10	2-Methyl-4-butyl	5.07	1.20					0.24					
11	2,6-Dimethyl	2.83		3.91					1.38				
12	N-Methyl	2.32		3.19		0.87	0.42		1.38			0.27	0.48
13	N-Ethyl	1.49		1.98		0.51	0.29		1.33			0.26	0.57
14	N-Propyl	1.06		1.50		0.41	0.25		1.42			0.27	0.61
15	N-*n*-Butyl	0.90		1.27		0.37	0.23		1.41			0.29	0.62

16	N-Phenyl	2.50	0.61	6.89	0.87*	4.01**	1.04***	0.24	2.76				
17	N-Benzyl	1.65		2.62		1.43	0.43		1.59			0.55	0.30
18	N-Methyl-2-methyl	1.43		2.01		0.58			1.41			0.29	
19	N-Ethyl-2-methyl	0.82		1.11		0.39			1.35			0.35	
20	N-Ethyl-2-ethyl	0.67		0.90		0.32			1.34			0.36	
21	N-Methyl-4-methyl	2.73	0.61		0.53			0.22		0.87			
22	N-Ethyl-4-methyl	1.72	0.39		0.38			0.23		0.97			
23	N,N-Dimethyl	0.65		0.73		0.44	0.14		1.12			0.60	0.32
24	N,N-Diethyl	0.50		0.48		0.24			0.96			0.50	
25	N,N-Diphenyl	0.39		0.52		0.46**	0.41 §		1.33			0.88	0.89
26	N,N-Dimethyl-2-methyl	0.45		0.42		0.15			0.93			0.36	
27	N,N-Diethyl-2-methyl	0.29		0.22		0.13			0.76			0.59	
28	N,N-Diethyl-2-ethyl	0.21		0.18		0.11			0.86			0.61	
29	N,N-Dimethyl-4-methyl	0.79	0.47		0.18			0.59		0.38			
30	N,N-Diethyl-4-methyl	0.90	0.24		0.15			0.27		0.63			
31	N,N-Dimethyl-2,6-dimethyl	0.14		0.18					1.29				

* 2,4′-Dibrominated.
** 4,4′-Dibrominated.
*** 2,4,4′-Tribrominated; 2,4,2′,4′-tetrabrominated; $k' = 0.44$.
§ 4,4′,4″-Tribrominated.

elution order on the nitrile phase of different kinds of anilines is tertiary, secondary and primary anilines and that the retention within each group is mainly determined by the number and size of *ortho*-situated alkyl groups and by alkyl groups substituted at the nitrogen atom.

The introduction of bromine into the aniline nucleus changes the elution order, and it is no longer possible to decide straight off, on the basis of k' values, if a brominated aniline is primary, secondary or tertiary. This fact is evident from the k' values presented in Table II. However, it can be seen that within each subgroup of brominated anilines, *i.e.* mono-*ortho*-bromo, mono-*para*-bromo-, etc., the k' values are to a great extent dissimilar and often allow the distinction between primary, secondary and tertiary bromoanilines of different kinds (Table III).

TABLE III

COMPARISON OF CAPACITY FACTORS, k', FOR BROMINATED PRIMARY, SECONDARY AND TERTIARY ALKYLANILINES IN THE STRAIGHT-PHASE LC SYSTEM

Column, Nucleosil CN; eluent: 0.2% (v/v) 2-propanol in isooctane.

Substituent	k'		
	Primary anilines	*Secondary anilines*	*Tertiary anilines*
Ortho-bromo	0.93– 2.65	0.39–0.61	0.24–0.47
Para-bromo	4.45–10.7	0.90–3.19	0.18–0.73
Di-*ortho*-bromo	0.54– 0.67	0.38–0.53	0.15–0.18
Ortho-para-dibromo	1.28– 3.96	0.32–0.87	0.11–0.44
Tribromo	0.87	0.23–0.42	0.14

The most striking nitrile phase retention effect of the introduction of bromine into an aniline is the great decrease in retention (with some exceptions) on *ortho*-bromination. This effect is primarily thought to be caused by steric hindrance due to the bromine atom, which restricts the interaction between the amino group and the nitrile group in the bonded phase. The substitution of bromine into the *para*-position of an aniline leads to either an increase in retention or to a small decrease, depending on the kind of aniline. This fact is further illustrated in Figs. 5b and 6b, which give the elution order on the nitrile phase of aniline and some methylanilines and their bromination products.

The semi-linear relationship between k' values of brominated and original anilines, previously established for the C_{18} phase, is also valid for the nitrile phase (Fig. 11). However, because of the increased selectivity of the nitrile phase, *o*-bromo- and *p*-bromoanilines fall on different lines and the same is true for *o,o*-dibromo- and *o,p*-dibromoanilines. The diagram in Fig. 11 is thus considerably more structurally informative than the corresponding diagram for the C_{18} phase in Fig. 7.

(a) Primary anilines. In Fig. 12a and b the separation factors for the brominated primary anilines on the nitrile phase are plotted against carbon numbers. The great difference in retention behaviour on *ortho*- and *para*-bromination, respectively, is clearly demonstrated.

(b) Secondary anilines. Mono-*ortho*- and mono-*para*-bromination of secondary anilines give rise to a similar separation factor picture as for primary anilines (Fig.

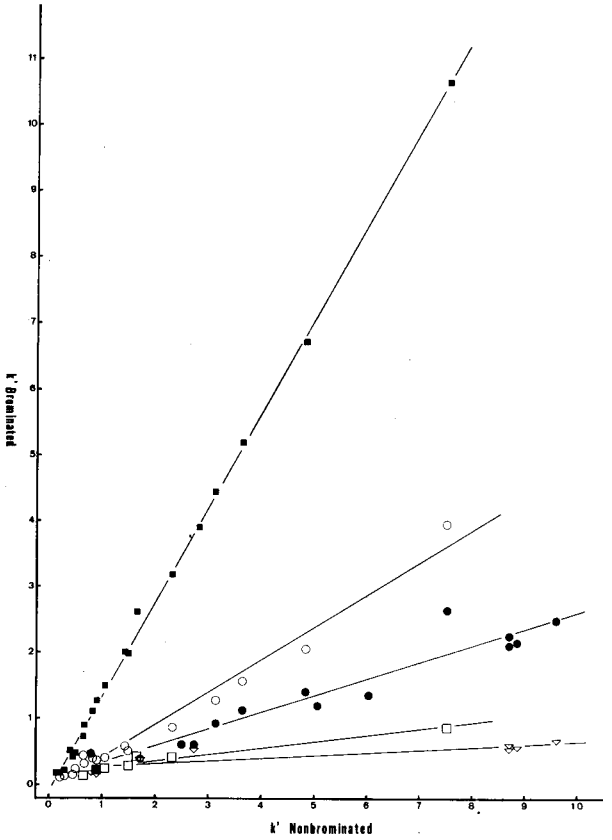

Fig. 11. Relationship between capacity factor, k', for alkylanilines and the corresponding bromination products in straight-phase LC. Column; Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. ■ = Mono-*para*-brominated; ● = mono-*ortho*-brominated; ○ = *ortho*-*para*-brominated; ▽ = di-*ortho*-brominated; □ = tribrominated.

13a). However, on introduction of a second and a third bromine atom the picture changes (Fig. 13b). While the lines for the conversion of *o*-bromo- to *o*,*o*-dibromo- and *p*-bromo- to *p*,*o*-dibromoanilines coincide for primary anilines, they are well separated for secondary anilines, and the retention change is far less for the former series. Hence, when 2-bromo-4-methyl-N-ethylaniline (No. 22) is brominated to 2,6-dibromo-4-methyl-N-ethylaniline the decrease in retention is very small.

The same tendency to a diminished change in retention on *ortho*-bromination of a secondary *o*-bromoaniline is shown on *ortho*-bromination of secondary *o*,*p*-dibromoanilines. Thus, when a third bromine atom is introduced into the *ortho*-position of these compounds the decrease in retention is considerably less than when 2,4-dibromoaniline is brominated to 2,4,6-tribromoaniline (Table II).

(c) Tertiary anilines. Most notable on bromination of tertiary anilines, is the behaviour on mono-*para*-bromination. On mono-*para*-bromination of primary and secondary anilines, *p*-bromoanilines with a greater retention than the original aniline resulted, but mono-*para*-bromination of tertiary anilines produced *p*-bromoanilines

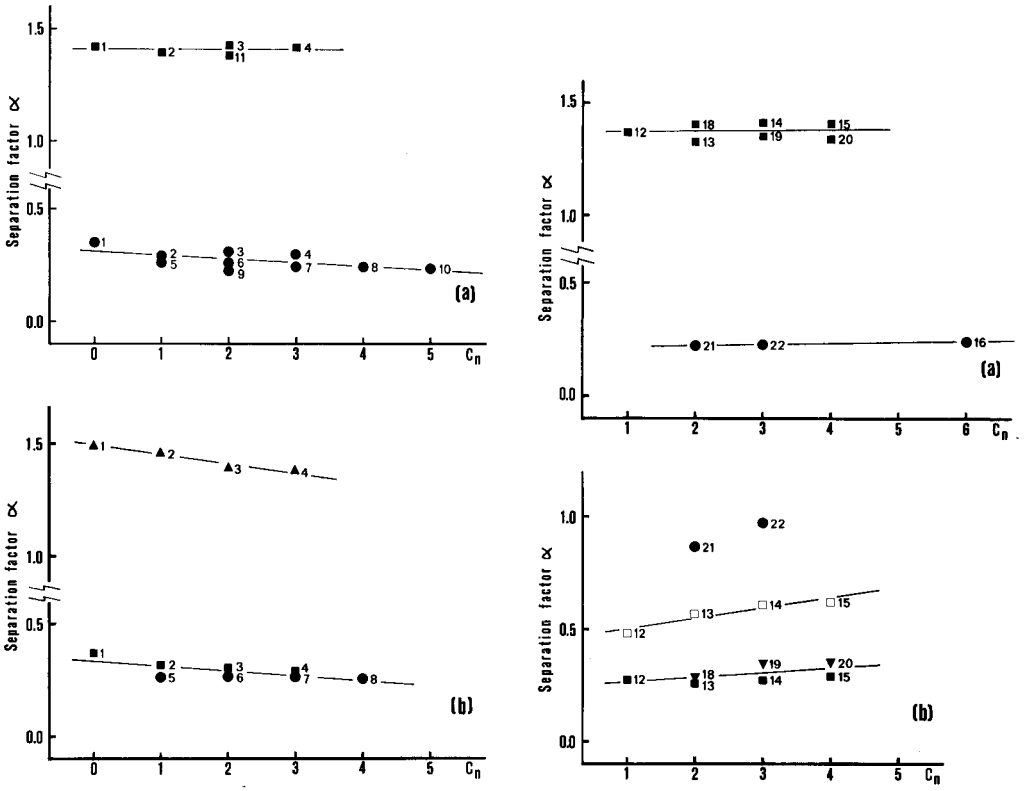

Fig. 12. Relationship between the separation factor, α, for monobrominated to non-brominated and dibrominated to monobrominated primary alkylanilines, and alkyl carbon number. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. (a) Mono-/non-brominated primary alkylaniline: ●, *ortho*-/non-brominated; ■, *para*-/non-brominated. (b) Di-/monobrominated primary alkylaniline: ●, di.*ortho*/*ortho*-brominated; ■, *ortho*-*para*-/*para*-brominated; ▲, *ortho*-*para*-/*ortho*-brominated. The numbers refer to Table II.

Fig. 13. Relationship between the separation factor, α, for monobrominated to non-brominated, dibrominated to monobrominated and tribrominated to dibrominated secondary alkylanilines, and alkyl carbon number. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. (a) Mono-/non-brominated secondary alkylaniline: ●, *ortho*-/non-brominated; ■, *para*-/non-brominated. (b) Di-/monobromined and tri-/*ortho*-*para*-brominated secondary alkylanilines: ●, di-*ortho*-/*ortho*-brominated; ■, *ortho*-*para*-/*para*-brominated (non-*ortho*-alkyl-substituted); ▼, *ortho*-*para*-/*para*-brominated (*ortho*-alkyl-substituted); □, tri-/*ortho*-*para*-brominated. The numbers refer to Table II.

which travelled either more slowly or more rapidly than the original aniline (Fig. 14a). The reason for this is discussed below.

Causes of retention change on bromination. In a previous study on retention behaviour of alkylanilines in straight-phase chromatography it was shown that retention was mainly governed by base strength and by the substitution pattern around the nitrogen atom[7]. This is partly true also for bromoanilines. Thus, the decrease in retention following *ortho*-bromination is considered to be due to a decrease in base strength and to increased steric hindrance to interaction between the amino group and the bonded-phase nitrile group.

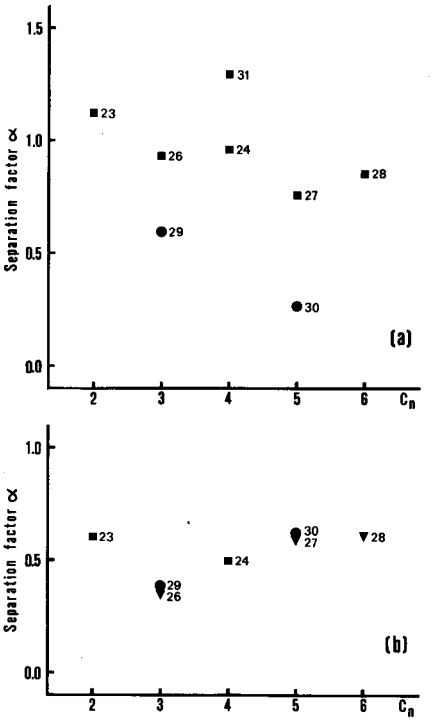

Fig. 14. Relationship between the separation factor, α, for monobrominated to non-brominated and dibrominated to monobrominated tertiary alkylanilines, and alkyl carbon number. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. (a) Mono-/non-brominated tertiary alkylaniline: ●, *ortho*-/non-brominated; ■, *para*-/non-brominated. (b) Di-/monobrominated tertiary alkylaniline: ●, di-*ortho*-/*ortho*-brominated; ■, *ortho*-*para*-/*para*-brominated (non-*ortho*-alkyl-substituted); ▼, *ortho*-*para*-/*para*-brominated (*ortho*-alkyl-substituted). The numbers refer to Table II.

The fact that *para*-bromination generally causes an increase in retention cannot be explained in this way. Since the environment of the nitrogen atom is not changed on *para*-bromination and the base strength decreases, one would rather expect the retention to decrease on *para*-bromination. There is obviously a third factor which influences retention of *p*-bromoanilines. It is suggested that this factor is a decreased solubility of the *p*-bromoaniline in the mobile phase in comparison with the original aniline, causing it to travel more slowly than this compound.

Among *para*-bromo-substituted tertiary anilines some compounds were eluted before the original aniline (Fig. 14a). This deviation from the general retention rule for *p*-bromoaniline is most likely a result of the interplay between the change in base strength and the change in solubility on bromination. In this case the decrease in solubility is not great enough to match the decrease in base strength, which causes the *p*-bromoaniline to travel more rapidly than the original aniline.

Although the base strength of the aniline, and especially steric effects, were considered mainly to govern retention change on *ortho*-bromination, solvent effects cannot wholly be left out of consideration. Thus, the different retention behaviour of certain secondary anilines on *ortho*-bromination compared to corresponding primary and tertiary anilines (Fig. 13b) may well be caused by solubility differences.

CONCLUSIONS

For the investigated anilines the coulometric bromination technique described by Truedsson and Smith[1] yields predictable, unambiguous products when the titration is continued to the end-point. In the reaction, hydrogen is exchanged for bromine at free *ortho*- and *para*-positions, with the formation of *o*- and *p*-bromoanilines.

Over-bromination of primary anilines leads to oxidation products, especially for *ortho*-alkyl-substituted compounds, whereas *para*-alkyl-substituted primary anilines show a far better stability against excess of bromine. The secondary and tertiary anilines studied in this work are generally not fully brominated at the end-point, but still contain vacant *ortho*-positions. On over-bromination, these free positions are substituted with bromine and, on further bromination, loss of N-alkyl groups occurs with the formation of primary and secondary anilines.

Introduction of bromine into the *ortho*- and *para*-positions of an alkylaniline causes an increase in retention in the reversed-phase LC system. There is a semi-linear relationship between k' values of brominated and non-brominated anilines, which is of value for identification purposes.

In the straight-phase LC system, *para*-bromination generally causes an increase in retention and *ortho*-bromination a decrease. As for the reversed-phase system, there is a semi-linear relationship between k' values of brominated and original anilines.

ACKNOWLEDGEMENT

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LIQUID CHROMATOGRAPHY STUDY OF BROMINATED ANILINES AND INVESTIGATION OF PRODUCT FORMATION IN THE BROMINATION REACTION

II. ANILINES WITH ALKYL GROUPS IN THE *meta*-POSITION

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SUMMARY

Straight- and reversed-phase liquid chromatography (LC) have been used to study product formation in the quantitative coulometric bromination of various anilines. The coulometric method yields, up to the end-point, predictable unambiguous products by exchange of hydrogen for bromine in free *ortho*- and *para*-positions. After the end-point, oxidation products may be formed from primary anilines and N-alkyl groups split off from secondary and tertiary anilines.

A detailed study of retention behaviour of some 30 bromoanilines in straight- and reversed-phase LC has been made. An increase in retention generally took place on the introduction of bromine into the aniline nucleus, except on formation of *o*-bromoanilines in straight-phase LC, where retention decreased. A semi-linear relationship was found to exist between capacity factors of brominated and non-brominated anilines in both the LC systems.

INTRODUCTION

In a previous paper[1] product formation in the quantitative coulometric bromination of anilines with alkyl groups in the *ortho*- and *para*-positions was studied, and retention behaviour of the formed bromoanilines in straight- and reversed-phase liquid chromatography (LC) was investigated. The present work is a continuation of this study applied to *meta*-alkyl-substituted anilines.

EXPERIMENTAL

The experimental conditions were described in detail in the previous paper in this series[1]. In order to identify certain of the anilines formed on bromination, 3-methyl-4,6-dibromoaniline, 3-ethyl-6-bromoaniline, N-ethyl-3-methyl-4,6-dibromoaniline, N,N-dimethyl-3-methyl-4,6-dibromoaniline and N,N-diethyl-3-methyl-4,6-dibromoaniline were prepared at this laboratory.

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RESULTS AND DISCUSSION

Choice of liquid chromatographic system

The reversed-phase octadecylsilane (C_{18}) system was used with methanol–aqueous buffer (80:20, v/v, pH 7.0) as eluent, and the straight-phase system on nitrile phase was used with isooctane containing 0.2% (v/v) 2-propanol as eluent.

Product formation in coulometric bromination

In the coulometric bromination method for the titration of anilines described by Truedsson and Smith[2], the reaction is carried out in a water–acetic acid medium and the reactivity is controlled by varying the water content and the bromide ion concentration and by the addition of pyridine. The reaction is believed to involve substitution with bromine at free *ortho*- and *para*-positions. For anilines with several such positions, the titration can be carried out to either the fully brominated stage or to a stage corresponding to the introduction of a smaller number of bromine atoms than the number of available free *ortho*- and *para*-positions. For a certain aniline, the outcome of a titration is dependent both on the structure and on the bromination-promoting properties of the titration medium.

In the present investigation the aim was to study product formation during titration in the pyridine-free medium III-1, containing acetic acid and water in the proportions 60:40 (v/v) and with a bromide concentration of 0.1 *M*. For this purpose samples were removed from the titration vessel at various stages of the titration and injected on to the LC column either directly (C_{18} phase) or after extraction (nitrile phase). A typical titration curve, with points of sample removal indicated, is given in Fig. 1 of ref. 1.

At the quantitative bromination in medium III-1 (point B on the titration curve), primary anilines studied in this work consume bromine corresponding to the number of free *ortho*- and *para*-positions, secondary anilines consume one bromine

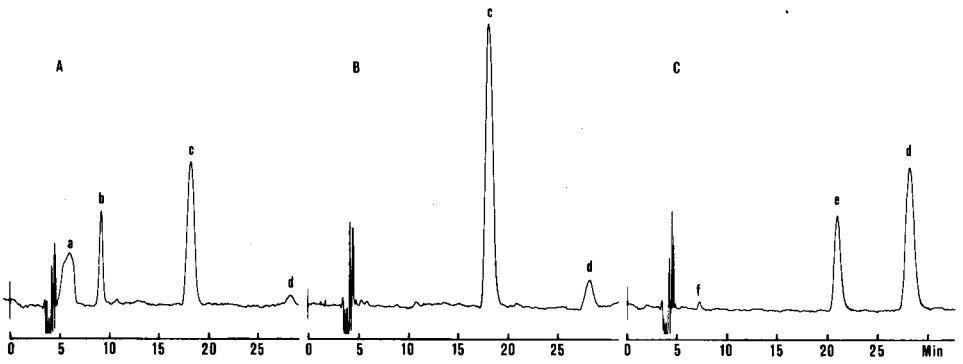

Fig. 1. Chromatograms of the product mixture after bromination of N-methyl-3-methylaniline. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}; wavelength, 280 nm; volume injected, 20 µl. Peaks: a = N-methyl-3-methylaniline; b = N-methyl-3-methyl-4-bromoaniline; c = N-methyl-3-methyl-2,4-dibromoaniline + N-methyl-3-methyl-4,6-dibromoaniline; d = N-methyl-3-methyl-2,4,6-tribromoaniline; e = 3-methyl-2,4,6-tribromoaniline; f = oxidation product. A, B and C refer to points at which samples were taken (see text).

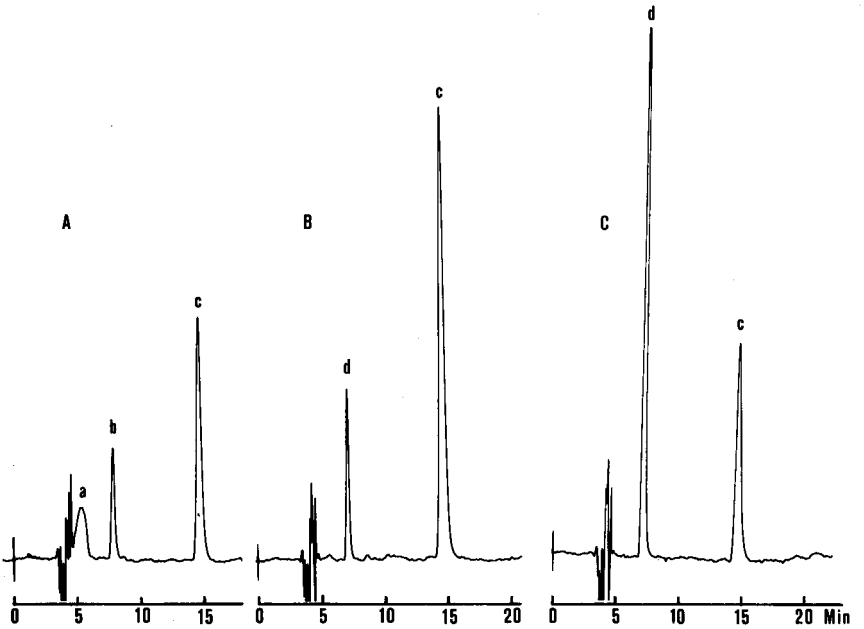

Fig. 2. Chromatogram of the product mixture after bromination of 2,5-dimethylaniline. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}; wavelength, 280 nm; volume injected, 20 μl. Peaks: a = 2,5-dimethylaniline; b = 2,5,-dimethyl-4-bromoaniline; c = 2,5-dimethyl-4,6-dibromoaniline; d = oxidation product. A, B and C refer to points at which samples were taken (see text).

atom less and tertiary anilines two bromine atoms less[2]. From the chromatograms of the samples taken at the end-point **B**, it was established that *e.g.* 3-methylaniline was converted into 2,4,6-tribromo-3-methylaniline, N-methyl-3-methylaniline into a mixture of the 2,4- and 4,6-dibromo-derivatives and N,N-dimethyl-3-methylaniline into the 4-bromo derivative.

Fig. 1 illustrates the bromination course for N-methyl-3-methylaniline. At point A, half-way to the end-point, the main bromination products formed are N-methyl-3-methyl-4-bromoaniline (b) and the corresponding 2,4- and 4,6-dibromo derivatives (c). In addition, a small amount of N-methyl-3-methyl-2,4,6-tribromoaniline (d) is present. At the end-point (B) only the mixture of the dibromo derivatives and somewhat of the tribromo derivative are present. The formation of the latter compound is in agreement with the slight overconsumption of bromine found for N-methyl-3-methylaniline on quantitative coulometric bromination in medium III-1[2].

As shown by Table I, a mono-*ortho*-brominated derivative is generally not formed on bromination of *meta*-alkyl-substituted anilines with a free *para*-position. There is only one example of such a compound being formed, *viz.* in the case of 3-ethylaniline. It appears that the steric hindrance, exerted by a more bulky substituent in the *meta*-position than methyl, decreases the speed of the *para*-bromination reaction enough to allow the formation of a 3-alkyl-6-bromoaniline.

On dibromination of 3-alkylanilines with free *ortho*- and *para*-positions two isomers can be formed, *viz.* 2,4- and 4,6-dibromo-3-alkylanilines. As shown by Table I, both isomers are formed from primary and secondary anilines, whereas tertiary

TABLE I

CAPACITY FACTORS AND SEPARATION FACTORS FOR *meta*-ALKYL-SUBSTITUTED ANILINES AND THEIR BROMINATION PRODUCTS IN THE REVERSED-PHASE LC SYSTEM

Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0)

No.	*Aniline (substituent)*	*Capacity factor, k'*							*Separation factor, α*					
		Non-brominated	*Monobrominated*		*Dibrominated*			*Tri-brominated*	*Mono-/non-*		*Di-/mono-para-*		*Tri-/di-*	
			Ortho	*Para*	*2,6-*	*2,4-*	*4,6-*		*Ortho*	*Para*	*2,4-*	*4,6-*	*2,4-*	*4,6-*
1	3-Methyl	0.51		0.88		2.17	2.17	5.14		1.73	2.47	2.47	2.37	2.37
2	3-Ethyl	0.67	1.31	1.12		2.57	2.73	6.24	1.96	1.67	2.29	2.44	2.43	2.29
3	2,3-Dimethyl	0.74		1.25			3.23			1.69		2.58		
4	2,5-Dimethyl	0.75		1.29			3.26			1.72		2.53		
5	3,4-Dimethyl	0.69	1.37		3.14				1.99					
6	N-Methyl-3-methyl	0.89		1.66		4.33	4.24	7.18		1.87	2.61	2.55	1.66	1.69
7	N-Ethyl-3-methyl	1.14		2.11		6.55	6.30	10.3		1.85	3.10	2.99	1.57	1.63
8	N,N-Dimethyl-3-methyl	1.82		3.42			5.18	17.7		1.88		1.51		3.42
9	N,N-Diethyl-3-methyl	3.08		5.83			10.1	31.9		1.89		1.73		3.16

anilines only yield the 4,6-dibromo derivative. This is undoubtedly caused by increased steric hindrance to bromine substitution at the 2-*ortho*-position.

Effect of over-bromination. Primary 3-alkylanilines without *ortho* alkyl groups are comparatively stable to excess bromine. At 50% excess (point C on the titration curve), small early peaks appear at separation on the C_{18} phase. Spectra evidence indicates that their structure is quinoidic.

If the aniline contains an *ortho* as well as a *meta* alkyl group, it becomes more sensitive to excess bromine, and quinoidic oxidation products can appear already at the end-point B. At 50% excess of bromine (point C), a considerable amount of the oxidation product has been formed. This is shown for 2,5-dimethylaniline in Fig. 2. Early peaks also appear on the straight-phase system, indicating the formation of substituted diphenylamines, as was reported in the previous paper on *ortho*-substituted anilines[1].

Secondary and tertiary anilines are not fully brominated at the end-point, but still contain vacant *ortho*-positions. On over-bromination these free positions are substituted with bromine, producing the fully brominated aniline (see Table I). However, this reaction cannot be utilized for quantitative purposes. The fully brominated secondary and tertiary anilines are very sensitive to further excess of bromine and react with loss of N-alkyl groups to the corresponding primary and secondary anilines. This is demonstrated in Fig. 1C where N-methyl-3-methylaniline on over-bro-

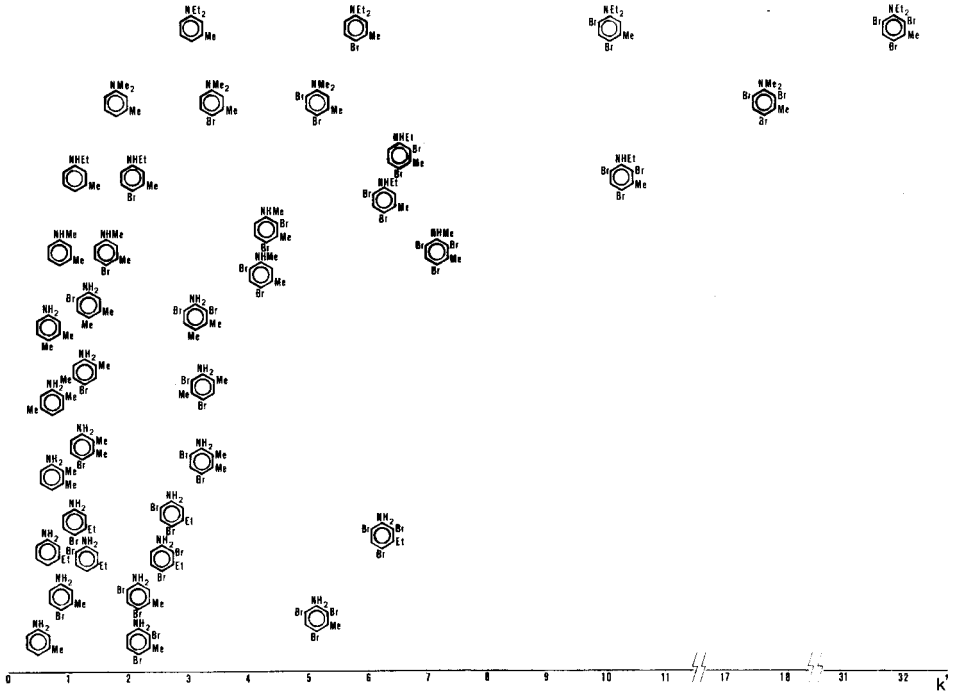

Fig. 3. Retention of primary, secondary and tertiary anilines and the corresponding bromination products in reversed-phase LC. Column, Nucleosil C_{18}; eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. Me = Methyl; Et = ethyl.

mination furnishes N-methyl-3-methyl-2,4,6-tribromoaniline (d) and 3-methyl-2,4,6-tribromoaniline (e). In addition a small amount of an oxidation product (f) is formed.

Retention behaviour of brominated anilines

Reversed-phase chromatography. The substitution of bromine into free *ortho*- and *para*-positions of *meta*-alkyl-substituted primary, secondary and tertiary anilines gives a considerable increase in retention in reversed-phase chromatography (Table I and Fig. 3). A semi-linear relationship exists between the k' values of the original anilines and those of the corresponding mono-, di-, and tribrominated derivatives, respectively (Fig. 4). Dibrominated tertiary anilines seem to fall on a line of their own (see broken line in Fig. 4).

The effect on retention of the successive introduction of bromine into a *meta*-

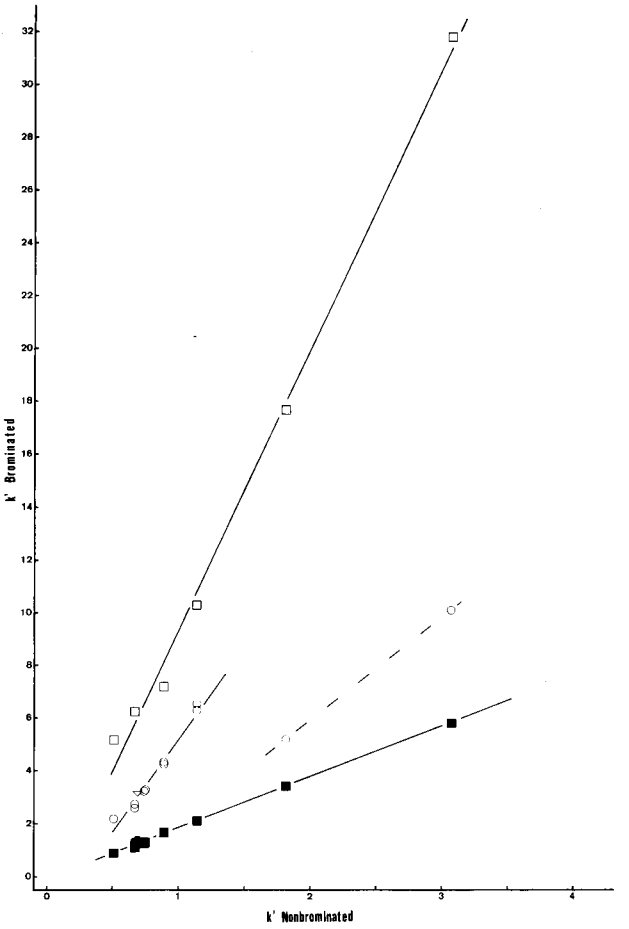

Fig. 4. Relationship between capacity factor, k', for alkylanilines and the corresponding bromination products in reversed-phase LC. Column, Nucleosil C_{18}, eluent, methanol–aqueous buffer (80:20, v/v, pH 7.0), 45 ml h^{-1}. ■ = Mono-*para*-brominated; ● = mono-*ortho*-brominated; ○ = *ortho*-*para*-brominated; ▽ = di-*ortho*-brominated; □ = tribrominated.

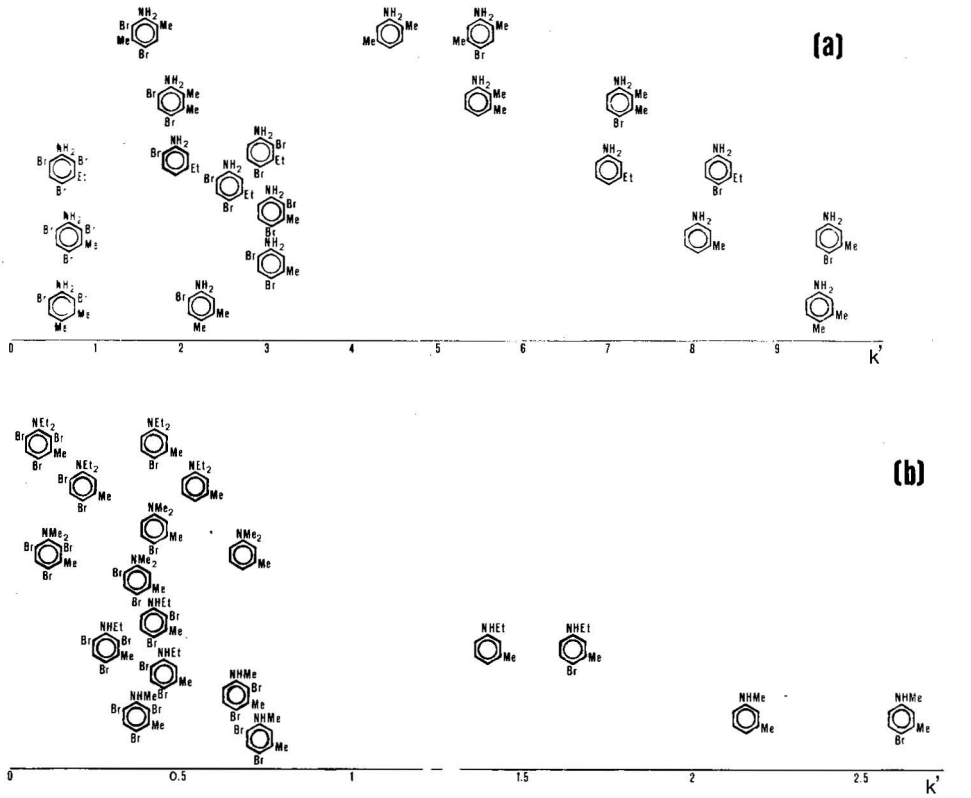

Fig. 5. Retention of primary, secondary and tertiary anilines and the corresponding bromination products in straight-phase LC. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. (a) Primary anilines. (b) Secondary and tertiary anilines.

alkyl-substituted aniline is demonstrated in Table I, where the separation factors, α, between the various bromination stages are given. As already established, the sensitivity of the values to the environmental structure is considerable. This is especially evidenced by their variation in the dibromo- to monobromo- and tribromo- to dibromo- series, respectively.

A kind of brominated aniline that has not been encountered previously is tribromoaniline containing a nuclear alkyl group. Comparison of k' values of these compounds with those of corresponding tribromoanilines without a nuclear alkyl group[1] indicates certain anomalies. Thus, it appears that the retention difference is abnormally high between N,N-dimethyl-3-methyl-2,4,6-tribromoaniline and N,N-dimethyl-2,4,6-tribromoaniline, the relationship between the k' values being 3.5. For the corresponding primary and secondary (N-methyl) aniline pairs the same relationship is only 1.4. Since the anilines, constituting a pair, must be considered to be of similar type, the retention differences between the members in each pair should, following Locke[3], be due mainly to solubility differences in the mobile phase.

Straight-phase chromatography. A great decrease in retention in straight-phase chromatography for *ortho*-brominated alkylanilines in comparison with correspond-

TABLE II

CAPACITY FACTORS AND SEPARATION FACTORS FOR *meta*-ALKYL-SUBSTITUTED ANILINES AND THEIR BROMINATION PRODUCTS IN THE STRAIGHT-PHASE LC SYSTEM

Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane.

No.	*Aniline (substituent)*	*Capacity factor, k'*							*Separation factor, α*					
		Non-brominated	*Monobrominated*		*Dibrominated*			*Tri-brominated*	*Mono-/non-*		*Di-/mono-para-*		*Tri-/di-*	
			Ortho	*Para*	*2,6-*	*2,4-*	*4,6-*		*Ortho*	*Para*	*2,4-*	*4,6-*	*2,4-*	*4,6-*
1	3-Methyl	8.07		9.62		3.06	3.06	0.68		1.19	0.32	0.32	0.22	0.22
2	3-Ethyl	7.02	1.93	8.31		2.93	2.57	0.60	0.27	1.18	0.35	0.31	0.20	0.23
3	2,3-Dimethyl	5.48		7.15			1.88			1.30		0.26		
4	2,5-Dimethyl	4.45		5.50			1.57			1.24		0.29		
5	3,4-Dimethyl	9.51	2.24		0.60				0.24					
6	N-Methyl-3-methyl	2.16		2.62		0.66	0.73	0.37		1.21	0.25	0.28	0.56	0.51
7	N-Ethyl-3-methyl	1.40		1.65		0.42	0.45	0.28		1.18	0.25	0.27	0.67	0.62
8	N,N-Dimethyl-3-methyl	0.68		0.42			0.37	0.11		0.62		0.88		0.30
9	N,N-Diethyl-3-methyl	0.54		0.42			0.21	0.08		0.78		0.50		0.38

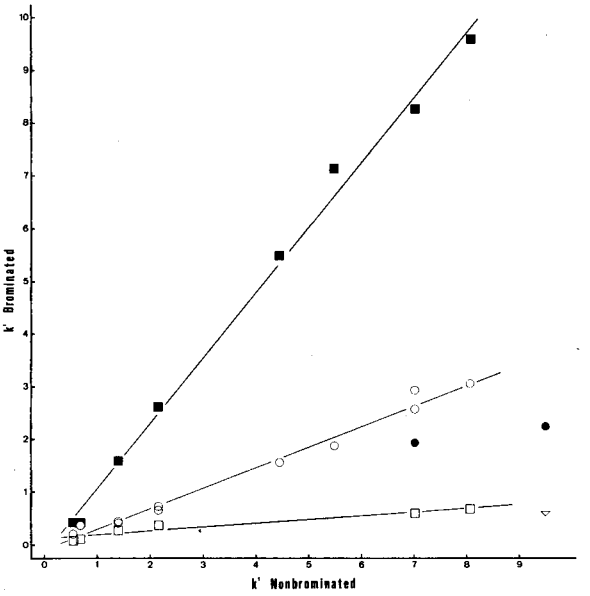

Fig. 6. Relationship between capacity factor, k', for alkylanilines and the corresponding bromination products in straight-phase LC. Column, Nucleosil CN; eluent, 0.2% (v/v) 2-propanol in isooctane, 45 ml h^{-1}. ■ = Mono-*para*-brominated; ● = mono-*ortho*-brominated; ○ = *ortho*-*para*-brominated; ▽ = di-*ortho*-brominated; □ = tribrominated.

ing non-*ortho*-brominated compounds, has already been established[1]. *Meta*-alkyl-substituted anilines behave similarly, as shown by the k' and α values given in Table II.

Substitution of bromine into the *para*-position leads to either an increase or a decrease in retention. An increase is observed for primary and secondary anilines and a decrease for tertiary anilines. A similar behaviour was previously noticed for other kinds of alkylanilines and for non-alkyl-substituted anilines and its cause discussed in terms of steric hindrance, base strength and solubility in the mobile phase[1].

Among the tribrominated *meta*-alkyl-substituted anilines the retention decreases in the order primary, secondary and tertiary anilines, *i.e.* with increased steric hindrance around the nitrogen atom. The very low k' values of the tribrominated tertiary anilines in Table II indicate that these compounds behave on the whole as hydrocarbons on the nitrile phase.

The elution order on the nitrile phase for some *meta*-alkyl-substituted anilines and their bromination products is further illustrated in Fig. 5. The semi-linear relationship between k' values of brominated and corresponding non-brominated anilines, previously established for the C_{18} phase, is also valid for the nitrile phase (Fig. 6). As can be seen, the position of a bromoaniline is governed by the number of *ortho*-situated bromine atoms and by the presence of a *para*-situated bromine atom.

CONCLUSIONS

For the investigated *meta*-alkyl-substituted anilines the quantitative coulomet-

ric bromination method described by Truedsson and Smith[2] yields, up to the end-point, mainly *o*- and *p*-bromoanilines by exchange of hydrogen for bromine at free *ortho*- and *para*-positions. Primary *m*-alkylanilines are not particularly sensitive to excess of bromine, unless *ortho*-situated alkyl groups are also present. In that case, oxidation products appear on over-bromination. Secondary and tertiary anilines are not fully brominated at the end-point, but still contain vacant *ortho*-positions. On over-bromination, these positions are substituted with bromine and, on further bromination, loss of N-alkyl groups occurs with the formation of primary and secondary anilines.

Introduction of bromine into the *ortho*- and *para*-positions causes an increase in retention in the reversed-phase LC system. In the straight-phase system, *ortho*-bromination occasions a decrease in retention and *para*-bromination leads to an increase for primary and secondary anilines and a decrease for tertiary anilines.

In both the LC systems studied, there is a semi-linear relationship between k' values of brominated and non-brominated anilines, respectively.

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The experimental assistance of Mrs. Kerstin Svensson is gratefully acknowledged.

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CONTRIBUTION TO THE ELUCIDATION OF THE MECHANISM OF SUGAR RETENTION ON AMINE-MODIFIED SILICA IN LIQUID CHROMATOGRAPHY

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SUMMARY

Liquid chromatography of reducing or non-reducing sugars results in single peaks on amine-modified silica with acetonitrile–water as eluent. In spite of the two anomeric forms of the reducing sugars, single peaks can be obtained because mutarotation is fast under these conditions. The bonded amine groups catalyse the mutarotation in such a way that triethylamine added to the eluent has no influence. The separation of the sugars is the result of their partition between two liquid phases, because the composition of the stationary liquid phase appears to be much richer in water than the eluent.

INTRODUCTION

Linden and Lawhead[1] and Palmer[2] were first to apply alkylamine-modified silicas for sugar analysis. A mixture of acetonitrile and water was used as eluent. Ever since, much has been written about the application of this system. Its popularity is related to the following facts:

(1) Silica is much more resistant to pressure than ion-exchangers (reviewed by Jandera and Churacek[3]).

(2) There is a quick exchange of mass between the mobile and the stationary phase, which enables high eluent flow-rates, without significant loss of resolution.

(3) No sample derivatization is needed.

Schwarzenbach[4] described the chemical modification of silica by reaction of aminopropyltriethoxysilane with the surface silanol groups. The separation process on this material was stated to be that of reversed-phase chromatography by Meagher and Furst[5], normal-phase chromatography (because increased polarity of the mobile phase leads to shorter elution times) by Rabel *et al.*[6] and competitive interaction of the water in the eluent and the sugar injected with the polar bonded phase by Hettinger and Majors[7]. Majors[8] reported that sugars are retained because their hydroxyl groups react with the bonded amine. Jones *et al.*[9] found that k' values were proportional to the amine loading becoming constant at higher loadings. Kahle and Tes-

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ařik[10] separated the anomeric forms of mutarotating sugars on an alkylamine column (SO_4^{2-}). The retention time for the mixed peak on the free amine column was the average of those for the separate peaks on the column in the sulphate form.

Physical modification of silica by coating with amine has also been investigated[11–14]. Silica impregnated *in situ* should be considerably cheaper than chemically modified silica, and, according to Aitzetmüller[13], is also more stable. Chemically modified silica can lose amine by hydrolysis, resulting in decreased sugar retention. Using the physically modified carrier, unchanged retention times are obtained after prolonged use if some amine is added with the eluent. Wheals[12] and White[14] and their co-workers investigated the influence of different amines on sugar retention. Both retention[12] and separation efficiency[14] appeared to be influenced by the type of amine.

Other relevant research has concerned the composition of the mobile phase, that most often employed being a mixture of acetonitrile and water. Müller and Siepe[15] successfully applied an eluent comprising acetone, ethyl acetate and water, which has the advantage that there is no need for acetonitrile (which is poisonous to man). Rabel *et al.*[6] obtained poor separations using methanol–water eluents, which they attributed to the high polarity of methanol causing poor solvation of the bonded phase.

We now describe the influence of amine in chemically modified silica on the mutarotation rate of sugars, and also experiments carried out to determine the composition of the stationary phases at different acetonitrile–water ratios in the eluent. The results contribute to the elucidation of the mechanism of sugar separation, and to the understanding of such phenomena as column ageing, influence of the type of amine and the eluent composition.

EXPERIMENTAL

Influences of triethylamine (TEA) in the eluent on the separation of sugars using amine-modified silica

A sample containing fructose, glucose, sucrose and lactose was injected on a Waters μCarbohydrate column (300 × 3.9 mm) at a column temperature of 20°C and an eluent flow-rate of 2 ml/min. Other conditions were as described previously[16]. The eluent was acetonitrile–water (80:20), respectively without TEA, with 0.001 *M* TEA and with 0.003 *M* TEA.

Influence of different parts of the chromatographic system on the mutarotation rate

Freshly prepared solutions of 1 g glucose in

(a) 75 ml acetonitrile + 25 ml water + 1 g LiChrosorb NH_2 (E. Merck, Darmstadt, G.F.R.)

(b) 75 ml acetonitrile + 25 ml water

(c) 100 ml water

(d) 75 ml acetonitrile + 25 ml water + 1 g Merckosorb SI 60 (E. Merck)

(e) 75 ml acetonitrile + 25 ml water + 1 g LiChrosorb RP-8 (E. Merck) were allowed to mutarotate at 20°C while stirring. Samples of 10 μl were injected at different times on an Aminex A-5 (Ca^{2+}) column (250 × 4.6 mm) at 45°C. Distilled water was used as the eluent at a flow-rate of 0.5 ml/min. Under these conditions the mutarotation rate can be determined[16].

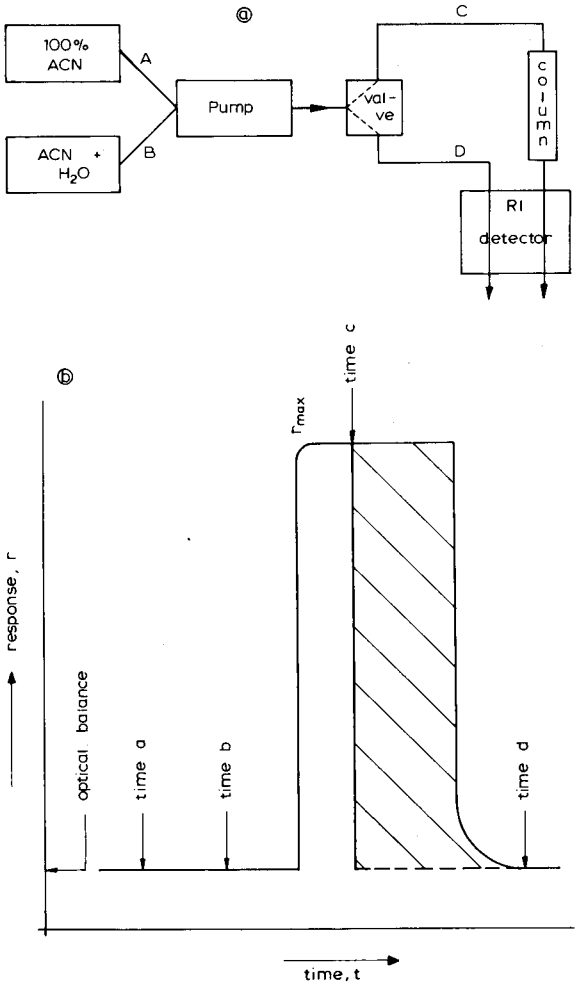

Fig. 1. a, Experimental set-up to determine the water hold-up in the column. b, Example of detector output from which the water hold-up could be calculated. ACN = Acetonitrile; RI = refractive index.

Carbon, hydrogen and nitrogen contents of LiChrosorb NH_2

These were determined with a Hewlett-Packard/F&M Model 185 CHN-analyzer. A known amount of sample was burned in an oxygen atmosphere. The nitrogen oxides formed were reduced, and the amounts were compared with those evolved from a reference sample.

Porosity of LiChrosorb NH_2

This was determined with a Carlo Erba Series 200 porosimeter. The amount of mercury which penetrated into the pores was determined as a function of the pressure applied.

Amount of water retained by LiChrosorb NH_2 in different acetonitrile–water eluents

A stainless-steel column (100 × 4.6 mm) was packed with LiChrosorb NH_2

and kept at 20°C. Eluent was pumped through this column at a flow-rate of 0.5 ml/min using a Waters 6000A pump. Between the pump and the column was placed a low-dead-volume valve to switch the eluent flow from the column to the reference channel of a Waters R 401 refractive index detector. The other channel of the detector was connected to the exit of the column, as shown in Fig. 1a. The delay in the attainment of equilibrium after changing from water-free eluent to eluents containing 10, 20, 30 and 40% (v/v) water was measured.

First, 100% acetonitrile was pumped via A and D, and after a constant detector signal was obtained, then via A and C, again up to a constant signal. By adjusting this constant signal to recorder zero with the optical zero of the detector, the situation reached was as depicted in Fig. 1b at time *a*. This corresponds to a completely water-free column and detector. At time *b* the eluent composition was changed by switching from A–C to B–D, and after some time a different but constant response was obtained, corresponding to a water-containing liquid in the reference and a water-free liquid in the measuring channel of the detector. Finally, at time *c*, the flow was changed from B–D tot B–C, and the water-containing eluent passed into the column. Some water is retained by the column and after some delay a new equilibrium in the detector is attained at time *d*.

The hold-up volume of water, v_w, on the column can be calculated as follows

$$v_w \text{ (ml)} = (q/r_{max} \int_c^d r dt - v_d) f_w$$

(for r, t, c and d see Fig. 1b), where q = eluent flow-rate; f_w = water fraction in the eluent, v_d = dead volume between valve and detector and $\int_c^d r dt$ = the surface underneath the response–time curve.

RESULTS AND DISCUSSION

With Aminex A-5 (Ca^{2+}) and water as eluent, the addition of TEA to the eluent causes a significant decrease in peak widths for mutarotating sugars[16]. However, in our experiments, there was no influence of amine addition on peak widths nor on retention times, for amine-modified silica with acetonitrile–water as eluent, not even at the highest (0.003 *M*) amine concentration. This may be caused by the α- and β-forms of the sugars having the same capacity factor (k') or by the fact that mutarotation proceeds rapidly in this system.

We thus determined the mutarotation rate under the conditions described under Experimental. The results are given in Fig. 2. Compared to the rate in pure water (c), a lower rate was found on addition of ACN (b). Further addition of *n*-octyl-modified SI 60 silica had hardly any effect (e), while unmodified silica had a slight positive effect (d). However, addition of LiChrosorb NH_2 greatly increased the mutarotation rate (a). It can be concluded that the amine groups are responsible for the much higher reaction rate. Under chromatographic conditions it is expected that the mutarotation rate will be even higher because the concentration of amine groups, as well as the ratio of these groups to the amount of glucose, is approximately 50 times higher.

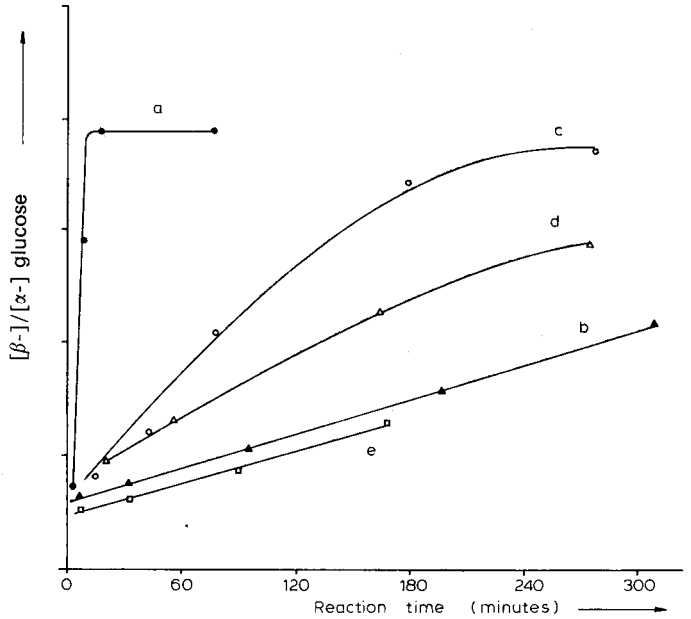

Fig. 2. Mutation rate of glucose under different conditions. (For conditions a–e, see the section *Influence of different parts of the chromatographic system on the mutarotation rate.*)

Kahle and Tesarik[10] separated anomeric α- and β-forms of sugars on an amine-modified column, neutralized with sulphate. The retention time, t_R, of the mixed peak on the unneutralized column corresponded to

$$t_{R(\alpha+\beta)} = t_0 [1 + k'_\alpha f_\alpha + k'_\beta (1 - f_\alpha)]$$

Where k' = capacity factor in the neutralized column, f_α = mol fraction of the α-anomer and t_0 is the elution time of an unretained compound. It can be concluded that the retention is not dependent on whether the amine is in the free or sulphate form, that the k' values for the α- and β-species are different and that when the amine is in the free form only one, mixed peak is observed, as a result of the high mutarotation rate.

By use of a CHN-analysis, the quantity and quality of the amine modification was determined. The results are in accordance with the manufacturer's statement that propylamine modification had been used. We found 3.51 % C, 1.10 % H and 1.40 % N which corresponds to an atomic ratio of 3:11:1. The hydrogen content is somewhat high due to loss of desorbed or dehydrated water. We calculated that the amount of propylamine per gram of LiChrosorb NH_2 was 1.0 mmol; or 0.6 mol/l in a packed chromatographic column.

The pore volume of LiChrosorb NH_2 was approximately 0.7 ml/g with an average pore diameter of 60 Å.

There is a remarkable similarity in elution patterns with the systems amine-

modified silica, acetonitrile–water and anion exchanger/ethanol–water as described by Samuelson[17]:

increased retention with increasing molecular size of the sugar
higher retention for aldoses than for ketoses
higher retention for β- than for α-anomers[10,18]

Based on measurements by Rückert and Samuelson[19], which showed that the stationary liquid in the anion exchanger/ethanol–water system contained more water than the mobile liquid, and, therefore, that the sugars preferred the stationary phase[20], Samuelson[17] concluded that the separation mechanism is one of liquid–liquid or partition chromatography.

TABLE I

WATER HOLD-UP AND DISTRIBUTION OF WATER OVER MOBILE AND STATIONARY LIQUID PHASES FOR DIFFERENT ELUENT COMPOSITIONS

In a column with a volume of 1.662 ml containing 1.1 g LiChrosorb NH_2.

Eluent composition, acetonitrile–water (%, v/v)		*90:10*	*80:20*	*70:30*	*60:40*
Eluent needed for water saturation (ml)		2.05	1.964	1.670	1.525
(% of the empty column volume)		123	118	100	92
Water hold-up (mg)		205	393	501	610
Water content in mobile phase (mg)	*	35	70	105	140
	**	66	133	199	266
Water content in stationary liquid phase (mg)	*	170	323	396	470
	**	139	260	302	344
(%, v/v)	*	22	42	52	61
	**	30	56	65	74

* At a mobile phase fraction of 0.21.
** At a mobile phase fraction of 0.40.

To attempt to explain the similarities in these two chromatographic systems, we determined the water hold-up of an amine-modified silica column (see Experimental). The results are presented in Table I and indicate a water enrichment of the stationary phase. In order to quantify this water enrichment the following estimates of the volume fractions for the different phase, were made:

the stationary phase (silica + propylamine): based on a specific density of 2.3 for silica, the volume fraction of silica is 0.27 (weight of LiChrosorb NH_2 silica fraction)/(specific density of silica × empty column volume); using a specific density of 0.7 for propylamine, its volume fraction can be calculated in the same way as 0.06. So the total for the stationary phase is 0.33

the stationary phase pore volume fraction: this can be estimated as 0.46 from the pore volume per gram LiChrosorb NH_2 as determined

the mobile phase fraction is the residual part of the column volume, *i.e.*, 0.21.

A mobile phase fraction of 0.21 is rather low. A more common value is 0.40, which is used for the calculation of the residence time of an unretained component.

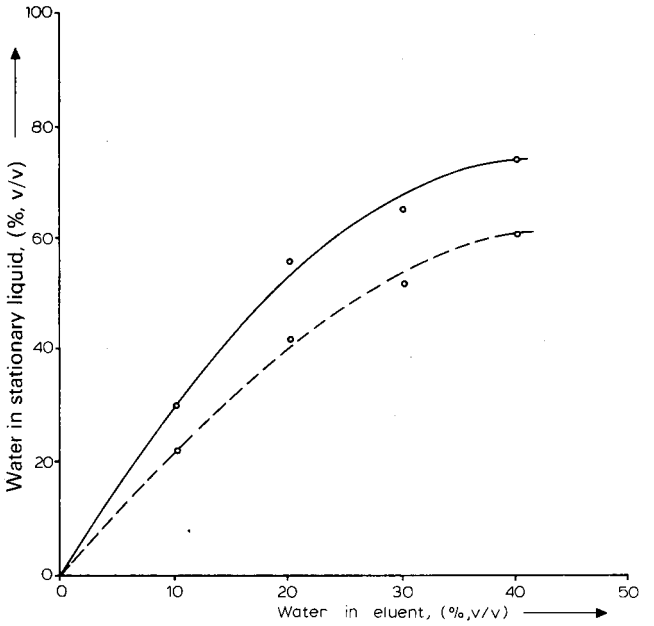

Fig. 3. Dependence of the water content of the stationary liquid on that of the eluent for different mobile phase values. – –, Low mobile phase value; ——, high mobile phase value.

Possibly, a part of the (superficial) pores should be taken into account when estimating the mobile phase fraction. Therefore, in further calculations, a low and a high value for the mobile phase fraction (0.21 and 0.40) and thus a high and a low value for the stationary liquid phase (0.46 and 0.27) were used.

The results are presented in Table 1 and Fig. 3. It is clear that the volume fraction of water in the stationary liquid is much higher than that in the eluent. This water enrichment of LiChrosorb NH_2 is caused by the hydrophilic nature of the amine groups; these groups also catalyse the mutarotation.

The amount of water enrichment is expected to be dependent on the nature and the amount of bonded amine. An illustration of the importance of the amine is the variation of the capacity factors for sugars when different amines are added to the eluent, as reported by Wheals and White[12]. The relationship between k' and the amine concentration, as mentioned by Jones *et al.*[9], can be explained if the water enrichment of the stationary phase, initially dependent on the amine content, subsequently becomes more or less constant because of water saturation, so that further addition of amine has no effect. Column deterioration, which results in shortening of elution times, can be interpreted in terms of a decreased water enrichment due to loss of amine. The original elution pattern can be restored simply by reducing the water content of the eluent, because at low water concentrations the extent of water enrichment of the stationary phase is relatively high, as can be seen in Fig. 3. The constant elution times found for physically modified columns, where water enrichment will also occur, are caused by the constant amount of amine added with the eluent.

Rabel *et al.*[6] ascribed the poor separations with methanol–water as eluent to

the low extent of "solvation" of the amine phase. However, probably no water enrichment occurred because of the strong hydrophilic nature of methanol. The eluent, used by Müller and Siepe[15], water–acetone–ethyl acetate, apparently does allow water enrichment of the stationary phase. Generally, the eluent composition should be such that, in addition to a reasonable solubility of the sugars, the affinity of each component for the amine is different.

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STRUCTURE–RETENTION RELATIONSHIP OF STEROLS AND TRITERPENE ALCOHOLS IN GAS CHROMATOGRAPHY ON A GLASS CAPILLARY COLUMN

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SUMMARY

The relative retention times and the methylene unit values of 168 acetate derivatives of sterols and triterpene alcohols, most of which are of higher plant origin, were determined on OV-1 and OV-17 glass capillary columns. Separation factors related to various types of double bond, alkyl substituent and other structural features were calculated from the retention data. These gas–liquid chromatographic retention characteristics are very useful for the identification and estimation of the structure of sterols and triterpene alcohols.

INTRODUCTION

Gas–liquid chromatography (GLC) is a vital tool for separating and identifying sterols and triterpene alcohols. A number of 4-desmethylsterols[1–16] have already been analyzed on several stationary phases and the correlation between the structure of these compounds and their retention data has been well discussed. Retention data on several stationary phases have also been reported for some 4-methylsterols[5,7–10,13], lanostane triterpene alcohols[2,4,8–10,13,16–18] and other tetracyclic triterpene alcohols[8–10,17,18] and pentacyclic triterpene alcohols[8–10,17–21]. Since 4-methylsterols and triterpene alcohols are closely related to 4-desmethylsterols, biogenetically as well as structurally, it seems of great value to study the GLC retention characteristics of large numbers of these compounds together with 4-desmethylsterols.

Such a study was therefore undertaken for a number of 4-methylsterols, tetra- and pentacyclic triterpene alcohols and 4-desmethylsterols. Although most previous work has been carried out on conventional packed columns, glass capillary columns were used in this study because such columns have recently been shown to have high resolving power even for the structurally closely related sterols and triterpene alcohols from natural sources[22–26]. Furthermore, it has also been shown that glass capillary columns can be used to differentiate C-24 epimeric 24-alkylsterols[27–29].

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The retention data ware expressed as the usual relative retention times, RRT, and as the methylene unit (MU) values[30,31], since the latter are independent of the operating temperature. Separation factors related to various types of double bond, alkyl substituent and other structural characteristics were calculated from the RRT data.

EXPERIMENTAL

A Shimadzu GC-4CM gas chromatograph equipped with a hydrogen flame ionization detector was used. Two support-coated open tubular (SCOT) glass capillary columns (30 m × 0.3 mm I.D.; Wako, Osaka, Japan) were coated either with OV-1 or OV-17 stationary phase. Sterol and triterpene alcohol samples as the acetate derivatives and internal standards were injected simultaneously as solutions in acetone. The sample size injected was adjusted so that the peak heights were comparable to that corresponding to 4–6 μg cholesterol acetate (*ca.* 60% of the full recorder response).

"Initial retention times"[32], based on the distances from the starting point of the solvent peak to the starting point of each sample peak on the chromatogram, instead of the usual retention times were determined since far less fluctuation in the former due to change in the amount of sample injected has been observed previously[32] and also in our preliminary experiments. The standard chart speed was 10 mm/min. RRT was expressed relative to cholesterol acetate, and MU value was determined using *n*-dotriacontane (C_{32}), *n*-tritriacontane (C_{33}), *n*-tetratriacontane (C_{34}), *n*-hexatriacontane (C_{36}) and *n*-tetracontane (C_{40}) as the standard hydrocarbons. The reproducibility of the values of RRT and MU obtained from several runs on the same column was satisfactory for a given substance.

Acetylation was performed in acetic anhydride–pyridine (1:1) at room temperature overnight. Sterols and triterpene alcohols were obtained from three sources: gifts from individuals; isolated from plants[8,23–26,33–35], and derived from available samples.

RESULTS AND DISCUSSION

The two stationary phases used in this work, non-polar OV-1 (dimethyl silicone; McReynolds' constant, $\chi' = 16$ (ref. 36)) and slightly polar OV-17 (50% phenyl-50% methyl silicone; $\chi' = 119$ (ref. 36)), are the ones most frequently used for sterol analysis. The acetate derivatives of the compounds were chosen because they are derivatives commonly used for the purification of naturally occurring sterols and triterpene alcohols by argentation chromatography[16,23–26], are very stable and can be quantitatively prepared from the free alcohols. Analysis of such derivatives on a capillary column gave satisfactory results, and thus it was unnecessary to prepare other derivatives solely for GLC analysis. The free alcohols were unsuitable for GLC on a capillary column because they were eluted as broad peaks with considerable tailing.

Table I shows the RRT and the MU values for the acetate derivatives of 168 sterols and triterpene alcohols including 68 4-desmethylsterols, 36 4α-methylsterols, 42 tetracyclic triterpene alcohols and 22 pentacyclic triterpene alcohols, most of

TABLE I

RELATIVE RETENTION TIMES AND METHYLENE UNITS OF THE ACETATES OF STEROLS AND TRITERPENE ALCOHOLS ON OV-1 AND OV-17 COLUMNS

Values are relative to cholesterol acetate; those in parentheses are taken from ref. 27. Unless otherwise specified in this and in the subsequent Tables, the acetoxy group at C-3 of all the compounds was β-oriented, and if not carrying a Δ^5-bond or not otherwise mentioned, all the compounds have a 5α-configuration. Conditions: OV-1, column temp. 260°C, N_2 flow-rate 0.55 ml/min, splitting ratio 120:1, scavenger gas (N_2) flow-rate 50 ml/min, net retention times of standard substances, cholesterol acetate (16.14 min, number of theoretical plates 17,689), *n*-dotriacontane (17.02 min), *n*-tritriacontane (22.10 min), *n*-tetratriacontane (28.84 min) and *n*-hexatriacontane (48.96 min); OV-17, column temp. 260°C, N_2 flow-rate 0.60 ml/min, splitting ratio 100:1, scavenger gas flow-rate 70 ml/min, net retention times of standard substances, cholesterol acetate (15.60 min, theoretical plates 19,712), *n*-tetratriacontane (13.60 min), *n*-hexatriacontane (23.16 min) and *n*-tetracontane (65.86 min).

Acetate	*Position of double bond**	*OV-1*		*OV-17*	
		RRT	*MU*	*RRT*	*MU*
I. 4-Desmethylsterol (cholestane group)					
C_{26} sterol					
24-Nor-5,22-cholestadienol	5,22*E*	0.65	30.26	0.66	32.96
C_{27} sterol					
Cholestanol	—	1.03	31.93	1.02	34.61
Epicholestanol (cholestan-3α-ol)	—	0.92	31.51	0.89	34.09
Coprostanol (5β-cholestanol)	—	0.86	31.25	0.82	33.77
Epicoprostanol (5β-cholestan-3α-ol)	—	0.89	31.38	0.84	33.87
20-Isocholesterol	5	0.91	31.47	0.89	34.09
Cholesterol	5	1.00	31.82	1.00	34.53
22-Dehydrocholesterol	5,22*Z*	0.88	31.34	0.90	34.13
22-Dehydrocholesterol	5,22*E*	0.92	31.51	0.93	34.25
Desmosterol	5,24(25)	1.09	32.14	1.21	35.24
27-Nor-5,22-ergostadienol (24*R*/β)	5,22*E*	0.89	31.38	0.89	34.09
7-Cholestenol	7	1.13	32.28	1.18	35.15
27-Nor-7,22-ergostadienol (24*R*/β)	7,22*E*	1.01	31.86	1.06	34.75
24-Dihydrozymosterol	8	1.07	32.07	1.06	34.75
Zymosterol	8,24(25)	1.16	32.37	1.28	35.45
C_{28} sterol					
Campestanol (24*R*/α)	—	1.33 (1.329)	32.89	1.33 (1.334)	35.61
Ergostanol (24*S*/β)	—	1.33 (1.325)	32.87	1.33 (1.329)	35.60
24-Methylcoprostanol (24*R,S*)	—	1.11	32.21	1.07	34.78
Pollinastanol	9:19-Cyclo	1.17	32.40	1.20	35.21
24-Dehydropollinastanol	9:19-Cyclo,24(25)	1.27	32.72	1.44	35.90
Campesterol (24*R*/α)	5	1.29 (1.293)	32.78	1.31 (1.312)	35.55
22-Dihydrobrassicasterol (24*S*/β)	5	1.29 (1.286)	32.76	1.31 (1.307)	35.54
Brassicasterol (24*S*/β)	5,22*E*	1.12	32.24	1.14	35.02
5,23-Ergostadienol	5,23*E*	1.26	32.69	1.35	35.65
24-Methylenecholesterol	5,24(28)	1.27	32.72	1.35	35.65
5,24-Ergostadienol	5,24(25)	1.46	33.23	1.63	36.36
5,25-Ergostadienol (24*S*/β)	5,25(27)	1.23	32.60	1.32	35.57
5,7-Ergostadienol (24*S*/β)	5,7	1.46	33.23	1.54	36.15
Ergosterol (24*R*/β)	5,7,22*E*	1.26	32.69	1.35	35.65
5,8,22-Ergostatrienol (24*R*/β)	5,8,22*E*	1.19	32.46	1.22	35.27
7-Ergostenol (24*S*/β)	7	1.46	33.23	1.55	36.17

(Continued on p. 68)

TABLE I *(continued)*

Acetate	*Position of double bond**	*OV-1*		*OV-17*	
		RRT	*MU*	*RRT*	*MU*
7,22-Ergostadienol (24*R*/β)	7,22*E*	1.26	32.69	1.36	35.68
7,24(28)-Ergostadienol	7,24(28)	1.43	33.16	1.61	36.32
Fecosterol	8,24(28)	1.36	32.97	1.43	35.87
C_{29} sterol					
Stigmastanol (24*R*/α)	–	1.65	33.69	1.65	36.41
		(1.648)		(1.651)	
5-Dihydroclionasterol (24*S*/β)	–	1.64	33.67	1.64	36.39
		(1.640)		(1.642)	
24-Ethylcoprostanol (24*R*/α)	–	1.38	33.03	1.32	35.57
22-Stigmastenol (24*S*/α)	22*E*	1.44	33.18	1.45	35.92
24-Ethyl-22-coprostenol (24*S*/α)	22*E*	1.21	32.53	1.16	35.09
Sitosterol (24*R*/α)	5	1.60	33.57	1.63	36.37
		(1.597)		(1.634)	
Clionasterol (24*S*/β)	5	1.59	33.55	1.63	36.35
		(1.588)		(1.626)	
Stigmasterol (24*S*/α)	5,22*E*	1.40	33.07	1.43	35.87
		(1.397)		(1.431)	
Poriferasterol (24*R*/β)	5,22*E*	1.40	33.09	1.44	35.90
		(1.404)		(1.439)	
5,23-Stigmastadienol	5,23*E*	1.53	33.41	1.62	36.34
23,24-Dimethyl-5,22-cholestadienol (24*R*/β)	5,22*E*	1.36	32.97	1.37	35.71
5,24-Stigmastadienol	5,24(25)	1.78	33.97	1.95	37.04
28-Isofucosterol	5,24(28)*Z*	1.67	33.74	1.81	36.76
Fucosterol	5,24(28)*E*	1.62	33.62	1.72	36.56
Clerosterol (24*S*/β)	5,25(27)	1.54	33.43	1.64	36.39
5,22,25-Stigmastatrienol (24*S*/β)	5,22*E*,25(27)	1.39	33.05	1.52	36.10
23-Demethylgorgosterol (22*R*,23*R*,24*R*)	5,22:23-Cyclo	1.65	33.69	1.73	36.59
22-Dihydrospinasterol (24*R*/α)	7	1.81	34.03	1.94	37.02
		(1.805)		(1.938)	
22-Dihydrochondrillasterol (24*S*/β)	7	1.80	34.01	1.93	37.00
		(1.797)		(1.928)	
Spinasterol (24*S*/α)	7,22*E*	1.58	33.53	1.70	36.52
		(1.582)		(1.698)	
Chondrillasterol (24*R*/β)	7,22*E*	1.59	33.55	1.71	36.54
		(1.589)		(1.705)	
7,22,25-Stigmastatrienol (24*R*/α)	7,22*E*,25(27)	1.55	33.46	1.77	36.66
		(1.549)		(1.765)	
7,22,25-Stigmastatrienol (24*S*/β)	7,22*E*,25(27)	1.57	33.50	1.80	36.71
		(1.567)		(1.793)	
Avenasterol	7,24(28)*Z*	1.89	34.20	2.15	37.40
28-Isoavenasterol	7,24(28)*E*	1.82	34.06	2.04	37.21
Peposterol (7,24-stigmastadienol)	7,24(25)	2.01	34.43	2.31	37.68
7,25-Stigmastadienol (24*S*/β)	7,25(27)	1.75	33.91	1.95	37.04
23,24-Dimethyl-7,22-cholestadienol (24*R*/β)	7,22*E*	1.53	33.41	1.62	36.34
Vernosterol	8,14,24(28)*Z*	1.72	33.85	1.94	37.02
8(14)-Stigmastenol (24*R*/α)	8(14)	1.62	33.62	1.67	36.47
		(1.618)		(1.674)	
8(14)-Stigmastenol (24*S*/β)	8(14)	1.61	33.60	1.66	36.45
		(1.610)		(1.664)	
14-Stigmastenol (24*R*/α)	14	1.59	33.56	1.64	36.39
C_{30} sterol					
Gorgosterol (22*R*,23*R*,24*R*)	5,22:23-Cyclo	2.19	34.75	2.32	37.69
Acanthasterol (22*R*,23*R*,24*R*)	7,22:23-Cyclo	2.46	35.19	2.74	38.32

TABLE I *(continued)*

Acetate	*Position of double bond**	*OV-1*		*OV-17*	
		RRT	*MU*	*RRT*	*MU*
II. 4α-Methylsterol					
4α-Monomethylsterol (4α-methylcholestane group)					
4α-Methylcholestanol		1.19	32.46	1.12	34.95
Lophenol	7	1.28	32.75	1.33	35.60
24-Methyllophenol (24,*R*,*S*)	7	1.66	33.72	1.73	36.59
24-Ethyllophenol (24*R*/α)	7	2.06 (2.062)	34.53	2.16 (2.156)	37.41
24-Ethyllophenol (24*S*/β)	7	2.05 (2.051)	34.51	2.15 (2.145)	37.39
24-Methyl-23-dehydrolophenol	7,23*E*	1.63	33.64	1.79	36.72
Gramisterol (24-methylenelophenol)	7,24(28)	1.63	33.64	1.79	36.72
24-Methyl-24(25)-dehydrolophenol	7,24(25)	1.87	34.16	2.17	37.44
24-Ethyl-23-dehydrolophenol	7,23*E*	1.97	34.35	2.16	37.42
24-Ethyl-24(25)-dehydrolophenol	7,24(25)	2.28	34.91	2.60	38.12
Citrostadienol	7,24(28)*Z*	2.16	34.70	2.41	37.84
28-Isocitrostadienol	7,24(28)*E*	2.08	34.55	2.29	37.65
24-Ethyl-25-dehydrolophenol (24*S*/β)	7,25(27)	1.99	34.39	2.19	37.47
4α-Methyl-8-cholestenol	8	1.21	32.53	1.18	35.15
4α-Methyl-8(14)-cholestenol	8(14)	1.15	32.35	1.14	35.02
4α-Methyl-8(14)-ergostenol (24*R*,*S*)	8(14)	1.48	33.28	1.49	36.03
4α-Methyl-8(14)-stigmastenol (24*R*,*S*)	8(14)	1.83	34.08	1.86	36.86
4α,14α-Dimethylsterol (31-norlanostane group)					
31-Norcycloartanol	9:19-Cyclo	1.33	32.89	1.31	35.54
31-Norcycloartenol	9:19-Cyclo,24(25)	1.45	33.21	1.58	36.26
24(28)-Dihydrocycloeucalenol (24*R*,*S*)	9:19-Cyclo	1.71	33.83	1.71	36.54
24-Methyl-31-nor-23-dehydrocycloartanol	9:19-Cyclo,23*E*	1.67	33.74	1.77	36.67
Cycloeucalenol	9:19-Cyclo,24(28)	1.69	33.78	1.77	36.67
24-Methyl-31-norcycloartenol	9:19-Cyclo,24(25)	1.94	34.30	2.14	37.39
31-Norcyclolaudenol (24*S*/β)	9:19-Cyclo,25(27)	1.64	33.67	1.72	36.56
31-Nor-7-lanostenol	7	1.32	32.86	1.33	35.60
24-Methyl-31-nor-7-lanostenol (24*R*,*S*)	7	1.70	33.81	1.74	36.62
31-Nor-8-lanostenol	8	1.18	32.43	1.10	34.87
31-Norlanosterol	8,24(25)	1.29	32.78	1.33	35.60
24(28)-Dihydroobtusifoliol (24*R*,*S*)	8	1.51	33.36	1.44	35.90
Obtusifoliol	8,24(28)	1.48	33.28	1.49	36.03
24-Methyl-31-norlanosterol	8,24(25)	1.72	33.85	1.79	36.72
24-Ethyl-31-nor-8-lanostenol (24*S*/β)	8	1.86	34.14	1.78	36.69
24-Ethyl-31-nor-8,25-lanostadienol (24*S*/β)	8,25(27)	1.79	33.99	1.80	36.74
31-Nor-9(11)-lanostenol	9(11)	1.28	32.75	1.26	35.39
24-Methyl-31-nor-9(11)-lanostenol (24*R*,*S*)	9(11)	1.65	33.69	1.64	36.39
24-Methylene-31-nor-9(11)-lanostenol	9(11),24(28)	1.63	33.64	1.69	36.50
III. Triterpene alcohol					
Tetracyclic triterpene alcohol					
4,4-Dimethylsterol (32-norlanostane group)					
4,4-Dimethylcholestanol	–	1.43	33.16	1.33	35.60
Lanostane group					
Lanostanol	–	1.76	33.93	1.71	36.54
24-Methyllanostanol (24*R*,*S*)	–	2.25	34.85	2.23	37.53
Cycloartanol	9:19-Cyclo	1.59	33.56	1.54	36.15
Cycloartenol	9:19-Cyclo,24(25)	1.74	33.89	1.86	36.86
24-Methylcycloartanol (24*R*,*S*)	9:19-Cyclo	2.04	34.49	2.01	37.15

(Continued on p. 70)

TABLE I *(continued)*

Acetate	*Position of double bond**	*OV-1*		*OV-17*	
		RRT	*MU*	*RRT*	*MU*
Cyclosadol(24-methyl-23-dehydrocycloartanol)	9:19-Cyclo,23*E*	2.00	34.41	2.08	37.28
24-Methylenecycloartanol	9:19-Cyclo,24(28)	2.01	34.43	2.07	37.26
Cyclobranol (24-methylcycloartenol)	9:19-Cyclo,24(25)	2.33	34.98	2.50	37.98
Cyclolaudenol (24*S*/*β*)	9:19-Cyclo,25(27)	1.95	34.32	2.03	37.19
24-Dihydrocimicifugenol	7,9:19-Cyclo	1.22	32.56	1.13	34.99
Cimicifugenol (7-dehydrocycloartenol)	7,9-Cyclo,24(25)	1.34	32.91	1.37	35.71
7-Lanostenol	7	1.58	33.54	1.57	36.23
24-Methyl-7-lanostenol (24*R*,*S*)	7	2.04	34.49	2.05	37.23
24-Dihydroagnosterol	7,9(11)	1.32	32.86	1.28	35.45
Agnosterol	7,9(11),24(25)	1.43	33.16	1.55	36.17
24-Dihydrolanosterol	8	1.39	33.05	1.30	35.51
Lanosterol	8,24(25)	1.52	33.39	1.57	36.23
24-Methyl-24-dihydrolanosterol (24*R*,*S*)	8	1.78	33.97	1.70	36.52
24-Methylene-24-dihydrolanosterol	8,24(28)	1.75	33.91	1.76	36.65
24-Dihydroparkeol	9(11)	1.54	33.43	1.49	36.03
Parkeol	9(11),24(25)	1.69	33.78	1.79	36.72
24-Methyl-24-dihydroparkeol (24*R*,*S*)	9(11)	1.97	34.35	1.94	37.02
24-Methylene-24-dihydroparkeol	9(11),24(28)	1.93	34.28	2.00	37.13
24,24-Dimethyl-9(11)-lanostenol	9(11)	2.54	35.31	2.39	37.81
24,25-Dimethyl-9(11)-lanostenol (24*R*,*S*)	9(11)	2.35	35.01	2.40	37.82
24,25-Dimethyl-9(11),23-lanostadienol**	9(11),23ξ	2.33	34.98	2.37	37.78
24,24-Dimethyl-9(11),25-lanostadienol	9(11),25(27)	2.37	35.05	2.49	37.96
Euphane-tirucallane group					
24-Dihydrobutyrospermol (20*R*)	7	1.44	33.18	1.40	35.80
Butyrospermol (20*R*)	7,24(25)	1.58	33.54	1.70	36.52
24-Dihydroeuphol (20*R*)	8	1.20	32.50	1.07	34.78
Euphol (20*R*)	8,24(25)	1.32	32.86	1.30	35.51
7-Tirucallenol (20*S*)	7	1.60	33.58	1.59	36.28
7,24-Tirucalladienol (20*S*)	7,24(25)	1.76	33.93	1.92	36.98
24-Dihydrotirucallol (20*S*)	8	1.34	32.91	1.22	35.27
Tirucallol (20*S*)	8,24(25)	1.46	33.23	1.47	35.97
Dammarane group					
Dammaradienol	20(21),24(25)	1.50	33.34	1.64	36.39
24-Methylenedammarenol	20(21),24(28)	1.70	33.81	1.78	36.69
Isoeuphenol (20*R*)	13(17)	0.91	31.79	0.93	34.25
Isotirucallenol (20*S*)	13(17)	1.12	32.24	1.01	34.56
Cucurbitane group					
10α-5-Cucurbitaenol	5	1.21	32.53	1.19	35.18
10α-5,24-Cucurbitadienol	5,24(25)	1.32	32.86	1.43	35.87
Pentacyclic triterpene alcohol					
α-Amyrin (12-ursenol)	12	1.64	33.67	1.84	36.82
Taraxasterol (20[30]-taraxastenol)	20(30)	2.06	34.52	2.50	37.98
ψ-Taraxasterol (20-taraxastenol)	20(21)	2.01	34.43	2.40	37.82
Bauerenol (7-bauerenenol)	7	1.98	34.37	2.26	37.59
Epibauerenol (7-baueren-3α-ol)	7	1.64	33.64	1.94	37.02
Isobauerenol (8-bauerenenol)	8	1.73	33.87	1.83	36.84
β-Amyrin (12-oleanenol)	12	1.52	33.39	1.65	36.41
Germanicol (18-oleanenol)	18	1.54	33.43	1.65	36.41
Glutinol (5-glutinenol)	5	1.59	33.56	1.91	36.96
Taraxerol (14-taraxerenol)	14	1.45	33.21	1.57	36.23
Friedelinol (5β-methyl friedelan-3α-ol)	–	2.10	34.59	2.66	38.21
Epifriedelinol (5β-methyl friedelanol)	–	2.09	34.57	2.55	38.04

TABLE I *(continued)*

Acetate	*Position of double bond**	*OV-1*		*OV-17*	
		RRT	*MU*	*RRT*	*MU*
Multiflorenol (7-multiflorenenol)	7	1.86	34.14	2.13	37.39
Isomultiflorenol (8-multiflorenenol)	8	1.61	33.60	1.73	36.59
Lupeol (20[29]-lupenol)	20(29)	1.66	33.72	1.93	37.00
Epilupeol (20[29]-lupen-3α-ol)	20(29)	1.35	32.94	1.63	36.36
Fernenol (9[11]-fernenol, 21*S*)	9(11)	1.98	34.37	2.28	37.63
Epifernenol (9[11]-fernen-3α-ol)	9(11)	1.58	33.54	1.94	37.02
Trematol (21-epi-9[11]-fernenol, 21*R*)	9(11)	2.27	34.89	2.79	38.39
Moretenol (21-epi-22[29]-hopenol)	22(29)	1.95	34.32	2.26	37.59
Simiarenol (5-adianenol)	5	1.92	34.26	2.40	37.82
Isoarborinol (9[11]-arborinenol)	9(11)	2.15	34.68	2.57	38.08

* Cyclopropyl group is also included and denoted by Cyclo.

** From *Quercus myrsinaefolia* (W. H. Hui and M. M. Li, *J. Chem. Soc., Perkin Trans. I*, (1977) 897, most probably has a 23*E*-configuration.

which are of higher plant origin or are derived therefrom. It is worth mentioning here that there are differences in the retention data of 24-alkylsterols epimeric at C-24 as reported recently[27–29]. The 24α-epimers of 24-methyl- and 24-ethylsterols with a saturated side chain showed slightly larger RRT and MU values than those of their 24β-counterparts, whereas the opposite elution order was observed for the 24-ethyl-Δ^{22}- and 24-ethyl-$\Delta^{22,25}$-sterols[27]. The use of more highly polar phases and longer capillary columns had resulted in GLC being a diagnostic tool in the differentiation and characterization of the epimeric 24-alkylsterols[28,29]. Chromatography of a 1:1 mixture of the C-24 epimeric pair of 24-alkylsterols with a saturated or a Δ^{22}-monounsaturated side chain afforded a single peak on OV-1 and OV-17 glass capillary columns in this study, instead of separated peaks, and hence several C-24 epimeric mixtures of 24-alkylsterols, which were derived from the corresponding dehydro-sterols by hydrogenation, are considered to be eluted at the midpoint of the peaks of the epimers of each epimeric pair.

The separation factors related to the presence of double bonds, steric effects and alkyl substituents, which were calculated from the RRT data given in Table I, are shown in Tables II–IV. As found previously[5], there is almost no interdependence of the individual separation characteristics related to the skeleton and to the side chain, and therefore, the two sets of features are presented separately in these tables.

Skeletal double bond and steric separation factors are shown in Table II. Since most of the compounds analyzed are of higher plant origin and such plants contain Δ^5, Δ^7 and 9β:19-cyclopropyl compounds as the major sterol constituents[37–39], these compounds were taken, for convenience, as the references in calculating the skeletal double bond separation factors. The separation factors related to the skeletal double bond of cholestane and of 4α-methylcholestane were almost identical, but markedly different from those of 31-norlanostane and lanostane which possess quite similar separation factors. This indicates that the skeletal double bond separation factor is affected solely by the angular methyl group at C-14α, whereas almost no influence is exerted by the methyl group at C-4. Several stationary phases have been shown to distinguish saturated 4-desmethylsterols from the corresponding Δ^5 sterols in packed

TABLE II

SKELETAL DOUBLE BOND AND STERIC SEPARATION FACTORS

Separation factors in this and in the subsequent tables refer to mean values when two or more examples are available.

Compounds compared		*Stationary phase*	
		OV-1	*OV-17*
Stanol/Δ^5	Cholestane	1.03	1.01
Δ^7/Δ^5	Cholestane	1.13	1.19
Δ^8/Δ^5	Cholestane	1.07	1.06
$\Delta^{8(14)}/\Delta^5$	Cholestane	1.01	1.02
Δ^{14}/Δ^5	Cholestane	0.99	1.01
$\Delta^{5,7}/\Delta^5$	Cholestane	1.13	1.17
$\Delta^{5,8}/\Delta^5$	Cholestane	1.06	1.07
Stanol/Δ^7	Cholestane	0.91	0.86
	4α-Methylcholestane	0.93	0.84
	Lanostane	1.11	1.09
Δ^8/Δ^7	Cholestane	0.95	0.90
	4α-Methylcholestane	0.95	0.89
	31-Norlanostane	0.89	0.83
	Lanostane	0.88	0.83
	Euphane-tirucallane	0.83	0.77
	Bauerenane	0.87	0.82
	Multiflorenane	0.87	0.81
$\Delta^{8(14)}/\Delta 7$	Cholestane	0.89	0.86
	4α-Methylcholestane	0.89	0.86
$\Delta^{9(11)}/\Delta^7$	31-Norlanostane	0.97	0.95
	Lanostane	0.97	0.95
Δ^{14}/Δ^7	Cholestane	0.85	0.88
$\Delta^{7,9(11)}/\Delta^7$	Lanostane	0.84	0.81
$\Delta^{8,14}/\Delta^7$	Cholestane	0.91	0.90
Stanol/9:19-cyclo	Lanostane	1.11	1.11
Δ^7/9:19-cyclo	31-Norlanostane	1.00	1.02
	Lanostane	1.00	1.03
Δ^8/9:19-cyclo	31-Norlanostane	0.89	0.84
	Lanostane	0.87	0.85
$\Delta^{9(11)}$/9:19-cyclo	31-Norlanostane	0.96	0.96
	Lanostane	0.97	0.97
$\Delta^{7,9(11)}$/9:19-cyclo	Lanostane	0.83	0.83
Δ^7,9:19-Cyclo/9:19-cyclo	Lanostane	0.77	0.74
Δ^{18}/Δ^{12}	Oleanane	1.01	1.00
$\Delta^{20(30)}/\Delta^{20(21)}$	Taraxastane	1.02	1.04
Stan-3α-ol/stanol	Cholestane	0.89	0.87
	Lupane	0.81	0.84
	Fernane ($\Delta^{9(11)}$)	0.80	0.85
	Bauerenane (Δ^7)	0.83	0.87
	Friedelane	1.00	1.04
5β-Stanol/stanol	Cholestane	0.84	0.80
5β-Stan-3α-ol/stanol	Cholestane	0.86	0.82
Euphane/lanostane	Δ^8	0.87	0.83
	Δ^7	0.91	0.88
Tirucallane/lanostane	Δ^8	0.96	0.94
	Δ^7	1.01	1.01
Ursane/oleanane	Δ^{12}	1.08	1.12
Bauerenane/multiflorenane	Δ^8	1.07	1.06
	Δ^7	1.06	1.06
20*R*(α-H)/20*S*(β-H)	Cholestane	1.10	1.12
	Euphane-tirucallane	0.90	0.88
	Dammarane($\Delta^{13(17)}$)	0.81	0.92
21*R*(α-H)/21*S*(β-H)	Fernane	1.15	1.22

columns[4,5,15,16], but none gave complete separations of these sterols[5]. The OV-1 glass capillary column used in this study, as well as in other studies[40,41], afforded almost complete separations. Δ^7-Lanostane was eluted simultaneously or slightly after its 9β: 19-cyclopropyl isomer, but the co-existence of the Δ^7-bond and the cyclopropyl group in the lanostane skeleton greatly reduced its retention time.

20-Isocholesterol (20*S*, β-H) was eluted before its 20*R* epimer, cholesterol (20α-H), as observed previously[42,43]. This is most probably due to the conformational difference in the side chain, since rotation of C-20 about the 17(20)-bond can place the side chain in significantly different positions[42–44]. Thus the most stable conformer at the 17(20)-bond should be the non-eclipsed one with the 20-H (the smallest group on C-20) close to C-18, and therefore presumably the preferred conformer for cholesterol is a "right-handed" structure (C-22, *trans*-oriented to C-13). When the configuration at C-20 is inverted as in 20-isocholesterol, the preferred conformation should be that with a "left-handed" side chain (C-22, *cis*-oriented to C-13). Based on these considerations, we can postulate conformation at C-20 of euphane-tirucallane and $\Delta^{13(17)}$-dammarane triterpenes from their retention behaviour. The 20*S*(β-H) epimers of these triterpenes were eluted after their 20*R*(α-H) counterparts, and therefore the former should have a "right-handed" side chain conformation and the latter a "left-handed" conformation.

Table III shows the separation factors associated with the side chain double bonds of sterols and tetracyclic triterpenes. The configurational isomerism at C-24 of 24-alkylsterols exerted almost no influence on the side chain double bond separation factors, while the separation factors related to the 22*E*,25(27)-double bonds of 24-ethylsterol differed appreciably according to the nature of the isomerism. The presence of

TABLE III

SIDE CHAIN DOUBLE BOND SEPARATION FACTORS

Relative to the RRT of the steryl acetates with the corresponding saturated side chain.

Position of double bond	*Alkyl or alkylene substituent*	*Stationary phase*	
		OV-1	*OV-17*
22*Z*	–	0.88	0.90
22*E*	–	0.92	0.93
	24-Methyl	0.86	0.88
	24-Ethyl	0.86	0.88
23*E*	24-Methyl	0.98	1.04
	24-Ethyl	0.96	1.00
24(25)	–	1.09	1.21
	24-Methyl	1.14	1.25
	24-Ethyl	1.11	1.21
24(28)	24-Methylene	0.98	1.03
	24*Z*-Ethylidene	1.04	1.12
	24*E*-Ethylidene	1.01	1.06
25(27)	24-Methyl	0.96	1.01
	24-Ethyl	0.97	1.02
	24,24-Dimethyl	0.93	1.04
22*E*,25(27)	24α-Ethyl	0.86	0.91
	24β-Ethyl	0.87	0.93

TABLE IV

ALKYL SUBSTITUENT SEPARATION FACTORS

Alkyl substituent	*Position of double bond**	*Stationary phase*	
		OV-1	*OV-17*
4α-Methyl	–	1.16	1.10
	7	1.14	1.12
	8	1.13	1.11
	8 (14)	1.14	1.11
4α,14α-Dimethyl/14α-methyl	9:19-Cyclo	1.14	1.10
4,4-Dimethyl/4α-methyl	–	1.20	1.19
4,4,14α-Trimethyl/4α,14α-dimethyl	9:19-Cyclo	1.19	1.18
	7	1.20	1.19
	8	1.18	1.18
	9 (11)	1.19	1.18
4,4-Dimethyl	–	1.39	1.30
4α,14α-Dimethyl/4α-methyl	7	1.03	1.01
	8	0.98	0.93
4,4,14α-Trimethyl/4,4-dimethyl	–	1.23	1.29
24-Methyl	–	1.29	1.31
	22*E*	1.22	1.23
	24 (25)	1.35	1.35
23,24-Dimethyl	22*E*	1.49	1.47
24,24-Dimethyl	–	1.65	1.60
24,25-Dimethyl	–	1.53	1.61
23,24-Dimethyl/24-methyl	22*E*	1.22	1.20
24,24-Dimethyl/24-methyl	–	1.29	1.23
24,25-Dimethyl/24-methyl	–	1.19	1.24
24-Ethyl	–	1.60	1.63
	22*E*	1.52	1.54
	24 (25)	1.65	1.63
24-Ethyl/24-methyl	–	1.24	1.24
	22*E*	1.25	1.25
	24 (25)	1.22	1.20
	25 (27)	1.25	1.25

* Cyclopropyl group is also included.

the 23*E*-double bond resulted in shorter retention times of sterols on the OV-1 column. whereas on the OV-17 column the retention times were increased or unaffected. The other separation characteristics listed are in reasonable agreement with those previously reported[4,5]. The alkyl substituent at C-24 affected the separation characteristics of the $\Delta^{22}E$, $\Delta^{23}E$, $\Delta^{24(25)}$ and $\Delta^{25(27)}$ sterols.

The separation factors for various alkyl substituents of sterol and lanostane triterpenes are listed in Table IV. Of all the methyl substituents examined, that at C-24 gave the largest retention time increment, whereas the smallest effect on the retention time was observed, as for Δ^7 and Δ^8 sterols, on introduction of the 14α-methyl group. In Δ^8 sterols, the 14α-methyl group actually resulted in a decrease in retention time, as observed previously[5]. The effect of this group was, however, exceptionally large for compounds with saturated skeletons. The increase of retention time attributed to an alkyl substituent was affected by the presence of an adjacent double bond,

most probably due to the interaction between the double bond and the alkyl group. For example, introduction of a $\Delta^{22}E$-bond decreased the retention time increment due to a methyl or ethyl substituent at C-24. Gas chromatography can be used to distinguish between 4-desmethyl-, 4-monomethyl- and 4,4′-dimethylsterols[5,8–10].

The RRT and MU values of the large number of sterols and triterpene alcohols given in Table I are very useful for the identification of these compounds. Several sets of "critical pairs" of sterols were encountered on both the column systems, but these can be distinguished by argentation thin-layer chromatography or by some spectroscopic techniques, or may be differentiated in GLC on other column systems. Using the RRT data, additional separation factors can be calculated for other structural features, and the probable RRT of unknown or undetected sterols and triterpene alcohols can be predicted.

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APPLICATION OF ELECTRONICALLY DIFFERENTIATED HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC DENSITOGRAMS TO THE ASSAY OF SOME PRESERVATIVES USED IN PHARMACEUTICAL FORMULATIONS*

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SUMMARY

The results of an extended study on the feasibility and scope of derivative high-performance thin-layer chromatography (HPTLC) for the assay of unresolved components are presented. It is shown that as the resolution decreases the accuracy of quantitative digital electronic integration, area and peak height measurements diminishe to a point where the differentiated signal becomes the only valid approach to the direct quantitation of unresolved HPTLC spots. Using the first derivative, the accuracy of these assays can be maintained within 2–3%, independently of the resolution, and the derivative techniques provide a very useful alternative to direct HPTLC analysis of small amounts of a component masked by major peaks. In practice, this simplifies the optimization of experimental parameters in HPTLC, and results in shorter analysis times.

The HPTLC spots are estimated in a spectrophotodensitometer, the signal of which is electronically differentiated by means of an analog circuit, capable of recording the first or second derivative spectrum. Instrumental possibilities for optimizing and enhancing the differential signal are discussed.

INTRODUCTION

It has already been shown[1] that the use of electronically differentiated first and second order signals in thin-layer chromatography (TLC) improves the quantitation of poorly resolved components. According to our experience, this technique could save a lot of time and effort while enhancing the scope of standard TLC or high-performance TLC (HPTLC) techniques, especially where overlapping of the ana-

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lytes precludes accurate measurement of the necessary parameters usually derived from a graphical evaluation of peak shape.

As a further check of the potential of derivative HPTLC, we have studied the performance of this procedure in the assay of a group of preservatives found in a wide range of pharmaceutical products and foods[2]. Relative to ultraviolet spectroscopic or gas chromatographic methods, quicker analysis of these products by TLC, is said to be possible if confirmation only is desired[3] and that for speed and quantitation high-performance liquid chromatography (HPLC) is more appropriate. Although this may generally be true, we demonstrate that the application of electronic differentiation of spectrophotodensitometric HPTLC signals is also very rapid and specific, sometimes obviating the need for tedious empirical adjustments of the mobile phases in order to quantitate poorly resolved compounds.

EXPERIMENTAL

Samples

The *p*-hydroxybenzoic esters used in this work were purchased from Lemmel S.A. (Barcelona, Spain). All are commercially available pharmaceutical preservatives.

Methods

The HPTLC separations were performed on E. Merck (Darmstadt, G.F.R.) reversed-phase nanoplates Sil-C_{18} 50 % and RP-18 F_{254} (10 × 10 cm). TLC runs were carried out on silica gel G-60 F_{254} plates (regular or silanized for reversed-phase TLC), also from E. Merck.

Samples were spotted with an Evachrom sample applicator and the plates were developed by the ascending technique in closed non-saturated glass chambers at 18–20 °C for 6–7 min. The reversed-phase plates were developed with methanol–water solutions of various compositions, depending on the degree of separation desired. The solvent front was allowed to rise 4 cm from the sample spots. The methanol was purchased from E. Merck.

Resolution was calculated from the expression $R = 2\,(x_2 - x_1) / (b_2 + b_1)$ where x_1 and x_2 are the distances from the starting point to the peak maximum for compounds 1 and 2, and b_1, b_2 are the corresponding peak widths at half-height. In cases where overlapping of peaks 1 and 2 did not allow the graphical measurement of these two parameters, the values were extrapolated from those of standards spotted alongside the mixture.

Spectrophotodensitometric detection

The plates (air dried in an oven for 5 min) were read on a Zeiss KM3 chromatography spectrophotometer (Carl Zeiss, Oberkochen, G.F.R.) operated in the reflectance mode at $\lambda = 270$ nm and variable slits of 3.5 × 2–0.2 mm, as discussed below. The scanning speed was usually set at 100 mm/min. The plates were read in the direction of solvent flow.

Derivative HPTLC

The derivative curves from the spectrophotodensitometric zero-order signals were directly recorded by coupling to the recorder unit (Perkin-Elmer Hitachi Model 200) a Perkin-Elmer Model 200-0628 derivative spectrum attachment.

RESULTS AND DISCUSSION

The components of a mixture of methyl (MHB), ethyl (EHB), propyl (PHB), butyl (BHB) and benzyl (BzHB) *p*-hydroxybenzoates cannot be resolved on standard silica gel HPTLC plates as shown in Fig. 1A. The only TLC approach yielding the complete separation of these five compounds implies the use of double development on properly activated and silanized silica G-60 plates, as illustrated in Fig. 2. This approach was described by Rangone and Ambrosio[2] who, for quantitative purposes, removed the bands and extracted the compounds from the silica gel with methanol. However, according to our experience, this procedure is limited in practice by the relatively short life of the silanized TLC surface, which tends to suffer serious disruption and cracking when wetted by the aqueous solvent front. Thus, reproducibility is rather poor for quantitative purposes.

A different approach is based on the use of modern reversed-phase systems using a relatively high water content in the mobile phase. In this context, two recent communications have described the use of circular HPTLC for the rapid separation of PHB, EHB and MHB[4] and the HPTLC quantitation of MHB and PHB[5] on reversed-phase layers. However, both of them do not deal with butyl and benzyl *p*-hydroxybenzoates which cannot be resolved on reversed-phase layers, even when the water content of the mobile phase approaches the maximum allowable by the hydrophobicity of these layers.

The first advice one would get from a TLC expert would be to "change or modify

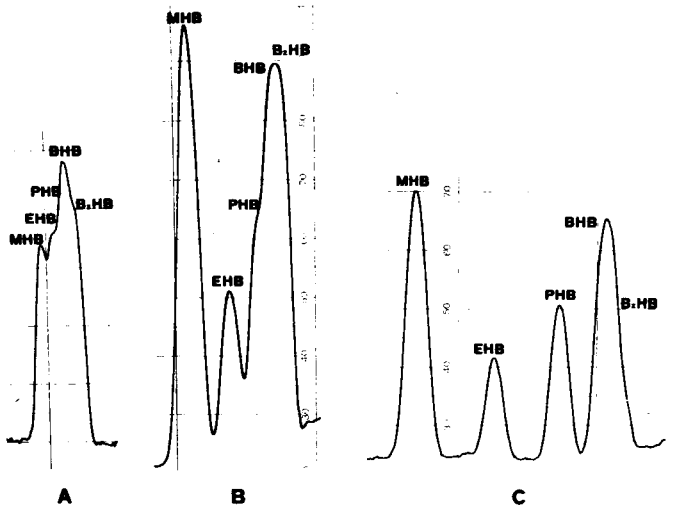

Fig. 1. TLC and HPTLC separations of different *p*-hydroxybenzoates (from low to high R_F): BzHB = benzoyl *p*-hydroxybenzoate (80 ng); BHB = butyl *p*-hydroxybenzoate (400 ng); PHB = propyl *p*-hydroxybenzoate (160 ng); EHB = ethyl *p*-hydroxybenzoate (80 ng) and MHB = methyl *p*-hydroxybenzoate (320 ng). The amounts shown in parentheses were dissolved in the 0.2 μl methanol deposited on the plates. A, Sorbent silica gel. G-60 F_{254}, 10 × 10 cm; solvent system pentane–acetic acid (22:3). B, Sorbent Sil-C_{18} 50%, 10 × 10 cm; solvent system methanol–water (40:60). C, Sorbent RP-18 F_{254}, 10 × 10 cm; solvent system methanol-water (85:15), triple development. Slit widths: A and B, 0.5 mm; C, 0.35 mm. Linear scanning at 100 mm/min. Recorder speeds: A and B, 120 mm/min; C, 240 mm/min.

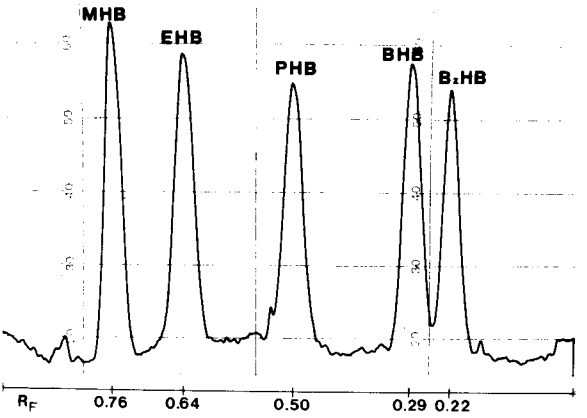

Fig. 2. Separation of the same mixture as in Fig. 1 on activated and silanized silica G-60 plates. Solvent system: borate buffer (pH 11)–dioxane (9:1). Double development. Spectrophotodensitometer conditions as in Fig. 1A and B. Volumes of 0.2 μl containing 240 ng of each compound were spotted.

the solvent", although occasionally this is not effective as illustrated in Fig. 1B and C. These HPTLC profiles show examples of the kind of results obtained in two of the multiple solvent systems tested. Changing the mobile phase did not result in a separation of the five compounds comparable to that of Fig. 2, but, as expected, it did affect the overall resolution so that we can merge or separate any of these components at will, except for BzHB and BHB which always overlap. Thus, in line with our recent experience on the application of derivative spectroscopic techniques to overlapped HPTLC spots, this particular system appeared to be an ideal model for the further study of the performance of the derivative technique.

One of the first aspects examined was the relative merits of conventional area and peak height measurements *vs.* first derivative determinations as a function of peak resolution. For this purpose, HPTLC of the ethyl and propyl esters was selected as a suitable working model since both compounds can be resolved by appropriate modifications of the methanol–water ratio in the mobile phase. The data in Fig. 3 summarize our experience of different laboratory applications over the past 2 years. That is, electronically differentiated signals consistently yield a higher precision and accuracy of the quantitative measurements at low resolutions. As shown in Fig. 3, for baseline-resolved peaks ($R > 1$), quantitation by peak area, peak height or first derivative provides in all cases a satisfactory degree of accuracy, irrespective of the method used. However, as resolution decreases, the accuracy of the peak height or peak area measurements also decreases to a point where quantitation of the merged peaks is no longer possible, Nevertheless, even when resolution is very poor or practically nil, the components can in many cases still be quantitated by application of derivative techniques, as illustrated in Fig. 4. In this example, the quantitation of EHB and PHB, even at a resolution of only 0.28, can readily be achieved by measuring the distances OA and OB in the first derivative curve of the unresolved HPTLC spot, relative to the corresponding standards.

Consequently, whenever peak area measurements cannot be used and peak height values are significantly affected by a lack of resolution, the electronically differentiated signals obtained directly from the overlapped peaks may provide the only

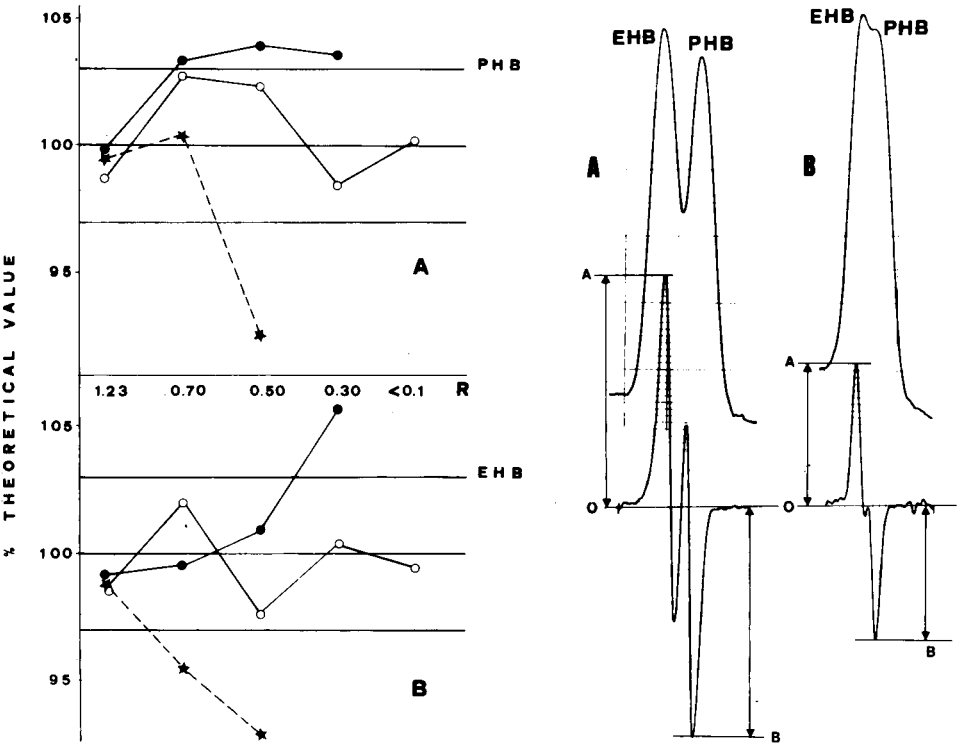

Fig. 3. Accuracy of peak height and first derivative procedures for the quantitation of unresolved HPTLC spots. The chromatographic resolution R, of PHB and EHB is dependent on the methanol–water ratio (in parentheses below), as follows: R = 1.23 (8:4); 0.70 (7:1); 0.50 (10:1); 0.30 (20:1); <0.1 (50:1). The data were obtained on RP-18 F_{254} plates with a slit width of 0.5 mm and a scanning speed of 100 mm/min. Mode 6, except when R = 0.30 and <0.1, where the modes used were 4 and 5, respectively. The 100% line represents the theoretical value corresponding to the actual content of propyl (A) and ethyl *p*-hydroxybenzoates (B). ★, Data points calculated from electronically integrated peak areas; ●, peak height values; ○, first derivative measurements of electronically differentiated signals. All data points represent the averages of four determinations.

Fig. 4. First derivative measurements of unresolved PHB and EHB at resolutions of 0.70; (A) and 0.30 (B). Conditions as in Fig. 3. The response shown corresponds to 320 ng of each compound.

practical means to perform rapidly the desired assay, thus avoiding the sometimes cumbersome and tedious process of finding another suitable mobile phase system. Also, as already demonstrated by us[1], the derivative approach in many cases provides valid quantitative information even when peaks are totally overlapped (R < 0.1 in Fig. 3).

In all of the present cases (R = 1.23 to <0.1), the values calculated from the first derivative curves approach the true theoretical value to within ± 3%, an accuracy which cannot be achieved by peak area or peak height measurements at resolutions lower than 0.71 (see Fig. 3). The corresponding precision values expressed as the coefficient of variation of replicate determinations at three different resolutions are given in Table I. These data clearly show that seriously overlapped peaks (R < 0.1) can be quantitated by the derivative technique with a relatively high degree of ac-

TABLE I

PRECISION OF DIFFERENT MEASUREMENTS AT DIFFERENT RESOLUTIONS

PHB = Propyl ester of *p*-hydroxybenzoic acid; EHB = ethyl ester of *p*-hydroxybenzoic acid. The values represent the coefficients of variation (%) corresponding to the points shown in Fig. 3. In all cases there were four replicate determinations per plate. The data were obtained by application of the data pair procedure[7]. The corresponding calibration curves and linear regression analysis are given in Table II.

	0.70		*0.50*		*0.30*		*<0.1*	
	PHB	*EHB*	*PHB*	*EHB*	*PHB*	*EHB*	*PHB*	*EHB*
Peak area (electronic integrator)	2.48	4.60	3.55	3.40	–	–	–	–
Peak height	0.24	0.48	0.73	0.75	1.94	2.44	–	–
First derivative	0.49	0.44	0.59	0.62	3.88	1.35	1.23	1.05

curacy and precision, whereas electronic peak area and peak height measurements become inoperative. It is also evident that the derivative technique shows its advantages in extreme situations where all other conventional quantitative approaches fail.

The calibration curves obtained for instance for PHB by application of three different types of regression formulae to the first derivative measurements confirm the higher quality of fit of the log/log and reciprocal $1/y$ *vs.* $1/x$ regression[6] according to Table II. The concentration of PHB spotted on the HPTLC plate ranged from 16 to 320 ng. As a test of the discriminating potential of the derivative technique, a series of parallel assays were carried out in the presence of a direct and constant interference of a fixed amount of EHB (320 ng added to all of the samples of PHB). These assays were carried out at a resolution of the order of 0.3. As shown in Table II, the accuracy of the calibration data obtained with and without EHB is maintained since the first derivative determination of PHB is not affected by the relatively high excess of unresolved EHB. In other words, the derivative spectra effectively discriminate against the overlapping effect of EHB.

The same concepts were next applied to three-component HPTLC spots with the aim of verifying the potential use of the derivative technique in more complex

TABLE II

FIRST DERIVATIVE CALIBRATION OF PHB BY THREE LINEAR REGRESSION METHODS COMPARED TO THE DATA OBTAINED FOR PHB IN THE PRESENCE OF EHB AT A RESOLUTION OF 0.30

		A	*B*	*r*	$S_{y/x}$
$y = Ax + B$					
	PHB	0.018	0.89	0.9817	0.22
	PHB + EHB	0.023	1.56	0.9796	0.23
$\ln y = A \ln x + B$					
	PHB	0.6518	1.57	0.9971	0.09
	PHB + EHB	0.630	1.44	0.9823	0.09
$1/y = A \cdot 1/x + B$					
	PHB	25.51	0.07	0.9997	0.03
	PHB + EHB	11.90	0.09	0.9989	0.05

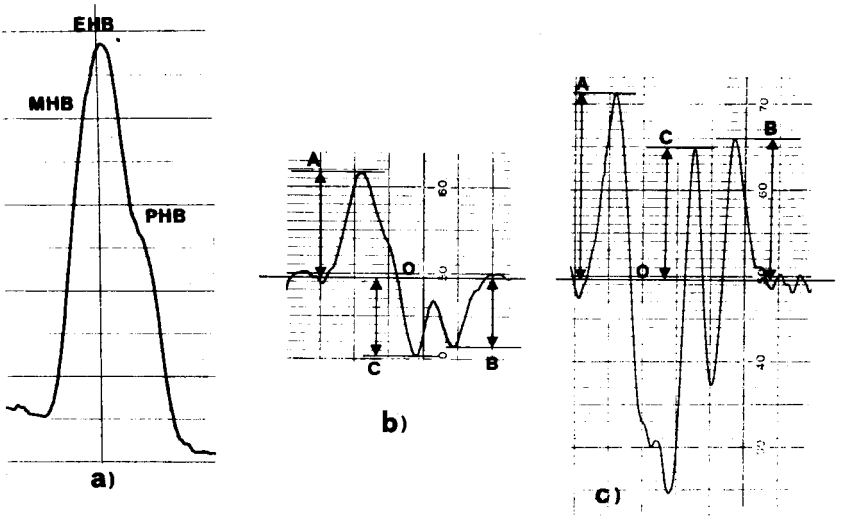

Fig. 5. A, Spectrophotodensitometric profile of an unresolved HPTLC spot containing MHB, EHB and PHB. Sorbent: RP-18 F_{254}. Solvent: methanol–water (9:0.7). Slit width: 0.2 mm. Scanning at 100 mm/min. B, First derivative scan of profile A. C, Second derivative scan of profile A. Derivative conditions: scan speed 240 mm/min; mode 5; absorbance scale 0.2.

situations. An example of the kind of results obtained for a peak representing a mixture of MHB, EHB and PHB is illustrated in Fig. 5. Whereas the components hidden behind the leading (PHB) and trailing edges (MHB) of the main peak (EHB) can readily be quantitated by measuring the distances OA and OB in the first derivative curves, as for the binary mixtures (see Fig. 4), the ascending and descending slopes of the first or second derivative curves corresponding to the middle component are significantly affected by the shape of those of the other two components. In other words, the middle component (EHB) would have to be quantitated using for example the distance OC in the derivative traces, a measurement clearly subject to significant deviations from the true values, as shown graphically in Fig. 6. Thus, while the MHB and PHB in this example can still be quantitated to within $\pm$ 10% by first or second derivative measurements this is not so for EHB. However, as shown by the higher deviations of the other points, this accuracy level applies only to the optimum 0.5-mm slit width of the spectrophotodensitometer. This leads to a consideration of the need to optimize the instrumental parameters.

To demonstrate the remarkable effect of the slit width on the accuracy of the derivative data, in Fig. 7A and B the first derivative values measured for PHB and EHB at different resolutions and with a slit of 0.5 mm (Fig. 3A and B) are compared to the corresponding values obtained at different slit widths. As shown, smaller or larger slits than 0.5 mm produce a remarkable decrease in accuracy. Thus, in derivative recording the slit width of the spectrophotometer is a critical parameter which must be adjusted in accordance with the peculiarities of each problem and instrument in order to optimize resolution.

Other instrumental parameters which may influence the accuracy of derivative measurements are the scan speed and the mode (differentiator time constant) selected.

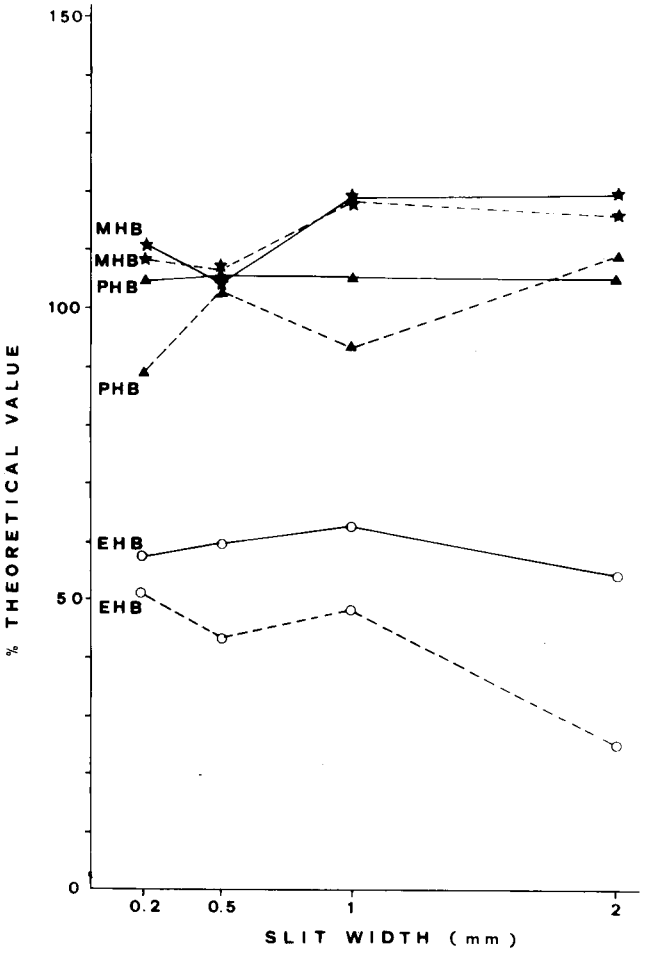

Fig. 6. Assay of an unresolved three-component peak (see Fig. 5) by first (———) and second (– – –) derivative measurements at different slit widths. PHB (▲) was measured using the distance OB (Fig. 5), MHB (★) using the distance OA and EHB (○) using distance OC.

This is illustrated in Fig. 8 for the case of the unresolved three-component peak of Fig. 5. The choice of scan speed (Fig. 8A) may be specially critical for the second derivative spectrum since in this case the instrument measures the differential changes of absorbance against time. An increase in scanning speed leads to an increased amplitude of the absorption derivative signal, although the resolution of the spectrum decreases. In this particular unit the mode selector acts as a series of frequency cut-off filters, with higher modes increasing the amplitude of the first and second derivative spectra and reducing the overall high frequency noise, although this is achieved at the expense of resolution. Thus, providing that all other instrumental parameters are fixed, in this case the optimum results would be obtained at mode 4 as shown in Fig. 8B. A plot of the mode setting *vs.* signal/noise ratio for the first and second derivatives of a mixture of PHB and EHB showed that this ratio increases at higher settings. For

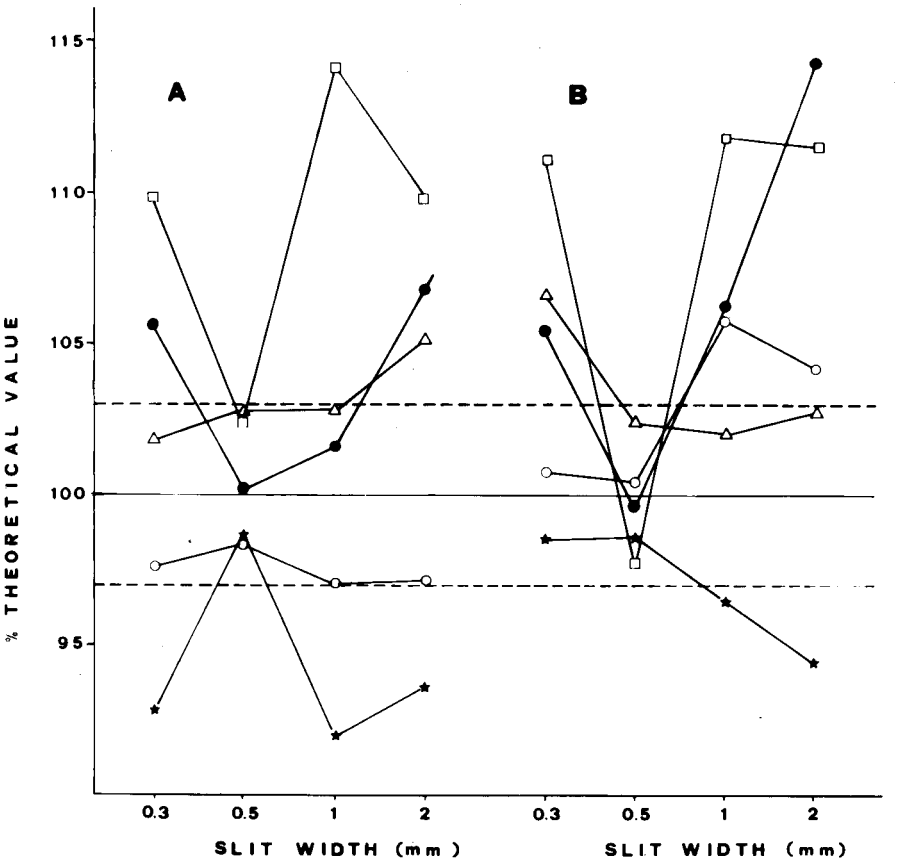

Fig. 7. Accuracy of first derivative determinations of the content of PHB (A) and EHB (B) in a mixture of both prepared in the laboratory, carried out at different resolutions and instrumental slit widths. ★, values obtained at $R = 1.23$; △, values at $R = 0.70$; □, values at $R = 0.50$; ○, values at $R = 0.30$; ●, values at $R = < 0.1$. Mode 6, scan speed 100 mm/min.

example, in the first derivative spectra of EHB and PHB, the signal/noise ratio increases from 6 and 4.5, respectively, at mode 3, to 14 and 12.5, respectively, at mode 6. However, the overall resolving power decreases as the mode setting increases; thus, a significant amount of the analytical information contained in the fine detail of these derivative curves is lost. For example, the detail seen in the negative part of the second derivative curve in Fig. 5C, which indicates overlapping of MHB and EHB, would be lost at mode 6 but enhanced at mode 2 or 3. Accordingly, the mode should be adjusted carefully depending on the type of problem. In this particular case, there was no need further to resolve these two second derivative minima by going to a lower mode, since the measurements taken from both minima to the derivative baseline zero (O in Fig. 5C) were not more accurate than those taken on the OA and OC distances, as indicated on this figure. Also, it should be noted that the loss of peak amplitude at the lower mode settings may be a disadvantage in trace analysis[1] or when dealing with very low amounts of sample.

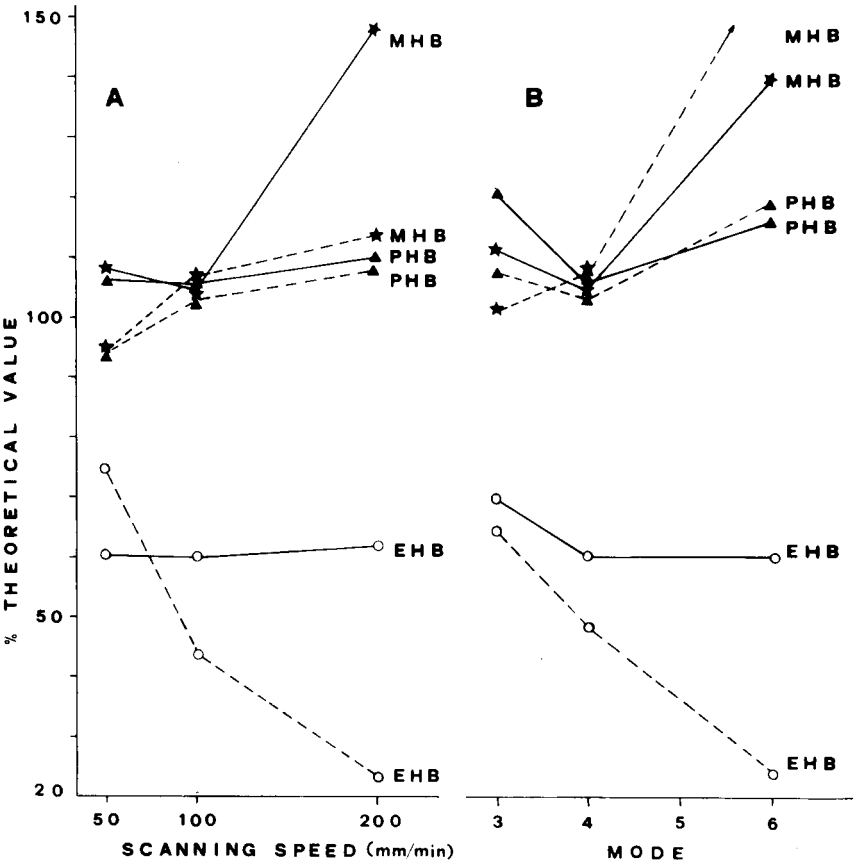

Fig. 8. First (———) and second (– – –) derivative spectrophotodensitometric determination of MHB (★), EHB (○) and PHB (▲) in an unresolved HPTLC spot (see Figs. 5 and 6) at different scan speeds (A) and modes (B). All measurements were carried out at a fixed slit width of 0.5 mm using the distances indicated in the caption of Fig. 6. Mode 6 was used for the readings in A. Data for B were obtained at a scan speed of 100 mm/min.

In this work one has to consider two kinds of resolutions, which should not be confused. On the one hand, the chromatographic resolution, which is fixed by the experimental parameters of plate development and on the other hand, the derivative resolution, which can be used, as shown herein, to improve a given TLC resolution without any further manipulation of the chromatographic parameters. This is why, at very low TLC resolutions, R, the discriminating power of the derivative readings taken on unresolved peaks (*e.g.*, Fig. 4) can be optimized by setting the mode of the differentiating instrument to lower values. For instance, in Fig. 3 the first derivative points corresponding to $R = 0.30$ and $R = <0.1$ were obtained at modes 4 and 5 respectively.

In conclusion, we believe that the application of electronically differentiated signals to the densitometric evaluation of TLC plates, especially for the resolution of seriously overlapping spots, could generate, without any further changes in the chro-

matographic systems, quantitative data of the same accuracy and precision as those obtained by more elaborate and time consuming procedures[8,9].

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OVERALL CHARACTERISTICS OF A LIQUID CHROMATOGRAPHIC DETECTION SYSTEM USING A SILICON PHOTODIODE ARRAY

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SUMMARY

The use of a silicon photodiode array as a multi-wavelength detector which allows the recording of complete UV spectra for each component in the effluent from liquid chromatography is described. This detector gives noise levels of the order of $6 \cdot 10^{-5}$ absorbance units in the UV range, and a linear calibration curve for 0.5–1000 ng biphenyl has been obtained.

INTRODUCTION

Several types of rapid scanning spectrophotometers for instantaneous recording of the spectra of eluted components have recently been introduced into liquid chromatographic (LC) systems. Denton *et al.*[1] employed an oscillating mirror rapid scanning spectrophotometer. Saitoh and Suzuki[2] designed a spectrophotometer to scan the 200–800 nm range in 375 msec. Rapid scanning spectrophotometers employing a silicon target vidicon have also been reported[3–5].

The recent availability of inexpensive silicon photodiode arrays has enabled the use of these devices in multi-wavelength UV detectors for LC. Applications of such arrays in LC have been reported by Dessy and co-workers[6,7] and Grushka and co-workers[8,9]. However, further work seems to be necessary to evaluate the overall system for use in high-performance liquid chromatography (HPLC).

The purpose of this study was to determine the overall performance characteristics of an LC system consisting of a reversed-phase column and a diode array detector (Reticon RL 1024C/17 or RL 512 S) and to demonstrate its ability to give lower noise levels of $6 \cdot 10^{-5}$ absorbance units (AU) at 250 nm.

EXPERIMENTAL

Optics

A single-beam spectrophotometer consisting of a light source, optics and a photodiode array detector was constructed as shown in Fig. 1. A deuterium lamp (L 613-03, Hamamatsu TV. Co.) was used as the light source. Light from this lamp is collimated, then passes through a flow cell (volume 8 μl, path length 1 mm) and forms

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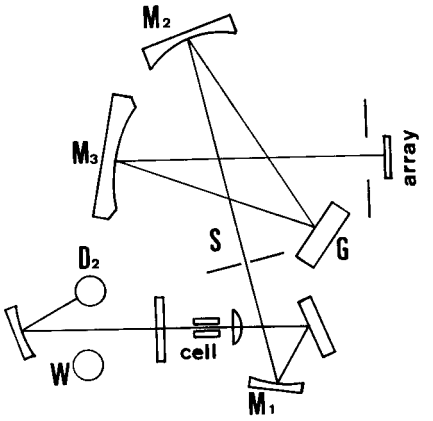

Fig. 1. Optical diagram. D_2 = Deuterium lamp; W = tungsten lamp; M_1, M_2, M_3 = mirrors; G = diffraction grating; S = slit.

an image on the entrance slit(s) of the polychrometer (Shimadzu Seisakusho Ltd.). An entrance slit width of 0.11 mm was used. A Bausch & Lomb grating G (600 lines per mm for the RL 1024 C/17 or 300 lines per mm for 512 S) was used. The wavelength range covered by each array is 220 nm. This range is focused on the sensing area of the array by a concave mirror M_3 (F/5.3). The RL 1024 C/17 array has 1024 pixcels (pitch 1 mil, aperture 17 mil), the RL 512 S array has 512 pixcels (pitch 1 mil, aperture 100 mil). The wavelength range focused on the sensing area can be adjusted within 200–800 nm region by mechanical rotation of the grating. The resolution of the polychrometer, measured by observing a line spectrum emitted from a pen Ray lamp, was 2.5 nm.

Liquia chromatograph

The liquid chromatograph consisted of a Model SF-0396-57 pump (Milton Roy), an injection port (Kyowa Seimitsu) permitting direct syringe injection on top of the column and a flow cell volume 8 μl. The column (150 × 4.6 mm I.D.) was packed with octadecyl silica (Zorbax ODS, particle diameter 5–6 μm). The mobile phase was methanol–water (90:10), flow-rate 1.0 ml/min or 0.5 ml/min.

Data acquisition system

Ssytem I. This system was used to evaluate the noise level of a single pixcel. It is a conventional fixed-wavelength detection system with which the desired wavelength can arbitrarily be selected within the 220 nm range by presetting a 10 bit digital switch. The system consists of the array (RL 1024 C/17 or 512 S), an evaluation circuit board (RL 103 for RL 1024 C/17, RC 1024 S for RL 512 S), a control logic section for selecting the pixcel number and an analogue interface section for recording the chromatogram. The last two sections are shown in Fig. 2. A clock pulse, generated by the evaluation circuit board, was fed to an asynchronous binary counter (SN 74197 × 4), and each bit of the counter was connected in parallel with a 10-bit preset switch. As soon as it is cleared by the start pulse provided by the board, the counter begins to count the clock pulses. When the counts reach a preset value, the

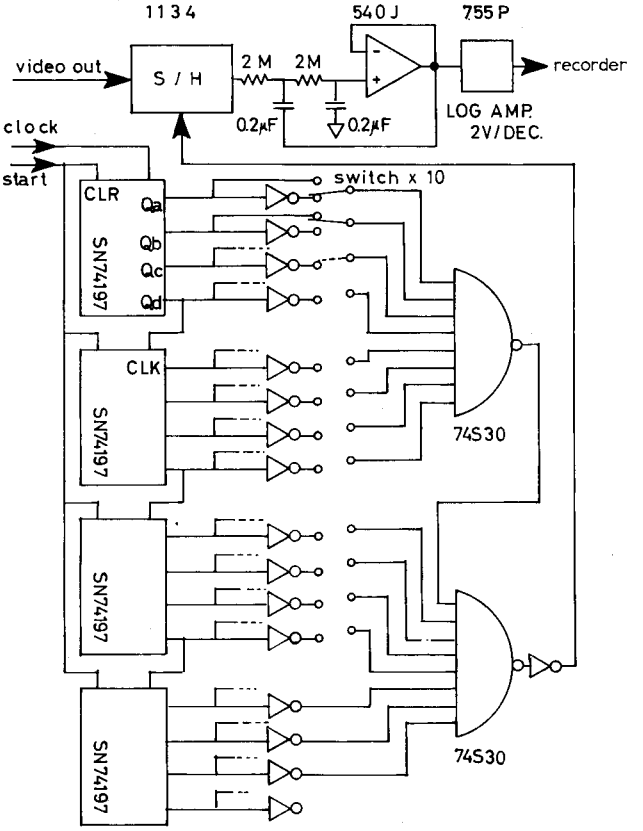

Fig. 2. Control logic and analogue interface for system I. M = MΩ; DEC. = decade; CLR = clear; CLK = clock; Qa, Qb, Qc, Qd = bit of counter.

control logic, which comprises switches and NAND gates (74530 × 2), is operated and a sampling pulse is transferred to an analogue sample/hold module (Analog Devices, SHA 1134) whose input is the spectral signal (video-out) from the array. The video-out signal of a certain pixcel, which is sampled and held during each scan, is recorded as a conventional chromatogram at the fixed wavelength after passing through a secondary Butterworth low-pass filter having a time constant of 0.4 or 1.0 sec and a logarithmic converter (Analog Devices, 755 P). If further control logic is installed in the counter, it is also possible to display the signals at additional wavelengths by using a multi-pen recorder.

System II. This system is interfaced to a microcomputer and can be used as an on-line multi-wavelength detection system. It is expected to have a lower noise level than system I because of the lack of analogue interface (logarithmic amplifiers, sample-hold amplifiers, etc.). As shown in Fig. 3, it consists of a microcomputer LSI-11 with a 27 k-word RAM, a cartridge disk, an analogue-to-digital (A/D) converter (Datel 12 bit ADC-EH12B), a digital-to-analogue converter and a timing circuit section, which is shown in Fig. 4. The last section consisting of random logic is

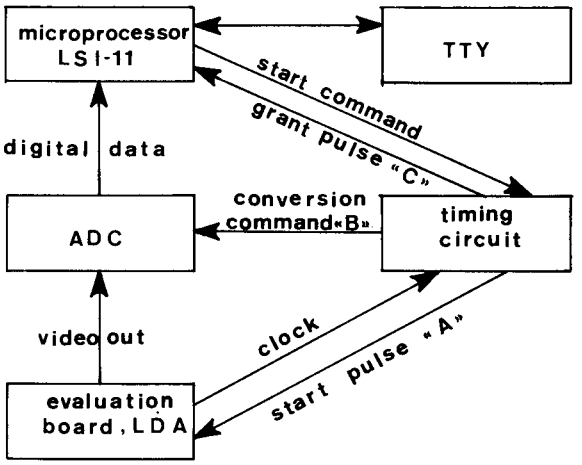

Fig. 3. Schematic diagram of system II. ADC = Analogue-to-digital converter; LDA = linear diode array; TTY = teletypewriter.

used to generate three kinds of pulses for commanding the array scanning (pulse A), the A/D conversion (pulse B) and the data acquisition by the computer (pulse C).

The whole system is initiated by a starting pulse generated by the software. This pulse from the computer is synchronized with the clock pulse from the board in the timing circuit by using two flip-flops and is fed back to the board as pulse A. After the array scanning is started by pulse A, the boxcar type video-out signal of each pixcel, which is pushed out of the array by the clock pulse, is converted into digital

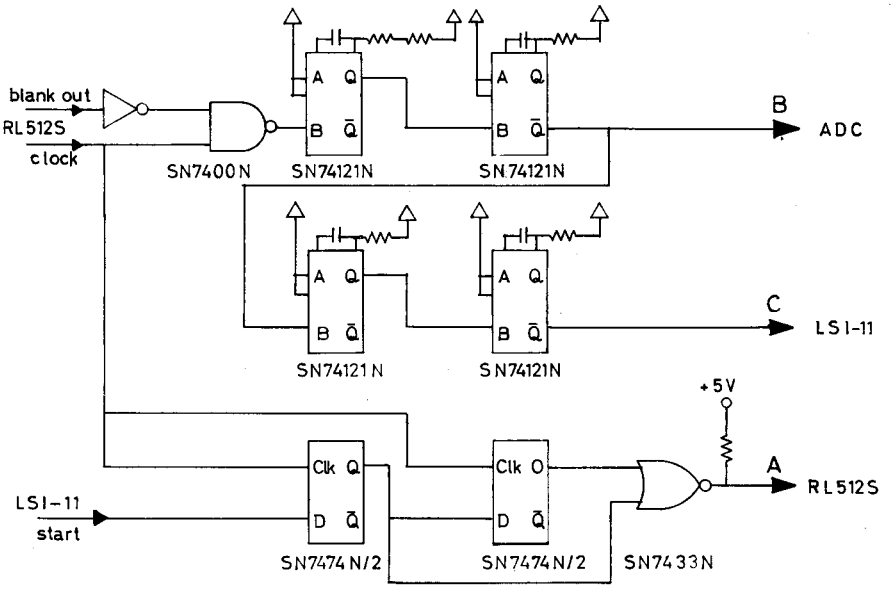

Fig. 4. Timing circuit. A, B, C, see text; Q = output of flip-flop in monostable multi-vibrator; $\bar{Q}$ = not Q; Clk = clock.

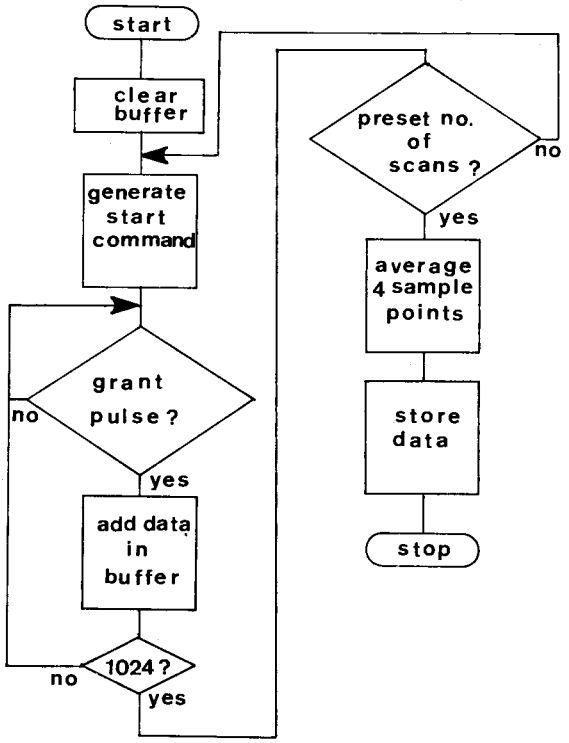

Fig. 5. Flow chart for data acquisition.

data and stored in the computer memory. The command pulse for the ADC (pulse B) and the grant pulse to the computer for data acquisition (pulse C) were produced by use of four monostable multi-vibrators in the timing circuit. Pulse B is generated by delaying the clock pulse from the board for a certain period required to stabilize the video-out signal of a single pexcel. Pulse C is generated by delaying the clock pulse for a certain period required for the completion of the A/D conversion. These two pulses are generated only for the scanning period by performing NAND operation between the clock pulse and the blanking pulse which blanks out the video signal of the board. Therefore, after a starting pulse has been sent to the timing circuit, the computer can store the 512 or 1024 digital data from all pixcels by sensing the grant pulse from the timing circuit.

The program for system II is composed of two parts; (i) acquisition of the sample data, S, the reference data, R, and the dark current data, D; (ii) floating point calculation (8 bit exponent and 24 bit mantissa) of the absorbance by using the expression $\log [(R-D)/(S-D)]$, and display of the pseudo three-dimensional chromatogram. The flow chart for the data acquisition is shown in Fig. 5. Because the single-beam type polychrometer is used, a spectrum is obtained with the light source blocked and this spectrum is subtracted from all subsequent spectra to correct for the finite dark current of the array. The reference spectrum is the video-out signal when the mobile phase passes through the flow cell. The initial variables specified by the program are the sampling rate, the exposure time of the array, which is the interval

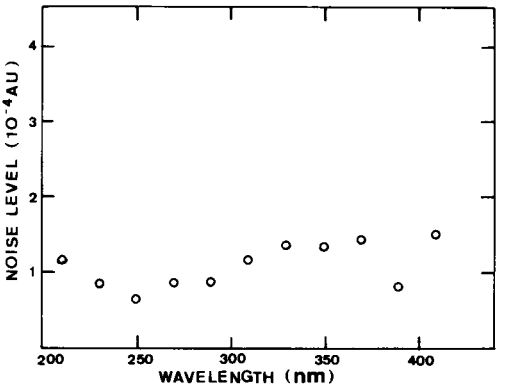

Fig. 6. Noise level of the array detector (512 S).

between two starting pulses, the number of accumulations, the starting point of the sampled video-out and the number of terms for quadratic/cubic least squares smoothing.

The processed data are displayed in various forms, *i.e.*, a plot of the absorbance at any single wavelength *vs.* elution time as in a conventional chromatogram, the spectrum of the eluting component at the selected time, the pseudo three-dimensional chromatogram (time *vs.* wavelength *vs.* absorbance) and the total integrated absorbance at all wavelengths *vs.* time.

RESULTS AND DISCUSSION

Noise level at a single pixcel

To determine the noise level of a single pixcel, the noise on the baseline, recorded by system I, was calibrated in absorbance units (AU) by using a metal wire net having a known absorbance of 0.66 AU as a reference. The noise level of a spectrophotometer generally depends on the slit width (0.11 mm in this work), the source intensity and the exposure time. Here, only the exposure time is variable.

The noise level of the RL 512 S measured with the cell at an exposure time of 30 msec (clock pulse 33 kHz) is shown in Fig. 6. It is seen that the noise level is $6 \cdot 10^{-5}$ AU at 250 nm. The present noise level of $0.6 \cdot 10^{-4}$–$1.4 \cdot 10^{-4}$ AU in the UV range is better

TABLE I

NOISE LEVELS AT THE INPUT TERMINAL OF THE LOGARITHM AMPLIFIERS *VS.* WAVELENGTH

Wavelength (nm)	*Input voltage (V)*	*Noise of input voltage (mV)*
210	1.15	0.30
230	2.38	0.31
270	1.70	0.30
310	1.37	0.28
350	0.88	0.31
370	0.83	0.31
410	0.72	0.32

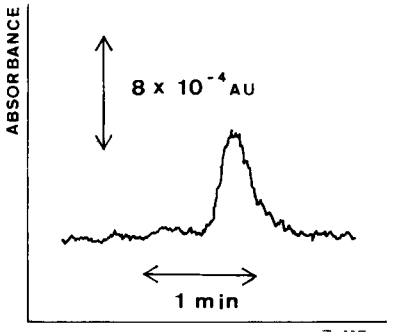

Fig. 7. Chromatogram at 230 nm of biphenyl (1 ng) measured by system I. LC conditions: Zorbax ODS, methanol–water (90:10), flow-rate 0.5 ml/min.

than those of $1.3 \cdot 10^{-4}$–$2.0 \cdot 10^{-4}$ AU in commercially available fixed-wavelength UV detectors for HPLC. On the other hand, the noise level of the RL 1024 C/17 can be ten times larger than that of the RL 512 S.

Some improvement in the noise level was achieved by decreasing the exposure time and increasing the number of accumulations. Such a dependence on integration time means that the source of the noise is predominantly in the detection system and not in the light source. Furthermore, it appears that the source of noise in the detection system is not in the array itself but in the electronic circuits. First, the noise levels observed at exposure times of 30 msec and 250 msec have the same waveforms (frequency) and amplitudes. Secondly, the noise in AU is correlated with wavelength, as shown in Fig. 6, but the noise in the input of the logarithmic converter at various wavelengths is constant regardless of input voltage as shown in Table I. These results

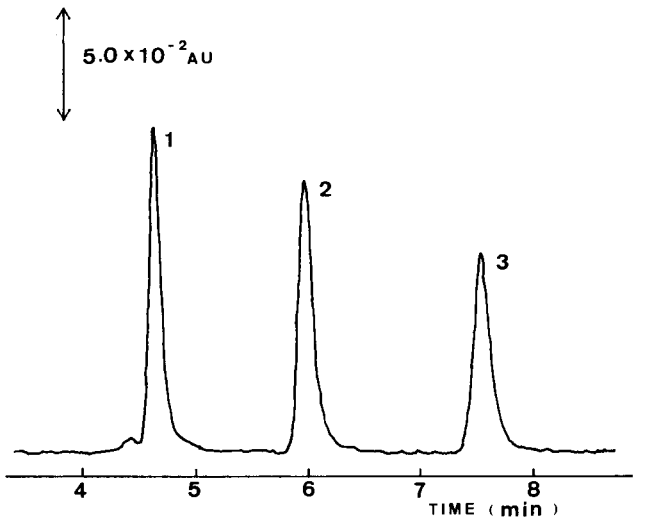

Fig. 8. Chromatogram at 230 nm of a mixture of acenaphthene (peak 1, 200 ng), acenaphthylene (peak 2, 100 ng) and fluoranthene (peak 3, 100 ng). LC conditions: Zorbax ODS, methanol–water (90:10), flow-rate 1.0 ml/min.

seem to indicate that the noise is associated not with the photodiode itself but with the electronic circuit of the evaluation board and/or the imperfect circuit built in our laboratory. Consequently, it may be possible to reduce the noise level below $6 \cdot 10^{-5}$ AU if a better circuit and a higher speed computer are employed.

Fig. 7 shows the chromatogram for 1 ng of biphenyl measured at 230 nm by using the RL 512 S as detector. Besides the high-frequency noise, there is a drift in the baseline. More precise stabilization of the light source is presumably necessary. Attempts to reduce the thermodynamic noise of the array would seem to be unwarranted until these factors are improved. However, it should be possible in this system to attain noise levels of the order of $1 \cdot 10^{-5}$ AU similar to that of recent highly sensitive UV spectrophotometers.

Chromatogram at a single wavelength

Fig. 8 shows the chromatogram of a mixture of acenaphthene, acenaphthylene and fluoranthene at 230 nm obtained by using system I equipped with the RL 1024 C/17 detector. Although the results are comparable to those obtainable by a conventional LC detector, in system I the wavelength can freely be selected and also the simultaneous monitoring of absorbances at two or more wavelengths is possible if a multi-pen recorder is used. Fig. 9 shows the linear response of system I equipped with the RL 512 S for $5 \cdot 10^{-1}$–$1 \cdot 10^{3}$ ng biphenyl measured at 230 nm. Each point was obtained by averaging three measurements. The dynamic range is about four decades for the RL 512 S and three decades for the RL 1024 C/17.

Fig. 10 shows a pseudo three-dimensional chromatogram obtained by using system II equipped with the RL 1024 C/17. Fig. 11 shows a plot of total integrated absorbance at all wavelengths *vs.* elution time (total absorbance chromatogram)

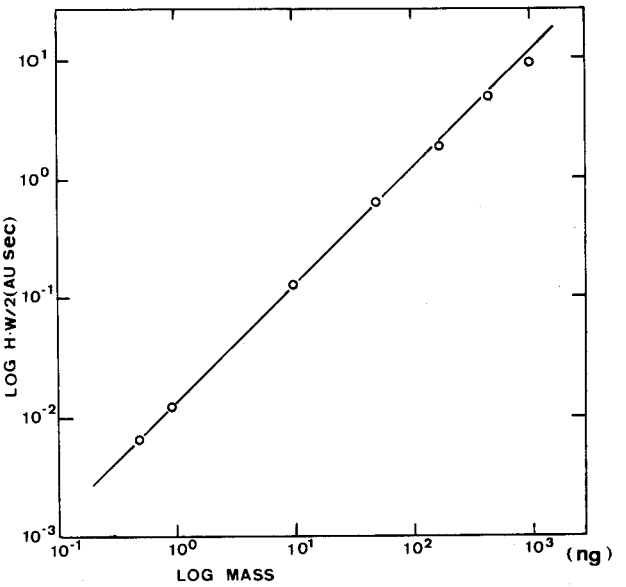

Fig. 9. Plot of peak area *vs.* mass of biphenyl injected.

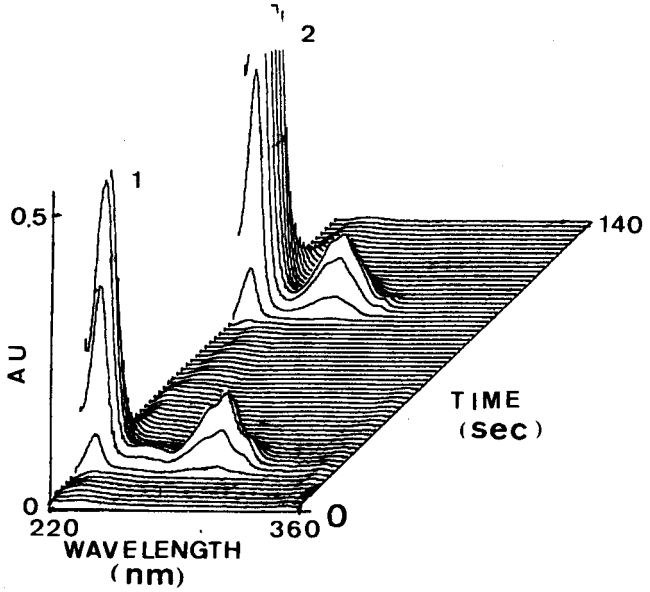

Fig. 10. Three-dimensional chromatogram for separation of acenaphthene (peak 1, 200 ng) and acenaphthylene (peak 2, 100 ng). LC conditions, as in Fig. 8.

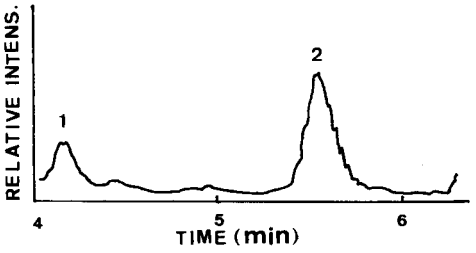

Fig. 11. Total absorbance chromatogram of acenaphthene (peak 1, 10 ng) and acenaphthylene (peak 2, 5 ng). LC conditions as in Fig. 8.

using the same system. In system II, all transmittance data obtained during an LC run are stored in the computer memory. Then, at the completion of the run, calculation of absorbance is undertaken, which takes about 5 min. Such post-run data processing is too time consuming for routine analysis and requires a large amount of computer memory. Work is therefore continuing in order to develop a real time display for three-dimensional chromatograms by using data compression techniques and real time retrieval by using an analogue–analogue correlator (charge coupled device).

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SEPARATION OF *cis/trans* ISOMERS OF 1,4-DISUBSTITUTED CYCLOHEXANES AND OF *Z/E*-MONOUNSATURATED FATTY ACID METHYL ESTERS BY GAS–LIQUID CHROMATOGRAPHY ON NOVEL LIQUID CRYSTAL PHASES

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SUMMARY

Three liquid crystals, *trans*-*p*-[5-(4-*n*-heptylcyclohexyl)-2-pyrimidinyl]benzonitrile (RO-CP-7870), *p*-[5-(*n*-butylphenyl)-2-pyrimidinyl]benzonitrile (RO-CP-7334) and 4′-*n*-pentyl-4-cyanoterphenyl (RO-CB-5515), have been applied as stationary phases for gas–liquid chromatography (GLC). These compounds have been utilized to solve several difficult separation problems, for example separation of the *cis/trans* isomers of 1,4-disubstituted cyclohexanes, the *Z/E*-isomers of monounsaturated fatty acid methyl esters and the xylene isomers. The described nematic phases can be applied as GLC phases in the range between 60°C and *ca.* 180°C.

INTRODUCTION

In 1963, Kelker[1,2] reported the application of nematic liquids as stationary phases for gas–liquid chromatography (GLC). Such phases have since been increasingly utilized for GLC determinations. For example, liquid crystal phases have been applied to separations of isomers of disubstituted benzenes or naphthalenes[3–6], to the determination of polycyclic hydrocarbons[6–9], to separations of epimeric steroids[10], aza-heterocyclic compounds[11] and steroids and bile acids[12]. Lester and Hall[13] utilized liquid crystals to investigate *Z/E* mixtures of fatty alcohols and their acetyl derivatives.

The Schiff base N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT) (Eastman-Kodak, Rochester, NY, U.S.A.) and its homologues[5–8], and a variety of azo and azoxy compounds[1–4,14], have most frequently been employed as stationary phases. In addition, many different types of nematic and smectic phases can be applied, *e.g.*, esters of benzoic acid or of cinnamic acid. BMBT and its homologues BBBT (butoxy), BHxBT (hexyloxy) and BPhBT (phenyl) are now commercially available (*e.g.*, from Analabs, Applied Science Labs, Chrompack, Supelco and other suppliers). Most users of azo and azoxy derivatives synthesize these compounds in their own laboratories. The high melting points ($\geqslant$ 180°C) and the corresponding temperature limits represent the main disadvantage of these nematic phases.

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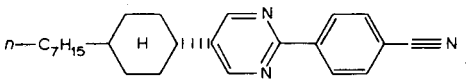
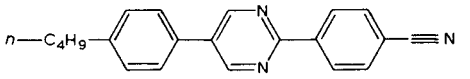
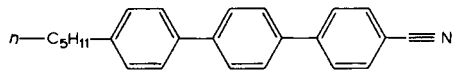
TABLE I

STRUCTURES, MELTING POINTS AND CLEARING POINTS OF THE LIQUID CRYSTALS

Structure		*Melting point (°C)*	*Clearing point (°C)*	*Nematic range (°C)*
I RO-CP-7870		80	226	80–226
II RO-CP-7334		95	244	95–224
III RO-CB-5515		129	238	129–238

In this report we describe GLC investigations of newly developed nematic compounds, the structures and some of the physical properties of which are given in Table I. Their systematic names are *trans-p*-[5-(4-*n*-heptylcyclohexyl)-2-pyrimidinyl]benzonitrile (RO-CP-7870, I), *p*-[5-(*n*-butylphenyl)-2-pyrimidinyl]benzonitrile (RO-CP-7334, II) and 4′-*n*-pentyl-4-cyanoterphenyl (RO-CB-5515, III). These compounds are currently utilized for digital displays. All three are commercially available or can be obtained on special request from Hoffmann-La Roche (Basle, Switzerland).

These liquid crystals can be applied as GLC phases between temperatures of 80°C and *ca.* 180°C. The upper temperature limit is determined by the boiling points of the compounds. In addition, RO-CP-7870 can be utilized in a supercooled condition with a lower limit of 60°C (separation properties and retention times for xylene isomers remained constant during 20 h of operation).

TABLE II

PARAMETERS OF THE COLUMNS

BMBT = N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (Eastman-Kodak);
BBBT = N,N′-bis(*p*-butoxybenzylidene)-α,α′-bi-*p*-toluidine (Eastman-Kodak).

Column no.	*Stationary phase*	*Percentage of stationary phase*	*Support material*	*Column length (m)*
1	I	2	Gas-Chrom Q, 120–140 mesh	3.6
2	I	2	Gas-Chrom Q, 80–100 mesh	1.8
3	II	5	Gas-Chrom Q, 80–100 mesh	1.8
4	II	2	Gas-Chrom Q, 80–100 mesh	1.8
5	III	2	Gas-Chrom Q, 80–100 mesh	1.8
6	BMBT	2	Gas-Chrom Q, 80–100 mesh	1.8
7	BBBT	2	Gas-Chrom Q, 80–100 mesh	1.8

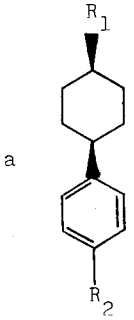
EXPERIMENTAL

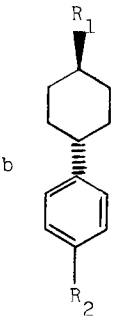
Liquid crystals were coated onto the carrier Gas-Chrom Q by conventional techniques (evaporation of a methylene chloride solution under reduced pressure on a rotavapor). Analyses were carried out with glass columns (2.2 mm I.D.) and Model 3920 or Sigma 3 B chromatographs (Perkin-Elmer). Table II summarizes the types of columns utilized.

RESULTS

Separation of cis/trans-1-phenyl-4-alkylcyclohexanes

The following *cis/trans* isomers of 1-phenyl-4-alkylcyclohexanes have been separated on stationary phases prepared with liquid crystals I, II or III (Table I):





1a, 1b $R_1 = C_3H_7$, $R_2 = CH_3$
2a, 2b $R_1 = C_4H_9$, $R_2 = H$
3a, 3b $R_1 = C_3H_7$, $R_2 = C_2H_5$
4a, 4b $R_1 = C_3H_7$, $R_2 = C_3H_7$
5a, 5b $R_1 = C_4H_9$, $R_2 = C_2H_5$

We were not able to separate these pairs of isomers on conventional stationary phases such as Apiezon L, SE-30, Carbowax 20M, Carbowax 20M + Bentone 34, XE-60 + Bentone 34, QF-1, Silar 10C, Poly-S-179, DC-560, OV-17, OV-225, OV-275, Reoplex 400, Polyphenylether 6 R or Dexsil 300. Utilization of packed columns with RO-CP-7870, RO-CP-7334 or RO-CB-5515 coated onto Gas-Chrom Q (80–100 mesh) yielded separations of *cis*- and *trans*-isomers with a relative retention, α, of 1.6–2.0. The *cis* isomer was always eluted prior to the corresponding *trans* compound.

Fig. 1 shows a separation of 4a and 4b, carried out on column 2 (Table II) at an analysis temperature of 165°C and a nitrogen flow-rate of 30 ml/min. Relative retentions for the *cis/trans* pairs 1–5 obtained on column 3 are given in Table III.

A mixture of the *cis/trans* isomers of 1-(4′-propylphenyl)-4-propylcyclohexane has been analysed on columns 2, 4 and 5. Approximately identical retention times for the *trans* isomer 4b were achieved by adjustment of the column temperatures, while the carrier gas flow-rate was kept constant. The nematic phases I, II or III were coated at 2% onto Gas-Chrom Q (80–100 mesh), and the column length was 1.8 m. Table IV shows the relative retentions obtained for 4b (α for *cis* isomer was set at

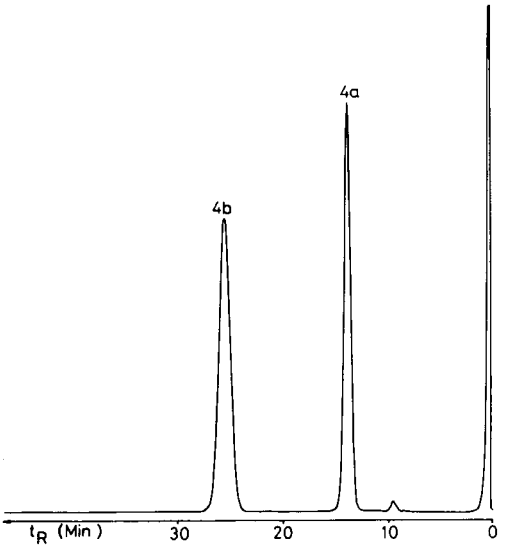


Fig. 1. Separation of *cis*- (4a) and *trans*- (4b) -1-(4′-propylphenyl)-4-propylcyclohexane on column 2 at 165°C. t_R = Retention time.

1.00). They are compared to the values obtained on similarly prepared columns of BMBT and BBBT. The BMBT column could be supercooled to 130°C (m.p. 181°C), but a similar treatment of BBBT was not possible.

Relative retentions for 4a and 4b are in the same range for four of the phases tested. BMBT behaves differently and yields a considerably higher α. However, it should be noted that BMBT was utilized in a supercooled condition, and that the GLC peaks exhibited some tailing. Determinations on BMBT columns are not possible without preliminary heating to 200°C, *i.e.*, above the melting point. In addition, prolonged application of this supercooled system may not be feasible. The retention time for the *trans* isomer on the BBBT column is very short, and consequently the corresponding α value is only an approximation. It is known that routine analyses of

TABLE III

RETENTION DATA (α) FOR *trans*-1-PHENYL-4-ALKYLCYCLOHEXANES

For *cis*-isomers α = 1.00. Column 3 (5% RO-CP-7334 on Gas-Chrom Q, 80–100 mesh).

Substance	α *(trans/cis)*	*Retention time of trans (min)*	*Analysis temperature (°C)*
1b	1.9	16.5	170
2b	1.6	10.0	170
3b	1.9	18.0	170
4b	2.0	34.4	170
5b	2.0	28.6	170

TABLE IV

RELATIVE RETENTION DATA (α) FOR *trans*-1-(4′-PROPYLPHENYL)-4-PROPYLCYCLOHEXANE

For *cis*-isomer α = 1.00.

Nematic phase	*Column*	*Analysis temperature (°C)*	*Retention time of trans (min)*	*α (trans/cis)*
RO-CP-7334	4	155	22.4	2.15
RO-CB-5515	5	155	21.2	2.30
RO-CP-7870	2	165	25.4	1.87
BMBT*	6	130	22.5	3.52
BBBT	7	200	2.7	2.25

* Supercooled.

compounds with retention times of 1 or 2 min are not reproducible due to overlap with the solvent front. In conclusion, the BMBT and BBBT phases are not suitable for this particular analytical problem.

Separation of methyl esters of (Z/E)-monounsaturated fatty acids

GLC separations of the methyl esters of oleic acid (*Z*) and elaidic acid (*E*) or of palmitoleic acid (*Z*) and palmitelaidic acid (*E*) are difficult. Of a number of conventional stationary phases on packed columns, only nitrile silicones have yielded satisfactory results. Ottenstein *et al.*[15] applied the cyanopropyl silicone OV-275 to separate the methyl esters of oleic acid and elaidic acid and obtained a relative retention

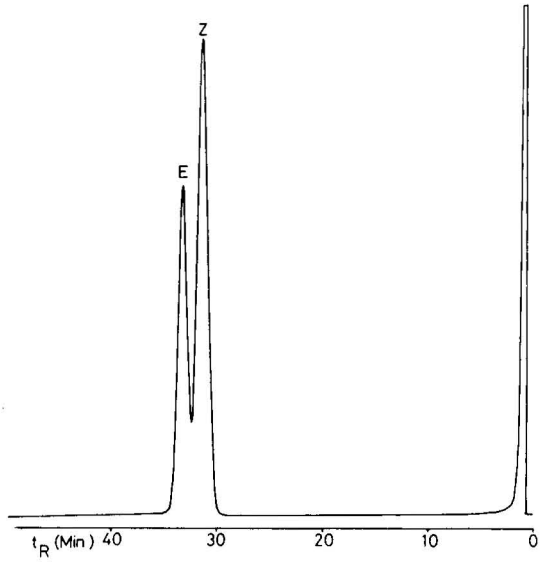


Fig. 2. Separation of methyl esters of palmitoleic acid (*Z*) and of palmitelaidic acid (*E*) on column 1 at 160°C; flow-rate 30 ml/min.

of 1.07. The (*E*)-ester was eluted in advance of the (*Z*)-ester. Investigations in our laboratory revealed that packed columns with OV-275 or similar phases have relatively low numbers of theoretical plates. Conventional gas chromatographs can accommodate glass columns with lengths of up to 3 or 4 m; accordingly we did not achieve satisfactory separations of these methyl esters on cyanopropyl silicone columns. However, utilization of column 1 (2% RO-CP-7870 on Gas-Chrom Q 120–140 mesh, length 3.6 m, 160°C, nitrogen flow-rate 30 ml/min) resulted in a separation of the methyl esters of palmitoleic acid and palmitelaidic acid (Fig. 2). About 35 min were required to complete the analysis, which yielded a relative retention of 1.06. Separation of the methyl esters of oleic acid and elaidic acid was accomplished under the same conditions, except at an increased carrier flow (90 ml/min). The analysis time was less than 1 h (Fig. 3), and a relative retention of 1.08 was obtained. In both cases the (*E*)-ester was eluted after the (*Z*)-ester.

Separation of xylene isomers

Separation of disubstituted benzenes and naphthalenes has been the most frequent application of liquid crystal phases in GLC[3–6]. Generally, the separation of *meta* and *para* isomers is quite difficult, and the pair *m*-/*p*-xylene represents a particularly demanding task. Accordingly, separation of this pair is often used as an indicator of the suitability of a given nematic phase for analyses of such isomers[2,14,16–20].

Fig. 4 shows the results obtained with column 1 (RO-CP-7870) and injection of about 1 μg of each of the xylene isomers and 0.5 μg of ethylbenzene. The column was operated at 60°C with a nitrogen flow-rate of 7 ml/min. The relative retentions were 1.14 for *p*-xylene (α for *m*-xylene set at 1.00) and 1.12 for *o*-xylene (α for *p*-xylene set at 1.00). Analysis was completed after 15 min. Application of column 3 (RO-CP-

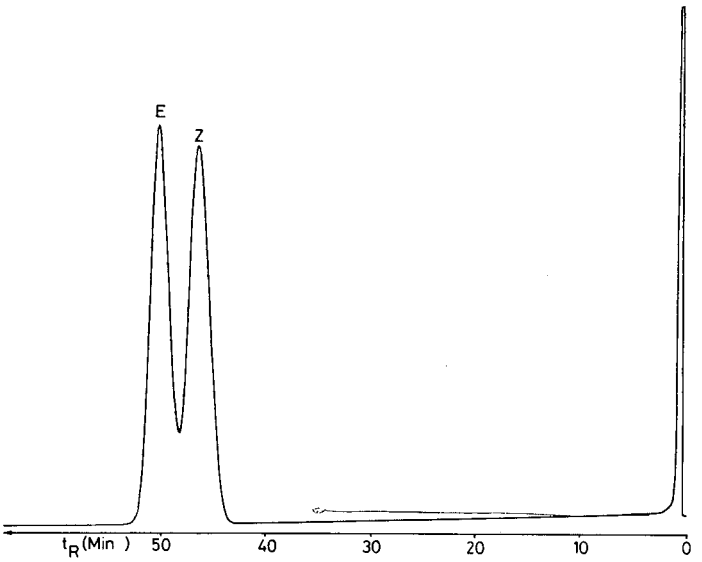


Fig. 3. Separation of methyl esters of oleic acid (*Z*) and of elaidic acid (*E*) on column 1 at 160°C; flow-rate 90 ml/min.

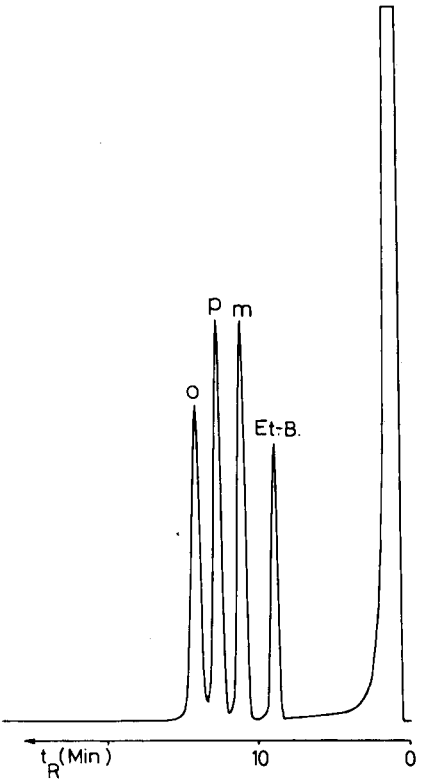


Fig. 4. Separation of *o*-, *m*- and *p*-xylene and ethylbenzene (Et.-B.) on column 1 at 60°C; flow-rate 7 ml/min.

7334, 60°C, nitrogen flow-rate 10 ml/min) yielded α values of 1.13 for *p*-xylene (α for *m*-xylene set at 1.00) and 1.18 for *o*-xylene (α for *p*-xylene set at 1.00). Since RO-CB-5515 cannot be supercooled, it is not suitable for determination of these isomers. xylene set at 1.00) and 1.18 for *o*-xylene (α for *p*-xylene set at 1.00). Since RO-CB-5515 cannot be supercooled, ti is not suitable for determination of these isomers. Until now the highest α value reported for the separation of *m*-/*p*-xylenes was 1.17[19].

The application of liquid crystals as stationary phases can be recommended only for separations that cannot be accomplished with conventional phases. The applicability of a given liquid crystal is restricted to a rather narrow temperature range, the lower temperature limit often being dictated by the melting point of the compound. In addition, liquid crystals have a relatively high vapour pressure–in comparison to silicone phases–which may limit their usefulness at higher temperatures.

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GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY OF PHENOLALKYLAMINES AFTER FLASH-HEATER DERIVATIZATION USING A DOUBLE INJECTION TECHNIQUE

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SUMMARY

Glass capillary column gas chromatography has been used for quantitative and qualitative analyses of some phenolalkylamines. The compounds are converted into the corresponding N-trifluoroacetyl-O-trimethylsilyl derivatives using a double injection technique. The phenolalkylamines are first flash-heater trimethylsilylated with N-methyl-N-(trimethylsilyl)trifluoroacetamide injected together with the sample, followed by N-acylation with N-methyl-bis(trifluoroacetamide) in a second injection. The N-acylation occurs on-column. The identity of the derivatives was confirmed by gas chromatography–mass spectrometry. The phenolalkylamines were also analysed using on-column derivatization combined with a packed column and a double injection technique. Several parameters were studied for both methods in order to get optimal response with good reproducibility. The injection technique proved to be the most important parameter in this study and had to be carefully controlled. For the quantitative analyses, the linearity, reproducibility and lower detection limit were controlled. A concentration range of 5–50 μg/ml was investigated using the capillary column and 25–1000 μg/ml for the packed column. The main advantages of this derivatization method are its speed and simplicity, which result in a substantial saving of time.

INTRODUCTION

High-resolution gas chromatography (GC) is finding increasing application for analyses of drugs and related compounds. The wide applicability and acceptance of this method have recently been summarized in a review article[1]. Although efforts to prepare adsorption-free columns have been made, the use of derivatization for compounds containing functional polar groups is still recommended, especially for analyses at the nanogram level.

The flash-heater derivatization technique has earlier been applied to glass capillary columns[2–4]. The reaction occurs in the heated injection port following simultaneous injection of the sample and the reagent. The method is applicable to heat-

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stable compounds that react rapidly with the derivatization reagent to form a single product.

Derivatization of compounds containing different functional groups may give two or more products, as the available reagents do not have optimum properties for reaction with different acceptor groups. Two products were obtained after flash-heater silylation of *p*-aminobenzoic acid[3]. A solution to the problem is to derivatize in two stages with different specific reagents. This has earlier been described for pre-column derivatization of steroids[5], amino acids[6] and catecholamines[7].

N-Methyl-bis(trifluoroacetamide) was developed by Donike for selective acylation of amine functions in the presence of hydroxyl and carboxyl groups[8]. Quantitative derivative formations were obtained if these groups were first trimethylsilylated[9,10].

The purpose of the present investigation was to determine whether compounds containing different functional groups could be quantitatively flash-heater derivatized with two specific reagents. The method was optimized for capillary columns. Some phenolalkylamines were selected as model substances and reagents capable of forming trimethylsilylderivatives were studied, followed by N-acylation with N-methyl-bis(trifluoroacetamide) in a second injection.

A packed column GC method with double on-column derivatization was also developed for the phenolalkylamines. When using a double injection technique, the reactions appear to be quantitative and reproducible for both column methods, giving the corresponding N-trifluoroacetyl-O-trimethylsilyl derivatives. The influence of the injection technique, the injection port temperature, the initial column temperature and the reagent volumes were studied.

For quantitative analyses, the linearity, reproducibility and lower detection limit were evaluated, and the results from both column methods were compared.

MATERIALS AND METHODS

Reagents

Phenylephrine hydrochloride, synephrine, isoproterenol sulphate and ephedrine were supplied by Norsk Medisinaldepot (Oslo, Norway). *p*-Hydroxyephedrine hydrochloride was obtained from Aldrich (Beerse, Belgium) and etilefrin hydrochloride was a gift from Boehringer Ingelheim (Ingelheim, G.F.R.). Nonadecane and eicosane were purchased from Koch-Light (Colnbrook, Great Britain). N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and N-methyl-bis(trifluoroacetamide) (MBTFA) in 5-ml vials were purchased from Fluka AG (Buchs, Switzerland). Ampoules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-trimethylsilylimidazole (TSIM) and Sylon BTZ (mixed BSA, TSIM and trimethylchlorosilane) were purchased from Supelco (Bellefonte, PA, U.S.A.). Analytical grade dimethylformamide (DMF) and pyridine were obtained from E. Merck (Darmstadt, G.F.R.).

Stock standard solutions of the phenolalkylamines and the alkanes used as internal standards contained 1 mg/ml in DMF. The concentration of the stock standard solutions used for packed column analyses was 2 mg/ml in DMF. The concentrations of the compounds available as salts were calculated as bases.

Gas chromatography

A Fractovap 2900 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector and a capillary column splitless injector was used. The glass capillary column (16 m × 0.35 mm I.D.) (H. and J. Jaeggi, Trogen, Switzerland) was wall-coated with OV-1. The injection port temperature was 275°C, and the samples were injected at an oven temperature of 130°C. The temperature was programmed at 10°/min up to 220°C. Nitrogen was used as the carrier gas at an inlet pressure of 0.4 kp/cm^2, which gave a flow-rate of 1.4 ml/min through the column. The sensitivity setting varied from 32 to 8, and a Spectra-Physics Autolab Minigrator was connected to the gas chromatograph for peak-area measurements. The samples were injected with a 5-μl Precision Sampling GC-130 microsyringe equipped with a 7.5-cm needle. The glass liner in the splitless injector was cleaned every other day.

A Fractovap 2300 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector was used with a packed glass column. The column (1 m × 2 mm I.D.) was packed with 3% SE-30 on Supelcoport (80–100 mesh). The injection port temperature was 275°C, and the samples were injected at an oven temperature of 130°C. The temperature was programmed at 10°/min up to 200°C. The flow-rate of the nitrogen carrier gas was 30 ml/min, and the sensitivity setting varied from 10 × 1 to 10 × 8.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS was carried out using a Micromass 7070F mass spectrometer (VG-Micromass, Altrincham, Great Britain) combined with a Fractovap 4200 gas chromatograph (Carlo Erba, Milan, Italy). The glass column (1 m × 2 mm I.D.) was packed with 3% SE-30 on Supelcoport (80–100 mesh).

Influence of the injection technique

A 1-μl volume of a test solution containing 50 ng of phenylephrine and 50 ng of nonadecane (internal standard) was injected together with 2 μl of MSTFA, followed by 2 μl of MBTFA in a second injection. The peak-area ratios were studied when the time between the two injections varied from 15 to 45 sec. The samples were injected splitless. The splitter was closed before the first injection and re-opened after the second injection (splitless time). This time was varied from 45 to 75 sec, and the peak-area ratios were calculated. The derivatization reaction was also studied when MBTFA was injected with MSTFA and the sample in one injection.

A 1-μl volume test solution containing 500 ng of phenylephrine and eicosane (internal standard) was injected on the packed column with 2 μl of MSTFA; 2 μl of MBTFA was injected in a second injection. The time between the two injections was varied from 15 to 60 sec. The peak-height ratios were calculated.

Testing of derivatization reagents and the influence of their volumes

Four different silylation reagents were tested: TSIM, Sylon BTZ, BSTFA and MSTFA and dilutions of MSTFA in pyridine (1:1) and DMF (1:1). MBTFA was used for the acylation reaction, undiluted and diluted with pyridine (1:1) and DMF (1:1). The concentration of the test solution was 50 μg/ml of phenylephrine and nonadecane.

The peak-area ratios were calculated after injection of 1, 2 and 3 μl of MSTFA

and 1 μl of the test solution, followed by 1, 2 and 3 μl of MBTFA in a second injection.

The different silylation reagents were also tested for on-column derivatization combined with the packed column.

Choice of initial column temperature

The derivatization reaction was studied by injection of 1 μl of test solution and 2 μl of MSTFA, followed by 2 μl of MBTFA in a second injection. The initial column temperature varied from 120 to 150°C. The peak-area ratios were calculated. The packed column initial temperature varied from 110 to 150°C, and the peak-height ratios were calculated.

Calibration graphs and reproducibility tests

Calibration graphs for the concentration range 5–50 μg/ml were constructed for ephedrine, phenylephrine and synephrine using nonadecane as internal standard (30 μg/ml). A 2-μl volume of MSTFA was injected splitless with 1 μl of the sample, followed in a second injection by 2 μl of MBTFA after 20–25 sec. The splitter was re-opened 50 sec after the first injection. The linearity was investigated up to 200 μg/ml.

For the packed column method, a calibration graph for phenylephrine was constructed for the concentration range 25–1000 μg/ml using eicosane as internal standard (500 μg/ml). A period of 20–25 sec was used between the two injections.

Five assays on each solution were carried out and the regression lines and the correlation coefficients were calculated for both methods.

For the reproducibility tests, solutions containing 10 and 50 μg/ml of ephedrine, phenylephrine and synephrine and 30 μg/ml of the internal standard were analysed (capillary column). Solutions containing 50 and 1000 μg/ml of phenylephrine and 500 μg/ml of the internal standard were analysed for the packed column method. The mean and the relative standard deviation (R.S.D.) of ten assays were calculated.

RESULTS AND DISCUSSION

The influence of the injection technique

The injection technique was the most important parameter in this study. Two products were formed when both derivatization reagents were injected together with the sample, but only one peak could be detected using the double injection technique. This was observed with both column methods.

The data in Table I indicate that the reproducibility of the derivatization reactions depends on the time between the two injections, especially for the capillary column method. Incomplete N-acylation and peak tailing may occur with increasing time between the injections. A period of 20–25 sec was therefore used for both columns.

The results were also influenced by the time when the splitter was closed (splitless time). From Table I it can be seen that poor reproducibility is obtained with increasing splitless time. For the quantitative analyses, the splitter was re-opened 50 sec after the first injection.

TABLE I

EFFECT OF THE TIME BETWEEN THE TWO INJECTIONS AND THE SPLITLESS TIME

The electrometer used for Tables I, II, V and VI is different from the one used for Tables III, VII and VIII.

Time between injections (sec)	*Capillary column*		*Packed column*	
	Peak-area ratio ($\bar{x}$, $n = 5$)	*R.S.D.* (%)	*Peak-height ratio* ($\bar{x}$, $n = 5$)	*R.S.D.* (%)
15	1.08	3.9	1.21	2.0
30	1.06	2.8	1.22	2.0
45	1.09	11.8	1.18	2.6
60			1.17	3.0
Splitless time (sec)				
45	1.09	2.4		
60	1.06	2.8		
75	1.03	4.6		

Derivatization studies with different reagents

Different reagents as TSIM, Sylon BTZ, BSTFA and MSTFA were investigated to find the most effective silylation reagent. The reagents were tested in combination with the acylation reagent and also for complete silylation of the phenolalkylamines. For all these reagents, two peaks were obtained when the acylation reagent was omitted. Non-quantitative reaction of amino groups in the complete silylation of catecholamines has earlier been reported for pre-column derivatization. The secondary amine groups are especially difficult to attack and acylation reaction has therefore been preferred[11]. TSIM and Sylon BTZ are the recommended reagents for all hydroxyl groups, but these reagents could not be used for flash-heater derivatization in combination with MBTFA. Broad extra peaks were obtained and, after a few injections, dark particles appeared in the injector part of the capillary column with a drastic loss of column efficiency. The injector part of the packed column also became dark and had to be repacked. MSTFA and BSTFA both seemed to react satisfactorily in combination with MBTFA. Of the two silylation reagents, MSTFA seemed to react more quantitatively than BSTFA and also with a noticably lower R.S.D. (Table

TABLE II

MSTFA COMPARED WITH BSTFA FOR TRIMETHYLSILYLATION

Reagent	*Capillary column*		*Packed column*	
	Peak-area ratio ($\bar{x}$, $n = 5$)	*R.S.D.* (%)	*Peak-height ratio* ($\bar{x}$, $n = 5$)	*R.S.D.* (%)
MSTFA	1.06	2.8	1.20	1.5
BSTFA	0.79	9.2	1.17	3.4

II). The difference was most marked for the capillary column and consequently MSTFA was selected as silylating reagent.

The separation of the N-TFA-O-TMS derivatives of a mixture of the phenolalkylamines is shown in Fig. 1. The trimethylsilylacyl derivatization combined with a glass capillary column is well suited for the identification of these compounds.

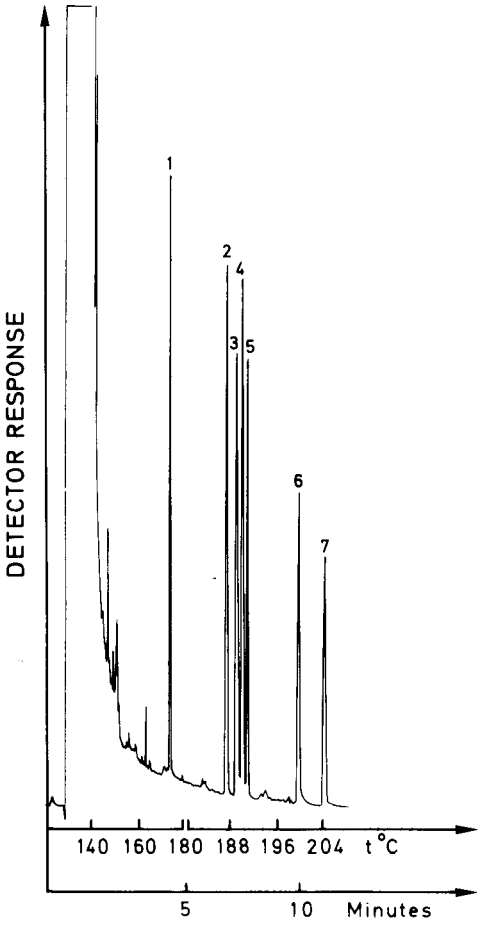


Fig. 1. Chromatogram of a sample containing 50 ng each of ephedrine (1), phenylephrine (2), etilefrin (3), synephrine (4), *p*-hydroxyephedrine (5) and isoproterenol (7) after flash-heater silylation followed by on-column acylation. Peak 6 is 50 ng of the internal standard eicosane. The temperature was programmed at 10°/min from 130 to 180°C then at 4°/min to 220°C. For other chromatographic conditions, see text.

Influence of the reagent volumes

The influence of the amount of the reagent volumes is shown in Table III. It is apparent that at least 2 μl of each reagent must be injected to obtain complete reaction. This volume also gave the best precision. With increasing volumes, the probability of interfering peaks will increase.

TABLE III

EFFECT OF VARIOUS REAGENT VOLUMES INJECTED (CAPILLARY COLUMN)

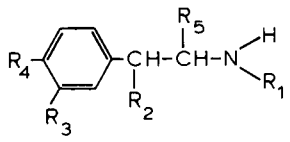
Volume (μl)	*MSTFA varied, 2 μl MBTFA*		*MBTFA varied, 2 μl MSTFA*	
	Peak-area ratio ($\bar{x}$, n = 5)	*R.S.D. (%)*	*Peak-area ratio ($\bar{x}$, n = 5)*	*R.S.D. (%)*
1	0.91	7.6	0.90	4.2
2	0.99	2.4	0.99	2.4
3	0.97	2.5	0.93	4.5

GC–MS investigation of derivatives

The derivatives were identified by GC–MS, and the major ions are listed in Table IV. These data support the formation of N-trifluoro-O-trimethylsilyl derivatives of the phenolalkylamines, and the ions are consistent with previously published data[9]. Only one peak could be detected when using the double injection technique, and it was concluded that the reaction was complete. Two products were obtained from phenylephrine when the second injection with MBTFA was omitted, and both

TABLE IV

PRINCIPAL MASS SPECTRAL DATA OF PHENOLALKYLAMINE DERIVATIVES



Compound	R_1	R_2	R_3	R_4	R_5	*Major ions with relative intensity in parentheses*			
									M − 15
Ephedrine	CH_3	OH	H	H	CH_3	73 (100)	179 (83)		318 (0.5)
Etilefrin	C_2H_5	OH	OH	H	H	73 (98)	267 (100)		406 (1.8)
Synephrine	CH_3	OH	H	OH	H	73 (84)	267 (100)		392 (0.9)
p-Hydroxyephedrine	CH_3	OH	H	OH	CH_3	73 (67)	267 (100)		406 (0.7)
Isoproterenol	C_3H_7	OH	OH	OH	H	73 (82)	267 (1.6)	355 (100)	508 (0.9)
Phenylephrine	CH_3	OH	OH	H	H	73 (99)	267 (100)		392 (1.7)
Phenylephrine (only with MSTFA)									
Main derivative						73 (37)	116 (100)	267 (2.1)	368 (1.4)
By-product						44 (100)	73 (96)	267 (28)	296 (2.6)
Phenylephrine (MSTFA and MBTFA in one injection)									
Main derivative						73 (100)	267 (82)		392 (1.5)
By-product)						73 (100)	291 (25)		416 (1.5)

were identified (Table IV). The mass spectral analysis of the main product indicated the presence of a third trimethylsilyl group introduced on the secondary amine nitrogen. A by-product with shorter t_r was identified as O,O-bis(trimethylsilyl)phenylephrine. Two products were obtained when MBTFA and MSTFA were simultaneously injected with phenylephrine. The main peak was identified to be the same product as the one obtained after a double injection technique, while the data obtained from the by-product indicated the formation of N,O-bis(trifluoroacetyl)-O-trimethylsilyl phenylephrine.

Column temperature effect

The effect of different initial column temperatures is shown in Table V. No significant difference in the peak-areas or the peak-height ratios was observed, but the R.S.D. was increased for the highest temperature. At 150°C, reduced response and broader peaks were observed for the capillary column. An initial column temperature of 130°C was used for both methods, which gave a satisfactory R.S.D. In order to obtain good solvent effect and to minimize the capillary column analysis time, an initial column temperature of 15–30°C below the boiling point of the solvent has been recommended[12]. The boiling point of DMF is 153°C. The results were not influenced by an isothermal period of 3 min before the start of the capillary column temperature programming.

The derivatization reaction was independent of the injection port temperature in the range 250–300°C.

TABLE V

THE EFFECT OF INITIAL COLUMN TEMPERATURE

Capillary column			*Packed column*	
Initial column temperature (°C)	*Peak-area ratio ($\bar{x}$, n = 5)*	*R.S.D. (%)*	*Peak-height ratio ($\bar{x}$, n = 5)*	*R.S.D. (%)*
110			1.15	1.9
120	1.05	3.4		
130	1.06	2.8	1.17	2.0
140	1.04	3.9		
150	1.10	6.9	1.16	6.0

Dilution of the derivatization reagents

With direct injection of derivatization reagent, by-products or generated impurities can interfere with the peak of interest at lower concentration levels[4]. Direct injection of the reaction medium after pre-column derivatization with MSTFA and MBTFA has been recommended[9]. In this investigation, excess reagents and by-products did not affect the packed column analyses significantly. Fig. 2 shows that 25 ng of phenylephrine could easily be detected without interferences. With the capillary column analyses, excess reagent by-products caused interference with sample sizes below 1 ng. Fig. 3 shows an injection of 1 ng each of ephedrine, phenylephrine and synephrine and 5 ng of nonadecane. Experiments with reagent dilutions did not make analyses at lower concentration levels possible as decreased peak-area ratios were

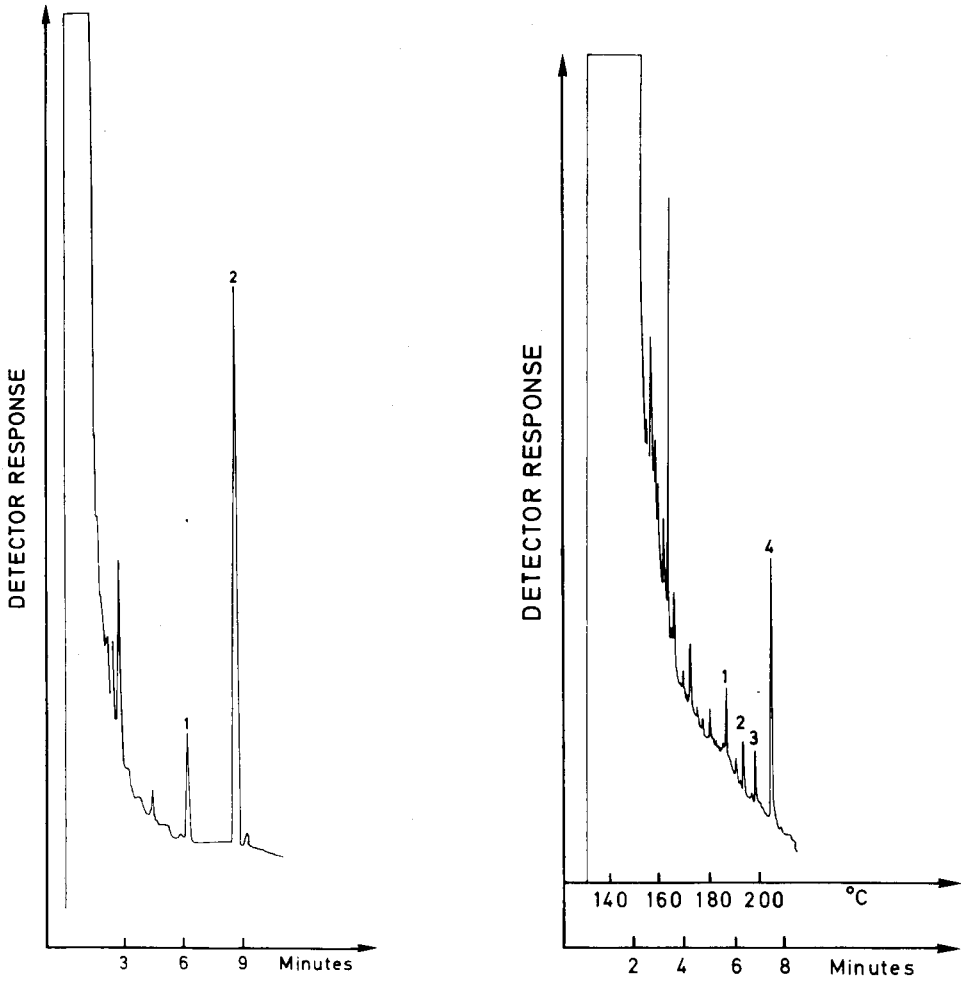


Fig. 2. Packed column chromatogram of a sample containing 25 ng of phenylephrine (1) and 125 ng of eicosane (2) as internal standard, after on-column silylation and acylation. For chromatographic conditions, see text.

Fig. 3. Chromatogram of a sample containing 1 ng each of ephedrine (1), phenylephrine (2) and synephrine (3) after flash-heater silylation followed by on-column acylation. Peak 4 is 5 ng of the internal standard nonadecane. For chromatographic conditions, see text.

observed (Table VI). As shown in Fig. 4, the reagent dilutions also gave unsatisfactory results when used for packed column derivatization. The same sample solution was injected with undiluted MSTFA and MBTFA, and with both reagents diluted with DMF (1:1).

The influence of the syringe needle handling technique

Previous papers have reported that the results obtained from injections into vaporizing GC injectors are dependent on the needle handling technique[13,14]. The technique used in our investigations is as follows:

TABLE VI

THE EFFECT OF REAGENT DILUTIONS (CAPILLARY COLUMN)

Reagent dilution	*Peak-area ratio*	*Comments*
MSTFA MBTFA	1.06	
MSTFA–DMF (1:1) MBTFA	1.03	double peaks occasionally observed
MSTFA MBTFA–DMF (1:1)	0.91	
MSTFA–DMF (1:1) MBTFA–DMF (1:1)	0.90	
MSTFA–pyridine (1:1) MBTFA	0.85	

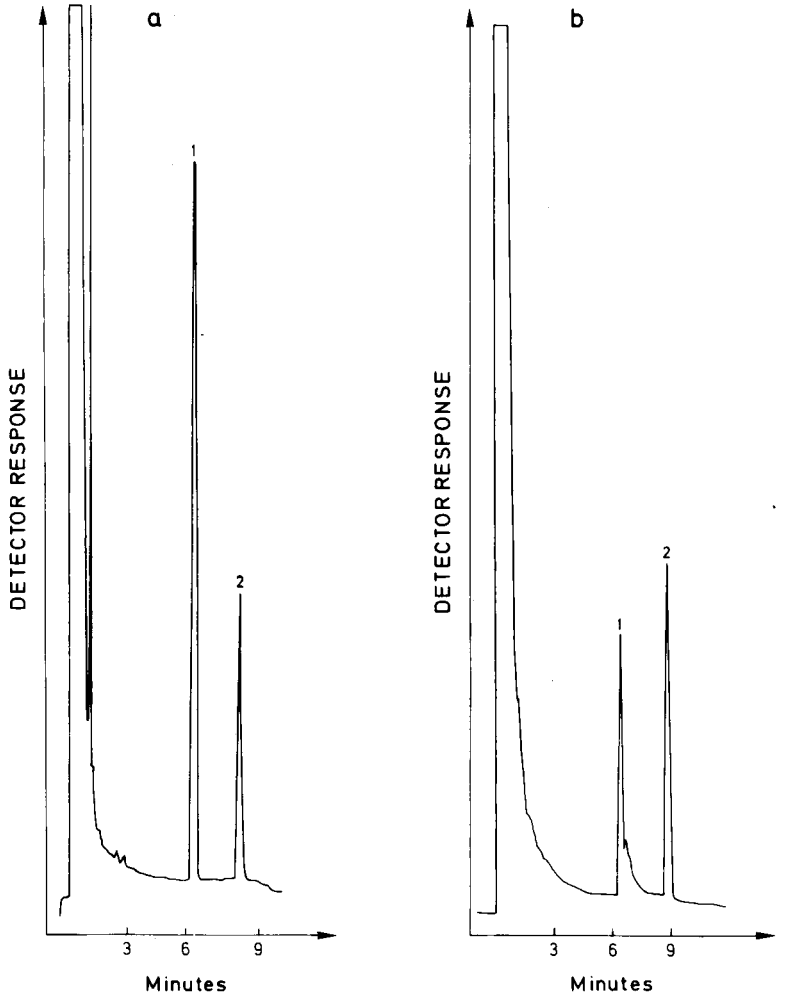


Fig. 4. The packed column chromatogram in (a) was obtained after on-column silylation with MSTFA followed by on-column acylation with MBTFA. The sample contained 1 μg of phenylephrine (1) and 0.5 μg of eicosane (2) as internal standard. Chromatogram (b) was obtained when the same solution was injected with a DMF dilution of both reagents (1:1). For chromatographic conditions, see text.

(1) The needle is filled with 1 μl of reagent, then 1 μl of sample and 1 μl of reagent. The solutions are withdrawn into the syringe barrel and injected without preheating over a period of 5 sec ("cold needle" technique). We have found previously that the rate of sample injection is important in order to ensure a uniform rate of sample vaporization[3]. Previously described techniques for handling syringe needles[13,14] were studied in this investigation.

(2) Same as (1), but injection after 5 sec of needle preheating ("hot needle" technique).

(3) Same as (1), but injection without withdrawing the sample from the needle ("filled needle" technique).

(4) Filling the needle with all reagent volume (2 μl) before picking up the sample.

(5) Filling the needle with sample before picking up the reagent (2 μl).

The data in Table VII show that the highest peak-area ratio combined with lowest R.S.D. was obtained for the usual needle handling technique ("cold needle"). In Grob's investigation, the "hot needle" technique was found to be superior to other methods, but most of his experiments were performed on alkanes[13,14]. Reduced syringe needle discrimination was observed for larger sample volumes[14]. Large sample volumes were also injected in this study. The results were most influenced by the syringe needle length. A microsyringe equipped with a 7.5-cm needle had to be used for Fractovap 2900 GC injections. With an ordinary 5-cm needle, double peaks were obtained.

TABLE VII

EFFECT OF DIFFERENT SYRINGE NEEDLE HANDLING TECHNIQUES

Technique	*Peak-area ratio* ($\bar{x}$, $n = 5$)	*R.S.D.* (%)
Cold needle (1)	0.97	2.4
Hot needle (2)	0.92	2.3
Filled needle (3)	0.93	3.8
All reagent behind the sample (4)	0.99	4.9
All reagent in front of the sample (5)	0.90	4.0

Calibration graphs

Calibration graphs in the concentration range 5–50 μg/ml were constructed for ephedrine, phenylephrine and synephrine in order to check the linearity of the derivatization method (Table VIII). The linearity was also checked up to 200 μg/ml and no change was observed. Fig. 5 shows a chromatogram of a sample containing 10 ng of the derivatized compounds and 30 ng of nonadecane.

A linear calibration graph was also obtained for phenylephrine in the concentration range 25–1000 μg/ml when analysed on the packed column (Table VIII).

The data from the reproducibility tests also show acceptable results for both columns (Table VIII).

TABLE VIII

DATA FROM THE CALIBRATION GRAPHS AND REPRODUCIBILITY TESTS AFTER DERIVATIZATION

Compounds	*Calibration graph equation over range 5–50 μg/ml and 25–1000 μg/ml*	*Correlation coefficient*	*R.S.D. (%)*		
			10 μg/ml	*50 μg/ml*	*1 mg/ml*
Capillary column					
Ephedrine	$y = 0.029x - 0.074$	0.998	4.2	2.7	
Phenylephrine	$y = 0.036x - 0.116$	0.999	3.9	2.2	
Synephrine	$y = 0.035x - 0.084$	0.997	3.8	2.2	
Packed column					
Phenylephrine	$y = 0.385x + 0.003$	0.999		1.4	1.2

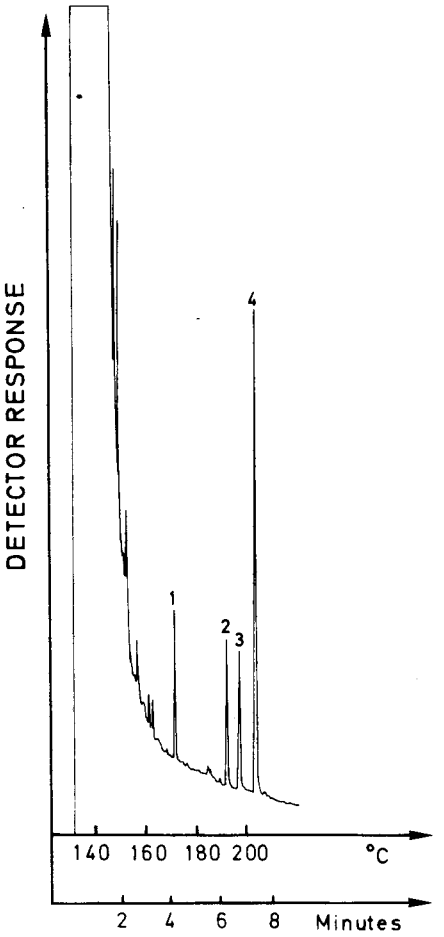
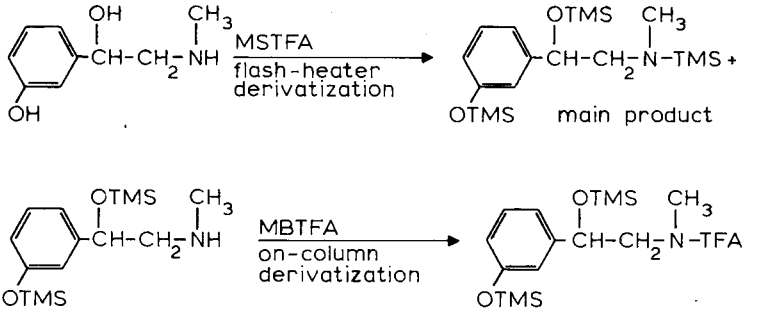


Fig. 5. Chromatogram of a sample containing 10 ng each of ephedrine (1), phenylephrine (2) and synephrine (3) after flash-heater silylation followed by on-column acylation. Peak 4 is 30 ng of the internal standard nonadecane. For chromatographic conditions, see text.

CONCLUSIONS

This study shows that compounds containing both hydroxyl and amino groups can be quantitatively derivatized using specific reagents combined with a double injection technique. The derivatization technique is applicable to both glass capillary and packed columns. With capillary columns, the silylation occurs in the flash-heater of the gas chromatograph. With phenylephrine as model substance the N,O,O-tris-(TMS) derivative was formed together with some of the N,O-bis-(TMS) derivative. The formation of these products was verified by GC–MS. After the injection of MBTFA, all N-TMS groups are replaced by N-TFA and unreacted secondary amine groups are acylated. These reactions occur on-column. The reaction scheme earlier proposed by Donike for pre-column derivatization[9] may also be applied to this study.



With packed column analyses, the silylation and acylation reactions both occur on-column. The main advantages of this derivatization technique are its speed and simplicity, which result in a substantial saving of time. Expensive reagents are saved, when only few microlitres are required for each analysis. The mixture shown in Fig. 1 could not be separated on the packed column. However, for the simplest type of separations, packed columns may be employed if adequate sensitivity is obtained. This column type is simpler to operate than capillary columns and is not so sensitive to the different parameters controlled in this study. The derivatization technique combined with capillary columns is a valuable tool for the identification of complex mixtures and for quantitative analyses of drugs at low concentrations.

ACKNOWLEDGEMENT

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBOXYLIC ACIDS USING 4-BROMOMETHYL-7-ACETOXYCOUMARIN AS FLUORESCENCE REAGENT

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(Received July 14th, 1981)

SUMMARY

A system for the high-performance liquid chromatography of carboxylic acids using 4-bromomethyl-7-acetoxycoumarin (Br-Mac) as the fluorescence reagent is described. Br-Mac reacts with carboxylic acids to give the ester derivatives, which are separated using reversed-phase liquid chromatography. The eluate from the column is mixed with an alkaline solution. The labelled carboxylic acids are hydrolysed to the fluorescent coumarin derivatives, which are introduced into a flow-through fluorimeter. In this system, the fluorescent hydrolysate, equimolar to a carboxylic acid, is detected and this fluorophor is common to every carboxylic acid. Only a slight variation is found in the peak areas for various carboxylic acids. A gradient elution technique is effectively used in this system because the fluorescence quantum yield of the fluorescent hydrolysate is not affected by the constitution of the mobile phase. Low femtomole levels of carboxylic acids can be detected.

INTRODUCTION

In general, non-aromatic organic acids do not produce strong fluorescence or absorption in the ultraviolet or visible regions. Therefore, derivatization with suitable labelling reagents has been used for the sensitive determination of carboxylic acids.

Recently 4-bromomethyl-7-methoxycoumarin (Br-Mmc)[1–3], 9,10-diaminophenanthrene[4] and 9-anthryldiazomethane[5,6] have been reported as fluorescence reagents for labelling carboxylic acids. When these reagents are used as pre-column labelling reagents for carboxylic acids, it is generally presumed that the smaller the

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molecular size of the labelling reagent, the better the separation of the labelled carboxylic acids would be. Therefore, Br-Mmc seems to be the most suitable of the above reagents for the analysis of carboxylic acids by high-performance liquid chromatography (HPLC).

The fluorescence quantum yields of carboxylic acids labelled with Br-Mmc are, however, severely influenced by the solvent environment[7]. 9,10-Diaminophenanthrene is also subject to the same solvent effect[4]. It is therefore considered that a gradient elution technique could not be used effectively for the HPLC separation of carboxylic acids labelled with these reagents.

Further, a wide variation of the fluorescence intensity of the carboxylic acid Mmc esters was found with equimolar amounts of different carboxylic acids[7]. Accordingly, various kinds of carboxylic acids could not be determined sensitively with Br-Mmc.

In this paper, a fluorimetric HPLC method for carboxylic acids using Br-Mac, which does not suffer from the above disadvantages, as the pre-column labeling reagent is described.

EXPERIMENTAL

Reagents and chemicals

All reagents were of analytical-reagent grade. The carboxylic acids and acetone were obtained from Wako (Osaka, Japan). Acetonitrile for use as the mobile phase was purchased from Kanto Chemicals (Tokyo, Japan) and was distilled prior to use. Dibenzo-18-crown-6 was purchased from Aldrich (Milwaukee, WI, U.S.A.) and ODS-6013 (10 μm) from Kyowa Seimitsu (Tokyo, Japan). Redistilled water was used throughout.

The coumarin derivatives related to Br-Mac were prepared according to the method of Baker *et al.*[8] with some modifications.

Preparation of Br-Mac

Br-Mac was prepared according to the following method. β-Methylumbelliferone (50 g) was refluxed with acetic anhydride (100 ml) for 1 h. After cooling, the resulting mixture was poured into cold water (500 ml). The solid product (4-methyl-7-acetoxycoumarin) was filtered, dried and recrystallized from ethanol. A mixture of 4-methyl-7-acetoxycoumarin (10 g), N-bromosuccinimide (9 g), a small amount of α,α′-azobisisobutyronitrile and carbon tetrachloride (100 ml) were refluxed for 20 h and, after cooling, the solvent was removed *in vacuo*. The residue was washed with water, filtered and dried. Recrystallization from ethyl acetate and cyclohexane gave pure Br-Mac, m.p. 184–185°C. The purity of the product was checked by HPLC and the structure of Br-Mac was confirmed by elemental analysis and infrared, ultraviolet and nuclear magnetic resonance spectroscopy.

Apparatus

The apparatus shown in Fig. 1 was constructed. All parts were obtained from Japan Spectroscopic Co. (Tokyo, Japan). For the separation of the labelled carboxylic acids, a TRI ROTAR I high-performance liquid chromatograph equipped with a Model GP-A30 solvent programmer was used. HPLC separations were carried

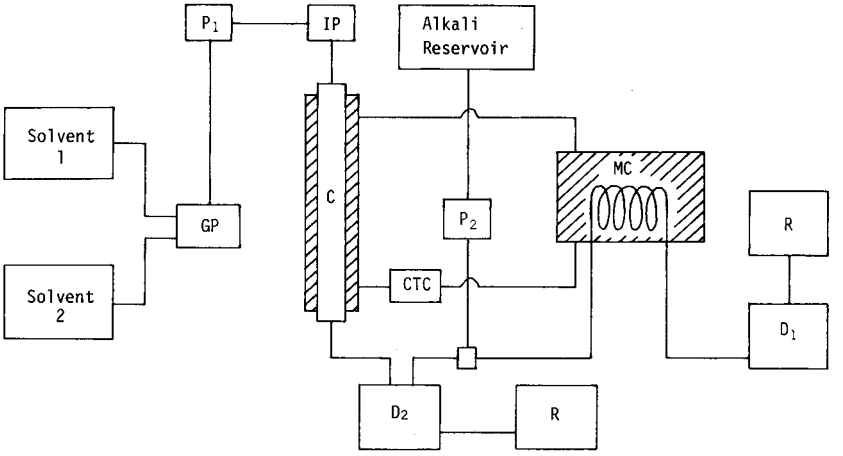


Fig. 1. Schematic diagram of the apparatus in the flow system. GP = Gradient programmer; P_1,P_2 = pumps; IP = inject port; C = column; CTC = constant-temperature circulator (50°C); MC = mixing coil; D_1 = spectrofluorimeter; D_2 = UV detector (connected only in the study of Br-Mac reactivity); R = recorder.

out with a stainless-steel column (250 × 2.1 mm I.D.) packed with ODS-6013 (10 μm) by a balanced-density slurry packing method. The temperature of the column and the mixing coil was maintained at 50°C. The operating conditions for HPLC are shown in Fig. 5. A Model FP-110 fluorescence spectrofluorimeter (excitation 365 nm, emission 460 nm) and a Model RC-225 strip-chart recorder were used. In the examination on the reactivity of BR-Mac with carboxylic acids, a UVIDEC 100 UV detector monitoring absorbance at 280 nm was introduced before mixing with an alkaline solution using a Model LCP 150 liquid chromatographic pump, and hydrolysis was performed through a coil made of 10 m × 0.5 mm I.D. stainless-steel tubing.

The fluorescence intensity of the coumarin derivatives related to Br-Mac was measured with a Model RF-510 spectrofluorimeter (Shimazu Seisakusho, Kyoto, Japan).

Derivatization

Each carboxylic acid (0.1–20 nmol), a 2.5-fold molar excess of Br-Mac and an equimolar amount of dibenzo-18-crown-6 were dissolved in 50 μl of acetone and the mixture was placed in a glass ampoule containing about 3 mg of a finely powdered mixture of potassium hydrogen carbonate and sodium sulphate (1:1). The ampoule was sealed and heated for 30 min at 50°C in the dark. After cooling, an aliquot of the resulting solution was injected on to the column.

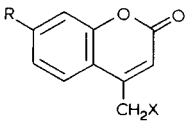
RESULTS AND DISCUSSION

Since Dünges and co-workers[1,2] used Br-Mmc as the fluorescence labelling reagent for various carboxylic acids, HPLC methods for organic acids using this reagent have been examined[8–13]. Recently other fluorescence reagents such as 9,10-

diaminophenanthrene[4] and 9-anthryldiazomethane[5,6] have been reported. In comparison with these reagents for pre-column labelling, Br-Mmc might be more suitable for the separation of labelled carboxylic acids, because it has the smallest molecular size. The fluorescence quantum yields of the carboxylic acids labelled with Br-Mmc are, however, subject to the solvent effect[7]. It has also been shown that 9,10-diaminophenanthrene had the same property[4]. Therefore, a gradient elution technique could not be used effectively with these reagents. In addition, it was reported that the fluorescence intensity of the carboxylic acid Mmc esters depended on the kind of carboxylic acid residues[7]. In this instance, various kinds of carboxylic acids could not be detected with high sensitivity using Br-Mmc. An initial effort was directed to overcoming these problems and to develop a fluorescence reagent without the defects mentioned above.

TABLE I

RELATIVE FLUORESCENCE INTENSITIES OF COUMARIN DERIVATIVES



1 μmol of each compound was dissolved in 10 ml of the solution shown and the fluorescence intensity was measured at the wavelength of the excitation and emission maxima. The results are given in arbitrary units.

X	*R*	*Acetonitrile–water (20:80)*	*Acetonitrile–water (50:50)*	*Acetonitrile–water (80:20)*	*Acetonitrile–0.1 M borate buffer (pH 11.0) (50:50)*	*Acetonitrile–0.1 M acetate buffer (pH 4.0) (50:50)*
H	OH	$1.2 \cdot 10^2$	$9.5 \cdot 10$	$4.0 \cdot 10^2$	$3.8 \cdot 10$	
H	$OCOCH_3$	1.0	0.5	0.2	$3.0 \cdot 10^2$	1.0
H	OCH_3	$1.8 \cdot 10^2$	$9.0 \cdot 10$	$3.4 \cdot 10$	$7.6 \cdot 10$	$8.6 \cdot 10$
Br	OCH_3	Precipitate	2.6	1.7	1.4	3.9
Br	$OCOCH_3$	0.5	0.4	0.3	$8.6 \cdot 10$	0.5
COOH	$OCOCH_3$	11.0	8.0	3.0	$3.8 \cdot 10^2$	8.7
COOH	CH_3	2.0	1.1	0.6	1.3	1.3

Table I gives the relative fluorescence intensities of several coumarin derivatives with different substituent groups at the 4- or 7-position in various solvents. Each fluorescence intensity was measured at the wavelength of the excitation and emission maxima. A substituent group at the 7-position in coumarin greatly affects the fluorescence quantum yield, whereas one in the 4-position has hardly any effect. The presence of an electron-donating group such as a hydroxy group tends to enhance the fluorescence intensity of the 7-hydroxy derivative, which is increased by making the pH of its solution more alkaline. The coumarin derivatives with acetoxy groups in the 7-position show strong fluorescence only in alkaline solutions. This phenomenon seems to be due to the hydrolysis of the acetoxy group to a hydroxy group. Based on these results, Br-Mac was adopted as the pre-column labelling reagent for carboxylic acids.

The reaction of Br-Mac with a carboxylic acid and the principle of the detection system are shown in Fig. 2 compared with those for Br-Mmc. With all of the fluorescence reagents reported previously, the reagent is allowed to react with carbox-

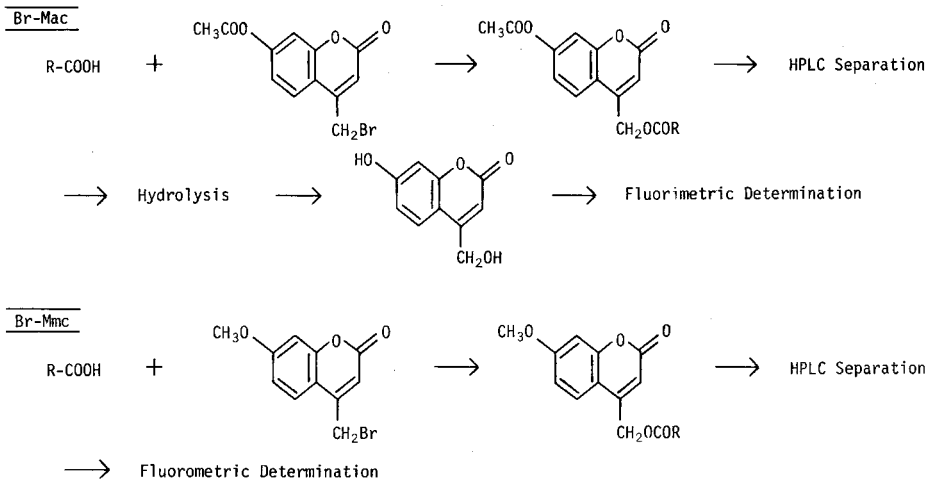


Fig. 2. Schemes for the reactions of carboxylic acids with Br-Mac and Br-Mmc and the analytical systems.

ylic acids first and, after column separation, each carboxylic acid labelled with the fluorophore is detected. Therefore, the fluorescence quantum yields tend to be influenced by the carboxylic acid residues. The fluorescence intensity of carboxylic acids labelled with Br-Mmc varies with the kind of carboxylic acid[7] and therefore different carboxylic acids could not be detected with similar sensitivities.

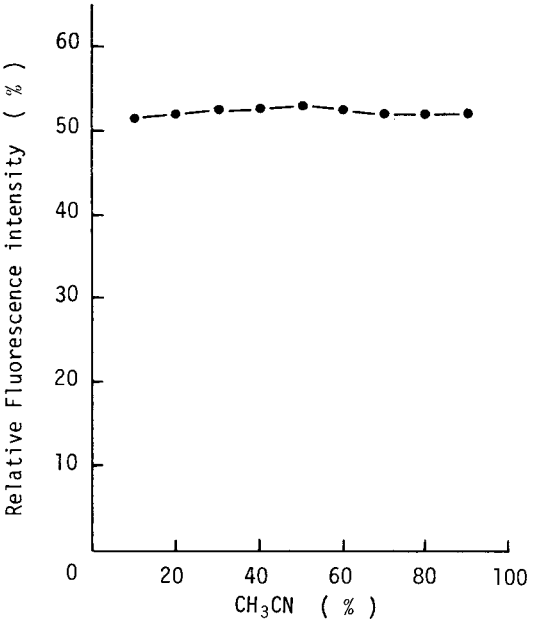


Fig. 3. Variation of the fluorescence intensity of the 7-hydroxycoumarin derivative with the concentration of acetonitrile in borate buffer. 4-Methyl-7-acetoxycoumarin was dissolved in acetonitrile (1 μmol/ml). To 1 ml of this solution, 5 ml of aqueous acetonitrile solution and 0.1 *M* borate buffer (pH 11.0) were added to give a total volume of 10 ml. After heating for 30 min, the fluorescence intensity was measured at the wavelength of the excitation and emission maxima.

On the other hand, Br-Mac reacts with carboxylic acids to give the ester derivatives, followed by HPLC separation. After separation, each compound is hydrolysed by addition of an alkaline solution, and then the resulting fluorescent compound is introduced into a spectrofluorimeter. Therefore, the fluorescence intensity is hardly influenced by the kind of carboxylic acid because not only the fluorescent hydrolysate, equimolar to a carboxylic acid, is detected, but also this fluorophore is common to all carboxylic acids. Further, the alkaline hydrolysis produces a much stronger fluorescence of the hydrolysate. For these reasons, highly sensitive detection of different carboxylic acids by HPLC using Br-Mac could be expected.

Fig. 3 shows the relationship between the fluorescence intensity of 4-methyl-7-hydroxycoumarin and the concentration of acetonitrile in alkaline solution. The fluorescence intensity was almost constant over the range of acetonitrile concentrations investigated. This result indicates that the detection system using Br-Mac might not be affected by changes in the acetonitrile concentration. Thus, a gradient elution technique could be used effectively in this system.

The results of the investigation of the hydrolysis time necessary for hydrolysis of the acetoxy group to a hydroxy group are shown in Fig. 4. To an aqueous acetonitrile solution of 4-carboxymethyl-7-acetoxycoumarin was added borate buffer (0.1 *M*, pH 11.0) and the mixture was pumped to the spectrofluorimeter. The fluorescence intensity became constant after 1–2 min, which suggests that the hydrolysis of carboxylic acid Mac esters proceeds to completion in this period. Therefore, a coil of length 10 m is sufficient to hydrolyse the Mac esters when tubing of 0.5 mm I.D. was used at a flow-rate of 1–2 ml/min.

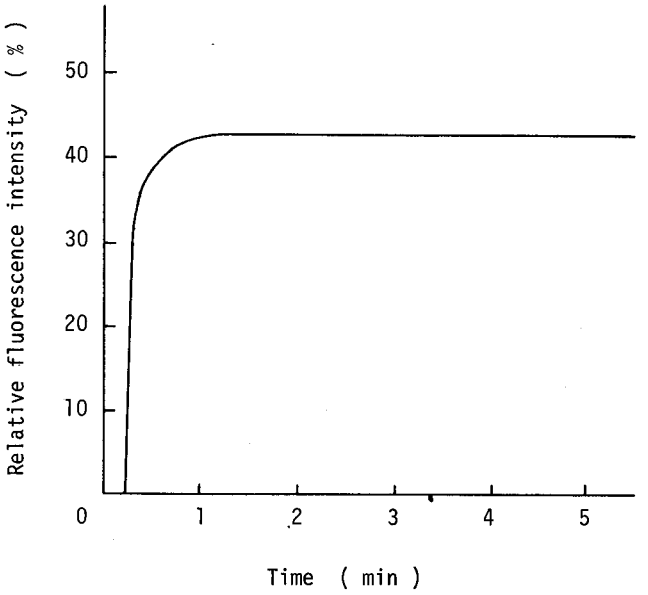


Fig. 4. Hydrolysis rate of 4-carboxymethyl-7-acetoxycoumarin. To 2 ml of 4-carboxymethyl-7-acetoxycoumarin in acetonitrile (0.5 mmol/ml), 18 ml of acetonitrile and 10 ml of water were added. This solution was mixed with 0.1 *M* borate buffer (pH 11.0) and pumped through the spectrofluorimeter, keeping the temperature at 50°C.

TABLE II

REACTIVITY OF 4-BROMOMETHYL-7-ACETOXYCOUMARIN WITH DIFFERENT CARBOXYLIC ACIDS

Carboxylic acid	*Reactivity**	*Carboxylic acid*	*Reactivity**
Aliphatic carboxylic acids:		Adipic acid	+
Saturated monocarboxylic acids:		Hydroxycarboxylic acids:	
Formic acid	+	Lactic acid	+
Acetic acid	+	Malic acid	+
Propionic acid	+	Citric acid	−
Butyric acid	+	Ketocarboxylic acids:	
Isovaleric acid	+	Pyruvic acid	−
Caproic acid	+	α-Ketoglutaric acid	−
Caprylic acid	+	Trichloroacetic acid	−
Capric acid	+	*Aromatic carboxylic acids:*	
Lauric acid	+	Benzoic acid	+
Palmitic acid	+	Phthalic acid	+
Stearic acid	+	Salicylic acid	+
Arachidic acid	+	*p*-Hydroxyphenylpropionic acid	+
Unsaturated monocarboxylic acids:		*p*-Aminobenzoic acid	+
Oleic acid	+	*o*-Aminobenzoic acid	+
Linoleic acid	+	Picolinic acid	+
Dicarboxylic acids:			
Succinic acid	+	Barbital	+

* + indicate carboxylic acids that give peaks with both UV and fluorescence detection; − indicate carboxylic acids that give neither a UV nor a fluorescence response.

In order to investigate the reactivity of Br-Mac with various carboxylic acids, each carboxylic acid listed in Table II was allowed to react with Br-Mac under the conditions described under *Derivatization*. The resulting solution was subjected to the chromatographic investigation. From the data shown in Table II, it is considered that the reactivity of Br-Mac is very similar to that of Br-Mmc.

Fig. 5 shows the separation of fatty acid Mac esters (C_4–C_{20}) using a gradient elution technique. A graph of peak area *versus* the carbon number of the fatty acids is shown in Fig. 6. Only a slight decrease in the peak areas occurred when the carbon number increased, which indicates that with Br-Mac the fluorescence intensity is hardly related to the kind of carboxylic acid, unlike Br-Mmc, and remains almost constant in spite of the variation of the acetonitrile concentration by using a gradient elution technique. As expected in the study using the compound related to Br-Mac, it was found that the fluorescence quantum yields of the hydrolysates of carboxylic acid Mac derivatives were hardly affected by the constitution of the mobile phase.

To ascertain the detection limit, the sample used in the experiment in Fig. 5 was diluted successively with acetone and injected on to the column. Fig. 7 shows a chromatogram in which each peak corresponds to 200 fmol of a fatty acid. Considering the signal-to-noise ratio, the detection limit is about 10 fmol. A further improvement in the sensitivity might be possible with optimization of the reaction and HPLC conditions.

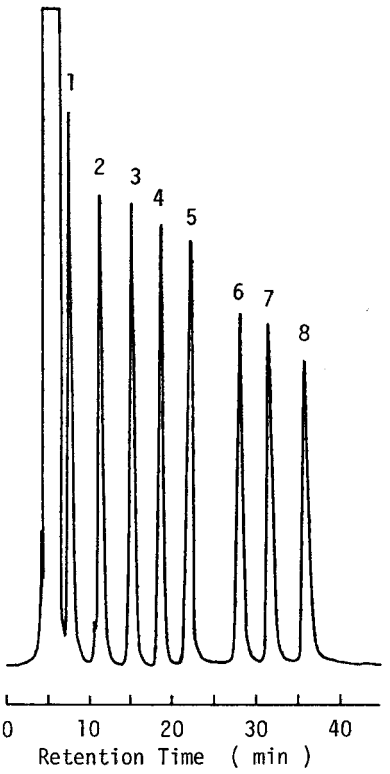


Fig. 5. High-performance liquid chromatogram of the Br-Mac derivatives of linear saturated fatty acids. 1 = $C_{4:0}$; 2 = $CC_{6:0}$; 3 = $CC_{8:0}$; 4 = $CC_{10:0}$; 5 = $CC_{12:0}$; 6 = $C_{16:0}$; 7 = $CC_{18:0}$; 8 = $CC_{20:0}$. Operating conditions: column, 250 × 2.1 mm ODS-6013 (10 μm); column and mixing coil temperature, 50°C; mobile phase, aqueous acetonitrile solution 40% (0)–90% (99). The gradient was prepared by use of a Model GP-A30 solvent programmer (Convex 1, 32 min); flow-rate, 0.8 ml/min; alkali flow-rate, 0.4 ml/min; detector, spectrofluorimeter (excitation 365 nm, emission 460 nm).

CONCLUSION

Compared with HPLC using other fluorescence labelling reagents, the present system with Br-Mac has several advantages. The fluorescence intensity depends neither on the kind of carboxylic acid nor on the concentration of acetonitrile in the mobile phase. In this system, a gradient elution technique can be effectively applied. Low femtomole levels of carboxylic acids can be detected. Methods for the determination of the organic acids involved in inborn errors of metabolism, bile acids and prostaglandins are at present under intensive investigation.

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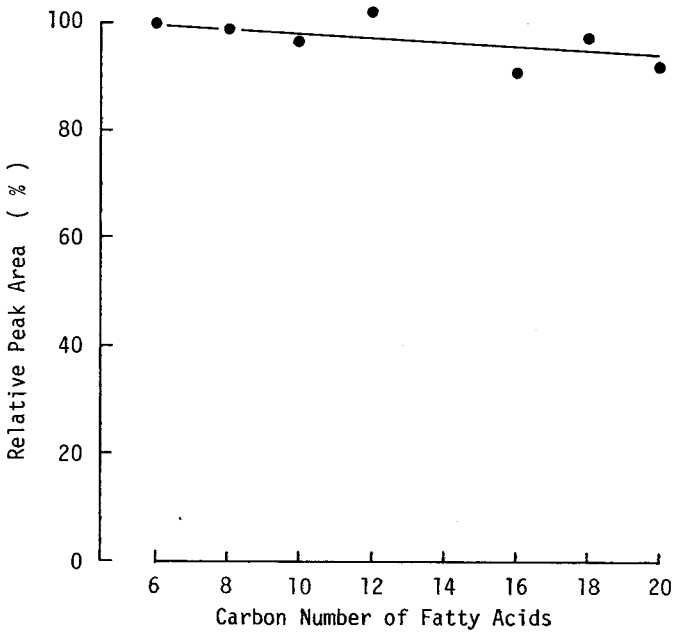

Fig. 6. Variation of peak area with carbon number of linear saturated fatty acids.

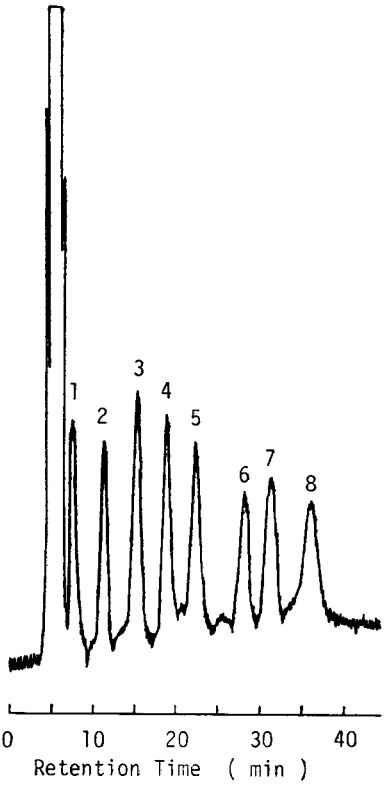

Fig. 7. High-performance liquid chromatogram of Br-Mac derivatives of linear saturated fatty acids. Each peak corresponds to 200 fmol of a fatty acid. Operating conditions and peaks as in Fig. 5.

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GENERAL SCHEME OF ANALYSIS OF PHENOLIC COMPOUNDS IN PLANT EXTRACTS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A general procedure of analysis of phenolic compounds by reversed-phase high-performance liquid chromatography with ternary elution gradients has been designed. The method was applied to the separation and estimation of phenolic acids, phenolic aldehydes and coumarins from standard mixtures or from plant extracts. Examples of its application to the variations in phenolic acids during grape juice storage, to studies of the monomeric composition of poplar lignins and to estimation of the coumarin contents of sweet clover are given.

INTRODUCTION

Phenolic compounds are widely distributed in plants and are frequently present in products of plant origin of economic importance.

Paper chromatography has been extensively used for qualitative research in phenolics, ever since its introduction in the 1940's by Bate-Smith[20]. In contrast, the quantitative analysis of these compounds has been little developed owing to the great diversity of their chemical structures and the absence of a general procedure.

Low-pressure liquid chromatography[1] gave excellent separations of phenolic compounds but was not adapted to systematic studies owing to the long analysis times. The separation of some phenolics has recently been achieved by gas–liquid chromatography. However, the low volatility of polyphenols and the poor selectivity of the detectors render this method inappropriate for plant extracts (for details see the review by Van Sumere *et al.*[2]).

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High-performance liquid chromatography (HPLC), first developed for phenolic compounds by Hostettmann and Jacot-Guillarmod[3] and Nagel and co-workers[4,5], and more recently by Murphy and Stutte[6], Hardin and Stutte[7] and Hartley and Buchan[8], seems more promising. We present here a general method of analysis of polyphenols by reversed-phase HPLC, and show, by direct applications, that this method can easily be used to solve problems concerning the analysis of the polyphenol contents of plants.

EXPERIMENTAL

Apparatus

A Spectra-Physics SP 8000 liquid chromatograph was used. A 250 × 4.6 mm stainless-steel column, packed with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R., was used for the chromatographic separations. The SP 8000 includes a ternary gradient generator, an automatic injector with a 10-μl sample loop and a peak integrator. A microprocessor corrects for the variations due to the changes in viscosity of the gradient mixtures and allows constant flow conditions, giving highly reproducible retention times (better than 2%).

Optical density was monitored with a variable-wavelength detector (Gilson Holochrom) equipped with a 10-μl flow cell.

Solvents

Only methanol, acetic acid and water were used. The two organic solvents were freshly distilled and filtered through a sintered glass filter (G 5). De-ionized water was filtered through a 0.45-μm Millipore filter and then degassed by boiling. All the solvents were stored under a helium atmosphere.

Isolation of phenolic compounds from plant materials

Phenolic acids. Phenolic acids were isolated from grape concentrate obtained from *Vitis vinifera* var. "Carignan noir" (I.N.R.A. Station of Pech Rouge, Gruissan, France). After harvesting, the grapes were crushed and 1 g/l of gaseous SO_2 was added. The juice was then decanted, and concentrated in a tubular evaporator. One aliquot was kept frozen and another stored for 6 months at 10°C.

Before use, 20 ml of each concentrate were first diluted with water to the initial concentration of the juice. Then 2% metaphosphoric acid, 20% ammonium sulphate and 20% ethanol were added and the phenolics extracted five times with 50 ml ethyl acetate. The combined extracts were taken to dryness. The residue was dissolved in water, adjusted to pH 4 and tannins were precipitated with 1% gelatine solution in 10% NaCl. The esters were then hydrolysed with 4 *M* NaOH for 4 h under nitrogen[9].

After adjustment of the pH to 2, phenolic acids were extracted by diethyl ether. The ether was then evaporated to dryness and the residue taken up in water and subsequently purified by extraction with diethyl ether, first at pH 8.5 (this extract was discarded) and then at pH 2. After evaporation of ether, the phenolic acids were dissolved in 1 ml methanol–1% HCl and centrifuged for 15 min at 10,000 *g* to eliminate undissolved salts.

Phenolic aldehydes. Phenolic aldehydes were obtained after nitrobenzene oxidation of lignins isolated from sclerenchyma and xylem tissues of stems of *Populus nigra* var. *Italica*[10,11]. The aldehyde fraction was purified by successive extraction

with diethyl ether, first at pH 11 to eliminate nitrobenzene, and then at pH 8.5. This second extract was evaporated to dryness, and the residue dissolved in 0.5 ml methanol and centrifuged for 15 min at 10,000 *g*.

Coumarins. The coumarins were extracted from the leaflets of two genotypes of *Melilotus alba:* the CuCu BB reported as coumarin rich and the cucu bb known as coumarin poor[12–14]. One gram of leaflets was ground in 20 ml of 80° ethanol with an Ultra-Turrax and the phenolic material was extracted with 500 ml of the same solvent, as described by Alibert *et al.*[15]. The solvent was evaporated, the dry residue dissolved in 10 ml of a 0.1 *M* acetate buffer, pH 5, and the glycosides hydrolysed for 4 h at 37°C by 0.3% emulsin (Nutritional Biochemicals)[16].

After acidification to pH 2, the coumarins were extracted by diethyl ether, the ethereal phase was evaporated and the residue dissolved in 0.5 ml methanol. This solution was centrifuged, as previously described for the other fractions, before HPLC analysis.

RESULTS

A HPLC separation of standard mixtures of phenolic compounds

During these experiments we tried to develop as simple as possible a method of analysis, allowing the separation of a wide variety of polyphenol classes without important modifications of the analytical system. After multiple preliminary essays we chose only one stationary phase: LiChrosorb RP-18 and methanol–acetic acid–water as solvent. Owing to the rapidity of the analysis, we preferred to separate the same sample twice under different conditions of elution and detection so as to obtain a complete separation, rather than to use a complicated elution mixture.

TABLE I

MOBILE PHASES FOR SEPARATION OF PHENOLIC ACIDS

Mobile phase	*Flow-rate (ml/min)*	*Elution (min)*	*Time (min)*	*Methanol (%)*	*Acetic acid (%)*	*Water (%)*
A	1	65	0	5	5	90
			2	5	1	94
			15	5	1	94
			16	5	5	90
			40	20	5	75
			41	65	5	30
			55	65	5	30
			56	100	0	0
			65	100	0	0
B	1	45	0	5	5	90
			2	5	1	94
			15	5	1	94
			16	5	5	90
			31	25	5	70
			32	100	0	0
			45	100	0	0

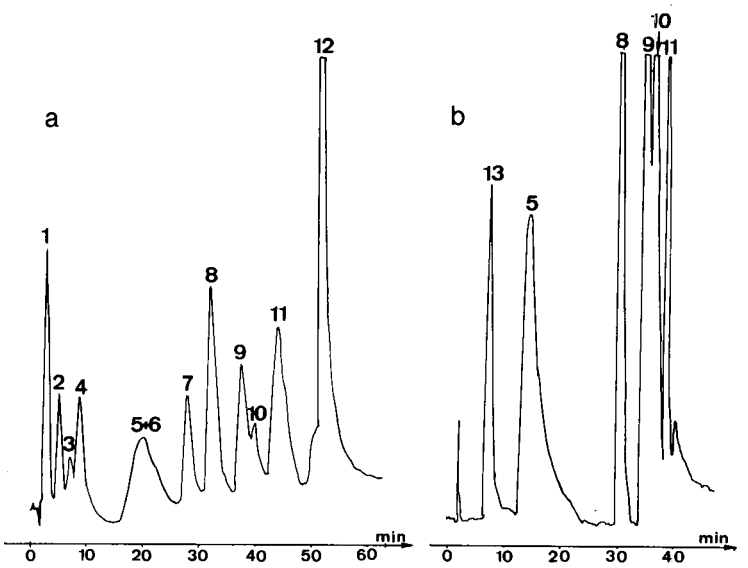

Fig. 1. Separation of phenolic acids: a, by mobile phase A with detection at 280 nm; b, by mobile phase B with detection at 335 nm. Numbers on the chromatograms correspond to the acids given in Table II.

Quantitative results are obtained directly by the data system of the SP 8000 liquid chromatograph. Different calibration methods are available; we used the external standard method where, in the "Cal Mode", the data system memorizes the values from successive standardization runs and corrects automatically the values of the analytical runs.

TABLE II

RETENTION TIMES (IN 0.1 min) OF PHENOLIC ACIDS

No.	*Phenolic acid*	*Mobile phase A, 280 nm detection*	*Mobile phase B, 335 nm detection*
1	Gallic	29	*
2	Protocatechuic	52	*
3	Salicylic	78	*
4	*p*-Hydroxybenzoic	85	*
5	Caffeic	193	144
6	Vanillic	193	*
7	Syringic	278	*
8	*p*-Coumaric	309	296
9	Ferulic	362	340
10	Sinapic	386	355
11	*o*-Coumaric	423	377
12	Cinnamic	482	*
13	Gentisic	*	76

* Not detected.

TABLE III

MOBILE PHASE FOR SEPARATION OF PHENOLIC ALDEHYDES

Flow-rate: 1 ml/min. Elution time: 25 min.

Time (min)	*Methanol (%)*	*Acetic acid (%)*	*Water (%)*
0	10	2	88
5	10	2	88
10	20	2	78
20	80	2	18
25	80	2	18

After 1 year of routine use, for amounts of 0.5–50 nmoles, the reproducibility of standards was better than 5%.

Phenolic acids. Phenolic acids are the most common phenolic compounds present in plants, and always as mixtures of benzoic and cinnamic acids.

Two mobile phase compositions were used to separate these substances (Table I). Mobile phase A, with detection at 280 nm, allows the quantitative analysis of most of the phenolic acids (Fig. 1a). However, gentisic acid does not absorb at this wavelength and caffeic and vanillic acids are poorly separated. Mobile phase B, with detection at 335 nm, allows quantitative analysis of these three acids (Fig. 1b)*.

Table II gives the retention times of the phenolic acids.

Phenolic aldehydes. Phenolic aldehydes, the main flavour fraction of various

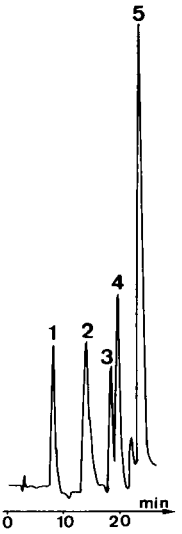

Fig. 2. Separation of phenolic aldehydes. Numbers on the chromatogram correspond to the aldehydes given in Table IV.

* The concentration of vanillic acid is given by $C_V = \left(A_{280} - \frac{K_C}{K'_C} \cdot A_{335}\right) \cdot \frac{1}{K_V}$

where A_{280} and A_{335} are the peak areas at 280 nm and 335 nm, respectively, K_C and K'_C are the response constants of the detector for caffeic acid at 280 and 335 nm respectively and K_V that of vanillic acid at 280 nm.

TABLE IV

RETENTION TIMES (IN 0.1 min) OF PHENOLIC ALDEHYDES

No.	*Aldehyde*	t_R
1	3,4-Dihydroxybenzaldehyde	78
2	4-Hydroxybenzaldehyde	133
3	Vanillin	176
4	Syringaldehyde	188
5	Benzaldehyde	225

TABLE V

MOBILE PHASES FOR SEPARATION OF COUMARINS

Mobile phase	*Flow-rate (ml/min)*	*Elution time (min)*	*Time (min)*	*Methanol (%)*	*Acetic acid (%)*	*Water (%)*
C	1	40	0	10	2	88
			30	50	2	48
			40	50	2	48
D	1	40	0	5	1	94
			12	20	1	79
			20	20	1	79
			30	80	1	19
			40	80	1	19

beverages, can easily be separated using the mobile phase given in Table III. Here the compounds are monitored at 290 nm (Fig. 2). The retention times of each compound are given in Table IV.

Coumarins. Coumarins constitute a large family of phenolic compounds particularly well represented in *Leguminosae*[17]. Most of them are separated on the RP-18 column using the solvent gradients reported in Table V. The coumarins were detected at 350 nm (gradient C) or 275 nm (gradient D). Table VI gives the retention times and the responses of the detector at these two wavelengths, while Fig. 3 shows the types of separation obtained under these conditions.

TABLE VI

RETENTION TIMES (IN 0.1 min) OF COUMARINS

No.	*Coumarin*	*Mobile phase C, 350 nm detection*	*Mobile phase D, 275 nm detection*
1	Aesculetin	203	
2	Umbelleferin	277	
3	Scopoletin	304	Not
4	5,7-Dihydroxy-6-methyl-8-ethylcoumarin	340	separated
5	4-Methyl-7,8-diethylcoumarin	395	
6	Coumarin	Not detected	267
7	4-Hydroxycoumarin	Not detected	317
8	4-Methylcoumarin	Not detected	368

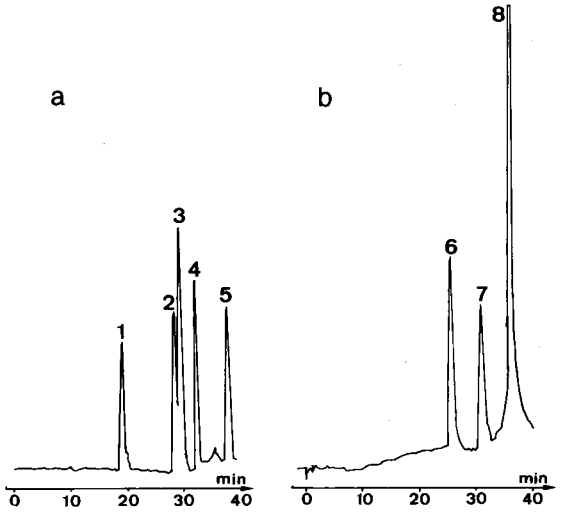

Fig. 3. Separation of coumarins: a, by mobile phase C with detection at 350 nm; b, by mobile phase D with detection at 275 nm. Numbers on the chromatograms correspond to the coumarins given in Table VI.

Application to the analysis of complex plant extracts

During our work on plant polyphenols we used this methodology to study the content or the variations of the phenolics. We present here some examples showing that the method can readily be adapted for various types of research.

Grape concentrate analysis. One of the main problems of the long-term storage of grape juice concentrate is the browning of the juices.

TABLE VII

RECOVERY OF PHENOLIC ACIDS USING THE EXTRACTION AND PURIFICATION DESCRIBED

In experiment 1, 10 nmoles of each acid were added to the sample; in experiment 2, 1 nmole of each acid was added.

Acids	*Percentage recovery*		*Mean*
	Experiment 1	*Experiment 2*	
Gallic	61	58	60
Protocatechuic	59	60	59
Salicylic	65	68	67
Gentisic	96	90	93
Caffeic	61	55	58
Vanillic	60	57	59
p-Hydroxybenzoic	64	58	62
Syringic	59	54	57
p-Coumaric	60	55	57
Ferulic	47	49	48
Sinapic	75	70	73
o-Coumaric	42	34	38
Cinnamic	83	80	81

TABLE VIII

AMOUNTS OF PHENOLIC ACIDS IN A GRAPE JUICE CONCENTRATE AFTER STORAGE FOR 6 MONTHS

Phenolic acid	*Amount (μmol/l)*	
	At −20°C	*At 10°C*
Gallic	12	12
Protocatechuic	37	36
Salicylic	36	23
p-Hydroxybenzoic	14	8.7
Caffeic	15	0
Vanillic	0.2	0
Syringic	48	25
p-Coumaric	37	29

Using our extraction and purification, the yield of phenolic acids was monitored by addition of standard mixtures to the concentrate after dilution with water. Table VII gives the percentage recovery of the different acids for two experiments and the mean values retained for automatic analysis. Table VIII shows that some phenolic acids (mainly caffeic, salicylic, *p*-hydroxybenzoic and vanillic) undergo degradation during a 6-month storage period while others (gallic, protocatechuic) seem to remain unchanged.

Studies on the monomer content of lignins. Next to cellulose, lignins are quantitatively the most important polymers in plants. The monomer content of lignin varies with the systematic classification of the examined plants[18], the age of the stems[19] or the nature of the lignified tissues[10]. The present technique has been applied to studies on lignins. As in the case of phenolic acids, the percentage recovery of the phenolic aldehydes was determined after addition of standard mixtures before nitrobenzene oxidation of a xylem sample of poplar stem. Table IX shows that the yields range from 70 to 84 % for the three major monomeric units of lignins (*i.e.*, vanillin, syringaldehyde and 4-hydroxybenzaldehyde), but 3,4-dihydroxybenzaldehyde is destroyed by the degradation process of the lignins. Mean correction factors are given in the table.

TABLE IX

RECOVERY OF PHENOLIC BENZALDEHYDE USING THE NITROBENZENE DEGRADATION OF LIGNINS DESCRIBED

In experiment 1, 10 nmoles of each aldehyde were added to the sample; in experiment 2, 1 nmole of each aldehyde was added.

Aldehyde	*Percentage recovery*		*Mean*
	Experiment 1	*Experiment 2*	
3,4 Dihydroxybenzaldehyde	6.9	10	8
4-Hydroxybenzaldehyde	84	85	84
Vanillin	67	68	68
Syringaldehyde	67	72	70

TABLE X

MONOMER CONTENTS OF THE LIGNINS OF XYLEM AND SCLERENCHYMA OF ONE POPLAR STEM

Aldehyde	*Amount (μmol/g)*	
	Xylem	*Sclerenchyma*
p-Hydroxybenzaldehyde	12	0
Vanillin (V)	78	50
Syringaldehyde (S)	42	30
Ratio, S/V	0.54	0.60

Table X gives data on the monomer content of sclerenchyma and xylem of one poplar stem. We can show that these two types of tissue are different: sclerenchyma contains three monomeric units (guaiacyl, syringyl and *p*-hydroxybenzoyl corresponding, after degradation by alkaline nitrobenzene, to coniferylic, sinapylic and coumarylic alcohols), while xylem has only two constituents (guaiacyl and syringyl). Furthermore, the relative proportions of the guaiacyl (vanillin) and syringyl (syringaldehyde) units differ from one tissue to another.

This rapid HPLC technique seems useful for routine analysis in such fields.

Coumarin analysis. Research was originally conducted on *Melilotus* with the aim of developing strains of sweet clover deficient in coumarins; these compounds are normally found in relatively high concentrations and render the plant unpalatable to herbivores. No attempt was made in this case to determine the quantitative yield of the coumarins using the proposed extraction and purification since the main object of the work was essentially a comparison between coumarin contents of two strains of sweet clover.

Compared to the common CuCu BB genotype, the cucu bb genotype contains much lower amounts of scopoletin, coumarin and 4-hydroxycoumarin, as shown in Table XI.

Comparative studies on coumarin biosynthesis, transport and storage could be performed using this technique.

TABLE XI

COUMARIN CONTENTS OF TWO STRAINS OF *MELILOTUS ALBA*

Coumarin	*Amount (μmol/g)*	
	Genotype (CuCu BB)	*Genotype (cucu bb)*
Scopoletin	121	17
Coumarin	32	0
4-Hydroxycoumarin	664	30

CONCLUSIONS

We have developed a general technique of separation and quantitative analysis of plant phenolic compounds by reversed-phase HPLC. It is shown, through examples, that this rapid and sensitive method is easily adapted to numerous fields of application such as phytochemistry, food control and fundamental research.

We believe that this method is not restricted to the three types of phenolic compound described here, but can easily be extended, with minor adjustments, to the analysis of other phenolic compounds such as esters, flavonoids or anthocyanins.

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SEPARATION OF SOME METABOLICALLY IMPORTANT AROMATIC N-ACYLAMINO ACIDS OF THE BENZOYL AND CINNAMOYL SERIES BY THIN-LAYER, GAS–LIQUID AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separations of 30 different N-acylamino acids and peptides from the benzoyl and cinnamoyl series have been studied by thin-layer (TLC), gas–liquid (GLC) and high-performance liquid chromatography (HPLC). TLC separations on three different layers and with four different solvent systems are described. GLC separations of the trimethylsilyl derivatives of some of the compounds were carried out on a glass column packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 + 1.5% SE-52 and using a temperature program. HPLC separations on a reversed-phase column (LiChrosorb RP-18, 10 μm, Knauer) employed a combination of isocratic and a linear gradient elution [solvent A, water–formic acid (95:5 v/v); solvent B, methanol; 35°C]. The few compounds which could not be separated by the latter system were separated on a second column (LiChrosorb Si 60, 7 μm, Knauer) by means of a combination of isocratic and linear gradient elution [solvent A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v); solvent B, methanol; 30°C]. These methods can easily be combined, and allow the separation of N-acylamino acids and N-acylpeptides from biological fluids, extracts and partial hydrolysates from phenolic acid-containing plant proteins.

INTRODUCTION

In addition to N-benzoylglycine (hippuric acid), which is normally present in urine as a metabolite of dietary components, several other N-acylglycines of the substituted benzoyl series (*viz.*, *o*-, *p*- and *m*-hydroxybenzoylglycine, vanilloylglycine and isovanilloylglycine, etc.[1–10]), the related phenylacetyl series (*viz.*, N-phenylacetyl-

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glutamine[11,12] and N-phenylacetylglutamic acid[13]) and the cinnamoyl series (*viz.*, N-cinnamoylglycine[13], N-*p*-hydroxycinnamoylglycine and N-feruloylglycine[1,2,7]) have been reported to occur in urine. Exposure of toluene or *p*- or *m*-xylene to man resulted in the production of *p*- and *m*-methylhippuric acid[14–16], while ingestion of L-DOPA gave rise to homovanilloylglycine[17].

Aliphatic N-acylamino acids may also occur in urine. Recently, N^{δ}-acetylornithine, a substance that has frequently been observed in plants[18], was identified as a minor component of both urine and deproteinized human or bovine blood[19]. Abnormal excretions of certain aliphatic N-acylglycine conjugates have been found in a number of organic aciduria[10,20,21], in a patient with D-glyceric acidimia[22] as well as in several other diseases[10], and according to the latter authors the discovery of new organic aciduria may well be promoted by the identification of "new" acylglycines.

Besides urine and blood plasma, other biological fluids and materials may also contain N-acylamino acids. In this context it should be mentioned that N-phenylacetylglutamine has been detected in bovine milk[23], while N-benzoylglutamic acid has been identified as a metabolite of benzoic acid in Indian fruit bats[24].

Conjugation of aromatic carboxylic acids can also occur in plants, because N-feruloylglycine has been identified as a building stone of barley and Medicago sativa proteins[25–27]. An analogous N-acylamino acid of the cinnamoyl series, *viz.*, N-*p*-coumaroylglutamic acid, has been reported to occur in black tea[28], and two aromatic amides of aspartic acid, namely N-benzoylaspartate and its homologue N-phenylacetylaspartate have been isolated from pea seeds[29].

It is therefore possible that certain aromatic N-acylamino acids may be of importance in the metabolism of phenolic acids and related compounds. N-acylamino acids such as N-benzoyl-L-leucine and N-phenylacetyl-L-leucine inhibit the growth of several plant pathogens[30], while N-benzoylaspartic acid reduces the relative germination rate of rice seeds[31]. It seems thus that N-acylamino acids (possibly also N-acylpeptides), especially those of the benzoyl, phenylacetyl and cinnamoyl series, are of great biological interest.

Unfortunately, with the exception of hippuric acid and its derivatives, little information on the chromatography of these compounds (especially those of the cinnamoyl series) is available. Usually, N-acylglycines and related substances (*e.g.*, hippuric acid and its derivatives) have been analysed by means of paper chromatography (PC) or thin-layer chromatography (TLC)[21,32–37]. There are few reports of the separation of these substances (with the exception of hippuric acid) by gas–liquid chromatography (GLC)[8–13,38,39] or by high-performance liquid chromatography (HPLC)[40–44].

For the above reasons, several N-acylamino acids and peptides of the benzoyl and cinnamoyl series have been synthesized[45–50] and studied. In this paper the TLC, GLC and HPLC separation and detection of some of these compounds will be described.

MATERIALS AND METHODS

With the exception of N-benzoylglycine (hippuric acid) which was purchased from Aldrich, (Beerse, Belgium), all other compounds were synthesized. N-*o*-, *m*- and *p*-hydroxybenzoyl-, N-vanilloyl-, N-syringoyl-, N-cinnamoyl-, N-*p*-coumaroyl-, N-

feruloyl- and N-sinapoylglycine and N-feruloyl-L-alanine were prepared from the corresponding N-hydroxysuccinimide esters[49]. N-Protocatechuoyl- and N-caffeoylglycine as well as N-caffeoylglycyl-L-phenylalanine were obtained from the corresponding N-(O-ethoxycarbonyl) derivatives using the same active ester method, the protecting O-ethoxycarbonyl groups being removed with hydrazine[50]. N-*p*-Hydroxybenzoyl-L-alanine, N-cinnamoyl- and N-*p*-coumaroyl-DL-alanine, N-*o*-coumaroyl-, N-*p*-coumaroyl- and N-feruloyl-DL-aspartic acid, as well as N-feruloyl-L-phenylalanine, N-cinnamoylglycyl-L-phenylalanine, N-*p*-coumaroylglycyl-L-phenylalanine and N-feruloylglycyl-L-phenylalanine were prepared by means of the acid chloride method[47]. N-*p*-Coumaroyl- and N-feruloylsarcosine were obtained analogously. N-Cinnamoyl-, N-*p*-coumaroyl- and N-feruloyl-L-methionine as well as N-feruloyl-L-phenylalanyl-L-phenylalanine were synthesized via the mixed anhydrides as described by De Pooter *et al.*[46].

MN cellulose powder 300 (average particle size *ca.* 10 μm) and N-methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) were purchased from Macherey, Nagel & Co. (Düren, G.F.R.). Silica gel G and pre-coated TLC plates (silica gel 60F-254) were obtained from E. Merck (Darmstadt, G.F.R.), and Chromosorb W AW DMCS (80–100 mesh), SE-30 and SE-52 from Varian Aerograph. The analytical HPLC column (250 × 4.6 mm I.D.), containing LiChrosorb RP-18 (10 μm) and the semi-preparative LiChrosorb Si 60 (7 μm, 250 × 8 mm I.D.) were obtained from Dr. H. Knauer (Wissenschaftliche Geräte, Oberursel/Taunus, G.F.R.). The solvents used for HPLC were formic acid (suprapur) and methanol (LiChrosolv for chromatography) (E. Merck). "Baker analysed HPLC reagent water" was obtained from Baker Chemicals (Deventer, The Netherlands).

TLC

Preparation of the thin layers. For the preparation of cellulose plates, MN cellulose powder 300 was used. The silica gel–cellulose layers were prepared as described previously[51]. In certain experiments the internal water phase of the plates was increased by keeping the plates, after spotting, but before irrigation, in an atmosphere saturated with water for 20 min[52] or by steaming[51]. However, this procedure, which gives excellent results with phenols, phenolic acids, coumarins, etc., did not improve the chromatography of the N-acylamino acids and peptides.

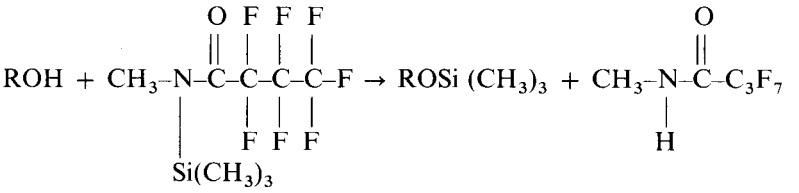
Chromatography. The compounds were dissolved in methanol–water (1:1 v/v) and 10–20 μg were applied on the thin layers. The following solvents were employed at room temperature: toluene–ethyl formate–formic acid (5:4:1) (TEF); *sec.*-butanol–water (4:1); 2% acetic acid and ethyl methyl ketone–pyridine–water–acetic acid (70:15:15:2) (EPWA).

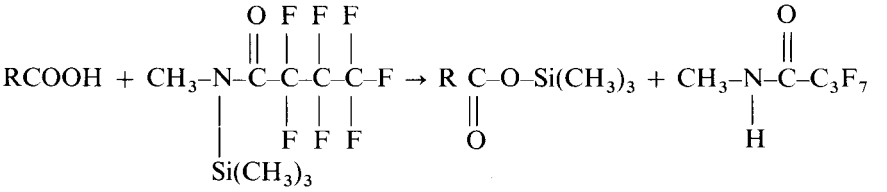
Detection. The compounds were detected by examination of the dried chromatograms under UV light (360 nm) before and after spraying the thin layers with 2 *M* sodium hydroxide. Thereafter the compounds were revealed by spraying with diazotized *p*-nitroaniline[51], treatment with Gaffney's reagent[32,34] or with 1% $KMnO_4$ in 0.1 *M* H_2SO_4.

GLC

Trimethylsilylation. Each component (± 0.4 mg) of a mixture was weighed. Subsequently, 400 μl MSHFBA were added and the reaction mixture was kept for 10

min at 125°C in a sealed vial. The possible reactions of the phenolic and acidic functions with MSHFBA are as follows:





Aliquots (20 μl) of the reaction mixture were injected directly in the gas–liquid chromatograph.

Chromatography. Analyses were performed on a Hewlett-Packard gas chromatograph 5730A equipped with glass columns and a thermal conductivity detector (temperature 350°C, filament current 150 mA). The glass column (3.0 m × 2 mm I.D.) was packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 plus 1.5% SE-52[53]. The carrier gas (helium) flow-rate was 30 ml/min. The injector port temperature was 300°C, and the column temperature was programmed at 8°C/min from 200°C to 310°C. The recorder (0–1.0 mV) chart speed was 1 cm/min. Attenuation:1.

HPLC

A Hewlett-Packard liquid chromatograph 1084B equipped with a Pye Unicam variable wavelength LC 3 UV-detector set at 280 nm was used throughout this work. Two different types of columns were employed. (a) An analytical LiChrosorb RP-18 (10 μm) prepacked column (250 × 4.6 mm I.D.). The eluent (gradient elution) and chromatographic conditions were: solvent A, formic acid–water (5:95, v/v); solvent B, methanol; gradient profile, linear; gradient range, 0–2 min, isocratic 7% B (% B in A), 2–8 min, 7–15% B, 8–25 min, 15–75% B, 25–27 min, 75–80% B, 27–29 min, isocratic 80% B; flow-rate 2.5 ml/min; oven and eluent temperatures 35°C; column pressure 80–100 bar; attenuation $6.4 \cdot 10^{-3}$ absorbance units per cm and recorder (0–1.0 mV) chart speed 0.5 cm/min. (b) A semi-preparative LiChrosorb Si 60 (7 μm) prepacked column (250 × 8 mm I.D.). Conditions: solvent A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v); solvent B, methanol; gradient profile, linear; gradient range, 0–5 min, isocratic 2% B (% B in A), 5–20 min, 2–17% B, 20–28 min, 17–35% B, 28–33 min, 35–60% B; flow-rate 4 ml/min; oven and eluent temperatures 30°C; column pressure and attenuation as above.

The N-acylamino acids and peptides were dissolved in methanol–water (1:1, v/v) and on both columns the sample size was 20 μl (loop-valve), 1–5 μg of each of the substances being injected.

RESULTS AND DISCUSSION

Table I shows the R_F values of 30 aromatic N-acylamino acids of the benzoyl and cinnamoyl series on cellulose (A), silica gel–cellulose (B) and precoated silica gel plates (C) (E. Merck), developed with TEF, *sec.*-butanol–water, 2% acetic acid or EPWA. Table II shows the fluorescence (UV light 360 nm) of the compounds before and after NaOH treatment as well as the colour reaction with diazotized *p*-nitroaniline. Also shown is the colour reaction of the compounds with either Gaffney's reagent[32] or 1% potassium permanganate in 0.1 *M* sulphuric acid. The colours produced were further standardized and matched against the "Derwent Colour pencils, series No. 19 (Cumberland Pencil Ltd., Keswick, Great Britain).

Table I shows that the R_F values of the compounds are generally higher on cellulose plates than on silica gel–cellulose and pre-coated silica gel layers, the values on these last two types of layers being relatively small. *sec.*-Butanol–water produces higher R_F values on cellulose layers than TEF, especially for compounds such as N-protocatechuoylglycine, N-caffeoylglycine and N-*p*-coumaroyl-DL-aspartic acid. The differences in R_F values obtained with the former solvent on silica gel–cellulose layers and precoated silica gel layers were less clear-cut. In the case of 2% acetic acid, only the R_F values on cellulose and silica gel–cellulose layers are given because separation of the compounds on pre-coated silica gel layers proved to be unsatisfactory. Finally, with EPWA the R_F values of the N-acylamino acids and peptides were again high, especially on cellulose layers. On the latter with TEF as solvent, the glycine derivatives separated according to the polarity and type of substitution of the phenolic moiety. The same holds true when compounds with the same acyl groups but different amino acid or peptide moieties are considered (*e.g.*, N-*p*-coumaroylglycine, N-*p*-coumaroylalanine, etc.).

Two-dimensional TLC of the compounds can be performed on cellulose or silica gel–cellulose layers with TEF and EPWA as solvents. Furthermore it should be noted that irrigation with 2% acetic acid gives rise to a separation of the pre-existing *cis* and *trans* isomers. This last effect can be prevented when the synthesis, spotting and chromatography are performed under an orange ICI Perspex filter No. 300.

As can be seen from Table II most of the substances were fluorescent at 360 nm, and with the exception of the cinnamoyl derivatives all compounds also showed a more or less pronounced colour reaction with diazotized *p*-nitroaniline. Some of the N-acylamino acids showed a further reaction with Gaffney's reagent, and all the compounds could be revealed with 1% $KMnO_4$ in 0.1 *M* H_2SO_4. GLC separations; especially GLC–mass spectrometry (MS) combinations, could also play a key rôle in the analysis of aromatic N-acylamino acids from the benzoyl or cinnamoyl series (see refs. 9–12, 14 and 39). Indeed, after trimethylsilylation and preparative GLC, N-feruloylglycine could be collected as a TMS derivative by means of a technique described by Vande Casteele *et al.*[53]. Overnight hydrolysis of the TMS compound with a drop of water and subsequent TLC showed that the GLC–TLC combination could be used for the purification and (or) identification of ferulic acid derivatives from complex mixtures.

Unfortunately, as shown in Fig. 1 and Table III, not all of the compounds studied could easily be derivatized and chromatographed. Moreover, the TMS com-

TABLE I

R_F VALUES OF THE COMPOUNDS STUDIED ON CELLULOSE (A), SILICA GEL–CELLULOSE (B) AND PRECOATED SILICA GEL PLATES (C) (E. MERCK)

Compound	*Toluene–ethyl formate–formic acid (5:4:1)*			*sec.-Butanol–water (4:1)*			*2% Acetic acid*		*Ethyl methyl ketone–pyridine–water–acetic acid (70:15:15:2)*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*A*	*B*	*C*
N-Benzoylglycine (hippuric acid)	0.54	0.37	0.25	0.86	0.43	0.31	0.93	0.92	0.77	0.57	0.66
N-*o*-Hydroxybenzoylglycine	0.51	0.38	0.33	0.69	0.35	0.36	0.76	0.84	0.90	0.76	0.64
N-*p*-Hydroxybenzoylglycine	0.25	0.17	0.14	0.73	0.18	0.33	0.76	0.91	0.69	0.58	0.61
N-*m*-Hydroxybenzoylglycine	0.23	0.18	0.15	0.68	0.29	0.33	0.86	0.91	0.73	0.64	0.63
N-Protocatechuoylglycine	0.05	0.06	0.09	0.53	0.15	0.29	0.67	0.83	1.00	0.64	0.42
N-Vanilloylglycine	0.30	0.22	0.16	0.60	0.22	0.28	0.75	0.84	0.66	0.58	0.60
N-Syringoylglycine	0.20	0.17	0.11	0.62	0.17	0.25	0.70	0.80	–	–	0.53
N-Cinnamoylglycine	0.60	0.43	0.28	1.00	0.36	0.33	0.66	0.72	0.83	0.62	0.67
N-*p*-Coumaroylglycine	0.27	0.21	0.17	0.70	0.28	0.37	0.49	0.67	0.77	0.64	0.63
N-Caffeoylglycine	0.08	0.09	0.10	0.58	0.18	0.32	0.38	0.46	0.59	0.53	0.57
N-Feruloylglycine	0.28	0.27	0.18	0.59	0.21	0.34	0.37*/0.64**	0.39*/0.64**	0.65	0.55	0.60
N-Sinapoylglycine	0.24	0.20	0.12	0.50	0.13	0.33	0.40*/0.79**	0.47*/0.77**	0.61	0.47	0.54

N-*p*-Coumaroylsarcosine	0.60	0.31	0.19	0.93	0.33	0.29	0.23*/0.98**	0.16*/0.90**	0.96	0.72	0.65
N-Feruloylsarcosine	0.66	0.34	0.20	0.84	0.24	0.25	0.58*/0.90**	0.77*/0.90**	0.93	0.68	0.60
N-*p*-Hydroxybenzoyl-L-alanine	0.44	0.57	0.43	–	0.93	0.77	0.86	0.75	1.00	0.78	1.00
N-Cinnamoyl-DL-alanine	0.71	0.42	0.33	0.92	0.34	0.38	0.72	0.57	1.00	0.84	0.77
N-*p*-Coumaroyl-DL-alanine	0.59	0.36	0.22	0.97	0.40	0.39	0.59	0.76	0.97	0.82	0.78
N-Feruloyl-L-alanine	0.64	0.39	0.23	0.94	0.34	0.33	0.53	0.76	0.96	0.79	0.74
N-Cinnamoyl-L-methionine	0.78	0.52	0.38	1.00	0.42	0.42	0.65	0.63	1.00	0.82	0.84
N-*p*-Coumaroyl-L-methionine	0.53	0.39	0.28	0.93	0.47	0.45	0.64*/0.94**	0.79*/0.98**	0.79	0.86	0.80
N-Feruloyl-L-methionine	0.54	0.44	0.31	0.88	0.37	0.40	0.87	0.59	1.00	0.79	0.80
N-*o*-Coumaroyl-DL-aspartic acid	0.25	0.19	0.51	0.97	0.76	0.66	0.67*/0.97**	0.37*/0.82**	0.86	0.67	0.58
N-*p*-Coumaroyl-DL-aspartic acid	0.12	0.14	0.12	0.83	0.16	0.22	0.65	0.91	0.91	0.67	0.55
N-Feruloyl-DL-aspartic acid	0.08	0.17	0.12	0.77	0.12	0.23	0.56	0.77	0.83	0.60	0.51
N-Feruloyl-L-phenylalanine	0.83	0.50	0.33	0.96	0.43	0.43	0.26	0.37	0.98	0.89	0.77
N-Cinnamoylglycyl-L-phenylalanine	0.72	0.42	0.28	1.00	0.39	0.38	0.50	0.57	1.00	0.68	0.77
N-*p*-Coumaroylglycyl-L-phenylalanine	0.48	0.31	0.19	0.93	0.45	0.36	0.55*/0.79**	0.72*/0.79**	0.94	0.74	0.72
N-Caffeoylglycyl-L-phenylalanine	0.28	0.23	0.13	0.79	0.35	0.38	0.46	0.61	0.88	0.64	0.70
N-Feruloylglycyl-L-phenylalanine	0.51	0.36	0.20	0.85	0.36	0.34	0.43	0.53	0.93	0.66	0.66
N-Feruloyl-L-phenylalanyl-L-phenylalanine	0.89	0.53	0.35	0.95	0.54	0.48	0.07	0.62	1.00	0.93	0.88

* *trans* compound.
** *cis* compound.

TABLE II

APPEARANCE OF THE COMPOUNDS STUDIED IN UV LIGHT AND AFTER SPRAYING WITH ONE OF THE CHROMOGENIC REAGENTS

With the exception of N-benzoylglycine (hippuric acid), all the compounds tested showed on cellulose or silica gel–cellulose plates a primrose yellow colour reaction with 1% $KMnO_4$ in 0.1 *M* H_2SO_4. On the pre-coated silica gel 60F-254 (Merck) plates, however, a zinc yellow colour was obtained with the same spray.

Compound	*Fluorescence (360 nm) before NaOH*		*Fluorescence (360 nm) after NaOH*		*Colour reaction with diazotized p-nitroaniline*		*Colour reaction with Gaffney's reagent*	
	Cellulose or silica gel–cellulose plates	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel–cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel–cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*	*Cellulose or silica gel–cellulose plates*	*Precoated TLC plates, silica gel (Merck) 60F-254*
N-Benzoylglycine (hippuric acid)	*	*	*	Blue-violet lake	Chinese white	Chinese white	Orange chrome	Deep chrome
N-*o*-Hydroxybenzoylglycine	Spectrum blue	Cobalt blue	Spectrum blue	Turquoise blue	Deep vermilion	Venetian red	Orange chrome	Spectrum orange
N-*p*-Hydroxybenzoylglycine	*	Blue-violet lake	*	Blue-violet lake	**	Deep vermilion	Orange chrome	Deep chrome
N-*m*-Hydroxybenzoylglycine	*	Blue-violet lake	Spectrum blue	Raw sienna	Crimson lake	Terra cotta	Orange chrome	Deep chrome
N-Protocatechuoylglycine	Dark violet	Raw sienna	Sky blue	Dark violet	French grey	Golden brown	French grey	**
N-Vanilloylglycine	Delft blue	Burnt carmine	Sky blue	Dark violet	Imperial purple-light violet	Burnt Carmine	Deep chrome	Spectrum orange
N-Syringoylglycine	Smalt blue	Middle chrome	Oriental blue	Raw sienna	Raw sienna	Straw yellow	**	**
N-Cinnamoylglycine	*	*	*	Blue-violet lake	**	Chinese white	Spectrum orange	Scarlet lake
N-*p*-Coumaroylglycine	Delft blue	Blue-violet lake	Blue-violet lake	Jade green	Dark violet	Raw umber	Orange chrome	Spectrum orange
N-Caffeoylglycine	Turquoise blue	Lemon cadmium	Lemon cadmium	Venetian red	Raw sienna	Raw umber	Raw umber	Spectrum orange
N-Feruloylglycine	Blue	Water green	Turquoise blue	Grass green	Prussian blue	Sepia	Orange chrome	Spectrum orange
N-Sinapoylglycine	Turquoise blue	May green	Water green	Grass green	Water green	Burnt yellow ochre	Orange chrome	Spectrum orange

N-*p*-Coumaroylsarcosine	Blue-violet lake	Blue-violet lake	Light blue	Jade green	Dark violet	Burnt carmine	Light violet	Indigo
N-Feruloylsarcosine	Smalt blue	Gold	Water green	May green	Bottle green	Red-violet lake	Light violet	Indigo
N-*p*-Hydroxybenzoyl-L-alanine	*	Blue-violet lake	*	Blue-violet lake	Madder carmine	Rose pink	**	**
N-Cinnamoyl-DL-alanine	*	*	*	Blue-violet lake	Chinese white	Chinese white	Orange chrome	**
N-*p*-Coumaroyl-DL-alanine	Blue-violet lake	Blue-violet lake	Light blue	Light blue	Gunmetal	Raw umber	**	**
N-Feruloyl-L-alanine	Spectrum blue	Water green	Turquoise green	Cobalt blue	Dark violet	French grey	**	**
NCinnamoyl-L-methionine	*	*	*	Blue-violet lake	**	Chinese white	**	**
N-*p*-Coumaroyl-L-methionine	Delft blue	Blue violet lake	Light blue	Venetian red	Dark violet	Raw umber	**	**
N-Feruloyl-L-methionine	Light blue	Water green	Turquoise green	Jade green	Delft blue	Gunmetal	**	**
N-*o*-Coumaroyl-DL-aspartic acid	Primrose yellow	Flesh pink	Lemon cadmium	Lemon cadmium	Red-violet blue	Madder carmine	**	**
N-*p*-Coumaroyl-DL-aspartic acid	Blue-violet lake	Blue-violet lake	Light blue	Kingfisher blue	Gunmetal	Raw umber	**	**
N-Feruloyl-DL-aspartic acid	Light blue	Water green	Turquoise green	Grass green	Bottle green	Sepia	**	**
N-Feruloyl-L-phenylalanine	Spectrum blue	Water green	Turquoise green	May green	Blue-grey	Blue-grey	**	**
N-Cinnamoylglycyl-L-phenyl-alanine	*	*	*	Blue-violet lake	**	Chinese white	**	**
N-*p*-Coumaroylglycyl-L-phenyl-alanine	Delft blue	Blue-violet lake	Light blue	May green	Dark violet	Raw umber	**	**
N-Caffeoylglycyl-L-phenyl-alanine	Light blue	Lemon cadmium	Raw sienna	Grass green	Raw umber	Burnt yellow ochre	Middle chrome	Middle chrome
N-Feruloylglycyl-L-phenyl-alanine	Light blue	Water green	Turquoise green	May green	Delft blue	Gunmetal	**	**
N-Feruloyl-L-phenylalanyl-L-phenylalanine	Smalt blue	Water green	Grass green	May green	Blue-grey	Blue-grey	**	**

* No fluorescence.
** No colour reaction.

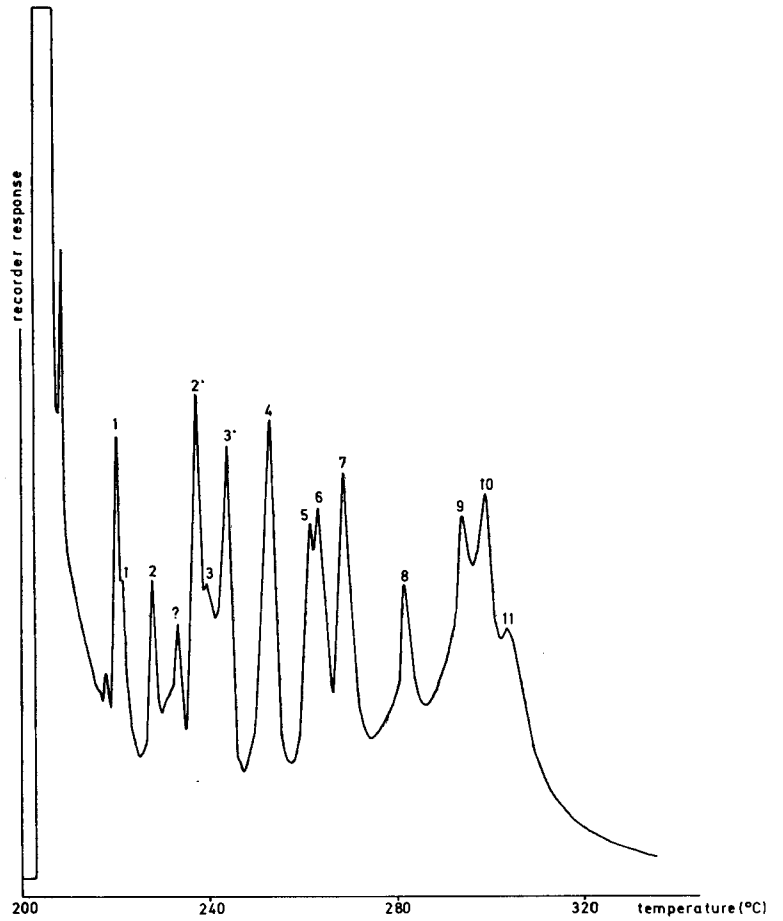


Fig. 1. Gas chromatogram of the TMS derivatives of some benzoyl and cinnamoyl amino acids. For peak identities see Table III.

pounds were very unstable, and could only be kept in the dark at room temperature for 24 h. In addition, most of these derivatives were extremely labile during GLC (stability: maximum of 15 min at higher temperatures) and subsequently the duration of the chromatography proved to be a limiting factor. The best results were obtained with an oven temperature of 200–310°C and a temperature program of 8°C/min. With a lower oven temperature (150°C) and (or) a program of 4°C/min the compounds could not be eluted, while an increase of the oven temperature at 16°C/min gave very poor separations. Thus, the GLC of these compounds is as yet not completely satisfactory. It should also be mentioned that certain of the substances gave rise to multiple peaks. This is a well known problem with N-acylglycines[10] and as indicated previously[54], single peaks (di- or tri-TMS) could not always be obtained by using longer reaction times.

For the above reasons, the complexity of the chromatograms containing N-acylglycines could not be reduced and although several silylating reagents and dif-

TABLE III

RELATIVE RETENTION TIMES AND COLUMN TEMPERATURES OF THE TMS DERIVATIVES OF SOME N-ACYLAMINO ACIDS

Retention time of N-cinnamoylglycine (5 min 27 sec) = 1.00.

Compound	*Relative retention*	*Column temperature (°C)*
1 Hippuric acid (N-benzoylglycine)	0.47	220
1′ Hippuric acid	0.50	222
2 *o*-Hydroxyhippuric acid	0.64	228
2′ *o*-Hydroxyhippuric acid	0.85	238
3 *m*-Hydroxyhippuric acid	0.91	240
3′ N-Cinnamoylglycine + *m*-hydroxyhippuric acid	1.00	244
4 *p*-Hydroxyhippuric acid	1.21	253
5 4-Hydroxy-3-methoxyhippuric acid	1.41	262
6 4-Hydroxy-3,5-dimethoxyhippuric acid	1.45	264
7 N-*p*-Coumaroylglycine, N-cinnamoyl-L-methionine	1.58	269
8 N-Feruloylglycine, N-caffeoylglycine	1.87	282
9 N-Sinapoylglycine	2.16	294
10 N-*p*-Coumaroyl-L-methionine	2.27	299
11 N-Feruloyl-L-methionine	2.38	304

ferent reaction conditions have been investigated, more research on the GLC separation of these compounds is required.

Since HPLC is more rapid and also much simpler than GLC, it has also been used for the separation of N-acylamino acids and peptides. HPLC techniques for hippuric acid and its derivatives are well known[15,16,42,44,55], but the separation of the present compounds has so far not been described. HPLC has the advantages that it does not require derivatization of the compounds, and when good separations can be obtained, a rapid quantitation of the substances is also possible.

For the HPLC separation of the N-acylamino acids of the benzoyl and cinnamoyl series, two different columns and eluting systems have been used (see Materials and Methods). The results obtained with the LiChrosorb RP-18 column, developed with a combination of isocratic and linear gradient elution [solvent A, water–formic acid (95:5, v/v) solvent B, methanol], were excellent because most of the 30 compounds investigated (with the exception of N-feruloylglycine and N-*o*-coumaroylaspartic acid; N-cinnamoyl-DL-alanine, N-*p*-coumaroyl-L-methionine and N-caffeoylglycyl-L-phenylalanine) could be separated (see Fig. 2 and Table IV).

The following conclusions can be drawn:

(a) Compounds with identical acyl groups but different amino acid or peptide moieties are eluted according to the polarity of the amino acid or peptide moiety. The retention times are in the order: aspartic acid derivatives < glycine derivatives < sarcosine derivatives ≈ alanine derivatives < methionine derivatives < glycyl-

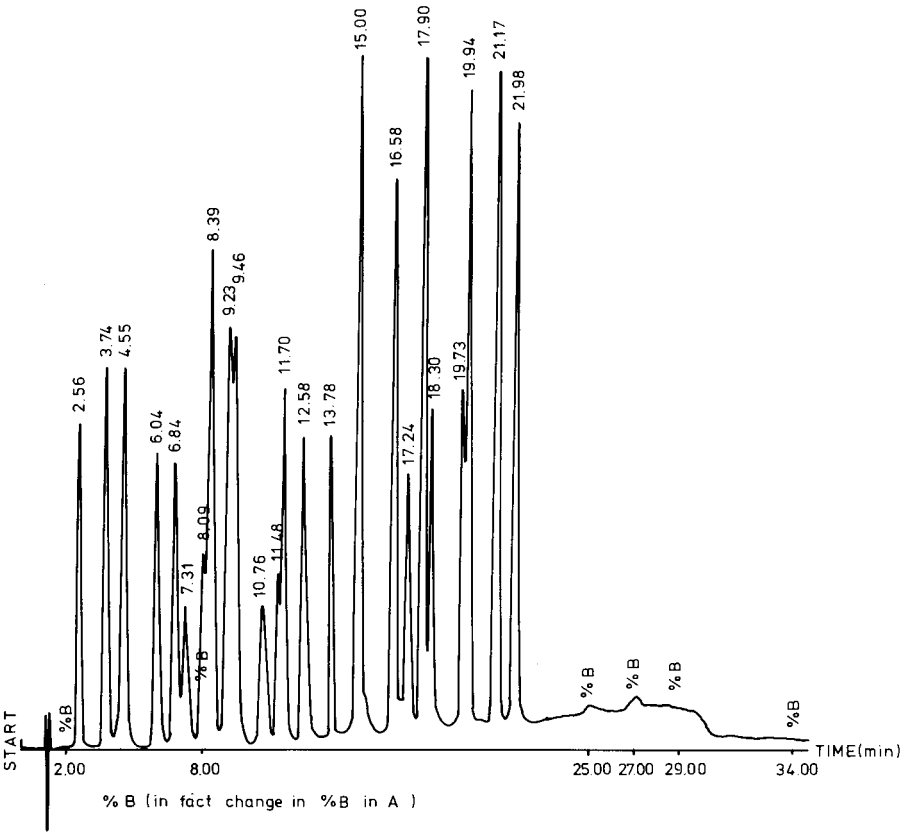


Fig. 2. Separation of N-acylamino acids and peptides of the benzoyl and cinnamoyl series on an analytical RP-18 column. For the eluting system see Materials and methods.

phenylalanine derivatives < phenylalanine derivatives < phenylalanylphenylalanine derivatives

(b) For compounds with the same amino acid or peptide moiety and the same substitution pattern of the acyl group, the order of retention is: N-benzoyl compounds < N-cinnamoyl compounds

(c) For compounds with acyl groups of the benzoyl type but different substitution patterns, the order of retention is: di-OH < *p*-OH < *m*-OH < –OH + $-OCH_3$ < *o*-OH < unsubstituted ring < –OH + di-OCH_3

(d) For compounds with acyl groups of the cinnamoyl type but different substitution patterns, the order of retention is: di-OH < *p*-OH < –OH + $-OCH_3$ < *o*-OH < –OH + di-OCH_3 < unsubstituted ring

Since certain of the above compounds could not be separated on the RP-18 column, a second system [LiChrosorb Si 60 (7 μm) (semi-preparative column); combination of isocratic and gradient elution with solvents A, dichloromethane–cyclohexane–formic acid (55:45:2, v/v/v) and B, methanol] has been investigated. With the latter system the separation of N-feruloylglycine and *o*-coumaroylaspartic acid, and of N-cinnamoyl-DL-alanine, N-*p*-coumaroyl-L-methionine and N-caffeoylglycyl-L-phenylalanine could be achieved (Fig. 3, Table V).

TABLE IV

RETENTION TIMES OF N-ACYLAMINO ACIDS AND PEPTIDES ON AN ANALYTICAL RP-18 COLUMN

For the eluting system see Materials and methods.

Compound	*Retention time (min)*
N-Protocatechuoylglycine	2.56
N-*p*-Hydroxybenzoylglycine	3.74
N-*m*-Hydroxybenzoylglycine	4.55
N-Vanilloylglycine	6.04
N-Caffeoylglycine	6.84
N-*p*-Hydroxybenzoyl-L-alanine	7.31
N-Benzoylglycine	8.09
N-Syringoylglycine	8.39
N-*p*-Coumaroyl-DL-aspartic acid	9.23
N-*p*-Coumaroylglycine	9.46
N-*o*-Hydroxybenzoylglycine	10.76
N-Feruloyl-DL-aspartic acid	11.48
N-Feruloylglycine	11.70
N-*o*-Coumaroyl-DL-aspartic acid	11.70
N-*p*-Coumaroylsarcosine + N-*p*-coumaroyl-DL-alanine + N-sinapoylglycine	12.58
N-Feruloylsarcosine + N-feruloyl-L-alanine	13.78
N-Cinnamoylglycine	15.00
N-Cinnamoyl-DL-alanine + N-*p*-coumaroyl-L-methionine + N-caffeoylglycyl-L-phenylalanine	16.58
N-Feruloyl-L-methionine	17.24
N-*p*-Coumaroylglycyl-L-phenylalanine	17.90
N-Feruloylglycyl-L-phenylalanine	18.30
N-Feruloyl-L-phenylalanine	19.73
N-Cinnamoyl-L-methionine	19.94
N-Cinnamoylglycyl-L-phenylalanine	21.17
N-Feruloyl-L-phenylalanyl-L-phenylalanine	21.98

TABLE V

RETENTION TIMES OF N-ACYLAMINO ACIDS AND PEPTIDES ON A SEMI-PREPARATIVE LICHROSORB SI 60 (7 μm) COLUMN

For the eluting system see Materials and methods.

Compound	*Retention time (min)*
N-Cinnamoyl-L-methionine	12.78
N-Feruloyl-L-phenylalanyl-L-phenylalanine	13.44
N-Feruloyl-L-phenylalanine	13.97
N-Feruloyl-L-methionine	14.78
N-*p*-Coumaroyl-L-methionine	16.61
N-Feruloylglycyl-L-phenylalanine + N-feruloylglycine	17.22
N-Vanilloylglycine	18.18
N-*p*-Coumaroylglycyl-L-phenylalanine	18.54
N-Feruloyl-DL-aspartic acid	19.09
N-Caffeoylglycyl-L-phenylalanine	19.67
N-Caffeoylglycine	20.02

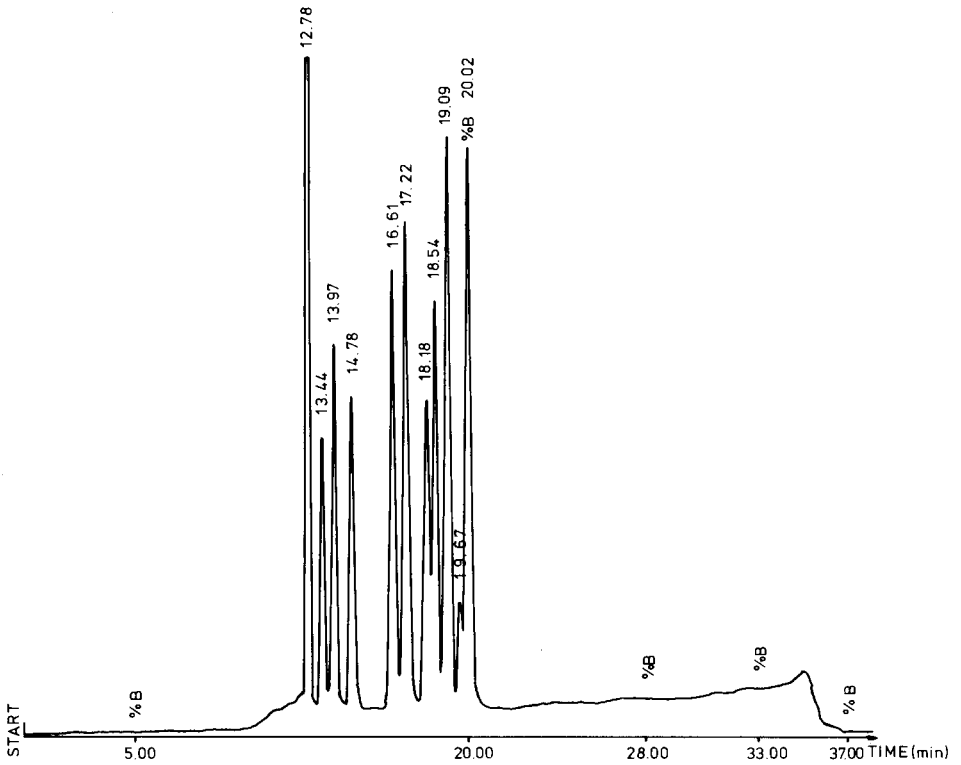


Fig. 3. Separation of N-acylamino acids and peptides of the benzoyl and cinnamoyl series on a semi-preparative LiChrosorb Si 60 (7 μm) column. For the eluting system see Materials and methods.

It can thus be concluded that with HPLC (possibly in combination with TLC or even, and where possible, TLC + GLC) all the N-acylamino acids and peptides studied can be separated. The methods described allow the separation and quantitation of such compounds in biological fluids and extracts, as well as in partial hydrolysates from plant proteins[27].

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GAS CHROMATOGRAPHY OF SOME METAL DIALKYLDITHIOPHOSPHATES WITH PACKED AND CAPILLARY COLUMNS USING FLAME PHOTOMETRIC DETECTION

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SUMMARY

Metal dialkyldithiophosphates are suitable for selective detection with a flame photometric detector operated in either the S_2 or HPO emission mode. Comparison of data for packed and capillary columns shows that the sensitivity in the HPO mode is only slightly greater than, and in the S_2 mode comparable with, that in the flame ionization detector. In addition, it is notable that the sensitivity to the chelates in the HPO mode is inferior to that for simple organophosphorus compounds by an order of magnitude. Background permeation of CS_2 leads to some improvement in detection limits for nickel dithiophosphate. Baseline resolution of palladium and platinum derivatives is readily achieved on wall-coated open-tubular columns.

INTRODUCTION

The search for new derivatising chelating agents that yield volatile metal compounds still continues, although the number of new systems that have been introduced in recent years does not match the rapid advances made in the early 1970's. The first metal chelates to come under gas chromatographic (GC) scrutiny were the acetylacetonates and their fluorinated analogues. Several review articles and books adequately cover this area[1–4].

Limitations associated with the β-diketonates resulted in efforts to increase the number of metals that could be suitably chromatographed by any one chelating system. Thus, ligands containing oxygen and/or sulphur or nitrogen have attracted considerable interest, specifically the thioacetylacetonates, bidentate and tetradentate β-ketoamines and their thio analogues, and dialkyldithiocarbamates. Such work, up to 1977, has been reviewed[5,6].

The exploitation of technological advances in chromatography must be expected to have some relevance to work on metal chelates, in which column adsorption

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effects may lead to significant on-column losses of compounds and render various derivatives unsuitable for GC analysis[3,7]. The intense efforts directed towards the preparation of capillary columns[8,9], especially with respect to the manufacture of neutral and non-adsorptive surfaces, offer a new vista in metal chelate chromatography; glass-surface preparations such as deactivation with Carbowax 20M[10], silylation[11] and polysiloxane degradation[12] appear to be foremost in this area. The acclaimed fused-silica flexible columns have been used to generate extremely good chromatograms of "difficult" compounds[13], including the bis-trimethylsilyl (bis-TMS) derivatives of the silicon(IV) porphyrins, Aetio^{I} Si(IV)$(OTMS)_2$ and OEPSi(IV)$(OTMS)_2$ (ref. 14).

Thus, the whole range of metal chelate derivatives needs to be re-evaluated in the light of such advances. Consequently, several papers have appeared on capillary chromatographic analysis of metal chelates. These have primarily featured the dithiocarbamates[15–17], but recent communications by Sucre and Jennings[18] and by Uden *et al.*[19] have involved studies of a broad range of derivatives. Thermal lability may still be a problem when flash-vaporisation injection techniques are used, but on-column capillary chromatographic methodology may overcome this.

Selective GC detection of metal chelates has not been widely employed, apart from the use of the electron-capture detector (ECD). Although the flame ionisation detector (FID) usually suffices for most analyses, some investigations involving use of atomic absorption, plasma emission sources, mass spectrometry and flame photometry have been reported. For the last-named technique, work has been concentrated on spectral emission (line or band) from the metallic species, and relatively high-temperature flames have been used[20–23], whereas little has been reported on flame photometric detection based on emission from elements in the ligand.

Recently, in continuation of earlier work[24], we reported a successful GC study of some volatile bis(dialkyldithiophosphato)metal(II) chelates on inert diatomaceous earth supports, with use of FID[25]. The suitability of specific detection of dithiophosphates by a flame photometric detector (FPD), with use of S_2 and HPO emission, was recognised and is investigated in the present study. Capillary column analyses of these derivatives was an important objective, since it was expected that significant improvements in the overall chromatographic behaviour could be realised. Apart from the paper by Sucre and Jennings[18], we are aware of no other reports on GC–FPD of thiochelates. It is intended, herein, to illustrate the general characteristics of the FPD as applied to metal dithiophosphates in comparison with simple organic compounds. The GC behaviour of the metal chelates on glass capillary columns will also be discussed for both the FID and the FPD.

EXPERIMENTAL

Metal chelates and organic standards

Metal dialkyldithiophosphates were prepared as described previously[25]. The abbreviations dedtp, dpr^{i}dtp, dprdtp and dbu^{s}dtp correspond to the diethyl-, diisopropyl-, di-*n*-propyl- and di-*sec*.-butyldithiophosphate ligand anions, respectively.

Organic standards used were triphenyl orthophosphate (TPOP), diphenyl disulphide (DPDS) and triphenylphosphine sulphide (TPPS).

The GC–FPD system

A Perkin-Elmer Model F30 gas chromatograph was used for these studies, with either a FID or a Perkin-Elmer single-flame (FPD) of spectral characteristics listed elsewhere[26]. The FPD was optimised for nickel di-isopropyldithiophosphate in the same manner as that reported for SO_2 and TPOP[26], and similar optimal conditions were obtained for the chelate and the appropriate standard in each detection mode. Thus, O–H ratios of 0.3–0.35 were generally used.

Columns

The individual columns used are indicated in the relevant chromatograms. Capillary-column analyses are reported for soda-lime-glass columns of length not exceeding 10 m. Surface preparations included etching with gaseous HCl, deactivation with Carbowax 20M, mild silylation with a dimethyldichlorosilane–trimethylchlorosilane solution, and dynamic coating with OV-101 or PS-300. A Perkin-Elmer injection splitter was used in the split mode. The F30 "plumbing" was modified by a length of glass-lined tubing (GLT) (0.3 mm I.D.) shaped to carry effluent to the detectors through the manifold; relocation of detectors was not undertaken. For the FPD, the GLT was pushed into the detector body as far as possible to help overcome void-volume problems. Make-up gas was added at the capillary–GLT connection as required.

Permeation tubes

The preparative procedures of O'Keeffe and Ortman[27] and of Kirsten[28] were used to make the permeation tubes; details have been given elsewhere[26].

The Carlo Erba capillary GC instrument

A Carlo Erba Model 4160 chromatograph was used to obtain some of the chromatograms of metal chelates. Columns used were a 10-m OV-1 statically coated capillary of persilylated borosilicate glass and a Hewlett-Packard flexible silica column (25 m × 0.3 mm I.D.) siloxane deactivated and coated with OV-1. The glass column was used in the split–splitless injection mode, whereas the fused-silica column was used with the on-column injector supplied as standard with this instrument.

RESULTS AND DISCUSSION

Except for the section *Capillary column analyses*, all results refer to data obtained with packed columns.

Examples of chromatography of metal dithiophosphates

A chromatogram of Ni, Pd and Pt diisopropyldithiophosphates with the FPD in the sulphur mode is shown in Fig. 1B; for comparison, flame ionisation detection of a mixture of the palladium and platinum chelates under the same experimental conditions produced the resolution shown in Fig. 1A. It is apparent that the peak-to-valley heights for Pd–Pt resolution are different for the two detectors. This effect has been discussed and quantified elsewhere[29] and will be further illustrated by the results for capillary columns (see below).

Examples of nickel chelate elution in which both the S_2 and HPO emission

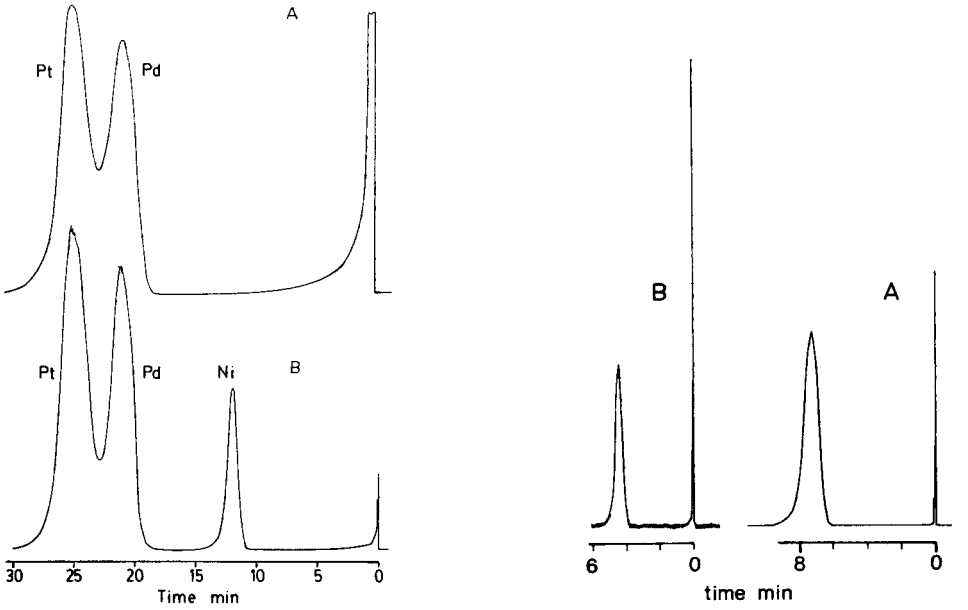

Fig. 1. Gas chromatographic traces of some metal dithiophosphates. Column: 0.4% of OV-101 on DMCS/TMCS-treated glass microbeads (60–80 mesh); stainless-steel column (40 cm × 2.2 mm I.D.; 170°C oven; N_2 carrier gas, flow-rate *ca.* 10 cm^3 min^{-1}. A, FID; B, FPD (S_2 mode), air = 14 p.s.i., H_2 = 24 p.s.i.; O/H = 0.32.

Fig. 2. Chromatography of Ni(dpr′dtp)$_2$ with FPD. Column: 2% of PS-300 on Chromosorb 750; stainless-steel column (40 cm × 2.2 mm I.D.); N_2 carrier gas, flow-rate *ca.* 25 cm^3 min^{-1}. A, HPO detection mode; air = 15 p.s.i., H_2 = 22 p.s.i.; O/H = 0.35; oven = 155°C. B, S_2 detection mode: air = 15 p.s.i., H_2 = 24 p.s.i.; O/H = 0.33; oven = 160°C.

modes were used for the FPD are presented in Fig. 2; the solid support used was Chromosorb 750. The peak symmetry is good for this sample, being better than that obtained for the glass microbead column used in Fig. 1. Solvent (chloroform) response varies markedly with change in the component gas flow-rates to the FPD, and appears to decrease with increasing hydrogen or decreasing air flow-rate to the detector (*i.e.* for a decreasing O–H ratio). This is probably a consequence of carbon formation, with subsequent luminescence in the flame, as the largest peak responses for solvent were invariably accompanied by venting of smoke from the detector. Emission from decomposition products of the organic molecule (such as C–H emission) may also occur, although we have not established this fact. Thus, a flame supported by hydrogen and air flow-rates of 66 and 125 cm^3 min^{-1} gave a solvent peak of 41745 integrated peak units, whereas a flame with respective flow-rates of 116 and 125 cm^3 min^{-1} gave an area of only 80 units. The solvent response appears to change monotonically with changing FPD component-gas flow-rate. For example, in the range of hydrogen flows listed above, the response uniformly decreases with increasing flow-rate. Within this range, however, S_2 emission response for diphenyldisulphide (DPDS) maximises then decreases.

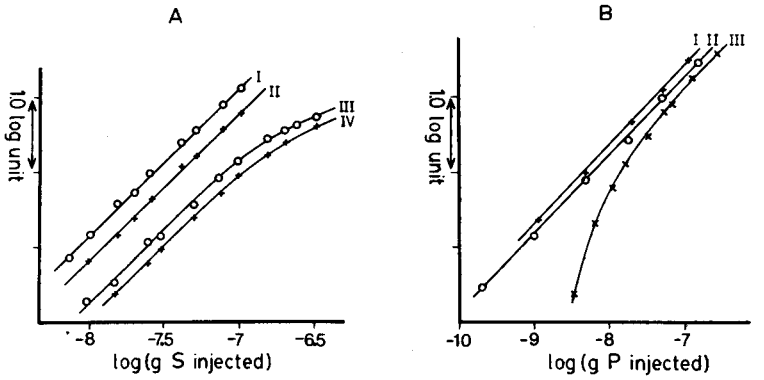

Fig. 3. Calibration curves for various compounds using FPD. A, S_2 detection mode: I, DPDS, response height data; II, DPDS, response area data; III, $Ni(dpr'dtp)_2$, response height data; IV, $Ni(dpr'dtp)_2$, response area data. B, HPO detection mode: I, TPPS; II, TPOP; III, $Ni(dpr'dtp)_2$.

Calibration curves and detection limits

The applicability of the FPD to the analytical determination of dithiophosphate chelates will be reflected by both the linearity of calibration curves and the absolute detection limits. Graphs of response against amount of sample injected are illustrated in Fig. 3; results for Ni $(dpr'dtp)_2$ in both S_2 and HPO emission modes are presented, together with curves for organosulphur and organophosphorus compounds. In the S_2 mode, the chelate shows good linearity of response, with a slope of *ca.* 1.97, although its relative response is lower when compared with the same injected mass of DPDS. This is due to the longer retention time of the chelate, with subsequent lower mass flow-rate into the detector. Log–log curves for the chelate tend to flatten (non-linearity) above *ca.* $2 \cdot 10^{-7}$ g injected sulphur. Since the organosulphur compound does not exhibit this phenomenon until much higher amounts have been injected, there could be increased contribution to interference from the non-organic component of the chelate during combustion of the compound in the FPD.

The HPO monitoring (Fig. 3) shows somewhat anomalous behaviour, with a negative deviation towards the ordinate at lower amounts of injected chelate. This contrasts with the rectilinear graphs obtained for the two organic compounds. This behaviour is attributed to interference in the production of HPO emission arising from either combustion products released when the chelate enters the flame or to formation of a refractory phosphorus-containing compound, which inhibits occurrence of the more usual decomposition processes. There must also be some degree of concentration dependency operating to cause this deviation.

Interest in the FPD was also directed towards determination of detection limits and comparison with those for FID. Results for minimum detectable quantities (MDQ), calculated for an injected mass that elicited a response equivalent to twice the noise level, are shown in Table I. MDQ values are quoted in terms of both nickel and the element responsible for the emission in the detector. For S_2 emission, similar results are obtained for both the chelate and the DPDS reference compound. In contrast, when HPO emission is monitored with triphenylphosphine sulphide (TPPS) as reference, the chelate gives a detection limit inferior by an order of magnitude (consistent with the shape of the calibration curve noted above).

TABLE I

LOWER LIMITS OF DETECTION FOR PACKED COLUMNS WITH THE FPD

Compound	*Column**	*Emission mode*	*Amount injected*	*MDQ*
$Ni(dpr'dtp)_2$	I	S_2	$6 \cdot 10^{-9}$ g Ni	$8.3 \cdot 10^{-11}$ g Ni sec^{-1} ($1.8 \cdot 10^{-10}$ g S sec^{-1})
DPDS	II	S_2	$7.5 \cdot 10^{-9}$ g S	$1.6 \cdot 10^{-10}$ g S sec^{-1}
$Ni(dpr'dtp)_2$	I	HPO	$2 \cdot 10^{-9}$ g Ni	$2.2 \cdot 10^{-11}$ g Ni sec^{-1} ($2.3 \cdot 10^{-11}$ g P sec^{-1})
TPPS	I	HPO	$2 \cdot 10^{-10}$ g P	$1.9 \cdot 10^{-12}$ g P sec^{-1}
$Ni(dpr'dtp)_2$	III	—**	$4.5 \cdot 10^{-9}$ g Ni	$7.5 \cdot 10^{-11}$ g Ni sec^{-1}

* Column I, 2% of OV-101 on Chromosorb 750; II, 2% of PS-300 on Chromosorb 750; III, 1% of OV-101 on Chromosorb 750.

** FID result.

The MDQ values obtained for the reference compounds are similar to those specified by the manufacturer. The values reported for the S_2 mode are at the upper limit expected[30–32] and other detectors may give better (*i.e.*, lower) limits. Comparison of MDQ values for sulphur compounds may be tenuous, as adsorption problems may persist[7]. The MDQ value for the dithiophosphate in the HPO mode is greater than expected. Sucre and Jennings[18] reported detection limits in the region of 20–30 pg of P sec^{-1} in the HPO mode, and, although no comment was made on this level, their results would appear to support our observations.

A small improvement in MDQ (about 3-fold) is possible when the HPO mode is used rather than FID, but the S_2 mode is similar in sensitivity to FID. The overall gain in sensitivity with FPD is not significant, but the enhanced selectivity towards sulphur and phosphorus may be considered as most desirable.

The use of permeation tubes with $Ni(dpr'dtp)_2$

Enhancement of detection limits by chemical means for sulphur compounds in photometric detectors is well recognised[32]. By pre-establishing a sulphur background in the detector, its additive effect on total sulphur concentration within the detector is subjected to a square-law response, which effectively increases the response to eluted sulphur-containing solutes. Response linearisation is also possible[33]. This phenomenon was investigated to observe if improvements in detection limits could be achieved and also to compare qualitatively the response of the chelate with those of organosulphur compounds.

Calibration curves were obtained with use of permeation rates of CS_2; response data with no permeation tube present were also collected. In Fig. 4 are shown the three curves, and relative background permeation levels are indicated (detector offset caused by the constant permeation rate). It is clear that absolute response levels at lower nickel concentrations are considerably increased at higher permeation levels, and this leads to an improved detection limit of $8 \cdot 10^{-11}$ g S sec^{-1} from curve II (noise increase results in lower signal-to-noise ratio at the higher permeation rate). The shape of the curves is rather unusual, as the slope in the lower concentration region is expected to be "linearised" to give a slope close to unity (*i.e.*, concave rather than convex shape)[34]. Again, it is necessary to postulate an interference effect when in-

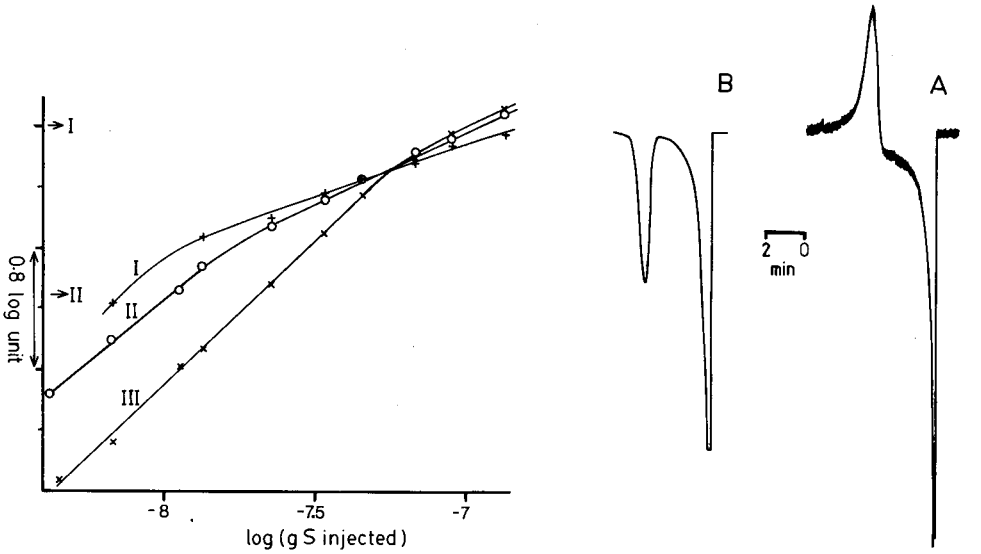

Fig. 4. Calibration curves for response against mass of sulphur [in $Ni(dpr'dtp)_2$] using a number of different CS_2 backgrounds as indicated. I, 'moderate' sulphur background; II, 'low' sulphur background; III, no background permeation.

Fig. 5. Representative chromatographic responses for $Ni(dpr'dtp)_2$ with different permeation rates of CS_2. A, 'moderate' CS_2 permeation (*ca.* 20% f.s.d. at 100 × 64 attenuation), 45 ng of Ni injected; B, 'high' CS_2 permeation (*ca.* 20% f.s.d. at 1000 × 128 attenuation), 68 ng of Ni injected.

creasing amounts of chelate enter the detector. The response with a high permeation rate adds support to this, as the chelate quenches the background to give a negative peak in a manner similar to that for a hydrocarbon[34], as shown in Fig. 5. Aue and Flinn[33] have touched on this point briefly by acknowledging that when both dopant (background gas) and analyte contain carbon, quenching of the S_2 emission must occur to some extent. The absolute degree of interference of this nature does not seem to have been studied in detail.

The peaks produced in the above experiment were analysed in an attempt to establish a correlation between mass of chelate injected (or relative level of background) and resulting peak width. We have previously shown[26] that this form of analysis may indicate the extent of linearisation of response for sulphur compounds and can also be used to estimate the exponential response factor, *n*. Generally, greater permeation rates produce broader peaks; however, as implied by the shape of the calibration curve for any one permeation rate, the greater amount of injected chelate produces a broader (and hence more linearised) peak response. This is manifested by a slope closer to unity in the upper regions of the curve, and this distinguishes the behaviour of the chelate from that of organosulphur compounds. Thus, it appears that interference associated with the metal chelate, from an as yet unidentified source, is much greater than any that could be ascribed to simple organosulphur compounds.

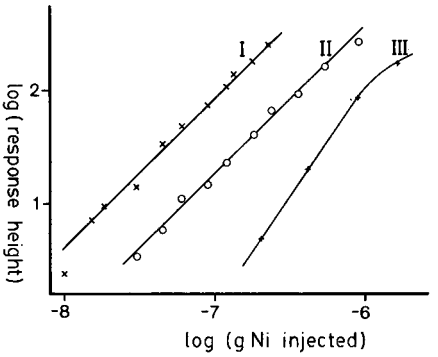

Fig. 6. Calibration curves for Ni(dpr'dtp)$_2$ with an HCl-etched, Carbowax 20M deactivated glass-capillary column dynamically coated with PS-300. I, FPD, (HPO mode), 100:1 split ratio. II, FID, 20:1 split. III, FPD, (S_2 mode), 30:1 split.

Capillary column analyses

Investigations on calibration response, detection limits and chromatographic-peak shapes were repeated using open tubular columns; Fig. 6 presents calibration curves of the nickel chelate using FID and both FPD modes. The HPO mode does not show the convex behaviour seen for packed columns (*cf.* Fig. 3), perhaps implying that some adsorption of chelate may have occurred in the packed column. Over the narrow range illustrated, the S_2 mode gives a slope of *ca.* 2.0 on the log–log graph, whereas the FID and the FPD /HPO mode calibrations both have slopes of *ca.* 1.2–1.3. Detection limits (Table II) show a definite improvement over those reported for packed columns. These values were obtained under conditions of split injection, and it is assumed that the amount of chelate delivered to the head of the column may be calculated on the basis of relative carrier flow-rates through the column and the splitter control. Again the MDQ values indicate that chelate detection based on HPO emissivity is about 5-fold poorer than Perkin-Elmer specifications and is about one order of magnitude inferior to that for triphenylorthophosphate (TPOP). Use of the S_2 mode leads to an MDQ of $3.8 \cdot 10^{-11}$ g Ni sec^{-1} ($8.4 \cdot 10^{-11}$ g S sec^{-1}) and this is comparable with that for the FID and also agrees with Sucre and Jennings' value[18].

TABLE II

LOWER LIMITS OF DETECTION FOR A CAPILLARY COLUMN WITH THE FPD AND INJECTION SPLITTING

Column as in Fig. 6.

Compound	*Emission mode*	*Amount injected*	*Split ratio*	*MDQ*
Ni(dpr'dtp)$_2$	S_2	$1.8 \cdot 10^{-7}$ g Ni	1:133	$3.8 \cdot 10^{-11}$ g Ni sec^{-1} ($8.4 \cdot 10^{-11}$ g S sec^{-1})
Ni(dpr'dtp)$_2$	HPO	$4 \cdot 10^{-8}$ g Ni	1:100	$1.1 \cdot 10^{-11}$ g Ni sec^{-1} ($1.2 \cdot 10^{-11}$ g P sec^{-1})
TPOP	HPO	$9.7 \cdot 10^{-9}$ g P	1:100	$1.8 \cdot 10^{-12}$ g P sec^{-1}
Ni(dpr'dtp)$_2$	—*	$6 \cdot 10^{-8}$ g Ni	1:35	$3 \cdot 10^{-11}$ g Ni sec^{-1}

* FID result.

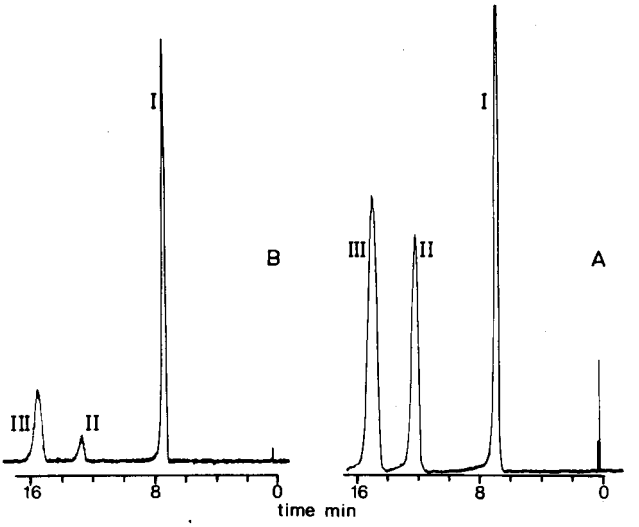

Fig. 7. Capillary chromatograms of a mixture of metal diisopropyldithiophosphates using FPD. Column: HCl-etched, dynamically coated with PS-300. Oven, 170°C isothermal; carrier gas flow-rate 67 cm sec^{-1}. A, HPO detection mode; B, S_2 detection mode. Chelates: I = Ni; II = Pd; III = Pt.

The compound dependency noted for the HPO mode may well be overcome by use of a double-flame photometric detector.

The favourable peak shapes produced by Ni, Pd and Pt chelates are illustrated in Fig. 7 for both FPD modes. The most noteworthy feature is the successful resolu-

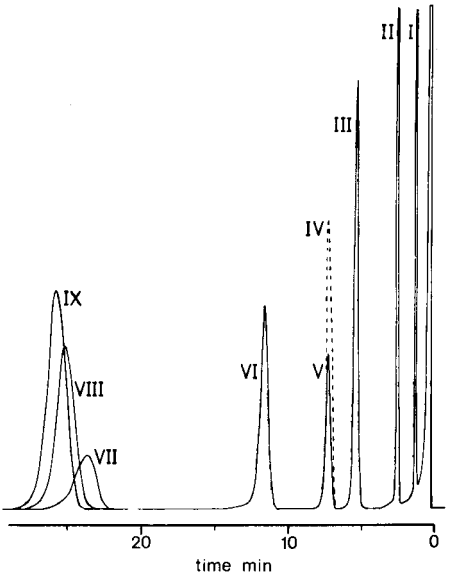

Fig. 8. Resolution of various Ni dialkyldithiophosphate chelates and *n*-alkanes using FID. Column as in Fig. 7; oven at 170°C; carrier gas flow-rate *ca.* 80 cm sec^{-1}. I = n-C_{20}; II = n-C_{22}; III = n-C_{24}; IV = $Ni(dedtp)_2$; V = $Ni(dpr^{i}dtp)_2$; VI = n-C_{26}; VII = $Ni(dprdtp)_2$; VIII = $Ni(dbu^{s}dtp)_2$; IX = n-C_{28}. Peak IV and peaks VII, VIII and IX were obtained on separate chromatograms.

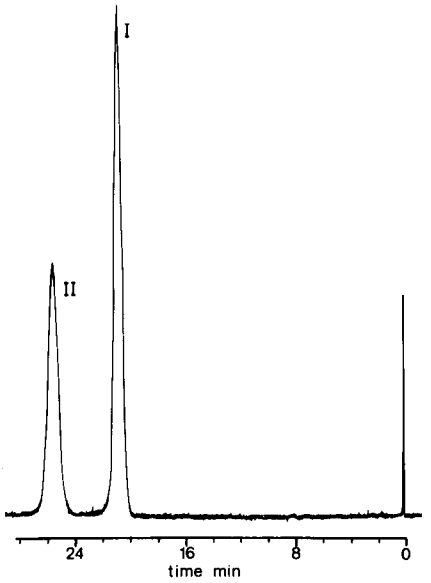

Fig. 9. Resolution of Pd and Pt chelates using FPD (S_2 mode). Column as in Fig. 7; carrier gas (He) flow-rate ~45 cm sec^{-1}. I = $Pd(dpr'dtp)_2$; II = $Pt(dpr'dtp)_2$.

tion of the palladium and platinum compounds. Since baseline resolution on packed columns was not achieved[24], this positive result is significant and indicates the prospects for resolution of derivatised metals of different chelating systems previously considered unresolvable. It is apparent that there is little difference in efficiencies and peak shapes for chelates and hydrocarbons that are eluted with similar retention volumes, as shown in Fig. 8 for a mixture of nickel chelates and some *n*-alkanes. The ethyl and isopropyl homologues essentially co-elute, and the *n*-propyl and *sec.*-butyl derivatives also show considerable overlap. These pairs have the same number of methylene units on their longest chain alkyl group, and, since the longest chain is directed towards the periphery of the molecule, the similar retention characteristics are not entirely unexpected (this consideration is based on expected solubility in the liquid phase). Baseline resolution of these chelate pairs would appear to be difficult and may require greatly improved column efficiencies.

The resolution of the Pd and Pt chelates with dpr'dtp (Fig. 9) allows us to test the resolution equation (Eqn. 1) previously derived for the S_2 mode of the FPD[29],

$$R_S = \frac{2\,\Delta t_R}{\sqrt{n_1}\,W_{bn1} + \sqrt{n_2}\,W_{bn2}} \qquad (1)$$

Exponential response factors of $n_1 = n_2 = 2.0$, when substituted into Eqn. 1 with the respective experimental values for Δt_R (the difference in retention time between solutes 1 and 2) and W_{bn1} and W_{bn2} (the basewidths of peaks 1 and 2 in the same units as retention time), produce a value for R_S of 2.32; n_1 and n_2 were assumed to have values of 2.0 on the basis of the observed response factor for the Ni chelate of 2.0. A successive trial of the same mixture with HPO monitoring was made to de-

termine the peak resolution by using the normal resolution equation (which is obtained by substituting n values of unity into Eqn. 1), and produced a calculated resolution of $R_S = 2.31$. This excellent agreement further illustrates, along with our previous supporting evidence, the applicability of our non-linear detection response model[26,29].

Stationary phase PS-300, a phenyl silicone, was used primarily in the capillary-column studies because it showed greater discrimination between the Pd and Pt chelates than was obtained with OV-101. Kovats retention indices for the Ni, Pd and Pt chelates of dpr'dtp were calculated as 2480, 2615 and 2665 at 170°C on a PS-300 capillary column; those for a column packed with 1 % of OV-101 on Chromosorb 750 were 2292, 2417 and 2457, respectively, at 155°C. Values of 2261, 2326 and 2474 have been quoted for Zn, Ni and Pd on a SE-30 coated capillary at 175°C[18]. The greater retention index difference, along with better column efficiency, certainly contributes to the enhanced (baseline) resolution observed with capillary columns. Overall, the number of effective plates obtained with the latter columns were not as good as expected; various instrumental considerations with the Perkin-Elmer F-30, requiring more extra-column connections than desired, probably account for this. Although there is considerable scope for improvement in the results reported above, this does not detract from the significance of the observations made.

Our limited experience with an improved capillary system (Carlo Erba Fractovap Model 4160 Chromatograph) incorporating on-column injection on to fused silica columns supports the above assertion. A representative chromatogram for a mixture of metal chelates and some hydrocarbons is illustrated in Fig. 10. Isothermal operation at 185°C after temperature programming from 60°C leads to an observed resolution of Pd and Pt chelates in excess of 10.0, and the peak shapes bear close resemblance to hydrocarbon standards of similar retention volumes.

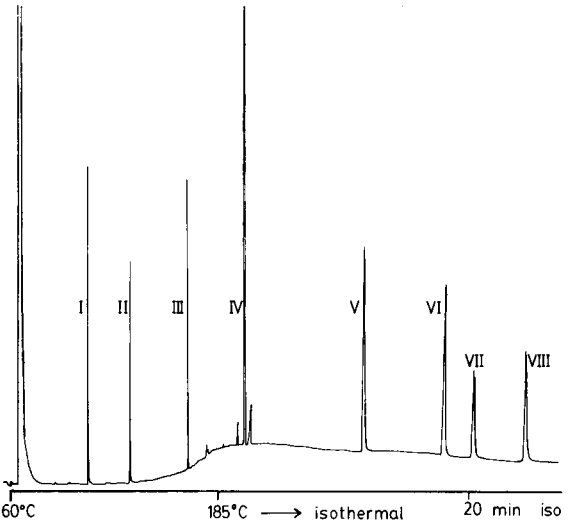

Fig. 10. Fused-silica capillary chromatogram of a mixture of n-alkanes and some metal dpr'dtp chelates. Carlo Erba 4160 instrument, with on-column injection. Carrier gas (H_2) 0.4 kg cm^{-2}; flow-rate, *ca.* 57 cm sec^{-1}; FID. I = n-C_{12}; II = n-C_{14}; III = n-C_{17}; IV = n-C_{20}; V = Ni(dpr'dtp)$_2$ (*ca.* 50 ng); VI = n-C_{24}; VII = Pd(dpr'dtp)$_2$ (*ca.* 20 ng); VIII = Pt(dpr'dtp)$_2$.

CONCLUSIONS

The results reported in this paper illustrate that metal dithiophosphates are well suited to selective detection based on either their S_2 or HPO molecular emission stimulated in fuel-rich reduced-temperature diffusion flames. The behaviour of the chelates in the S_2 detection mode appears to be similar to that for simple organosulphur compounds, whereas the HPO mode reflects that the process probably responsible for the emission is more complex (degradative) than that operative for organophosphorus compounds.

Analysis on open-tubular columns shows that the chromatographic integrity of the chelates is good in such systems, and improved capillary technology should enhance the analysis of the range of dithiophosphates with different alkyl substituents.

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TWO IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF BIOLOGICALLY SIGNIFICANT FORMS OF FOLATE*

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SUMMARY

Two new separations of folate derivatives by *in situ* paired-ion high-performance liquid chromatography are described. The first utilizes two C_{18} phenyl columns and the second utilizes a C_8 radial compression cartridge. Folic acid, dihydrofolic acid, folinic acid, tetrahydrofolic acid, 5-methyltetrahydrofolic acid and a decomposition product, *p*-aminobenzoylglutamic acid are separated. A comparison is made between these two methods and with the standard microbiological *Lactobacillus casei* assay for folates. The first method offers improved resolution and *in situ* sample clean-up. The second method accomplishes this in one-fifth the time. Both methods provide information not obtainable by the microbiological assay.

INTRODUCTION

Interest in a satisfactory method for the determination of folacin in biological samples stems from the critical role that this B-vitamin plays in the biosynthesis of nucleic acids. Accordingly, it is a prime requisite for cellular division and growth. Methodological problems arise from the fact that folacin actually comprises a large family of natural products of the parent folic acid (pterolylglutamic acid). At least three reduced states of the pyrazine ring, six different one-carbon substituents present in the position N^5 and/or N^{10}, and the glutamyl residue linked in gamma peptide linkage to a poly-γ-glutamyl side-chain of unknown and likely, varied length, exist in nature[1].

The method of choice of folacin assay has been microbiological[2], using *Lactobacillus casei* because it allows measurement of total folates at the nanogram level, the typical physiological concentration. This method, however, has several drawbacks, most notably the length of time required to complete the assay, poor reproducibility of separate assays, the extreme care that must be taken to avoid erroneously high or low values, and the lack of specificity for the various folate forms that are present in

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biological samples[3–5]. The necessity to separate the forms of folate is central because of the evidence that they differ in biological activity and bioavailability.

We recently reported a technique to separate folate forms by *in situ* paired-ion high-performance liquid chromatography (HPLC)[6]. Although we are able to apply this method to some biological samples, it became clear that there was still a great deal of chromatographic interference from other sample components. To facilitate the removal of interfering sample components, we have developed two improved techniques. These both employ *in situ* generation of the paired-ion derivatives of folate to separate folic acid, folinic acid, 7,8-dihydrofolic acid, 5,6,7,8-tetrahydrofolic acid and 5-methyltetrahydrofolic acid. The first of these methods utilizes two C_{18} phenyl columns as a direct extension of our previous separation. The second method uses a C_8 radial compression cartridge. A comparison is made between these methods and with the microbiological assay.

Those forms of folate, regardless of the state of reduction or nature of the single carbon substituent, which contain more than two additional glutamyl residues, are not reliably detected by any of the standard microbiological procedures[1]. In addition, those folylpolyglutamates which support *L. casei* growth do not do so uniformly. Previously, it was assumed that chromatographic separations of the folate forms was a function of the pteridine ring, independent of the number of glutamic acid residues[7]. Thus, it was assumed that a complete chromatographic assay could be done on the native folates in biological samples. We have, however, separated folyltriglutamate and folylpentaglutamate from folic acid by both HPLC methods reported herein. Therefore, it is clear that biological samples containing a mixture of conjugated folates must be treated with conjugase to cleave the glutamyl residues prior to the determination of the total folate content.

EXPERIMENTAL

Two C_{18} phenyl column separation

An HPLC system composed of a Tracor Model 980A solvent programmer, Tracor Model 950 chromatographic pump, Rheodyne Model 9120 injector, Waters Associates Model 440 absorbance detector, and Hewlett-Packard Model 3380A integrator recorder was used. The two columns (each 30 cm × 3.9 mm I.D.) were packed with μBondapak phenyl (10 μm) (Waters Assoc., Milford, MA, U.S.A.). In addition, a precolumn (12.5 cm × 3.0 mm I.D.), packed with μBondapak phenyl/Corasil (30 μm), was used (Waters Assoc.). Injections were made at ambient temperature from a 20-μl or 100-μl loop of the Rheodyne injector. Two consecutive isocratic eluents were employed. Initially, the columns were equilibrated with a mobile phase containing a phosphate buffer and composed of 0.10 *M* potassium dihydrogen phosphate, 0.082 *M* sodium hydroxide, 0.005 *M* tetrabutylammonium phosphate (PIC A), and 1.2% methanol, pH 7.0. Six minutes after injection, the second mobile phase was initiated. It had the following composition: 0.036 *M* sodium perchlorate, anhydrous (G. Frederick Smith Co., Columbus, OH, U.S.A.), 0.0013 *M* potassium dihydrogen phosphate, 0.0009 *M* potassium hydroxide, 0.5% methanol, pH 7.0 adjusted with 1.0 *M* potassium hydroxide. Mobile phases were filtered through a 0.22-μm filter prior to use. All water used in the preparation of mobile phases was treated to remove organic and inorganic impurities using a nanopure water purification system (Barnstead

Sybron, Boston, MA, U.S.A.). A flow-rate of 1.0 ml/min was maintained through the columns with a resultant pressure of *ca.* 2000 p.s.i.

Radial compression cartridge separation

The HPLC system was composed of a Rheodyne Model 5302 3-way slider valve, Tracor Model 950 chromatographic pump, Rheodyne Model 7125 injector, Waters Assoc. Model 440 abosrbance detector, and Hewlett-Packard 3380A integrator recorder. A radial compression cartridge (either 5 mm I.D. or 8 mm I.D.) packed with C_8 (10 μm) in a radial compression module model RCM-100 (Waters Assoc.) was used with a precolumn (5.0 cm × 3.0 mm I.D.), packed with μBondapak phenyl/-Corasil (30 μm). Injections were made at ambient temperature from a loop-column fitted with a C_{18} cartridge on the Rheodyne injector (Rheodyne, Cotati, CA, U.S.A.). Two consecutive isocratic eluents were used. Initially the cartridge was equilibrated with the phosphate–PIC A buffer used with the two C_{18} phenyl column separation. Three minutes prior to injection, the second mobile phase was used, of the following composition: 0.042 *M* sodium perchlorate, anhydrous (G. Frederick Smith Co.), 0.0015 *M* potassium dihydrogen phosphate, 0.001 *M* potassium hydroxide, 1.6% methanol, pH 7.0 adjusted with 1.0 *M* potassium hydroxide. Mobile phases were filtered as described above. A flow-rate of 2.0 ml/min for the 5 mm I.D. cartridge or 3.0 ml/min for the 8 mm I.D. cartridge was maintained through the cartridge with a resultant pressure of *ca.* 700 p.s.i.

Standard compounds

Folic acid (FA), 7,8-dihydrofolic acid (FH_2), folinic acid (CHO-FA), 5,6,7,8-tetrahydrofolic acid (THF), and 5-methyltetrahydrofolic acid (5-CH_3THF) were purchased from Sigma (St. Louis, MO, U.S.A.). Folylpentaglutamate (PG_5), and folyltriglutamate (PG_3) were purchased from the laboratory of Carlos L. Krumdieck, University of Alabama, Birmingham, AL, U.S.A. Standard solutions were prepared by dissolving each compound in 0.01 *M* potassium dihydrogen phosphate, pH 7.5, containing 0.003% sodium ascorbate and a trace of 2-mercaptoethanol to inhibit oxidative decomposition. A trace of ammonium hydroxide was added to facilitate the dissolution of 5,6,7,8-tetrahydrofolic acid. Standards were prepared immediately prior to use, protected at all times from light, and kept cold.

Conjugase treatment

Chicken pancreas (Difco Labs., Detroit, MI, U.S.A.) was purified by the method of Iwai *et al.*[8]. An aliquot of this purified conjugase was then added to each sample to be analyzed for total folate content. The contents of the tubes were evenly dispersed mechanically on a "Vortex" mixer and then incubated at 37°C for 24 h. After centrifugation, aliquots of the supernatant were frozen for analysis by HPLC or *L. casei*.

Folate analysis by L. casei

The method of folate assay of Scott *et al.*[2] which employs *L. casei* as the test organism was used to determine the amount of free and total folates present in the samples (prepared above) for HPLC analysis.

Response of L. casei growth to various folate forms

Aliquots of the standard FA, FH_2, CHO-FA, THF, 5-CH_3THF, PG_5 and PG_3 solutions were analyzed by the *L. casei* method[2] to determine the relative microbiological activity of the various folate forms on an equal molar basis. Folic acid was used as the standard, its activity placed at 100%.

Determination of the amount of chicken pancreas conjugase required to convert folylpentaglutamate to folic acid

Folylpentaglutamate was prepared as described above onder *Standard compounds*. An aliquot of 50 µl containing 0.005 µmole of PG_5 was combined with extracts of chicken pancreas in purified water (see *Conjugase treatment*). Extracts of 10–60 mg per ml of water were used, 100-µl aliquots were added to the reaction mixture, and the volume was brought to 200 µl with purified water. The reaction mixture was then incubated at 37°C for 24 h and frozen for later HPLC analysis on the two C_{18} phenyl column system.

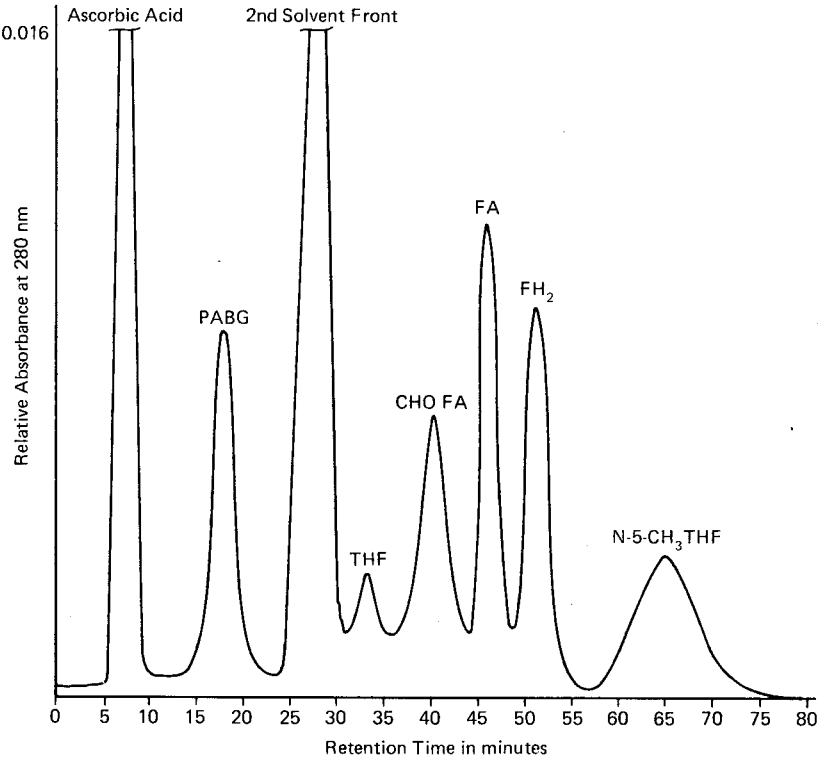

Fig. 1. Chromatogram of six folate forms obtained by isocratic elution at pH 7.0 on the two C_{18} phenyl column system pre-equilibrated with PIC A phosphate buffer. Elution, after 6 min delay, with 0.036 *M* sodium perchlorate, 0.0013 *M* potassium dihydrogen phosphate buffer, pH 7.0, 0.5% methanol; flow-rate, 1.0 ml/min; inlet pressure, 2000 p.s.i.; ambient temperature; UV detection at 280 nm; 0.5 nmole each of *p*-aminobenzoylglutamate (pABG), 5,6,7,8-tetrahydrofolic acid (THF), folinic acid (CHOFA), folic acid (FA), 7,8-dihydrofolic acid (FH_2), and 5-methyltetrahydrofolic acid (5-CH_3THF), containing 0.003% ascorbic acid and traces of 2-mercaptoethanol and ammonium hydroxide.

RESULTS AND DISCUSSION

The approach we use here to separate folic acid, folinic acid, 7,8-dihydrofolic acid, 5-methyltetrahydrofolic acid and 5,6,7,8-tetrahydrofolic acid is a modification of our previously reported single C_{18} phenyl column separation[6]. By equilibrating the column or columns with PIC A prior to injection, ion-pairs of the various folate forms are prepared *in situ* and retained on the column or columns while endogenous interfering sample components are removed. The decision was made to use two C_{18} phenyl columns instead of one because of the extra time margin gained to remove these chromatographically interfering substances without appreciable loss in peak resolution or broadening of folate peaks. This is because the ion-pairs are tightly held on the column for 5–10 min as the PIC A buffer is being used. Beyond this time, broadening of the folate peaks is seen; thus, we switch to the second mobile phase 6 min after injection to begin the elution of the folate ion-pairs. The entire separation requires 75 min with *ca.* 15 min for re-equilibration of the column between separations.

The two C_{18} phenyl columns were used to separate five forms of folate (see Fig. 1) and a decomposition product of the folates, *p*-aminobenzoylglutamic acid (pABG). A reproducible and quantitative analysis of FA, FH_2, 5-CH_3THF, THF and pABG can be obtained with this approach (Table I). Standard curves were obtained for each folate form by plotting peak area *versus* the amount of compound injected on the column. The curves for all five compounds were linear over the following ranges: FA, 0.008–0.906 nmole, $r = 0.99$; FH_2, 0–0.902 nmole, $r = 0.99$; PABG, 0–1.500 nmole, $r = 0.98$; 5-CH_3THF, 0–0.672 nmole, $r = 0.95$; THF, 0.092–0.462 nmole, $r = 0.82$. In the case of THF, the amount injected was adjusted for the presence of FA and FH_2 in the sample, as determined from the chromatograph.

TABLE I

QUANTITATIVE DETERMINATION OF FIVE FOLATE FORMS USING TWO C_{18} PHENYL COLUMNS

Quantitative determination of five folate forms obtained by isocratic elution at pH 7.0 on the two C_{18} phenyl column system pre-equilibrated with tetrabutylammonium phosphate buffer. Eluent, 0.036 *M* sodium perchlorate, 0.0013 *M* potassium phosphate buffer, pH 7.0; flow-rate, 1.0 ml/min; inlet pressure, 2000 p.s.i.; ambient temperature.

Folate form	*Range of linearity (nm)*	*Regression**	
		Equation	*Coefficient (r)*
FA	0.008–0.906	$y = 1207.510x - 9.714$	0.99
FH_2	0–0.902	$y = 1063.796x + 3.144$	0.99
pABG	0–1.500	$y = 872.132x + 19.218$	0.98
5-CH_3THF	0–0.672	$y = 1133.689x + 60.503$	0.95
THF**	0.092–0.462	$y = 527.430x - 48.463$	0.82

* y = area count/1000 as reported on Hewlett-Packard Model 3380A integrator recorder; x = nmoles of folate form injected.

** The amount injected for tetrahydrofolic acid has been adjusted for the presence of folic acid and 7,8-dihydrofolic acid in the sample.

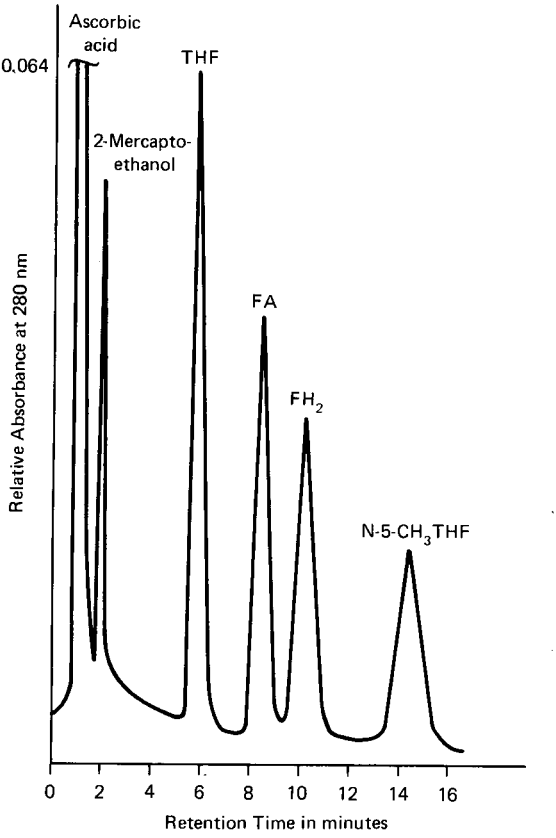

Fig. 2. Chromatogram of four folate forms obtained by isocratic elution at pH 7.0 on the radial compression C_8 cartridge, 5 mm I.D., pre-equilibrated with PIC A phosphate buffer until 3 min prior to injection. Eluent, 0.042 *M* sodium perchlorate, 0.0015 *M* potassium dihydrogen phosphate, 1.6% (v/v) methanol buffer, pH 7.0; flow-rate, 2.0 ml/min; inlet pressure, 680 p.s.i.; ambient temperature; UV detection at 280 nm; 0.5 nmole each of folic acid (FA), 7,8-dihydrofolic acid (FH_2), and 5-methyltetrahydrofolic acid (5-CH_3THF) and 1.0 nmole of 5,6,7,8-tetrahydrofolic acid (THF), containing 0.003% ascorbic acid and traces of 2-mercaptoethanol and ammonium hydroxide.

A very similar technique was used to separate the folate forms on a C_8 radial compression cartridge (Fig. 2). Modifications were required in the composition of the perchlorate–phosphate mobile phase and in the injection-buffer change sequence to accommodate the characteristics of the C_8 cartridge. Separation of the four folate forms requires 15 min with *ca.* 6 min of re-equilibration time between separations. The quantitative chromatographic parameters are presented in Table II. Standard curves were obtained as in the two C_{18} phenyl column system. The curves were linear over the following ranges: FA, 0–1.36 nmole, $r = 0.99$; FH_2, 0–0.90 nmole, $r = 0.99$; 5-CH_3THF, 0–1.34 nmole, $r = 0.99$; THF, 0.02–0.81 nmole, $r = 0.89$. As before, the amount of THF injected was adjusted for the presence of FA and FH_2 in the sample.

Folylpentaglutamate, folyltriglutamate and folic acid are separated on either the two C_{18} phenyl column system or the C_8 cartridge system. Although this was not

TABLE II

QUANTITATIVE DETERMINATION OF FOUR FOLATE FORMS USING A RADIAL COMPRESSION C_8 CARTRIDGE

Quantitative determination of four folate forms obtained by isocratic elution at pH 7.0 on the C_8 radial compression cartridge system pre-equilibrated with tetrabutylammonium phosphate buffer. Eluent, 0.042 *M* sodium perchlorate, 0.0015 *M* potassium phosphate buffer, pH 7.0; flow-rate 2.0 ml/min; inlet pressure, 680 p.s.i.; ambient temperature.

Folate form	*Range of linearity (nm)*	*Regression**	
		Equation	*Coefficient (r)*
FA	0–1.36	$y = 745.113x + 5.291$	0.99
FH_2	0–0.90	$y = 644.786x + 12.833$	0.99
5-CH_3THF	0–1.34	$y = 599.558x + 3.706$	0.99
THF**	0.02–0.81	$y = 375.129x - 7.706$	0.89

* y = area count/1000 as reported on Hewlett-Packard Model 3380A integrator recorder; x = nmoles of folate form injected.

** The amount injected for tetrahydrofolic acid has been adjusted for the presence of folic acid and 7,8-dihydrofolic acid in the sample.

previously seen in HPLC separations of folates[7], the folylpolyglutamates are readily separated by conventional chromatography on DEAE-cellulose chloride[1]. In both of the HPLC separations presented here, the order of elution is PG_5, PG_3, FA.

Folylpentaglutamate was incubated with chicken pancreas conjugase which hydrolyzes the folate polyglutamates to monoglutamate forms. Using varying amounts of conjugase, one can then determine the minimum amount of conjugase necessary to completely hydrolyze the folylpentaglutamate to folic acid (see Fig. 3). The peak areas corresponding to PG_5, PG_3 and FA were monitored as a function of the amount of conjugase used. The chicken pancreas conjugase must first be purified by the method of Iwai *et al.*[8]. Both the *L. casei* assay and the HPLC separation show amounts of folate present in the unpurified extracts comparable with that in foods and biological fluids.

A comparison was made of the *L. casei* response to the various forms of folate used as standards on the HPLC system. The growth response of *L. casei* to FA was set at 100%, giving a relative response to FH_2, THF, CHOFA, and 5-CH_3THF of 75.4%, 43.9%, 54.8% and 87.1%, respectively. These relative values are comparable with those reported previously[9,10]. Because the *L. casei* response to the different folates is not uniform, any assay using this microorganism will give spurious results for a sample containing a mixture of folates. Thus, the need for another method of analysis, *e.g.* the HPLC separation, is even more apparent.

In summary, the need to supplement or replace the *L. casei* assay for folates is greatest because of the lack of specificity for the various folate forms. This specificity is obtained in a reliable and reproducible manner by either of the HPLC methods presented here. The two C_{18} phenyl column method is readily adapted to any HPLC system. Two shortcomings are inherent, however, in this system compared to the radial compression system. First, the relative cost of column replacement is four times that of the radial compression cartridge beyond the initial investment for the system.

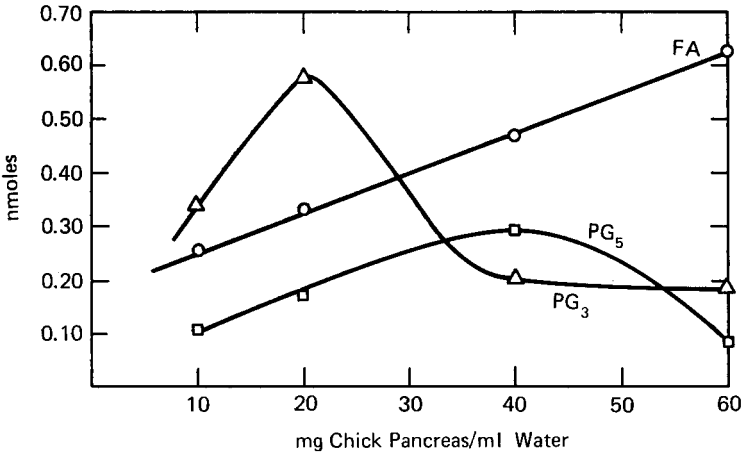

Fig. 3. Products of varying amounts of conjugase in incubation with folylpentaglutamate. Folylpentaglutamate (0.005 μmole) was incubated for 24 h at 37°C with varying amounts of chicken pancreas conjugase. At the end of the incubation period, the reaction mixture was frozen. Aliquots (20 μl) were subsequently thawed and injected for HPLC separation and quantification on the two C_{18} phenyl system: ○, FA; △, PG_3; □, PG_5.

Secondly, the time required for separation of the folates is five times greater on the two C_{18} phenyl column system than on the radial compression system. The re-equilibration time is twice as long on the two C_{18} phenyl column system compared with the radial compression cartridge. However, it has not been possible to obtain a good separation of folinic acid from folic acid on the radial compression cartridge. Also, one must consider the initial investment necessary for a radial compression system.

Recently, Allen and Newman[11] have separated folic acid, dihydrofolic acid, folinic acid, 5-methyltetrahydrofolic acid and *p*-aminobenzoylglutamate using a tetrabutylammonium phosphate containing water–methanol gradient on an ODS column. This system was developed for direct serum analysis. The folate in serum is in a non-conjugated form. We have developed our systems for more complex biological fluids and tissues, for example, human milk. In such samples the folates are conjugated and typically the samples contain many more interfering substances. Our separations are specifically designed to overcome the problems involved with the more complex sample matrix, allowing removal of interfering substances before elution of the folate forms. The two systems reported herein also include separation and quantification of tetrahydrofolic acid. In addition, these improved separations have much lower detection limits and a two- to three-fold increase in the range of linearity.

The absolute necessity to determine the total folate content of biological samples by treatment with conjugase is shown here. We have been able to apply this method to some infant foods in our laboratory, but we are still working on problems arising from sample preparation. The *in situ* formation of folate ion-pairs facilitates the removal of most chromatographically interfering sample components. Some interference is observed, however, from by-products of conjugase treatment. One very promising approach is the rapid filtration of the biological sample using immersible 10,000-dalton filters (Millipore, Bedford, MA, U.S.A.) prior to conjugase treatment.

We have used the HPLC methods and the *L. casei* assay in tandem. For standard compounds the HPLC methods agree completely with the *L. casei* assay and also supplement this technique by identifying the forms of folate. For biological samples the HPLC determinations, although not fully refined, provide critical information which is obscured by the microbiological assay. To obtain these data microbiologically, one would need simultaneous differential analyses using several organisms. Using a single sample injection, the HPLC technique is clearly more direct, rapid and offers the opportunity to analyze for many more forms of folate.

ACKNOWLEDGEMENTS

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PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE BY COVALENT CHROMATOGRAPHY ON REDUCED THIOPROPYL-SEPHAROSE 6B

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SUMMARY

Disulfiram-modified cytoplasmic aldehyde dehydrogenase binds covalently to the thiol groups of reduced thiopropyl-Sepharose 6B under conditions in which mitochondrial aldehyde dehydrogenase does not bind. After washing the resin, the uncontaminated form of the enzyme is eluted by dithiothreitol solution.

INTRODUCTION

Previous studies[1] have shown that the cytoplasmic aldehyde dehydrogenase of sheep liver is very much more sensitive to the inactivatory effect of disulfiram than is the mitochondrial form of the enzyme. (Disulfiram, or tetraethylthioperoxydicarbonic diamide, is a drug used in the treatment of chronic alcoholics[2].) However, detailed interpretation of the results of studies with the cytoplasmic enzyme has been complicated by the fact that published isolation procedures for this enzyme lead to material contaminated to a significant extent with mitochondrial aldehyde dehydrogenase[3]. The idea behind the present work was to utilise the pronounced specificity of disulfiram for cytoplasmic aldehyde dehydrogenase as a means of purifying this enzyme from contamination by the mitochondrial species. Thus it was proposed that the disulfiram-modified enzyme would bind covalently to an insoluble resin carrying thiol groups whereas other proteins (including the mitochondrial enzyme) would not bind. After washing the resin, pure cytoplasmic aldehyde dehydrogenase would be eluted by the reductive action of a low-molecular-weight thiol, such as dithiothreitol. As discussed below this proposal was shown to be correct.

As well as furnishing a method for removing mitochondrial contamination, the present study is of theoretical interest from the points of view of the versatility of covalent affinity chromatography and of the nature of the disulfiram-reactive groups in cytoplasmic aldehyde dehydrogenase. Moreover, the technique may lead to identification of the amino acid residues within the enzyme which carry the disulfiram-sensitive thiol groups.

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EXPERIMENTAL

Materials

NAD^+ was purchased from Boehringer (London, Great Britain). Disulfiram and dithiothreitol were obtained from Sigma (London, Great Britain). Thiopropyl-Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). All other chemicals were analytical-reagent grade whenever available, purchased from Fisons (Loughborough, Great Britain) or BDH (Poole, Great Britain). Solutions of acetaldehyde were made up daily from 1 *M* stock solutions (kept frozen), which were prepared from freshly redistilled acetaldehyde.

Methods

Protein concentrations. For purified cytoplasmic aldehyde dehydrogenase, protein concentration was determined spectrophotometrically using a specific extinction coefficient at 280 nm of $A^{1\%}_{1\,cm} = 11.3$.

Enzyme assay. This was performed fluorimetrically as described by Hart and Dickinson[4].

Preparation of sheep liver aldehyde dehydrogenase. The preparation of the cytoplasmic enzyme was carried out essentially by the procedure of Crow *et al.*[5]. Dickinson and Berrieman[3] have shown that this method results in cytoplasmic aldehyde dehydrogenase which usually contains some contaminating mitochondrial enzyme, but which is otherwise pure. A sample of the mitochondrial enzyme (prepared by the method of Hart and Dickinson[4]) was a generous gift from Dr. G. J. Hart.

Reduction of thiopropyl-Sepharose 6B. This was carried out using 2-mercaptoethanol according to the instructions in the booklet *Thiopropyl-Sepharose 6B — immobilised thiol reagent*, available from the manufacturers.

The binding of disulfiram-modified aldehyde dehydrogenase to reduced thiopropyl-Sepharose 6B. All operations involving the enzyme were carried out at 0–4°C. All buffers contained 0.3 m*M* EDTA. Thiopropyl-Sepharose 6B (1 g dry weight) was reduced to the free thiol form and washed well with 20 m*M* sodium phosphate buffer, pH 8.0. The resin was centrifuged gently and excess buffer poured off. Aldehyde dehydrogenase was dialysed against the same buffer; to 4 ml of the resulting enzyme solution (8–15 mg/ml) was added 20 μl of freshly made 20 m*M* disulfiram in ethanol. The enzyme activity was assayed before and after the addition of disulfiram. The disulfiram-treated enzyme solution ws added to the resin prepared as above, the resin was dispersed gently with a glass rod, and the mixture was allowed to stand for *ca.* 4 h with occasional gentle stirring. The resin was then lightly compacted by centrifugation, the supernatant was decanted and the resin was washed thoroughly with several changes of 20 m*M* phosphate buffer, pH 8.0, by dispersal with a glass rod, centrifugation and decantation. The resin was then dispersed in 3 ml of 20 m*M* dithiothreitol in 40 m*M* phosphate buffer, pH 8.0, and left overnight. After centrifugation, the eluted aldehyde dehydrogenase solution was collected by decantation. (In calculating the recovery of enzyme activity from the resin, the volume of the decanted solution was corrected for the volume of solution contained in the compacted wet resin; 1 g dry weight of resin occupies 3 ml when wet.)

Attempted binding of disulfiram-modified aldehyde dehydrogenase to reduced thiopropyl-Sepharose 6B on a large scale. A preparation of sheep liver cytoplasmic

aldehyde dehydrogenase was carried out to just after the first $(NH_4)_2SO_4$ fractionation stage, at which point the enzyme solution is grossly impure. The solution was dialysed against 40 m*M* sodium phosphate buffer, pH 8.0. To the enzyme solution (370 ml, 58 mg/ml) was added sufficient 4 m*M* disulfiram in ethanol (7.5 ml) to cause a substantial degree of inactivation (the remaining activity was 29% of the starting figure). Then 14 g (dry weight) of reduced thiol-resin (which had been washed with 40 m*M* phosphate buffer, pH 8.0) was added and the mixture was stirred gently for 4 h. (All buffers had been deaerated by standing under suction to minimise non-specific oxidative coupling of protein thiol groups to the resin.) The mixture was centrifuged and the supernatant decanted. The resin was thoroughly washed on a sintered-glass funnel with 40 m*M* phosphate buffer, pH 8.0, and then packed into a small glass column (15 × 2 cm I.D.). 15 m*M* Dithiothreitol in 40 m*M* phosphate buffer, pH 8.0, was then run through the column until it was just detectable in the eluate [from its effect on a sample of 5,5′-dithiobis-(2-nitrobenzoic acid)]. The column was left overnight, and the first 40 ml of eluate were then collected and assayed for enzymic activity.

RESULTS AND DISCUSSION

The concept underlying the experiments reported here is that there should be some way of using the highly specific reaction between disulfiram and cytoplasmic aldehyde dehydrogenase to furnish a purification of this enzyme from non-disulfiram-sensitive protein (such as the mitochondrial form of aldehyde dehydrogenase). With this view in mind, the feasibility of using the sequence of reactions shown in Fig. 1 was investigated. First is depicted the straightforward reduction of commercially available thiopropyl-Sepharose 6B to a resin with a high concentration of free thiol groups. Secondly, the reaction between enzyme thiol groups and disulfiram is shown. Previous work[6] has shown that this reaction is very rapid with the cytoplasmic enzyme and that no more than two molecules of disulfiram are required per tetrameric enzyme molecule for maximum inactivation; however, the mitochondrial enzyme only reacts significantly in the presence of high concentrations of disulfiram over a relatively long period of time[1,4]. Thus in a mixture of cytoplasmic aldehyde dehydrogenase with the mitochondrial enzyme (or other protein) only the former enzyme species should become modified when limiting amounts of disulfiram are added. Reaction 3 shows the reaction between reduced thiopropyl-Sepharose 6B and the disulfiram-modified enzyme, a reaction which theoretically should go to completion since the diethyldithiocarbamate ion is a good leaving group. At this stage non-covalently bound protein (including the mitochondrial enzyme) would easily be removed physically from the insoluble polymeric matrix. Elution of pure cytoplasmic aldehyde dehydrogenase would then be effected by reductive disulphide-interchange with an excess of a small molecular weight thiol such as dithiothreitol, as shown in reaction 4.

Of course, the disulfiram-modified cysteine residues in cytoplasmic aldehyde dehydrogenase might be sterically inaccessible to the resin's thiol groups, in which case reaction 3 would not proceed and the purification scheme would fail. Conversely, the reaction might go too far; reaction 5 shows one of the excess thiol groups on the resin displacing the bound enzyme. This would result in the reactivation of the di-

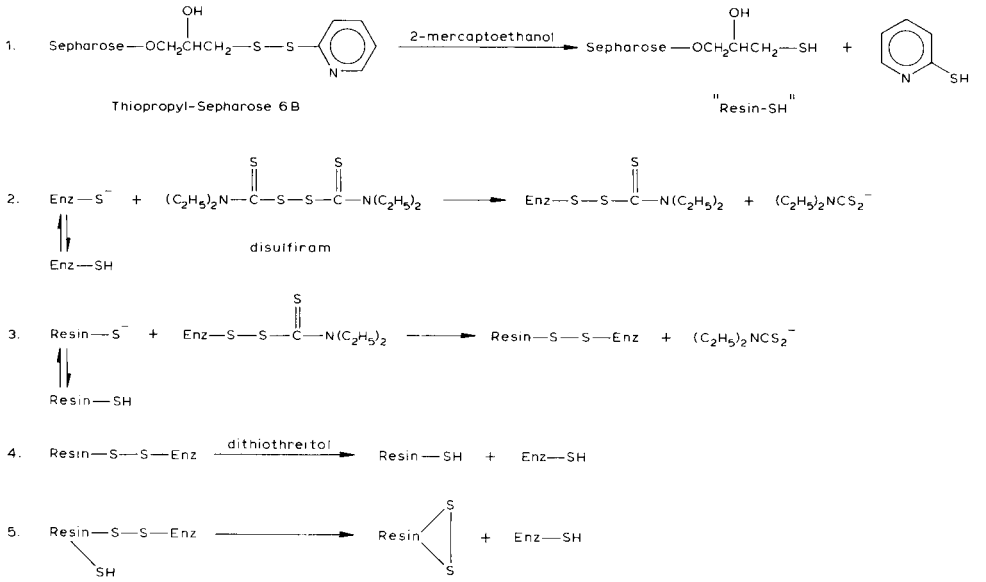

Fig. 1. The chemical reactions involved in the purification of cytoplasmic aldehyde dehydrogenase by covalent chromatography on reduced thiopropyl-Sepharose 6B.

sulfiram-modified enzyme, but this species would then find itself back in solution with the contaminating mitochondrial form or other impurities. (The sum of reactions 3 and 5 is analogous to the reactivation of disulfiram-modified cytoplasmic aldehyde dehydrogenase, which is brought about by high concentrations of a low molecular weight thiol, such as 2-mercaptoethanol[6].)

The results in Table I show to what extent the various possible reactions referred to above proceed in practice. Experiments A–C show that on a small scale there is moderate success in the amount of cytoplasmic aldehyde dehydrogenase which can be bound to and subsequently eluted from the thiol-resin (Between 35 and 44% of the units of activity which were abolished upon disulfiram-modification are recoverable from the resin.) A rather more variable amount of the disulfiram-modified enzyme is reactivated in solution, presumably according to reactions 3 and 5. The facts that under similar conditions virtually no mitochondrial enzyme is bound to the resin (Experiment E) and, in the absence of disulfiram-treatment, very little cytoplasmic enzyme is bound (Experiment D) confirm that the positive results in Experiments A–C must arise from operation of the reactions shown in Fig. 1 as predicted (and not through any non-specific binding by, for example, interaction of the resin's thiol groups with protein disulphide bridges).

When the large-scale purification of very impure aldehyde dehydrogenase was attempted using this method, no activity was recovered from the resin (Experiment F; repetition of the experiment gave the same result). Presumably the presence of large amounts of other proteins interferes in some way with the reaction between the thiol-resin and the modified enzyme. Even on a small scale using much purer aldehyde dehydrogenase the recovery of enzyme did not exceed 44%. Nevertheless, since the mitochondrial enzyme does not bind under these conditions (Experiment E), the method is useful and important in providing pure samples of cytoplasmic aldehyde

TABLE I

PURIFICATION OF CYTOPLASMIC ALDEHYDE DEHYDROGENASE BY COVALENT CHROMATOGRAPHY ON REDUCED THIOPROPYL-SEPHAROSE 6B

Enzyme activity was assayed as described under *Methods*; 1 unit is defined as the amount of enzyme catalysing the formation of 1 nmol of NADH per minute in the standard assay. The first four columns represent, respectively, (i) the total number of units of activity at the start of the experiment, (ii) the remaining activity after disulfiram treatment, (iii) the activity found in the supernatant after standing the disulfiram-modified enzyme in the presence of reduced thiopropyl-Sepharose 6B for 4 h, (iv) the activity which was eluted from the resin by dithiothreitol after having been covalently attached. "Recovery" is the total of columns 3 and 4 expressed as a percentage of column 1. (A large difference between "recovery" and 100% signifies disulfiram-modified enzyme which was neither bound to the resin nor reactivated in solution.) The last two columns represent, respectively, the activity which was covalently bound to the resin and the activity which reappeared in solution, expressed as a percentage of the number of units of activity which had been abolished by disulfiram treatment. Experiments A–C are small-scale experiments with the cytoplasmic enzyme; D is an analogous experiment without disulfiram treatment; E is an experiment analogous to A–C, but using the mitochondrial enzyme; F is a large-scale attempt using impure cytoplasmic enzyme.

Expt.	*Units of enzyme activity*				*Recovery (%)*	*Fate of the disulfiram-modified enzyme*	
	Original activity	*After disulfiram treatment*	*Supernatant*	*Bound and eluted*		*Bound and eluted (%)*	*Reactivated in solution (%)*
A	2072	456	953	563	73	35	31
B	2488	624	1536	826	95	44	49
C	1640	62	271	693	59	44	13
D	2004	–	1816	50	93	–	–
E	916	916	859	7	95	–	–
F	111,000	31,860	49,059	0	44	0	15

dehydrogenase for experiments in which mitochondrial enzyme contamination is unacceptable.

The purification of an enzyme by affinity chromatography usually relies on the affinity which the native enzyme has for some grouping which is covalently attached to an inert support. The affinity is often expressed in a non-covalent binding such as that between many dehydrogenases and AMP-Sepharose, but it may result in a covalent attachment such as that which occurs between papain or urease and activated thiol-Sepharose. The sequence of reactions in Fig. 1 (which we have seen is experimentally supported by the results in Table I) constitutes an unusual and interesting variation of affinity chromatography, partly because two separate affinities are involved and partly because it entails the deliberate inactivation of the enzyme which it is desired subsequently to purify. Thus initially, the affinity of cytoplasmic aldehyde dehydrogenase for disulfiram ensures that only this species becomes modified (reaction 2) and secondly, the affinity of the resin's thiol groups for the resulting reactive diethylthiocarbamoyl disulphide linkage ensures that such modified protein becomes bound to the resin (reaction 3).

The thiol groups on reduced thiopropyl-Sepharose 6B are separated from the polymeric matrix by a relatively short spacer group (see Fig. 1). Thus the fact that reactions 3 and 5 occur at all (and the results in Table I show that they both do to a substantial extent) must mean that the disulfiram-modified groups of cytoplasmic

aldehyde dehydrogenase are on or close to the enzyme's surface and not buried in some sterically inaccessible position within the enzyme molecule.

A further potential value of the work described here is that it allows the immobilisation of pure cytoplasmic aldehyde dehydrogenase specifically through the cysteine residues which react with disulfiram. This means that the sequencing of the peptide(s) containing the disulfiram-reactive cysteine residues may prove to be relatively simple. The isolation procedure would involve protease digestion of the immobilised enzyme, the washing away of free protease and interfering peptides, followed by the elution of the pure peptide(s) of interest by dithiothreitol solution. (The cysteine-containing peptides of human ceruloplasmin have been isolated from an activated thiol-Sepharose in this manner[7].) The importance of disulfiram to an understanding of the enzymology of aldehyde dehydrogenase would make this a desirable achievement.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION AND QUANTIFICATION OF A DILACTONIC ANTIBIOTIC MIXTURE (ANTIMYCIN A)*

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SUMMARY

High-performance liquid chromatographic (HPLC) conditions are presented for the separation and quantitative determination of a homologous antibiotic complex (antimycin A). Combined HPLC and chemical ionization mass spectrometry proved to be exceptionally useful for the structural identification of chromatographic components. Using electrochemical, fluorescence, and ultraviolet detectors, the minimum detectable amounts of the antibiotics were found to be in the ranges 0.10–1.12, 0.31–1.69, and 4.10–28.2 ng, respectively. Advantages of the preparation of Dns derivatives for use in fluorescence detection are discussed. Application of the HPLC technique to the analysis of the antibiotic mixture in organic tissues is demonstrated.

INTRODUCTION

A cluster of closely related antibiotic substances produced from Steptomyces by fermentation is known as antimycin A and has been used for many years as a general piscicide in fishery management. It consists of a group of chemically labile nine-membered ring dilactonic compounds that differ merely in the homologous alkyl substituents on the 7- and/or 8-side-chains (Fig. 1). In recent years interest in the chemistry, synthesis, and biological activities of the antimycin A complex has stimulated much chemical and biochemical research[1–5]. Despite earlier extensive studies on analytical methodology utilizing pyrolysis gas chromatography[6], semi-quantitative mass spectrometry[7], and gas–liquid chromatography of derivatives[8] for the determination of antimycins in samples from various sources, there existed severe limitations due to lack of selectivity, sensitivity, and specificity in association with separation as well as detection techniques. The published pyrolytic gas chromatographic and mass spectral procedures possess one disadvantage in common that involves sample decomposition prior to assays. A few reported methods of separation by fractional crystallization, paper chromatography, and countercurrent distribution[9–11] are too

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crude to be quantifiable, and yield only partial resolution of the antimycin constituents. We now describe a new method based on the use of octadecylsilica bonded reversed-phase high-performance liquid chromatography (HPLC) in the speciation of the complex titel mixture, and simultaneous quantitative measurement of the series of resolved component homologues. Some applications of various commercial detectors coupled with an HPLC system are presented, and the detection sensitivity and linearity are evaluated. In addition, a method for prior sample cleanup of organic tissue extracts has been developed which embodies an adsorption and thin-layer chromatographic (TLC) procedure.

EXPERIMENTAL

Materials

Solvents and buffer salts for HPLC were of high purity spectral grade purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Other chromatographic solvents were acquired from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) in "distilled-in-glass" quality. Mixtures of antimycin standards were gifts from Aquabiotics Corp. (North Brook, IL, U.S.A.). A sample of the radioactive antibiotics (Ayerst Research Laboratories, Montreal, Canada) that contains mixed homologues of [^{14}C]antimycin A uniformly labeled at the benzene ring was first recrystallized from hexane–methylene chloride and then purified by TLC (silica gel plate, 20 × 20 × 0.025 cm; benzene–chloroform–methanol–acetic acid, 7:5:1:0.7). The pure ^{14}C-labeled antimycin A complex obtained in this manner had a specific activity of 2.82 μCi/mg. 1-Dimethylamino-naphthalene-5-sulfonyl chloride (Dns chloride) and pyridine were products of Aldrich (Milwaukee, WI, U.S.A.) and used as purchased. Silica gel (40–140 mesh) for column adsorption chromatography was of "Baker analyzed reagent" grade. Precoated TLC uniplates were supplied by Analtech (Newark, DE, U.S.A.). All other chemicals used were of "analytical reagent" purity.

Equipment

The general procedure for this study, unless otherwise stated, employed a Varian Model LC-5000 liquid chromatograph with an ultraviolet detector (Varian Varichrom) set at 254 nm, and a reversed-phase, high efficiency, ultrasphere ODS column (5 μm) (25 cm × 4.6 mm I.D.) (Altex, Berkeley, CA, U.S.A.). The mobile phase was composed of 75:25 methanol–acetate buffer (0.25 *M*, pH 5) at a flow-rate of 2 ml/min. The injection system consisted of a Valco CV-6-UHPa-N60 injection valve and a 10-μl loop (Valco, Houston, TX, U.S.A.). Samples to be analyzed were introduced into the HPLC column via a guard column (5 cm × 4 mm I.D.) packed with Varian Vydac reversed-phase hydrocarbon (40 μm) as the stationary phase.

The electrochemical detector used in this study was a Model LC-3 detector controller attached to a Model TL-5 glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The electrode potential was maintained at +1.00 V *versus* a silver–silver chloride reference electrode. The HPLC-fluorescence measurements were performed with a Varian Fluorichrom detector equipped with a tungsten halide light source, a flow cell, and selected optical filters. The HPLC column effluent was monitored at an excitation wavelength of 365 nm and an emission wavelength of 418 nm.

In all analyses, the output signal was fed into a Varian Model 9176 strip chart recorder and the peak area and retention time were automatically computed by a Varian Model CDS-111L data system.

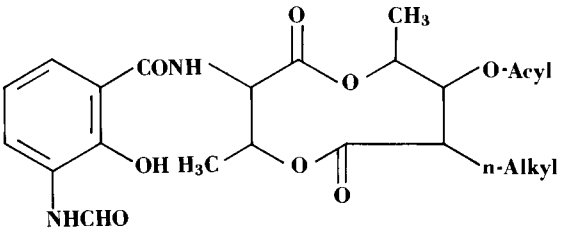
Mass spectra characterization of the antimycin components isolated from the preparative reversed-phase HPLC column (Varian MicroPak-MCH, 10 μm, 30 cm × 8 mm I.D.) was done on a Finnigan Model 4021-T quadrupole mass spectrometer interfaced to a direct probe. The combined HPLC–mass spectrometry (LC–MS) system used was a Finnigan Model 3200 quadrupole mass spectrometer coupled with the Varian Model LC-5000 instrument through a moving belt transport device and a continuous extraction interface. The mass spectrometers were operated in the chemical ionization mode using methane as the reagent gas. The data were collected and processed by an INCOS data system operating on a Data General Nova 3/12 computer.

Isolation of antibiotic components by preparative HPLC

Depending on the amount of the sample (10–50 mg) employed in each injection, preparative HPLC was run on either a Varian MicroPak-MCH reversed-phase column (10 μm, 30 cm × 8 mm I.D.) or a Whatman Partisil Mag 9 ODS column (10 μm, 50 cm × 10 mm I.D.). In a typical run, a 50-mg sample was chromatographed on the latter column with a mobile phase consisting of 70:30 acetonitrile–phosphate buffer (0.2 *M*, pH 3.5) at a flow-rate of 0.5 ml/min. The eluent was monitored with a Varian refractive index detector, and the separated antimycin components were collected into 0.5-ml fractions utilizing a Buchler linear automatic fraction collector, Twenty injections, which constituted a total of 1.0 g of the sample of the antimycin A complex, were made. Prior to their extraction workup, analytical HPLC–UV chromatograms were obtained for all the fractions collected to check purity of each fraction. The homogeneous fractions that contained identical single antibiotic components were combined. The above process generally yielded highly pure antimycins resolved into ten fractions. The organic matter from the HPLC fractions was isolated by careful removal of acetonitrile by evaporation on a rotary evaporator under a reduced pressure followed by extraction of the remaining aqueous layer with methylene chloride. Upon addition of hexane to the chilled concentrate of the methylene chloride extract, there was obtained a pure antimycin component as white crystals. All specimens were estimated to be greater than 99% pure by analytical HPLC–UV. The amounts of the individual antimycins isolated from a sample of the antimycin A complex were as follows: A_6, 6.3 mg; A_5, 13.0 mg; A_4, 75.7 mg; A_3, 60.9 mg; A_2, 103.4 mg; A_1, 136.6 mg; A_{0b}, 8.2 mg; A_{0a}, 4.5 mg; A_{0d}, 5.7 mg; A_{0c}, 11.8 mg (see Fig. 1 for structures). As the composition of components varied among the antibiotic samples originated from dissimilar fermentation batches, some of the pure compounds isolted above were added to the analytical samples to serve as internal standards for the HPLC quantitation of different lots of the antimycin samples in which a component having the same structural identity as the internal standard was absent, or present in negligible amount.

Dns derivatives

The preparation of Dns antimycins by the conventional dansylation technique was initially attempted, but the yield was too low to be of analytical signifi-



Component	n-Alkyl	Acyl
A_0	(a) n-Hexyl	Hexanoyl
	(b) n-Butyl	Heptanoyl
	(c) Octyl	Butyryl
	(d) Heptyl	Isovaleryl
A_1	n-Hexyl	Isovaleryl
A_2	n-Hexyl	Butyryl
A_3	n-Butyl	Isovaleryl
A_4	n-Butyl	Butyryl
A_5	Ethyl	Isovaleryl
A_6	Ethyl	Butyryl

Fig. 1. Structural formulas of the antimycins isolated by HPLC.

cance. However, a better alternative method was developed at this laboratory. A sample (100 mg) of the antimycin A complex in 100 ml of acetone was treated with 40 ml of 1 % Dns chloride in acetone and 5 ml of pyridine. This mixture was stirred at room temperature for 30 min. The excess solvent was evaporated on a Buchi rotary evaporator (Flavil, Switzerland). Then 50 ml each of water and ethyl acetate were added to the residue. After shaking for a few minutes, the layers were separated. Extraction of the aqueous solution was repeated twice with 50 ml of ethyl acetate. The combined organic extract was dried over anhydrous sodium sulfate, decanted, and evaporated to leave a light yellow solid. The residual material was purified by silica gel dry column chromatography (hexane–methylene chloride–methanol, 7:6:2) followed by recrystallization from cold methanol to give ultrapure leaf crystals (m.p. 147–151°C; overall yield: 90 %). These dansylated antimycins were suitable for use as calibration standards. Their structures were ascertained by LC–MS. The micro-scale technique for the derivatization of tissue samples spiked with the antibiotics is described in the following section.

Procedure for cleanup and analysis of organic tissue samples

Each of the frozen (− 70°C) ground fish tissue samples (10–50 g) fortified with the antimycin mixtures at various levels and the ^{14}C-labeled analogues (10 nCi) was placed in an erlenmeyer flask containing 200 ml of ethyl acetate–methanol–acetic acid (3:4:1). The suspension was shaken in a Lab-Line 3535 shaker for 40 min at 25°C. The mixture was then centrifuged at 34,575 *g* for 5 min in an IEC 3401 centrifuge. After the complete withdrawal of the supernatant, the tissual matter was quantitatively transferred into a soxhlet thimble and extracted continuously with 200 ml of ether–methylene chloride (1:1) for 60 min and subsequently with 150 ml of

acetone–methanol (1:1) for 30 min. These tissue extracts, including the foresaid supernatant, were combined. Removal of the solvents at room temperature in the usual manner afforded an oily residue which was taken up in 100 ml of methylene chloride. The latter solution was extracted with three 50-ml portions of 0.5 *M* pH 5.5 phosphate buffer. The pooled aqueous solution was back-washed with 60 ml of benzene. Then the concentrate (*ca.* 3 ml) of the combined methylene chloride solution and the benzene washing was chromatographed onto an adsorption column prepared by packing in sequence 50 g of silica gel and 10 g of anhydrous sodium sulfate (as the top layer) in a glass tube (50 × 2.5 cm I.D.). The column was eluted sequentially with 200 ml each of hexane, benzene, methylene chloride–methanol (1:1) and methanol. The eluate was dripped into 20-ml test-tubes at a flow-rate of 2.5 ml/min. Most of the radioactivity was found in the methylene chloride–methanol (1:1) fractions, as determined by liquid scintillation counting with a Beckman LS-7500 liquid scintillation counter. The bulk of the pooled radioactive eluate was reduced to 2 ml, which was efficiently pipetted into a 15-ml test-tube with a PTFE-lined screw cap. The contents of the tube were brought to dryness under a stream of nitrogen. The residue was redissolved in 0.5 ml of acetone and 0.2 ml of 1% Dns chloride in acetone and 0.1 ml of pyridine was added to it. The tube was sealed and the mixture was thoroughly agitated on a vortex mixer for 10 min. Acetone was driven off by purging with nitrogen. The remaining liquor was mixed with 3 ml of water and 5 ml of ether. After separation of the ether phase, the aqueous layer was extracted twice with 3 ml of methylene chloride. The combined organic solution was concentrated to *ca.* 0.5 ml and purified on a preparative TLC plate (silica gel, 20 × 20 × 0.05 cm) using hexane–ethyl acetate–acetic acid (2:2:0.2) as the developing solvent. The TLC band whose R_F value (0.53) corresponded to that of the Dns antimycin standards was scraped from the plate and extracted successively with 25 ml of ether–methanol (2:1) and 15 ml of methylene chloride–methanol (3:2). To prepare analytical samples ready for HPLC measurements in the reversed-phase system with a mobile phase of 63:37 acetonitrile–water at a flow-rate of 2 ml/min, the extract collected from TLC cleanup was evaporated to *ca.* 0.2 ml. This was diluted with methanol to exact volume (1 ml). Aliquot samples (10 μl) were then injected into the HPLC column.

RESULTS AND DISCUSSION

Earlier attempts to derivatize antimycins through mild alkylation with an intention to analyze the intact molecule by gas chromatography resulted in sample decomposition. In consideration of the chemical and thermal instability inherent with the structures of these dilactonic antibiotics, HPLC would, therefore, appear to be the most suitable method for maintaining structural integrity and permitting adequate differentiation of the components on account of its proven record of merits in this regard. Under optimal HPLC conditions as specified in the Experimental sections, all antimycin components including four major and six minor compounds were well resolved. The structure of each component was examined and identified by direct chemical ionization mass spectrometry of the isolated material from preparative HPLC and by LC–MS (chemical ionization). The two separate mass spectral analyses gave identical fragmentation patterns exhibiting pseudo molecular ions at (M_1+1) *m/e* 480, $(M_2 + 1)$ *m/e* 494, $(M_3 + 1)$ *m/e* 508, $(M_4 + 1)$ *m/e* 522, $(M_5 + 1)$ *m/e* 536,

$(M_6 + 1)$ *m/e* 550, $(M_7 + 1)$ *m/e* 550, $(M_8 + 1)$ *m/e* 564, $(M_9 + 1)$ *m/e* 564, and $(M_{10} + 1)$ *m/e* 564, respectively, for the HPLC peaks from 1 to 10 in sequence (Fig. 2). The latter peaks corresponded unambiguously to the antimycin components A_6, A_5, A_4, A_3, A_2, A_1, A_{0b}, A_{0a}, A_{0d}, and A_{0c} (Fig. 2) on the basis of mass spectral information.

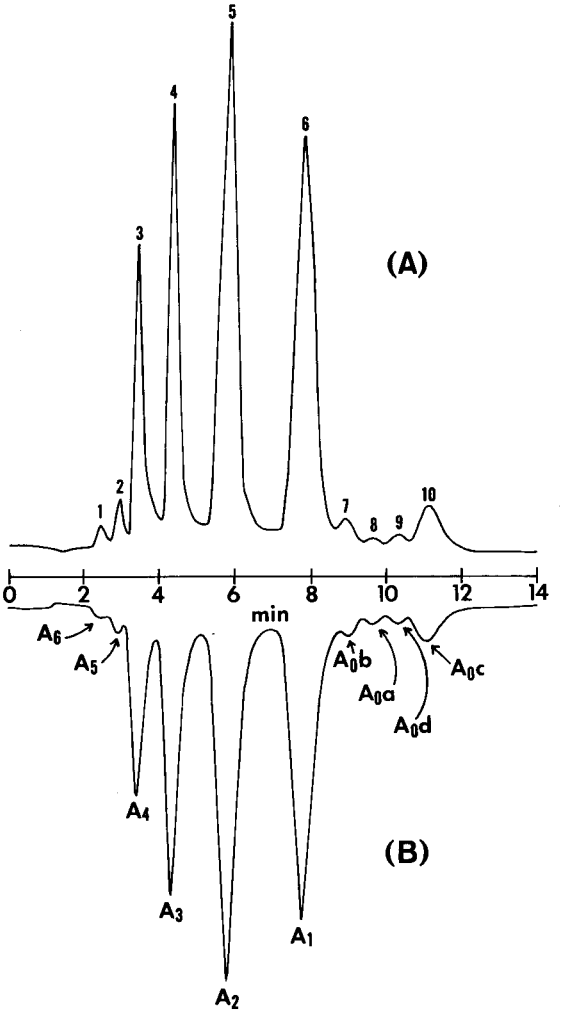


Fig. 2. HPLC separation of antimycin components from a recrystallized commercial sample: (A) UV detector at 254 nm; (B) electrochemical detector at 1.00 V (flow-rate 2 ml/min).

Results of the comparative evaluation of minimum detection limits of the ten isolated antimycins using electrochemical, UV, and fluorescence detectors interfaced independently with the HPLC instrument are shown in Table I. Since a phenolic function group contributes to the primary structural features of an antimycin, it is not surprising to learn from the data that the antibiotic compounds of fishery interest are electroactive. Their detectability in the electrochemical system is comparable with (or

even better than) that in the fluorescence detection system. Inspection of the voltammograms as depicted in Fig. 3 for the smallest and largest peaks disclosed that the optimum voltage on the electrochemical detector is 1.00 V on the glassy carbon electrode *versus* the silver chloride reference electrode. At this voltage, the normalized peaks showed the least changes in reproducibility. With a fluorescence detector, the sample was chromatographed on the same reversed-phase column as the one employed in the electrochemical and UV detection except that the mobile phase was modified to contain 0.05 *M* acetate buffer at pH 6 in 75% methanol (Fig. 4). Under these conditions, the sample fluoresced with five times greater intensity than in the lower pH buffers. A maximum fluorescence detector response was observed upon subjection of the sample to an excitation wavelength of 365 nm and an emission cut-off filter wavelength of 418 nm. Calibration curves for all three detectors studied were linear from *ca.* 1 mg/ml to the minimum detection limit values. Some typical examples are presented in Fig. 5 to illustrate the linear relationship between the detector (electrochemical and UV) response and the concentration of samples injected.

TABLE I

MINIMUM DETECTION LIMITS OF ANTIMYCINS DETERMINED BY HPLC INTERFACED WITH VARIOUS DETECTORS

A standard solution containing equal molar concentrations of antimycin components was used. Values were obtained for the injected amount at a signal-to-noise ratio of 2:1. Coefficient of variation for three replicate injections ranged from 3.8 to 7.1%.

Antimycin	*Minimum detection limit (ng)*		
	Electro-chemical	*UV*	*Fluorescence*
A_6	0.10	4.1	0.31
A_5	0.10	4.3	0.35
A_4	0.13	5.5	0.42
A_3	0.17	7.9	0.50
A_2	0.23	10.4	0.58
A_1	0.41	15.3	0.65
A_{0b}	0.67	18.6	0.77
A_{0a}	0.83	21.0	0.91
A_{0d}	0.90	25.1	1.19
A_{0c}	1.12	28.2	1.69

If the mobile phase consists of identical proportions of methanol and water instead of buffers (pH 3–7) or, synonymously, if it is devoid of any modifying organic and inorganic salts (Table II), the component peaks tend to be poorly resolved and display peak broadening accompanied by loss of peak symmetry. Evidently, small variations in mobile phase conditions have a large effect on peak resolution. Table II shows the retention (capacity factor k') and resolution data obtained with a number of chosen mobile phase conditions. There was distinct improvement in resolution by the addition of either acetate buffer or tetrabutylammonium phosphate to the straight methanol–water system, as the chromatographic separation was preferably controlled by the ionic suppression and ion-pair formation processes. The effect of pH on

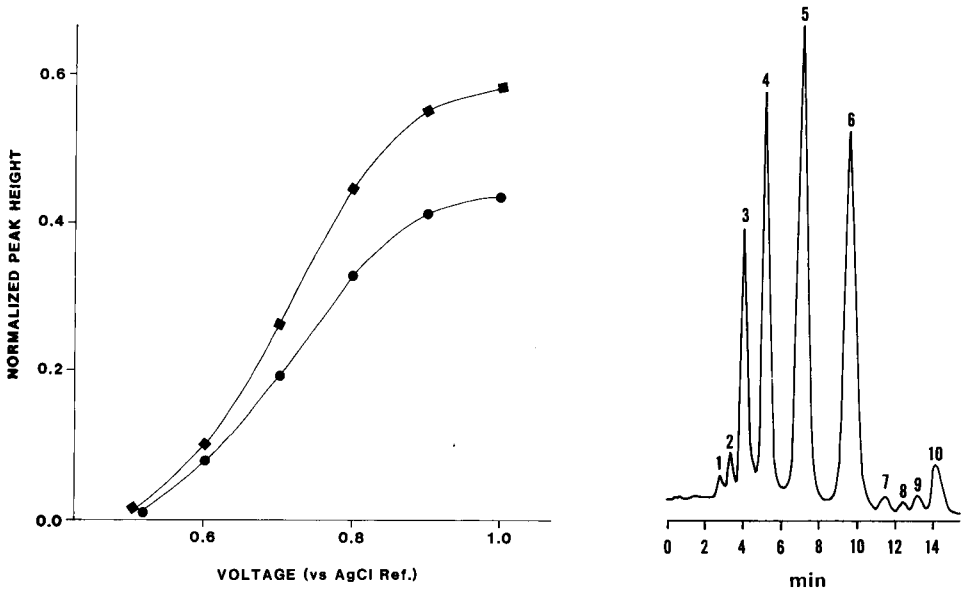


Fig. 3. Voltammograms (normalized peak height *versus* applied voltage) for the smallest and largest antimycin component peaks as represented respectively by the lower and upper curves.

Fig. 4. HPLC separation of antimycin components from a recrystallized commercial sample: fluorescence detector at 365 nm (ex.) and 418 nm (em.) (flow-rate 1.5 ml/min).

retention behavior and selectivity (α) (Table III) follows the general trend that both capacity factor (k') and selectivity (α) values decrease with the increase in pH. This is expected with reversed-phase, because the weakly acidic analyte solutes have higher affinity for the non-polar hydrocarbonaceous silica stationary phase at lower pH by virtue of ionic suppression. Such influence of pH on the retention and selectivity parameters seems to be remarkably effective in the methanol–acetate buffer system in comparison to that in the acetonitrile–phosphate buffer system. Table IV shows the results of retention measurements at various buffer concentrations. While the UV absorption detector response gradually inclines to a maximum value with the change in buffer concentration from 0.05 *M* to 0.3 *M* (not shown here), no significant change of the capacity factors was noted to occur as a result of increasing the buffer concentration at constant pH 5.

Attempted separations of the antimycin mixture on two normal-phase cyano- and amino-bonded silica columns by use of a wide variety of solvent pairs including added modifiers were unsuccessful. In all instances, only one ill-defined peak, presumably representing all unresolved components, appeared early on the liquid chromatogram with little retention.

A summary of the HPLC analysis of four different batches of the antimycin A complex as supplied from commercial sources is given in Table V to demonstrate the compositional diversity of the antibiotics concerned, which is apparently governed by the fermentation origin.

Our experience with the derivatization of the piscicidal antibiotics under study suggests that the rapid alkaline degradation of these compounds precludes forma-

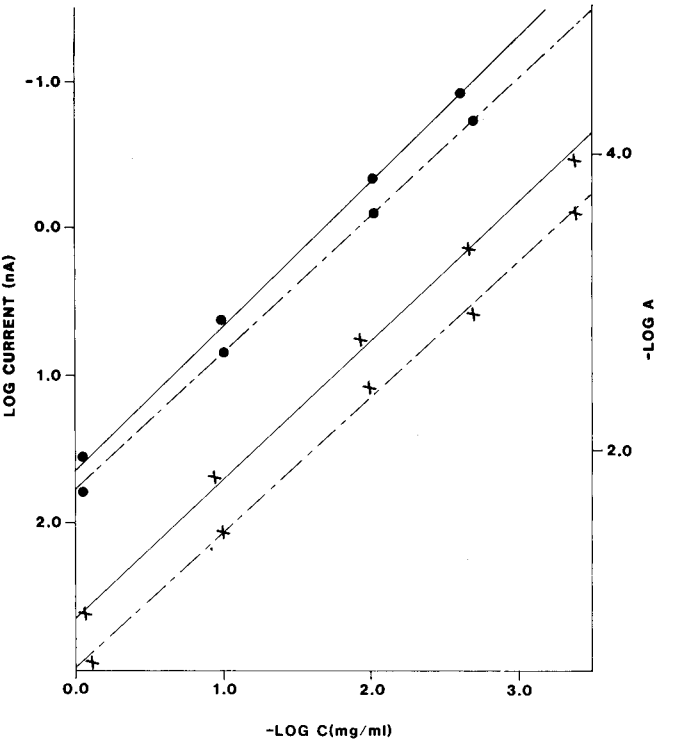


Fig. 5. Plot of detector response as a function of concentration for antimycin analytes. Electrochemical detection, lower two lines; UV detection, upper two lines; smallest component peak, solid line; largest component peak, broken line.

tion of any useful derivatives without undergoing rupture of the dilactone rings by the bases present and eventually giving the decomposition products. Following a carefully controlled procedure developed in this laboratory, we were able to carry out fluorescence labeling of antimycins with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Dns chloride) in a non-aqueous solution using pyridine in place of the conventional alkaline salts. The crude derivatized material was purified by silica gel dry column chromatography and recrystallization. Confirmatory examination of the pure derivatives by LC–MS (chemical ionization) established their structural identities as the fluorigenic dansylated antimycins. In the individual mass spectra of the HPLC components, there were relatively intense signals assignable to the pseudo molecular ions as predicted on the basis of structural formulas. The detected pseudo molecular ions recorded herein are listed according to the order of increasing retention time on the liquid chromatogram: (M_1+1) *m/e* 713, (M_2+1) *m/e* 727, (M_3+1) *m/e* 741, (M_4+1) *m/e* 755, (M_5+1) *m/e* 767, (M_6+1) *m/e* 783, (M_7+1) *m/e* 783, (M_8+1) *m/e* 797, (M_9+1) *m/e* 797, $(M_{10}+1)$ *m/e* 797. The compositional distribution of parent antimycins in the mixture remained unaffected by the derivatization process. This is indicative of the non-discriminative reactivity among the homologous components toward the derivatizing agents. The chromatographic conditions described so far for the separation of the underivatized antimycins were found to be equally applicable to the separation of the dansylated derivatives. Excellent baseline resolu-

TABLE II

THE EFFECT OF MOBILE PHASE CONDITIONS ON CHROMATOGRAPHIC RETENTION (k') AND RESOLUTION (R) CHARACTERISTICS (HPLC–UV)

Component peak	*Methanol–water*				*Methanol–acetate buffer**				*Methanol (TBA)–Water (TBA)***			
	63:37 (%)		*65:35(%)*		*63:37(%)*		*65:35(%)*		*63:37(%)*		*65:35(%)*	
	k'	R	k'	R	k'	R	k'	R	k'	R	k'	R
1	0.36		0.29		0.44		0.38		0.42		0.20	
		0.00		0.00		1.20		1.11		1.60		1.18
2	0.36		0.29		0.68		0.58		0.67		0.31	
		0.00		0.00		1.14		1.00		1.52		1.29
3	0.36		0.29		0.96		0.77		1.00		0.56	
		0.53		0.49		1.16		1.13		1.57		1.23
4	0.64		0.48		1.40		1.15		1.27		1.00	
		0.95		0.78		1.53		1.36		1.98		1.75
5	1.21		1.15		2.16		1.69		2.60		1.71	
		0.89		0.83		1.33		1.26		1.93		1.57
6	1.86		1.74		3.04		2.35		3.93		2.56	
		0.00		0.00		0.75		0.72		1.06		1.01
7	1.86		1.74		3.55		2.68		4.54		2.95	
		0.00		0.00		0.69		0.66		1.00		0.97
8	1.86		1.74		3.82		2.96		4.99		3.29	
		0.00		0.00		0.68		0.65		1.03		0.99
9	1.86		1.74		4.23		3.32		5.58		3.42	
		0.00		0.00		0.81		0.77		1.13		1.07
10	1.86		1.74		4.56		3.59		5.93		3.87	

* Acetate buffer: 0.05 M, pH 7.

** TBA, tetrabutylammonium phosphate: 0.05 M, pH 7 (adjusted with NaH_2PO_4).

tion of all the components was easily achieved. In a mobile phase of acetonitrile–water (70:30), the derivatized antimycins showed superior chromatographic characteristics over the underivatized counterparts. The use of buffer salts in the mobile phase was not essential and could be omitted in most cases. The benefits of derivatization were fully realized, as the observed one-hundred-fold increase in sensitivity by fluorescence detection was undoubtedly accredited to the incorporation of the dansyl fluorotag into the antimycins. By this method of detecting the antibiotics as the Dns derivatives, the lowest detectable amounts were estimated to be 5–10 pg. The fluorescence detector was found to respond linearly with the injected amounts of analytes (Dns antimycins) over the range from 10 μg to lower picograms (minimum detection limit). The latter observation has a crucial bearing on the success of accurate and reliable analyses of traces of antimycins, which often coexist with a host of degradation products.

As frequently is the case encountered with the analysis of a complex sample for analytes of trace concentration, a somewhat elaborate cleanup procedure is needed to achieve the analytical goal with high precision. In the method developed for the determination of the antimycin A complex in fish tissues, an adsorption chromatographic purification step was interposed between the tissue extraction and HPLC quantification procedures to facilitate sample quantitation with considerable reduction in interferences from the endogenous substances and consequently with im-

TABLE III

THE EFFECT OF pH ON CAPACITY FACTOR (k') AND SELECTIVITY (α)* (HPLC–UV)

Component peak	*Methanol–acetate buffer***				*Acetonitrile–phosphate buffer***			
	pH 3	*pH 4*	*pH 5*	*pH 6*	*pH 3*	*pH 4*	*pH 5*	*pH 6*
	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)	k' (α)
1	1.55(1.00)	1.36(1.00)	0.91(1.00)	0.64(1.00)	2.53(1.00)	2.49(1.00)	2.42(1.00)	2.38(1.00)
2	2.27(1.46)	1.94(1.43)	1.27(1.40)	0.87(1.36)	3.57(1.41)	3.50(1.41)	3.36(1.39)	3.25(1.37)
3	3.00(1.94)	2.61(1.92)	1.73(1.90)	1.09(1.70)	4.58(1.81)	4.47(1.80)	4.35(1.80)	4.25(1.79)
4	4.36(2.81)	3.67(2.70)	2.45(2.69)	1.55(2.42)	6.85(2.71)	6.61(2.65)	6.29(2.60)	6.13(2.58)
5	6.55(4.23)	5.73(4.21)	3.73(4.10)	2.36(3.69)	9.79(3.87)	9.58(3.85)	9.20(3.80)	8.88(3.73)
6	9.36(6.04)	8.09(5.95)	5.05(5.55)	3.36(5.25)	13.3(5.25)	12.9(5.19)	12.5(5.15)	12.0(5.03)
7	11.1(7.16)	9.66(7.10)	6.30(6.92)	4.08(6.38)	15.9(6.28)	15.5(6.24)	15.0(6.18)	14.7(6.16)
8	12.7(8.19)	10.2(7.52)	6.67(7.33)	4.44(6.94)	16.8(6.64)	16.4(6.58)	15.8(6.51)	15.3(6.43)
9	14.4(9.29)	11.4(8.35)	7.45(8.19)	4.82(7.53)	18.7(7.40)	18.1(7.27)	17.5(7.23)	17.0(7.14)
10	16.6(10.7)	12.3(9.02)	8.18(8.99)	5.09(7.95)	20.0(7.90)	19.5(7.83)	18.9(7.82)	18.5(7.77)

* All selectivity values were determined based on the capacity factors of the least retained component peak 1.

** The buffer concentration was 0.2 *M*. The mobile phase was composed of 75:25 (%) methanol (or acetonitrile)–acetate (or phosphate) buffer.

TABLE IV

RETENTION MEASUREMENTS OF ANTIMYCIN COMPONENTS WITH VARIOUS ACETATE BUFFER CONCENTRATIONS IN TWO MOBILE PHASE SYSTEMS (HPLC–UV)

Buffer concentration (M)	*Capacity factor k'*									
	Component peak									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Methanol–acetate buffer (70:30)*										
0.05	1.08	1.48	2.10	2.79	4.07	5.71	6.64	7.15	7.86	8.93
0.1	1.07	1.47	1.97	2.71	3.93	5.57	6.58	6.99	7.82	8.76
0.2	1.06	1.43	1.93	2.60	3.89	5.50	6.55	6.96	7.74	8.48
0.3	1.08	1.41	1.87	2.58	3.87	5.47	6.43	6.94	7.58	8.31
0.5	1.03	1.40	1.88	2.53	3.83	5.46	6.33	6.90	7.45	8.00
Acetonitrile–acetate buffer (60:40)*										
0.05	2.25	3.22	4.00	5.04	6.84	9.02	10.5	11.4	12.2	13.4
0.1	2.19	3.15	3.93	5.00	6.82	9.00	10.3	11.4	12.0	13.1
0.2	2.20	2.91	3.82	4.90	6.50	8.56	9.94	11.1	11.7	13.0
0.3	2.16	2.90	3.79	5.00	6.46	8.33	9.87	10.6	11.4	12.7
0.5	2.17	2.88	3.72	4.97	6.37	8.30	9.83	10.4	11.1	12.5

* Ratio of the mobile phase solvent composition.

proved sensitivity. Table VI gives the results of recovery studies of fortified tissue samples using the ^{14}C-labeled radiotracer technique. The data indicate that the homologous antibiotic compounds added to tissue samples at various concentrations can be completely extracted from the tissues. They can be quantitatively adsorbed on

TABLE V

HPLC DETERMINATION OF THE COMPOSITION OF FOUR DIFFERENT BATCHES OF THE ANTIMYCIN A COMPLEX

Component	*Composition (%)*			
	Batch			
	1	*2*	*3*	*4*
A_6	0.29	1.37	1.61	TR*
A_5	0.92	8.10	3.05	4.29
A_4	10.84	10.18	12.19	11.30
A_3	19.23	34.51	19.07	22.46
A_2	32.30	15.78	26.25	25.08
A_1	29.57	30.14	30.96	33.71
A_{0b}	2.39	ND**	2.89	TR
A_{0a}	0.68	ND	0.38	TR
A_{0d}	1.12	ND	2.18	0.99
A_{0c}	2.66	ND	1.42	2.07

* TR = trace amount (less than 0.10%)
** ND = none detected.

TABLE VI

RECOVERY OF AN ANTIMYCIN MIXTURE FROM TISSUE SAMPLES (HPLC–FLUORESCENCE DETECTION)

Amount added (μg/g)	*Stepwise sample clean-up*			*Overall*		*Amount found (μg/g)*
	*A**	*B***	*C****	*Recovery (%)*	*C.V.§ (%)*	
	Recovery (%)	*Recovery (%)*	*Recovery (%)*			
0.45	95.3	89.4	90.2	76.7	7.3	0.35
1.10	96.4	90.2	91.2	79.3	6.1	0.87
2.35	96.0	90.4	90.9	78.6	7.3	1.85
6.05	95.8	91.3	91.4	79.9	7.2	4.83
10.25	96.4	89.8	92.5	79.7	5.7	8.17
16.00	95.9	90.5	92.0	79.8	5.4	12.77
20.40	96.6	93.4	91.8	82.8	4.9	16.89
40.30	95.5	92.9	92.3	81.9	4.8	33.01
80.15	94.8	94.1	91.8	81.9	4.2	65.64
100.65	96.1	94.0	93.5	84.5	4.1	85.05

* A = tissue extraction step.

** B = column adsorption chromatography step.

*** C = derivatization step (dansylation).

§ C.V. = coefficient of variation; analytical values are mean values of three determinations.

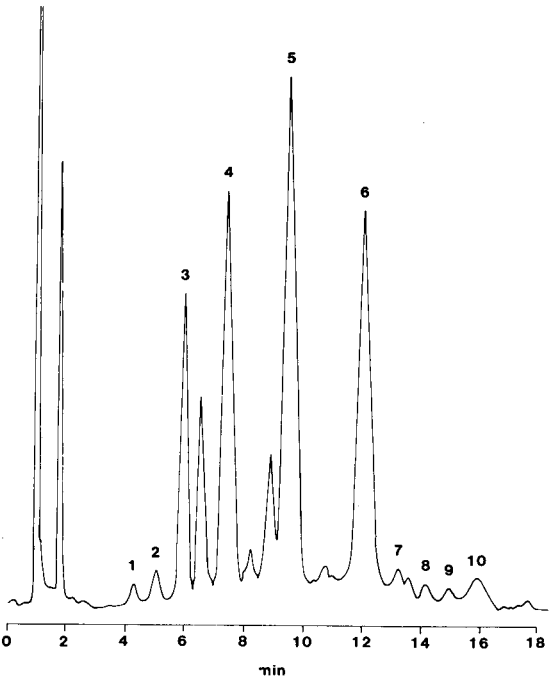


Fig. 6. HPLC separation of dansylated antimycin components in a purified tissue sample spiked with 1.10 μg/g of the antimycin standards. The chromatogram was obtained with a fluorescence detector; mobile phase, acetonitrile–water (70:30); flow-rate, 2 ml/min.

the silica gel column using a non-polar hydrocarbon solvent (hexane or benzene) as the eluent, and quantitatively eluted from the column with methylene chloride–methanol (1:1). Initial attempted use of more polar solvents gave unsatisfactory results, since these solvents expedited column elution at the expense of eluting impurities which interfered with subsequent HPLC measurements. The overall recoveries including the Dns derivatization step are as shown within the acceptable range (77–85%). In the low level antimycin calculations, it was necessary to make a correction for the amount of [^{14}C]antimycin tracer used as the internal standard in the individual tissue sample analysis. An HPLC chromatogram of a purified tissue sample spiked with antimycin standards is provided in Fig. 6 to show efficient separation of the dansylated antimycin components. The chromatogram exhibits the characteristic homologous components similar to that of the underivatized antimycin homologues.

CONCLUSIONS

The findings in this study demonstrate the potential utility of HPLC for the effective resolution and sensitive detection of a mixture of compositional complexity that has hitherto remained unexplored by this chromatographic technique. The method developed here facilitates for the first time the isolation of each individual antibiotic in high purity and provides a non-destructive means of separation and quantitative measurement. Inclusion of a sample purification procedure to the HPLC quantification technique presents a viable method for the practical analysis of the antimycin antibiotics in organic tissues.

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MARIJUANA METABOLITES IN URINE OF MAN

X. IDENTIFICATION OF MARIJUANA USE BY DETECTION OF Δ^9-TETRAHYDROCANNABINOL-11-OIC ACID USING THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Marijuana use can be determined by detecting Δ^9-tetrahydrocannabinol-11-oic acid (THC-11-oic acid) in urine. For this, we describe a procedure for its chemical detection by using sequential thin-layer chromatography on a single plate for rapid isolation and identification. A volume of urine containing 50 mg of creatinine is concentrated by evaporation to 10 ml, the concentrate is enzymically hydrolyzed for 30 minutes and extracted with ether, and the extract is purified by treatment with $NaHCO_3$, then chromatographed in an alkaline and an acidic solvent sequence. The plate is sprayed with Fast Blue Salt B, and THC-11-oic is identified by its characteristic mobility and its characteristic colour reaction. The sensitivity is 0.5 μg. THC-11-oic acid has been detected in urines collected after the smoking of one standard cigarette containing 16–18 mg of Δ^9-tetrahydrocannabinol and in 34 of the first 100 tests of spontaneously collected urines of patients in a hospital drug-abuse treatment program. Multiple samples are easily carried through this extraction procedure. Evaporative concentration takes about 20 min per sample, and the analysis of eight concentrated samples takes about 5.5 h.

INTRODUCTION

Recent use of marijuana can be determined by testing for Δ^9-tetrahydrocannabinol (THC), but only by using the extreme sensitivity of radioimmunoassay (RIA), mass spectrometry or far-ultra-violet spectrophotometry coupled with high-performance liquid chromatography (HPLC)[1,2,3]. In this report, we show that marijuana use can be determined by testing for Δ^9-tetrahydrocannabinol-11-oic acid (THC-11-oic acid), the major urinary metabolite of THC in man, using equipment normally found in the clinical laboratory. Although the presence of THC-11-oic acid in urine cannot establish the time of ingestion of marijuana, as it is excreted for periods varying from hours to several days, depending on the amount and the route of ingestion of the drug[1,4], its detection is of value as a definite indication that

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marijuana has been used. Thus, THC-11-oic acid in urine can support other evidence of intoxication.

Previously published methods for the determination of THC-11-oic acid are primarily research-oriented. To ensure specificity, some workers use multi-step extraction procedures to separate the THC-11-oic acid from other metabolites of THC, others use mass fragmentography preceded by derivatization with silylating agents, still others employ HPLC followed by RIA and some use RIA alone[1,4–7]. Of these methods, only RIA and the enzyme-multiplied immunoassay technique (EMIT), each of which measures THC-11-oic acid and other THC metabolites, are suitable for clinical work[8].

The RIA method requires a liquid scintillation counter and readily available antisera. Conditions to set up and perform the EMIT procedure are far less restrictive, but the initial outlay for the equipment may still be prohibitive. The procedure we present has none of these disadvantages. The most extraordinary laboratory equipment required is a vacuum rotary evaporator. Thus, it is the most easily set up of any currently available means for determination of cannabinoid use.

TEST PROCEDURE

A volume of urine containing 50 mg of creatinine is adjusted to pH 4.7 to 6.3 with 6 *N* HCl and concentrated to approximately 5 ml in a Buchi rotary evaporator at 50°C. The concentrate is transferred to a 25 × 150 mm screw-cap culture tube (scct) and diluted to 10 ml with water, then the tube is stoppered with a PTFE-lined screw cap, and the contents are mixed and incubated with 0.1 ml of enzyme (Boehringer-Mannheim, β-glucuronidase–aryl sulfatase) at 55–60°C for 30 min. The hydrolysate is cooled to room temperature and, without further adjustment of pH, is extracted with anhydrous ethyl ether (2 × 15 ml). After each extraction, the tube is centrifuged at *ca.* 2000 RCF for 3 min to quickly separate the two phases and the ether extracts are transferred to another 25 × 150 mm scct. Between the extractions, the ether of the first extract is evaporated in a stream of nitrogen in a water bath at 50°C. After being washed with 5% $NaHCO_3$ solution and dried with anhydrous sodium sulfate, the ether is evaporated. The residue is streaked across 1.5 cm of a pre-coated silica gel G TLC plate, (250 μm, Analtech) with two 50-μl volumes of dichloromethane and chromatographed sequentially in two saturated TLC chambers, first with acetone–chloroform–triethylamine (80:20:1), then with petroleum ether–ether–glacial acetic acid (50:50:1.5). Between developments, the TLC plate is placed in a fume hood for 5 min. Approximately 5 min after the second development, the plate is sprayed with a freshly prepared cold 0.1% solution of Fast Blue Salt B in 2 *N* NaOH. A positive response is indicated by a magenta colored zone of R_F approximating 0.1 or corresponding to that of a reference standard.

EXPERIMENTAL

The experimental work was initially done with silylated glassware to ensure maximum recovery of THC-11-oic acid[6,9]. Untreated glassware was adopted for the test procedure when it was determined that it did not have a detectable effect on the recovery of THC-11-oic acid.

Sample and sample size

After removing an aliquot for determination of creatinine, the urine is stored either refrigerated, if the analysis will be done within 4 or 5 days, or frozen if analysis will be delayed longer.

Creatinine content rather than volume is used as the reference for amounts of sample, due to wide variations in concentration of urine. The use of creatinine content rather than volume as a reference for the sample vitiates the deliberate dilution of urine, which can lead to falsely negative tests.

A volume of urine containing 50 mg of creatinine approximates the maximum that can be used for analysis. Larger amounts frequently yield emulsions during extraction and "overloading" of the plate during TLC. Smaller amounts, such as 10 to 15 mg of creatinine, applied to the plate in a spot in order to obtain the same sensitivity as a 50-mg sample applied in a streak, gave poor results.

Concentration by evaporation rather than by column chromatography is used because our previous experience indicates that the solute content of the aqueous phase significantly affects the extraction of THC-11-oic acid by ether[4].

Hydrolysis

At pH 4.5–5, in which range β-glucuronidase of *Helix pomatia* has maximum activity, and with larger amounts of enzyme and incubation temperatures above 37.5°C[10–12], we found that conjugated THC-11-oic acid was completely hydrolyzed in 30 min at 55–60°C using 0.1 ml of enzyme per 10 ml of aqueous phase. These conditions were used for the experimental work until recovery and sensitivity were evaluated. Then we determined that, at 55–60°C, hydrolysis was complete over the pH range 4.5 to 6.5. Thus, 4.7 to 6.3 is recommended in the test procedure and is used routinely.

Ether extraction

In an experiment similar to those previously reported[4], we showed that metabolic THC-11-oic acid, like THC-11-oic acid added to urine, was completely extracted into ether at pH values up to 10. Thus, the extraction could be done at the pH of hydrolysis.

After finding that successive prolonged extractions with ether[13] were not necessary, as almost all of the THC-11-oic was recovered in the first extraction, 10-ml aliquots of a hydrolyzed concentrated urine were extracted twice with 15-ml portions of ether by shaking on a reciprocal shaker with the long axis of the scct in the direction of shaking both vigorously (225 cycles per min) and gently (140 cycles per min) for various periods. All of the THC-11-oic acid was extracted by shaking vigorously for 1 min or gently for 5 min.

Purification of ether extract

Ether extracts of a hydrolyzed urine containing a moderate amount of metabolic THC-11-oic acid in order to have a high ratio of background to analyte were purified by shaking vigorously on the reciprocal shaker with 8 ml of 2% $NaHCO_3$ solution for various periods. The results showed that three extractions for 2 min each gave chromatograms as clean as those obtained after extraction for longer periods, and that three extractions for 30 sec each gave chromatograms that were almost as

good. Shaking the ether extracts with 10 ml of 5% $NaHCO_3$ solution for 1 min twice effected an adequate and slightly better clean-up than three extractions with 2% $NaHCO_3$ solution for 30 sec each, as evidenced by more intense coloring and slightly better defined spots in the chromatograms, and was still quite rapid.

After each extraction, the tube was centrifuged briefly to separate the two phases quickly. The aqueous layer was removed easily by inserting a 10-ml pipette through the ether layer, ejecting the small volume of ether in the pipette by hand-warming the pipette and drawing up the $NaHCO_3$ solution using a 3-ball valve pipette-filler.

To ensure that THC-11-oic acid was not taken up by 5% $NaHCO_3$ solution as reported for Δ^6-THC-7-oic acid[14] (monoterpenoid numbering for Δ^8-THC-11-oic acid), an ether solution of 200 ng of THC-11-oic acid was extracted with 5% $NaHCO_3$ solution, and the extract was treated with anhydrous Na_2SO_4 and chromatographed as described in the test procedure. The chromatogram was compared with that of an equivalent ether solution that had not been treated with $NaHCO_3$ and Na_2SO_4. There was no difference in the intensity of the spots, thus indicating that 5% $NaHCO_3$ solution did not extract THC-11-oic acid from ether. This experiment also demonstrated that no loss occurred due to the use of Na_2SO_4.

Drying the purified ether extract

Approximately 1 g of anhydrous Na_2SO_4 was found to be an adequate amount to dry the purified ether extract. After it has been added, the tube is capped, inverted several times and centrifuged briefly, and the ether extract is poured through a small glass funnel into a 20 × 125 mm scct. The Na_2SO_4 is washed once with 5 ml of ether, and the combined ether solutions are evaporated as previously described. The inner wall of the scct is washed down with 0.5 ml of absolute ethanol, which is also evaporated.

Thin-layer chromatography

A blank urine that, after hydrolysis, had a great deal of chromogenic material and other substances that gave non-cannabinoid color reactions with Fast Blue Salt B was selected for this phase of the work to ensure that optimum TLC conditions would be devised. Different chromatographic conditions were tested with residues of ether extracts of this urine "spiked" with 500 ng of THC-11-oic acid before chromatography. A single-development procedure for distances of both 15 and 10 cm using mixtures of acetone, chloroform and triethylamine in proportions ranging from 80:10:1 to 50:50:1 were tried. Mono- and dihydroxy derivatives of THC migrate at this range of polarity and specific alkalinity and have R_F values greater than that of THC-11-oic acid, which remains at the origin uncontaminated by non-acidic hydroxy compounds[15]. Background material made it difficult to evaluate results in single-development procedures with samples containing less than 1 μg of added THC-11-oic acid. Additionally, the single-development procedure was tried with acetone–chloroform (80:20) containing 0.5 or 2% triethylamine. The separation of THC-11-oic acid from endogenous material was poor with either concentration of triethylamine; with 0.5% of the amine, the mobility of THC-11-oic acid increased, and with 2% the mobility of the endogenous material was suppressed.

The two-development procedure overcame the disadvantages of the single-

development procedure. A definitive band of appropriate R_F value and truer color was obtained, and, with a 10-cm development each time, the total time was only slightly greater than for one 15-cm development. An acetic acid concentration of 1.5% in the second solvent system was essential; 1% acetic acid produced little movement of the THC-11-oic acid, and 2% caused the THC-11-oic acid to move into a zone of endogenous material.

Standard

A 1-μg amount of pure THC-11-oic acid, dissolved in absolute ethanol, is mixed with the dried ether extract of a blank urine. This adjusts for the slight increase of mobility of THC-11-oic acid due to the effect of background material on the binding sites of the silica gel.

Recovery and sensitivity

Ethanolic solutions of THC-11-oic acid added to 50-ml aliquots of the highly chromogenic blank urine containing 50 mg of creatinine were analyzed. The intensity of the reaction of a 500-ng spot of THC-11-oic acid and Fast Blue Salt B was faint, but definitely positive, and approximately half of that obtained by adding 500 ng of THC-11-oic acid to the final residue of the extract of an aliquot of the same urine.

Evaluation of the effect of each step in the procedure showed that none contributed disproportionately to the apparent low recovery. Background material had a large effect on sensitivity. We found that 250 ng of THC-11-oic acid were required to give a detectable spot in the presence of urinary background material. This was approximately equal to the intensity of 80 ng applied directly to the TLC plate.

Duplicate samples of a urine "spiked" with THC-11-oic acid were refrigerated overnight, then frozen for 5 days in silylated and in untreated glass bottles before being routinely processed. Silylated and untreated glassware, respectively, were used throughout. No difference could be detected between the results; thus, non-silylated glassware is used in the recommended procedure.

Comparison between TLC and EMIT assay methods[8]

For 39 of 43 samples analyzed by both methods, results were in agreement (see Table I); as 3 of the 4 samples that did not check were in the borderline range of the EMIT procedure, the correlation is excellent. The correlation was absolute among the 19 samples that were either zero or exceeded the EMIT detection limit (50 ng/ml), but was less among the 17 that were greater than zero but less than 20 ng/ml. In this range, results by EMIT are negative[16]. By the TLC procedure, 16 were negative and one was positive. The correlation was poorest among the 7 samples that were in the range 20–50 ng/ml. In this range, results by EMIT are positive[16]; by the TLC procedure, 3 were negative and 4 were positive. Such mixed results can be expected in the borderline range, as the TLC method measures only THC-11-oic acid, whereas the EMIT procedure measures total cannabinoids.

CLINICAL RESULTS

Fig. 1 shows the results of analysis for THC-11-oic acid in spontaneously voided urines from six subjects participating in a study for which "heavy" and "light"

TABLE I

COMPARISON OF RESULTS WITH THOSE OF EMIT ASSAY FOR TOTAL CANNABINOIDS IN URINE*

The EMIT assays were carried out as described in ref. 16.

TLC result	*EMIT test values*			
	Negative	*< 20 ng/ml***	*20–50 ng/ml****	*> 50 ng/ml*
Negative	11	16	3	
Positive		1	4	8

* See ref. 8.
** Interpreted as negative[16].
*** Interpreted as positive[16].

users were recruited[17]. In this series, there were three positives and three negatives; thus, three were considered as being "heavy" users and three "light" users.

We have also used the method to assay urines from patients on several of our psychiatric wards to determine whether or not some of their unexpected aberrant behavior could possibly be attributed to marijuana[18–20]. Of 31 tests, 12 were negative and 16 positive (the 16 positives included four patients who were positive on each of two trials and one who was negative on one trial and positive on another).

Currently, we are using the test routinely in conjunction with a hospital drug-abuse treatment program. Of the first 100 tests, 34 have been positive. In contrast,

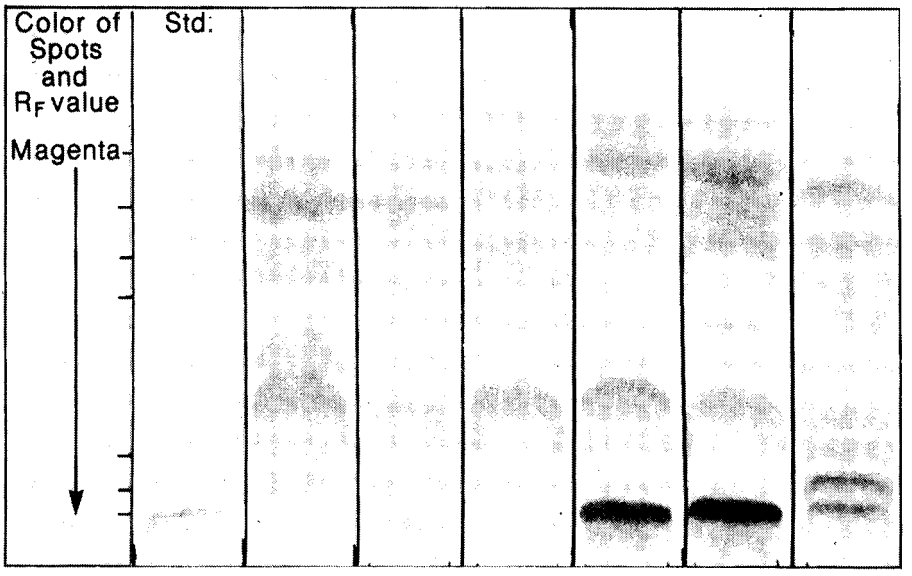

Fig. 1. Chromatograms of spontaneously voided urines from six volunteers for a study on "heavy" and "light" users of marihuana analyzed by the test procedure. Of these six, three are positive and three are negative. Only colors characteristic of the reaction of metabolites of THC and Fast Blue Salt B noted. Std. = Δ^9-THC-11-oic acid.

screening for methadone, morphine, barbiturates, codeine, amphetamine and methamphetamine yielded less than 2% positive samples. This may be due in part to use of urine as delivered, which, in many instances, is quite dilute.

Clinically, THC-11-oic acid can be detected after the smoking of one marijuana cigarette, depending on potency, smoking efficiency and metabolism. Among spontaneously voided urines tested after the smoking of one standard marijuana cigarette containing 16–18 mg of THC, THC-11-oic acid was detected as early as 1.5 h after smoking. Among the urines tested from the patients in the hospital drug-abuse treatment program cited above, we have obtained positive results as much as 1 week after admission and, in one instance, 28 days, from a self-admitted user of 20–30 marijuana cigarettes a day for approximately 3 months. This exceeds the 48 and 72-hour excretion periods previously described for this metabolite[1,5] and is most likely due to the amount ingested and prolonged use.

DISCUSSION

At present, immunoassays are the only means available for the relatively routine detection of urinary cannabinoids. The proposed procedure makes available an independent method that can be used in some laboratories routinely and in others as a simple confirmatory test.

Laboratories performing drug-abuse screening on a large scale would obviously find it more feasible to use a set volume and eliminate the determination of creatinine and the concentration of the urine. But such an approach reduces the effectiveness of this test, and of other tests involving use of spontaneously voided urine samples, because of the wide range of concentration. Among the samples tested for the drug-abuse treatment program we have used a wide range of sample volumes and have obtained a moderately strong positive result (250 ng) with a 250-ml sample.

A possible source of false positives might be due to the non-specificity of Fast Blue Salt B, which reacts with phenols and amines and with various herbs, such as rosemary, sage and thyme[21,22]. However, it is highly unlikely that these materials would be taken in quantities sufficient to produce urinary metabolites or that such metabolites would be extracted and chromatographed in the same way as cannabinoids. Additionally, of 150 urines tested by this procedure, none has shown interference due to metabolites of cannabidiol and cannabinol and only two have shown interference due to background.

The procedure has been used to detect current cannabis use among psychiatric patients and those being treated for drug abuse. A positive test allows physicians to confront patients with reliable support information. On the other hand, a negative test alleviates suspicion that patients are abusing privileges.

Some evidence suggests that use of cannabis may also aggravate the psychosis of schizophrenics, as well as the depressed mood of patients with affective disorders[18–20]. Thus, any measure that might discourage use of this drug in such patients might be clinically useful.

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Fine technical assistance was given by Miguel Williams. We thank Syva Corp. Palo Alto, CA, for the EMIT assays. This study was supported in part by grant DA-00424 and the Research Services of the Veterans Administration.

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Note

Retention behaviour of alkylene oxides in reversed-phase high-performance liquid chromatography

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(First received May 11th, 1981; revised manuscript received June 26th, 1981)

Alkylene oxide type non-ionic surfactants are widely used as emulsifiers, wetting agents and detergents. Many procedures for chromatographic analysis of non-ionic surfactants have been reported. For example, the molecular weight distribution of ethylene oxide adducts has been determined[1–8], and the determination of polyethylene glycols (PEG)[9–13] and of homologous alkyl chain distribution[14] in ethylene oxide adducts have been reported. However, there have been few reports on other types of non-ionic surfactants, such as poly(oxypropylene) alkyl ethers, poly(oxypropylene) poly(oxyethylene) alkyl ethers and copolymers of ethylene oxide (EO) and propylene oxide (PO), using high-performance liquid chromatography (HPLC), although their use as surfactants is increasing.

In this study, retention data of non-ionic surfactants in reversed-phase HPLC were collected for a wide range of EO and/or PO adducts. The logarithm of the capacity factor, log k', was used as an index of retention time, and the retention behaviour of non-ionic surfactants was elucidated in terms of the hydrophilic or hydrophobic properties of the EO or PO chains. The separation of non-ionic surfactants can be predicted from a linear relation between log k' and the degree of polymerization of EO or PO. It was also possible to estimate the degree of polymerization or alkyl chain length from the log k' value.

EXPERIMENTAL

Chemicals

Poly(oxyethylene) alkyl ethers (R-EO) were purchased from Kao-Atlas (Tokyo, Japan), and the block copolymers of EO and PO and PPG 4000 (polypropylene glycol) from Asahi Denka (Tokyo, Japan). PEG 4000 (polyethylene glycol) was obtained from Wako (Tokyo, Japan). Poly(oxypropylene) alkyl ethers (R-PO) and poly(oxypropylene) poly(oxyethylene) alkyl ethers (R-PO/EO) were synthesized in our laboratories.

HPLC

Analyses were performed with a liquid chromatograph (TRI Rotar; Jasco, Japan) equipped with variable loop injector (VL-611, Jasco), differential refractom-

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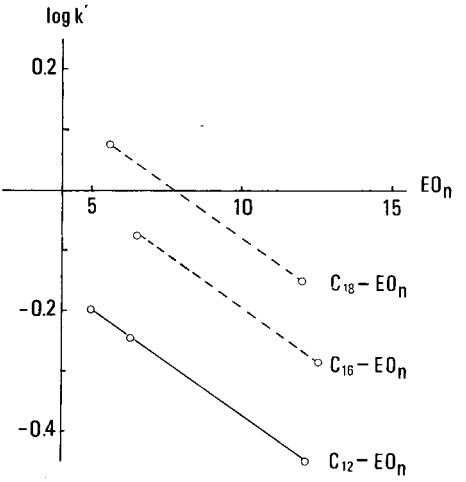
eter (SE-11, Shodex) and Hewlett-Packard 7101 BM strip chart recorder. All results were obtained on stainless-steel columns (250 × 4.0 mm I.D.) packed with porous polymer gel (No. 3011; Hitachi, Japan). An acetone–water mixture was used as mobile phase at a flow-rate of 1.0 ml/min.

Sample solutions of *ca.* 10% were prepared in the mobile phase and 10 μl of each solution were injected into the chromatographic system. The logarithm of the capacity factor, log k', of each sample was calculated from the chromatogram.

RESULTS AND DISCUSSION

Fig. 1 shows the linear relation between log k' and n, the degree of polymerization, for C_{12}-EO_n. Here, C_{12}-EO_n denotes the adduct between an average of n moles of EO and 1 mole of lauryl alcohol. The log k' value decreases by 0.2 between $n = 5$ and 12. Thus, the retention time of C_{12}-EO_5 is 1.5 times longer than that of C_{12}-EO_{12}, the longer EO chain enhancing the hydrophilic property of C_{12}-EO_{12}. For the EO adducts of C_{16} and C_{18} alcohols, parallel lines (broken lines in Fig. 1) can be drawn although only two data points are available, because the only difference between C_{12}-EO_n and C_{16}-EO_n or C_{18}-EO_n is in the alkyl chain length. The effect of the EO chain on the retention behaviour will not change for adducts with different alkyl chain lengths. The retention times of the adducts increase in the order C_{12}-EO_n < C_{16}-EO_n < C_{18}-EO_n in accordance with the hydrophobic property of the alkyl chain.



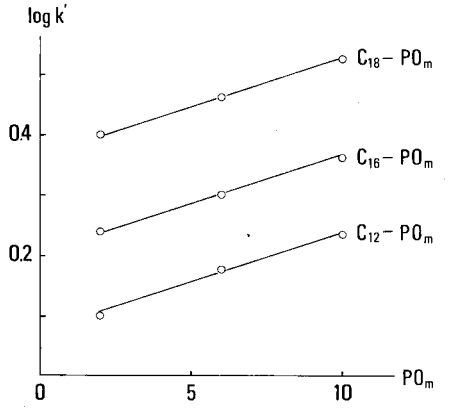


Fig. 1. Reations between log k' and the degree of polymerization of EO for (R-EO_n).
Fig. 2. Relation between log k' and the degree of polymerization of PO for (R-PO_m).

Fig. 2 shows a similar relation between log k' and the degree of polymerization of PO in R-PO. Although the relation is linear, the slope is positive due to the hydrophobic property of the PO chain.

Fig. 3 shows a linear relation between log k' and n of EO/PO adducts of lauryl alcohol. Again, C_{12}-PO_mEO_n denotes an adduct between an average of m moles of PO, n moles of EO and 1 mole of lauryl alcohol. The linear relation holds only for certain values of m. In Fig. 3, the data for $m = 0$, 3, 5 and 10 are shown. Although

there are only two points each for $m = 3$ and 5, we assume linear relations (broken lines) because linear relations are found for $m = 0$ and 10 and the effect of the EO chain on the retention behaviour of C_{12}-PO_mEO_n will not change for constant values of m. The straight lines of negative slope are similar to those in Fig. 1. The elution of C_{12}-PO_mEO_5 is delayed by the hydrophobic PO chain, and C_{12}-EO_5 is eluted first, followed by C_{12}-PO_3EO_5 and C_{12}-$PO_{10}EO_5$. The retention times of C_{12}-$PO_{10}EO_n$ increase in the order C_{12}-$PO_{10}EO_{15}$ < C_{12}-$PO_{10}EO_{10}$ < C_{12}-$PO_{10}EO_5$.

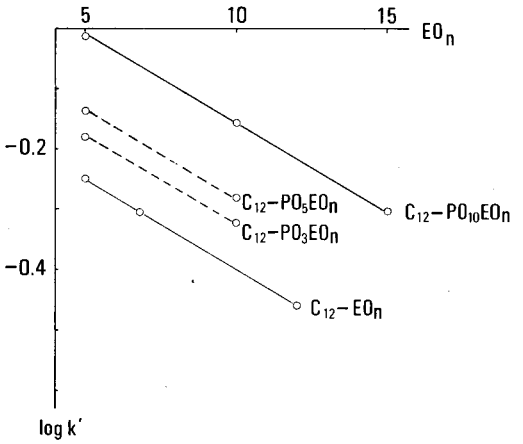


Fig. 3. Relation between log k' and the degree of polymerization of EO for (R-PO_mEO_n).

The elution pattern of block copolymers of PO and EO is similar to that of EO/PO adducts of alcohols (Fig. 3) although these copolymers have no alkyl chain. Fig. 4 shows chromatograms of copolymers with PEG and polypropylene glycols (PPG). The weight percentages of the EO chain (EO%) in the copolymer are 100 (a), 80 (b), 50 (c) and 0 (d), respectively. The retention time of the copolymer increases and the shape of its peak becomes broader as the EO% decreases from 80 to 40, *i.e.*, a copolymer of small EO% has an elution behaviour like PPG, while a copolymer of large EO% behaves like PEG.

The chromatographic separation of non-ionic surfactants can be predicted from the elution data described above. Practically, a complete chromatographic separation is possible if the difference in log k' values is greater than 0.5. For instance, the separation of R-EO and R-PO can easily be performed because, as shown in Fig. 1 and 2, the difference in log k' is large in most cases. Fig. 5a shows the separation of C_{12}-EO_{12} and C_{12}-PO_{10}. In this example, the difference in log k' is 0.69 and the resolution is calculated from the chromatogram to be 96%. As shown in Figs. 1 and 3, the values of R-PO/EO are close to those of R-EO and a complete separation of these types of non-ionic surfactants not be achieved. The separation of C_{12}-PO_mEO_n and C_{18}-PO_m was achieved fairly easily.

The separation of block copolymers from other non-ionic surfactants was investigated. The separation of PEG and R-EO has already been reported[9–13]. The block copolymers should behave like PEG when the weight percentage of EO in the molecule is very large. As expected, the behaviour of the copolymer was similar to PEG, but its peak is broader and the retention time is slightly longer than that of

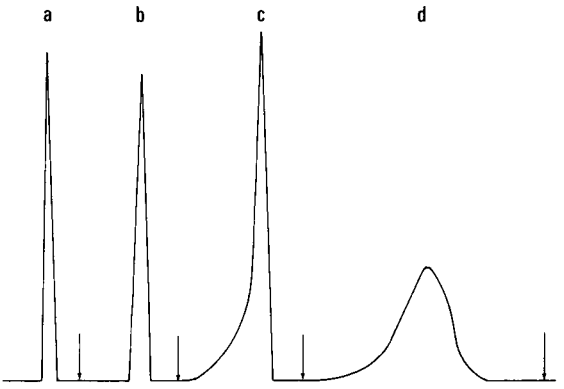


Fig. 4. Typical chromatogram of PEG, the block copolymer, and PPG. a, PEG 4000; b, copolymer (EO% = 80); c, copolymer (EO% = 50); d, PPG 4000.

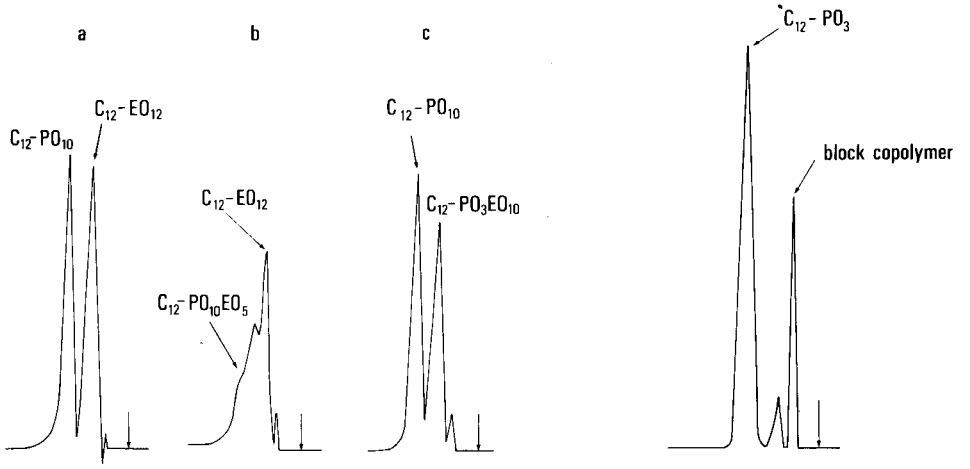


Fig. 5. Separation of alkylene oxide type non-ionic surfactants. a, C_{12}-EO_{12}, C_{12}-PO_{10}; b, C_{12}-EO_{12}, C_{12}-$PO_{10}EO_5$; c, C_{12}-PO_3EO_{10}, C_{12}-PO_{10}.

Fig. 6. Separation of C_{12}-PO_3 and the block copolymer (EO% = 80).

PEG. Fig. 6 shows the separation of the block copolymer and C_{12}-PO_3. The small peak between the peaks of the block copolymer and C_{12}-PO_3 is PPG, a by-product.

As described above, the relation between log k' and the degree of polymerization of EO or PO adducts is found to be linear. This relation is useful not only for the prediction of chromatographic separation but also for the estimation of the degree of polymerization and of the alkyl chain length of alkylene oxide type non-ionic surfactants.

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Note

Determination of precise retention indices of steroids using an all-glass solid injection system

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Accurate and precise retention index data of steroids are required for their identification and the determination of physicochemical properties. The experimental factors that affect the determination of precise retention are the accuracy of the carrier gas pressure, column temperature and the method of "dead time" determination.

The use of a precise pressure controller has minimized the effect of pressure fluctuations on retention[1], and temperature variations have been decreased to 0.2°C by placing the column inside an aluminium block[2]. Methods of "dead time" determination have been summarized by Kaiser[3] and Wainwright and Haken[4]. Ezrets and Vigdergauz[5] estimated the error in the retention index due to the solubility of gases used for measuring the "dead time".

Since the introduction of gaseous samples by all-glass solid injection systems is not possible, the "dead time" has to be determined indirectly. The three equidistant *n*-alkanes method[6] may result in considerable errors due to extrapolation through more than twenty carbon atoms when steroid samples are used. The solvent front or *n*-pentane peak maxima, although used extensively, are limited to analyses at high column temperatures[7,8].

The present paper describes the determination of precise retention indices of steroid derivatives, directly by introducing methane through a solid injection system for "dead time" measurement, and indirectly by converting arithmethic or linear retention indices, I_A, into retention indices, I, by the method developed in this laboratory[9].

EXPERIMENTAL

A Pyrex glass capillary column (30 m × 0.25 mm) was deactivated with 1% benzyltriphenylphosphonium chloride solution in methylene chloride and coated by the static procedure[7] with 0.5% (w/w) SE-30 in *n*-hexane.

A self assembled gas chromatograph and a flame ionization detector with well controlled pneumatics (Becker, Delft, The Netherlands) was fitted vertically with an all-glass solid injector. Shrinkable PTFE tubing was used to connect the glass capillary column which was housed inside an aluminium block designed by Krupcik *et*

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al.[2]. Temperature gradients of 0.5, 0.6 and 0.7°C inside and 6.5, 7.0 and 7.5°C outside the aluminium block were observed, when the column oven was maintained at 221, 231 and 241°C respectively. The inlet pressure of the nitrogen carrier gas was maintained within 0.005 atm by using two fine pressure regulators in series.

In order to introduce methane and other *n*-alkane (C_5–C_8) vapours directly, a Swagelok T-joint with a self sealing septum was connected to the carrier line prior to the solid injector.

A misture of *n*-alkanes (C_{22}–C_{30}) dissolved in *n*-hexane and methoxime trimethylsilyl (MO-TMS) derivatives of steroids, prepared as described elsewhere[8], were injected. The retention data measured were averages from three injections.

RESULTS AND DISCUSSION

Accurate retention indices of steroids can be determined either directly by using adjusted retentions from methane injection or indirectly by unadjusted retentions from an arithmetic index system[9].

Direct methane injection

In order to determine the methane retention time using the solid injection system, a T-joint was attached as mentioned earlier. The vapours of lower *n*-alkanes (C_5–C_8) and methane were injected individually in the T-joint and the retention times of alkanes and methane (t_{CH_4}) were measured. Liquid *n*-alkanes (C_5–C_8) were injected using the solid needle after reduction in volume by evaporation, in order that the respective peaks fitted on the scale. The difference between the retention times of the vapour and liquid of each alkane was measured and found to be constant (say t_c). Therefore, the column "dead time" *i.e.*, the retention time of methane injected using a solid needle, can be calculated: dead time = $t_{CH_4} - t_c$. The "dead time" determined in this way was found to be 165, 174 and 183 sec at 221, 231 and 241°C respectively and the retention indices for some standard steroids derivatives are given in Table I.

Indirect arithmetic index

It has been demonstrated[9,10] that linear or arithmetic retention indices can be measured more accurately than retention indices. The arithmetic retention indices for MO-TMS derivatives of steroids were calculated using the non-adjusted retention times (see Table I). In order to determine the retention indices from arithmetic indices, the graphical and analytical method developed by Mitra[9] has been utilized. The mathematical relation employed is

$$\frac{I_A - 100\,n}{100} = \frac{\sigma^{(I-100n)/100} - 1}{\sigma - 1} \tag{1}$$

where

$$\sigma = \frac{t_{Rn+2} - t_{Rn+1}}{t_{Rn+1} - t_{Rn}} = \frac{t_{Rn+1} - t_{Rn}}{t_{Rn} - t_{Rn-1}}$$

Here t_{Rn+2}, t_{Rn+1}, t_{Rn} and t_{Rn-1} are the unadjusted retention times of the standard *n*-alkanes of carbon number, *n*, between which the components are eluted.

TABLE I

RETENTION INDEX DATA OF MO-TMS DERIVATIVES OF STEROIDS ON AN SE-30 GLASS CAPILLARY AT VARIOUS TEMPERATURES

Compound	*Retention time (sec) at*			*Arithmetic indices at*			*Retention indices from methane at*			*Retention indices from I_A at*		
	221°C	*231°C*	*241°C*	*221°C*	*231°C*	*241°C*	*221°C*	*231°C*	*241°C*	*221°C*	*231°C*	*241°C*
Methane	165.0	174.0	183.0	100.0	–	–	100.0	–	–	100.0	–	–
n-C_{24}	759.0	574.0	459.5	2400.0	–	–	2400.0	–	–	2400.0	–	–
Androsterone	909.5	690.0	560.5	2473.4	2487.9	2509.8	2476.2	2489.3	2511.2	2476.1	2489.2	2511.2
Etiocholanolone	947.0	712.0	574.5	2491.7	2503.5	2521.7	2492.7	2504.0	2524.3	2492.1	2504.7	2524.1
n-C_{25}	964.0	706.0	549.0	2500.0	–	–	2500.0	–	–	2500.0	–	–
Dehydro-epiandro-sterone	1080.5	804.5	641.5	2542.4	2557.3	2578.7	2546.0	2560.6	2580.2	2546.2	2560.6	2580.1
n-C_{26}	1239.0	878.0	666.5	2600.0	–	–	2600.0	–	–	2600.0	–	–
Pregnanolone	1338.0	962.5	746.5	2626.8	2637.1	2652.5	2629.8	2640.4	2656.0	2629.1	2640.3	2655.6
Testosterone	1363.5	990.0	772.5	2633.6	2649.1	2669.5	2637.0	2652.6	2672.4	2636.9	2652.4	2672.2
Estradiol	1460.0	1040.5	798.5	2659.7	2671.3	2686.6	2663.2	2674.0	2688.1	2662.9	2674.1	2688.0
11-β-Hydroxyetio-cholanolone	1607.5	1133.0	861.5	2699.6	2708.9	2721.3	2700.0	2710.2	2723.8	2700.0	2710.2	2723.4
n-C_{27}	1609.0	1106.0	819.0	2700.0	–	–	2700.0	–	–	2700.0	–	–
Allo-Pregnanediol	1919.5	1334.0	1001.5	2762.2	2775.5	2791.4	2765.6	2777.9	2792.7	2765.6	2778.0	2792.4
Pregnanediol	2005.0	1378.5	1025.0	2779.4	2790.2	2802.3	2781.6	2791.4	2803.1	2781.7	2791.3	2802.6
n-C_{28}	2108.0	1408.0	1018.0	2800.0	–	–	2800.0	–	–	2800.0	–	–
Estriol	2678.0	1784.0	1290,5	2884.9	2894.2	2903.7	2886.7	2894.9	2904.2	2886.6	2894.6	2904.3
n-C_{29}	2799.0	1807.0	1278.0	2900.0	–	–	2900.0	–	–	2900.0	–	–
n-C_{30}	3690.0	2337.0	1616.5	3000.0	–	–	3000.0	–	–	3000.0	–	–

The σ values determined for all the sets of alkanes are generally found to be constant for a given stationary phase and column temperature. In this case, the average σ values were found to be 1.349, 1.325 and 1.304 at 221, 231 and 241°C on SE-30, and have been used for the calculation of retention index values (see Table I).

The retention index values determined by using the solvent front peak as "dead time" were comparable with those obtained by the direct and indirect methods as the column temperatures were quite high.

In conclusion, the measurement of the arithmetic retention indices of steroid derivatives when working with a solid injection system and lower column temperature yields precise and reproducible results, and has the added advantage of giving accurate retention index values, indirectly, if needed.

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Note

Novel system for separation of phospholipids by high-performance liquid chromatography

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Only a few methods for the separation of phospholipids by high-performance liquid chromatography (HPLC) have been reported[1–3] and none of them resolve all the major phospholipids found in cell membranes. We have developed a method which accomplishes this and also separates the newly characterized platelet-activating factor[4,5] from other phospholipids.

Retention times were determined by using radioactive phospholipids and a flow-through radioactivity detector. Use of a stream splitter enabled part of the effluent from the column to be diverted to a fraction collector for confirmation of radioactivity measurements. The system has advantages over thin-layer chromatography (TLC) because resolution of phosphatidylinositol from phosphatidylserine is obtained, recovery of individual phospholipids is excellent and there is no need to elute material from zones on TLC plates for subsequent analyses.

METHODS

A Waters Assoc. liquid chromatograph equipped with a Model 6000A solvent delivery system was used for HPLC. The column employed was a 250 × 4.6 mm I.D. LiChrosorb Si60 (10-μm particle size) purchased from Rainin Instrument, Woburn, MA, U.S.A. The radioactive flow detector was purchased from Radiomatic, Tampa, FA, U.S.A. The system was set up as shown in the flow chart (Fig. 1). It consisted of two solvent delivery pumps, an injector port and a solvent programmer. The effluent from the column was divided by means of a stream splitter. Half went into the radio flow detector and half into the fraction collector for liquid scintillation counting. Miniscint (Radiomatic) was continually pumped through the radio flow detector which was equipped with a built-in integrator. A chart recorder was connected to the above device which recorded the profile of radioactive components as they eluted from the column. HPLC grade solvents were obtained from Fischer Scientific, King of Prussia, PA, U.S.A. Deionized water was filtered through a millipore filter (0.45 μm). All organic solvents were degassed by sonication. Phosphati-

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dylcholine (dipalmitoyl-1-^{14}C, SA 60–100 mCi/mmole), phosphatidylethanolamine (dipalmitoyl-1-^{14}C, SA 95.0 mCi/mmole), sphingomyelin (choline-methyl-^{14}C, SA 40–60 mCi/mmole), prostaglandin-E_2, (5,6,8,11,14,15-^{3}H(N))-, SA 165 Ci/mmol) and [^{3}H]6-keto-prostaglandin $F_{1\alpha}$ (SA 120 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Uniformly labelled [2-^{3}H]arachidonyl phosphatidylinositol (SA 30 Ci/mmole) and ^{3}H-platelet-activating factor (1-O-alkyl[2-^{3}H]acetyl glyceryl-3-phosphorylcholine, SA 50 Ci/mmole), were gifts from New England Nuclear. Phosphatidyl-L-^{14}C-serine (SA 25 mCi/mmole) and [1-^{14}C]arachidonic acid (SA 54 mCi/mmol) were purchased from Amersham, Arlington Heights, IL, U.S.A. [^{14}C]Lysophosphatidylcholine was prepared from [^{14}C]phosphatidylcholine by the methods of Wells and Hanahan[6]. [^{14}C]Lysophosphatidylcholine was purified by TLC on silica gel GH plates (Analtech, Newark, NJ, U.S.A.) using a modification of Skipski's solvent system[7], chloroform–methanol–acetic acid–water (100:44:10:3, v/v).

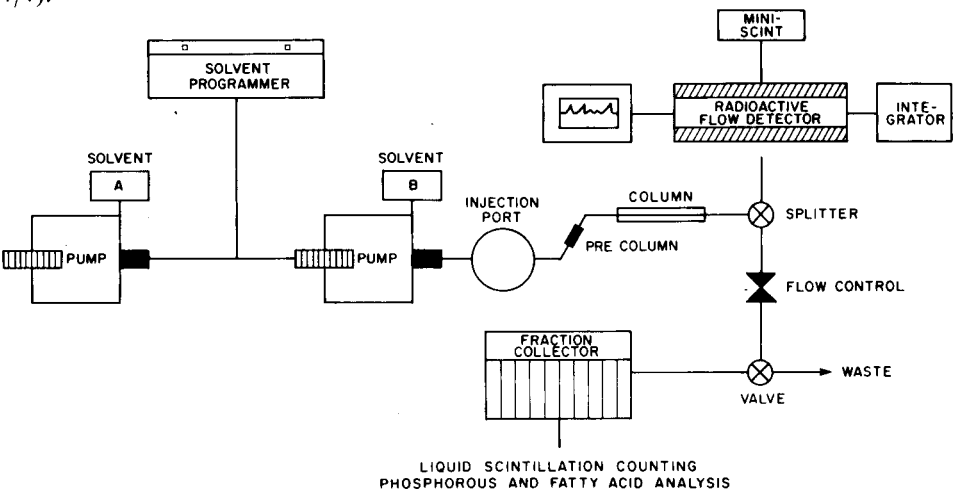

Fig. 1. Flow chart of HPLC system.

RESULTS AND DISCUSSION

The elution profile of the different phospholipids studied is shown in Figs. 2 and 3. Fig. 2 shows the recording of radioactivity (in arbitrary units) obtained directly from the Radio-Flow-One detector. Fig. 3 shows the radioactivity in individual fractions collected at 1-min intervals and measured by liquid scintillation counting. These separations were obtained on a normal-phase LiChrosorb Si60 column under isocratic conditions. The mobile phase consisted of two solvents A and B. Solvent A was a mixture of propanol–ethyl acetate–benzene–water (130:80:30:20, v/v) and solvent B was propanol–toluene–acetic acid–water (93:110:15:15, v/v). Solvent A was pumped at a flow-rate of 1 ml/min for 20 min. Prostaglandins (PGs) and arachidonic acid (AA) were eluted by 7 min (Fig. 3) and a clean phosphatidylethanolamine (PE) fraction came off by 20 min. A change in flow-rate from 1 to 1.5 ml/min enabled the elution of phosphatidylinositol (PI) by 30 min. Elution with solvent B, at 1 ml/min, was started at 36 min to elute phosphatidylserine (PS), followed by phosphatidyl-

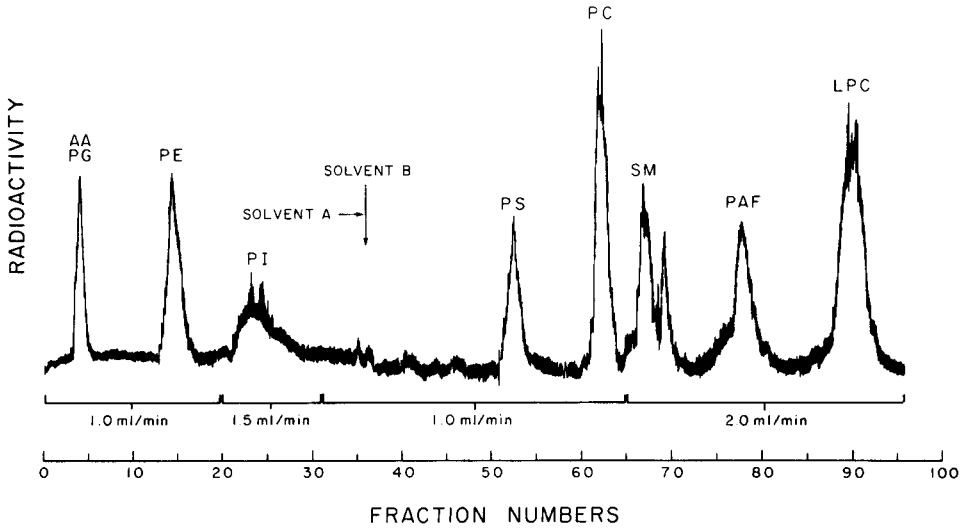

Fig. 2. Radioactivity recording from the Radio-Flow-One detector. For abbreviations, see text.

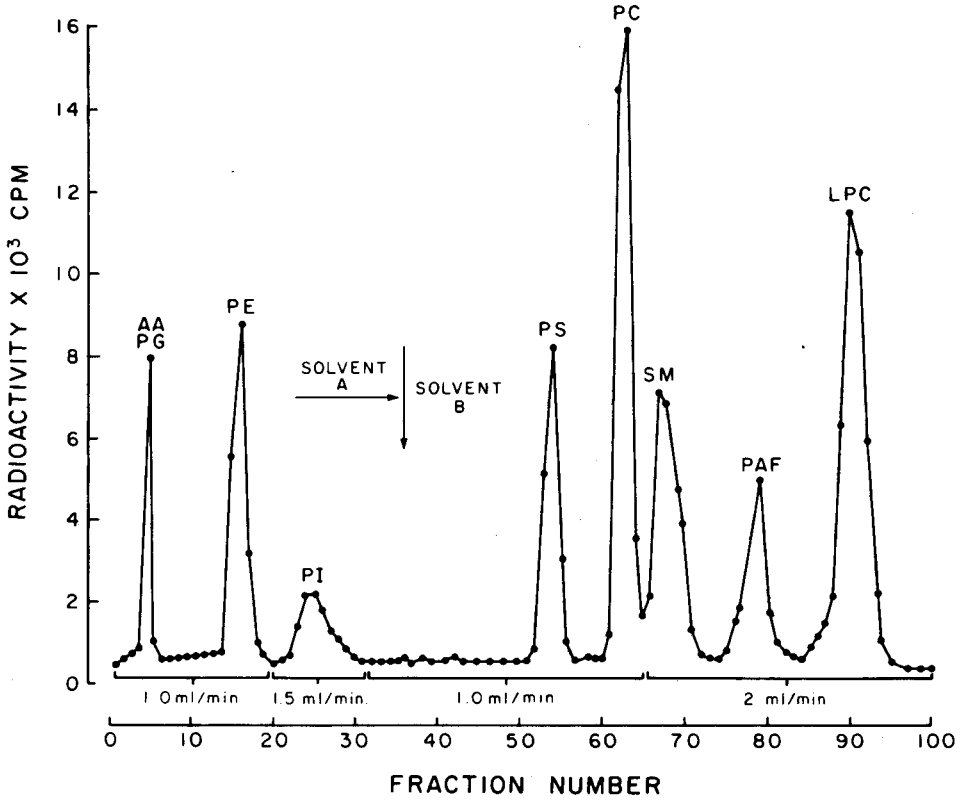

Fig. 3. Radioactivity recording from liquid scintillation counting. For abbreviations, see text.

choline (PC). At 65 min, after the elution of phosphatidylcholine, the flow-rate was increased to 2 ml/min to obtain complete resolution of sphingomyelin (SM), platelet activating factor (PAF) and lysophosphatidylcholine (LPC). The total analysis time was 100 min. The retention times of individual phospholipids analysed alone or in mixtures were highly reproducible and rarely varied by more than 1 min. Recovery of radioactivity was always greater than 90%.

Our approach for the complete separation of PE from PS and PI was based on the use of the non-amphoteric solvent ethylacetate in combination with polar solvents on a column of deactivated silica. Although detection of phospholipids by UV absorption has been used in combination with HPLC it is inappropriate when solvents such as benzene and toluene, which are excellent for separation of phospholipids but which strongly absorb UV light, are used. We obviated this difficulty by using a flow-through detector to monitor continuously the radioactivity in the effluent from the HPLC column.

The use of a stream splitter allowed us to collect fractions and count the radioactivity to validate the results obtained with the flow-through detector. This analysis revealed that the flow-through detector was superior because it monitors radioactivity every 4 sec, whereas samples were collected only at 1-min intervals by the fraction collector. For example, the radio-labelled sphingomyelin standard, which was 99% homogeneous by TLC and appeared as a single peak using the fraction collector (Fig. 3) was resolved into two components by the flow-through detector (Fig. 2). The stream splitter also will allow for collection of samples, free of silicic acid or TLC binders, which can be used for phosphorus, fatty acid or other analyses. Finally, this system should be of value in experiments involving radioactive labelling of cell membrane phospholipids as well as for the purification of individual phospholipids.

ACKNOWLEDGEMENT

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Note

Separation of optical isomers by zwitterion-pair chromatography

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Pirkle *et al.*[1] reminded chromatographers that, to achieve chiral recognition or resolution of a chiral solute, it is necessary to arrange for a three-point interaction between the solute and an optically active component of the eluent or stationary phase. So far, attempts to use chiral ion-pairing agents or chiral bonded phases have been unsuccessful, except when an extremely rigid chiral ion-pairing agent has been used[2,3], or alternatively when one is chromatographing what are essentially diastereoisomeric complexes, such as the copper complexes of amino acids[4,5].

Recently, we developed the technique of zwitterion-pair chromatography, which has proved particularly applicable in the separation of zwitterionic solutes such as nucleotides[6,7], coenzymes[7] and drugs[8]. Our studies[7,8] have indicated that the enhanced retention provided by addition of a zwitterion-pairing agent arises from the formation of quadrupolar ion pairs. This interaction occurs within a "pH window" where both the pairing agent and solute exist as zwitterions and is maximal towards the centre of this window. We have postulated[8] that, since the formation of quadrupolar ion pairs provides two strong interactions between solute and eluent, it should be possible with only one further interaction, say through hydrogen bonding or Van der Waals' repulsion, to achieve chiral recognition using an optically active zwitterion-pairing agent added to the eluent.

Following this line of thought, we now report the separation of enantiomers of tryptophan and of glycylphenylalanine using L-leucyl-L-leucyl-L-leucine as a zwitterionic pairing agent.

EXPERIMENTAL

Liquid chromatography was carried out using a thermostatted photometer/column oven unit (Shandon Southern Products, Runcorn, Great Britain), maintained at 25°C. The eluent was pumped by a single-piston reciprocating pump (Model 110; Altex, Berkeley, CA, U.S.A.). Columns were 125 × 5 mm I.D. (Shandon Southern Products) and were packed with ODS-Hypersil (Shandon Southern Products) using isopropanol as the slurry liquid followed by 200 ml of hexane pumped at the pressure of 7000 p.s.i.

Eluents were water–methanol (88:12), the pH being adjusted with 1 m*M* phosphate buffer (Na_2HPO_4 + H_3PO_4). Methanol was of HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). L-Leucyl-L-leucyl-L-leucine (LLL), DL-tryp-

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tophan, D-tryptophan, L-tryptophan, glycyl-DL-phenylalanine, glycyl-D-phenylalanine and glycyl-L-phenylalanine were obtained from Sigma (Poole, Great Britain): other reagents were of AnalaR grade (BDH, Poole, Great Britain).

Samples of solutes were dissolved in water–methanol (88:12) and introduced into the column by Rheodyne injection valve (Model 7120; Rheodyne, Berkely, CA, U.S.A.).

RESULTS AND DISCUSSION

We reported earlier[8] that LLL is strongly adsorbed from the above eluent by ODS-Hypersil, giving a surface concentration of 115 μmol/g or *ca.* 0.6 μmol/m^2, and that it could successfully be used as zwitterion-pairing agents for nucleotides and coenzymes (NADH, NaDPH). Accordingly, LLL has been used in this study as a chiral ion-pairing agent in an attempt to separate racemic mixtures of tryptophan and glycylphenylalanine.

When LLL is added to the eluent (pH 6.4) at a concentration of 2 m*M*, the single peaks for DL-tryptophan and glycyl-DL-phenylalanine split into doublets of equal area, as shown in Fig. 1A. The separation factors are 1.14 for tryptophan and

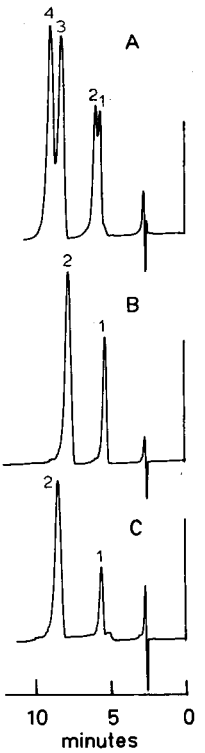

Fig. 1. A, Separation of racemic mixtures of tryptophan and glycylphenylalanine: Packing, ODS-Hypersil; eluent, water–methanol (88:12) containing 2 m*M* LLL and 1 m*M* phosphate buffer (pH 6.3); temperature, 25°C; flow-rate, 0.6 ml/min; detector, UV photometer, 254 nm. Peaks: 1 = glycyl-L-phenylalanine; 2 = glycyl-D-phenylalanine; 3 = L-tryptophan; 4 = D-tryptophan. B, Separation of L-tryptophan (2) and glycyl-L-phenylalanine (1). C, Separation of D-tryptophan (2) and glycyl-D-phenylalanine (1).

1.11 for glycylphenylalanine. Fig. 1B and 1C show chromatograms of the individual L- and D-forms, respectively, and establish that the L-form of each compound is eluted first.

It should be noted that, during this work, we observed that the presence of heavy metal ions seriously impaired the resolution of the enantiomers. This could have arisen from the formation of amino acid–metal ion complexes as reported elsewhere[4,5,9]. This view was supported by the observation that addition of copper ions to the eluent completely destroyed the capability of added LLL to enhance solute retention in normal zwitterion-pair chromatography. We have also noted that chiral resolution is impaired if reagents are not of AnalaR quality.

CONCLUSIONS

Our results provide the first example of enantiomer separations with use of ion-pairing agents in the absence of complexing metal ions. They add strong further confirmation to our basic hypothesis that zwitterion-pair chromatography is effected through formation of quadrupolar ion pairs in the stationary phase.

We believe that the method can be exploited in the resolution of a wide range of chiral amino acids.

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We wish to acknowledge the valuable assistance of Dr. R. A. Wall, who made the original suggestion that LLL might be used as a zwitterion-pairing agent for chiral resolution. We also gratefully acknowledge the support for this work provided by the Science Research Council of Great Britain.

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Note

Rapid concentration and analysis of short chain carboxylic acids: variation on a theme

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Short-chain carboxylic acids (C_1–C_5) are ubiquitous in natural systems, such as food and drink[1–5], living organisms[6–19] and water bodies and sediments[20–22], and are widely studied. However, problems can arise in analysis, since these are polar, comparatively volatile compounds, which, in biological systems, may make up only a small proportion of the total organic content. The most popular analytical technique is gas chromatography (GC) of the underivatized acids, and whilst most problems associated with GC of such polar compounds (reviewed by Ackman[23]) can be overcome with care, the exception is insensitivity to the flame-ionization detector (FID). Thus an appropriate derivative of an acid can be used to either increase GC sensitivity or facilitate use of high-performance liquid chromatography (HPLC). Formation of the *p*-bromophenacyl ester[30] was chosen from the methods available, *e.g.* refs. 24–37, as this reaction is easy to use, tolerant to water and the ester formed has a strong UV absorbance.

However, the literature provides few satisfactory ways for rapid preparation of aqueous samples ready for derivatization. Solvent extraction with diethyl ether has been used previously[38–43], and was adapted for this work to provide a concentrated, salt-free aqueous solution of short-chain carboxylic acids, which can be analysed directly by GC, or derivatized for GC or, here, HPLC. This technique is currently in use to study concentrations of carboxylic acids in the snail hosts of schistosomiasis, and in decaying plants on which they feed.

EXPERIMENTAL

Glassware

All glassware was scrubbed in detergent, then rinsed in hot and cold tapwater and finally in double distilled (DD) water (the second distillation from alkaline potassium permanganate solution).

Reagents

p-Bromophenacyl bromide (Aldrich) and 18-Crown-6 (Aldrich) were co-crystallized (10:1 ratio) after dissolution in HPLC-grade acetonitrile (Rathburn Chemicals) by addition of excess DD water, then filtered and dried *in vacuo*. The dried mixture was made up as a 10^{-2} *M* solution of the bromide/10^{-3} *M* of the crown

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ether in acetonitrile, and stored in the dark. Potassium hydrogen carbonate (AnalaR; BDH) was recrystallized after dissolution in DD water, by addition of excess methanol (AnalaR; BDH). The purified chemical was used either as the solid, or as a 0.1 *M* solution in DD water. Hydrochloric acid (1 *M*) was prepared by dilution of a concentrated solution (AnalaR; BDH) and was extracted with diethyl ether (May & Baker) before use. Carboxylic acid standards (acetic and propanoic acids: BDH; butanoic acid: Sigma) were made up, without purification, as concentrated solutions (0.1 *M*) in DD water and stored frozen.

Apparatus

The HPLC system comprised a Milton Roy Minipump (5000 p.s.i.g. maximum) with pulse dampening, a variable volume loop injector (typically 10 μl injected) coupled to a μBondapak C_{18} column (30 cm × 3.9 mm I.D.; Waters Assoc.). Column effluent passed through a 8-μl flow cell in a Carl Zeiss UV/Vis variable wavelength spectrophotometric detector set at 254 nm. The mobile phase was acetonitrile–water (50:50), flow-rate 1 ml/min.

Procedure

To an aqueous sample (1–2.5 ml, pH $\approx$ 7) in a centrifuge tube (*ca.* 10 ml) was added sufficient 1 *M* HCl (0.1–0.25 ml) to make the pH $<$ 2. Diethyl ether (2–3 × aqueous volume) was added and the two layers mixed (30 sec) using a Whirlimixer (Fisons). If necessary, the solutions were centrifuged briefly (1500 *g*; 2 min) to separate the two layers. The diethyl ether layer was transferred, using a pasteur pipette, to a vial ($\geqslant$7 ml total volume). Extraction with ether was repeated once or twice more (see Table I for exact conditions). The ether was reduced, if necessary, to *ca.* 6 ml under a stream of nitrogen, and then placed in a deep-freeze (-20°C, 30 min)

TABLE I

RECOVERY OF BUTANOIC ACID (0.5 μMOLES) FROM (i) WATER (1 ML) CONTAINING KNOWN CONCENTRATIONS OF INORGANIC SALTS[44] AND (ii) NEUTRALIZED 2 *M* PERCHORIC ACID (2.5 ML)

	Experiment	*No.*	*Recovery of butanoic acid (μmoles)***	*Recovery (%)*	*Mean*	±	*Std. dev.*
(i)	Water + salts*	1	0.41	83			
		2	0.42	83			
		3	0.37	75	79		4
		4	0.38	76			
		5	0.39	77			
(ii)	Neutralized	1	0.31	62			
	perchloric acid**	2	0.29	57			
		3	0.22	43			
		4	0.16	32	46		13
		5	0.18	35			

* Extracted with 2 × 3 ml diethyl ether.
** Extracted with 3 × 6 ml diethyl ether.
*** Determined by HPLC of *p*-bromophenacyl ester and comparison with standard.

so that trace amounts of water (containing high concentrations of HCl and salts) crystallized out. The dry and inorganic salt-free ether remained liquid and was decanted or filtered in the deep-freeze into a small vial with gas-tight screw top [either a 7-ml McCartney vial with rubber liner, or preferably a 3.5-ml Reactivial (Pierce), with PTFE/silicone liner]. Aqueous $KHCO_3$ ($\leqslant$0.1 ml of a 0.1 *M* solution) was added to the ethereal solution; the vial was capped and Whirlimixed (1 min). At the end of this period, the pH of the water layer was tested by applying 0.2 μl (using a 1 μl SGE syringe) to pH paper (1–14 range). If the pH was 7–8, the aqueous layer was made up to 0.1 ml total with DD water. If pH < 7, more aqueous $KHCO_3$ was added up to a total volume of 0.1 ml. The addition of $KHCO_3$ converts any carboxylic acid present into the potassium salt necessary for the derivatization. Finally, the ether layer was evaporated off under a stream of nitrogen.

The carboxylic acids (K^+ salts) in the aqueous layer (0.1 ml) were esterified by addition of excess of the *p*-bromophenacyl bromide/18-crown-6 solution in acetonitrile, made up to 1 ml total volume with acetonitrile. After heating (80°C, 20–30 min), the *p*-bromophenacyl esters were analyzed by HPLC. Structures were confirmed in two ways: (i) by co-injection and/or comparison of retention times of authentic standards on HPLC; (ii) by separation of the esters on thin-layer chromatography (TLC) (silica gel G; hexane–diethyl ether, 7:3, eluent). The following R_F values were obtained: *p*-bromophenacyl (*p*-bpa) bromide, 0.82; *p*-bpa butanoate, 0.68; *p*-bpa propanoate 0.56; *p*-bpa acetate, 0.38. Individual bands were removed, the ester recovered and re-analyzed by HPLC.

RESULTS AND DISCUSSION

The results of two solvent extraction experiments, chosen as typical of different sample origins, are presented in Table I. Sample i, an inorganic salt solution[44] spiked with butanoic acid (0.5 μmoles), represents a marine or freshwater source, and here contained calcium chloride (2 m*M*) as the major salt. Sample ii was perchloric acid (2 *M*), again spiked with butanoic acid (0.5 μmoles), which was neutralized with solid $KHCO_3$. Perchloric acid was chosen because it is commonly used for digesting organisms since it degrades proteins, thus preventing enzymatic action in tissues after death. Both experiments gave good recoveries of the small amount of the standard: *ca.* 79% for sample i and *ca.* 46% for sample ii (determined by HPLC: see Fig. 1 for example analysis of standard compounds). The lower recovery and high standard deviation in the latter case is not surprising because of the larger number of steps involved in sample preparation; in particular, loss through volatilization could occur when perchloric acid is neutralized with $KHCO_3$. It is worth noting that the acids are never reduced to dryness by this method (in contrast to typical solvent extraction, and also freeze drying, a common concentration technique for aqueous samples). In particular, adding aqueous $KHCO_3$ before removing the diethyl ether under nitrogen, added significantly to the efficiency of recovery.

The main departure, however, from standard solvent extraction is the removal by freezing of trace quantities of inorganic salt-containing water carried over with the diethyl ether. This step is important, particularly if the acids are to be derivatized. Even with direct analysis by GC (*i.e.*, acids not derivatized), high concentrations of inorganic ions can contaminate injectors and/or columns. In this case, the standard

was esterified to form the *p*-bromophenacyl ester[30]. This reaction has the advantage of tolerance to quite high proportions of water (> 10 %); but it was found here to be sensitive to chloride ion, whether as hydrochloric acid or potassium chloride. With chloride ion present, *p*-bpa bromide (starting material) "decays" to form a compound which co-elutes with propanoate ester on the reversed-phase column used here (Fig. 1). This "decay" product may be *p*-bpa chloride as (i) it is non-reactive for esterification purposes, (ii) it cannot be separated on silica gel G TLC (hexane–diethyl ether, 7:3, eluent) from the bromide ($R_F \approx 0.82$), although readily separable from the propanoate ester ($R_F \approx 0.56$) under these conditions. In fact, TLC was found to be an excellent method for purification of samples, or for structure confirmation, since the acids (as esters) can be separated into individual compounds (see Experimental for R_F values).

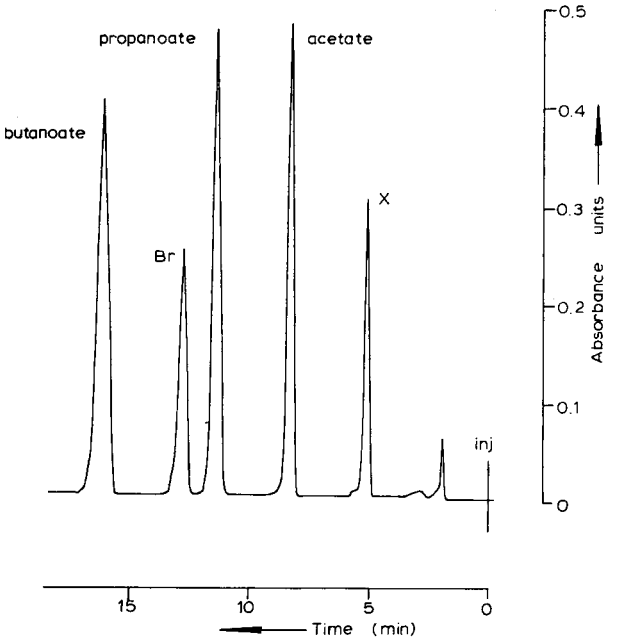

Fig. 1. HPLC separation of standard carboxylic acids (C_2–C_4) as *p*-bromophenacyl esters. Conditions: 10 µl (5 nmoles per component) loop injection on µBondapak C_{18} column; acetonitrite–water (50:50) elution; flow-rate 1 ml/min; UV detection, 254 nm. Br = *p*-bromophenacyl bromide; X = reaction by-product from excess $KHCO_3$.

A number of different tests were undertaken to confirm the origin of the "decay" compound. It was formed under all the following experimental conditions, when chloride was present, and never when chloride was excluded: (i) µmole amounts of neutral KCl were added to a reaction mixture containing only the bromide and crown ether; (ii) samples containing chloride were heated in stirred and unstirred sealed vials or (iii) in an open system under reflux; (iv) samples were treated to preliminary ion exchange (K^+ form) only, without addition of HCl or solvent extraction; (v) an acidified aqueous medium was extracted/esterified in a two phase, single-step process using dichloromethane/phase transfer reagent $[(C_4H_9)_4NBr]$/*p*-bromophenacyl bromide (similar to procedure of L'Emeillat *et al.*[45]).

Because the presence of this "decay" product can be taken as evidence for propanoate on reversed-phase columns, exclusion of inorganic salts generally and chloride especially proved essential. Solvent extraction, then removal by freezing of traces of salt-containing water, is an efficient, reliable and speedy way of concentrating comparatively volatile carboxylic acids in good yield, free from both inorganic salts and ether-insoluble organic matter.

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Note

Simple chromatographic separation of *para*- and *ortho*-hydrogen and -deuterium

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From time to time the need arises to analyse the relative *para* and *ortho* concentrations of hydrogen and deuterium. The most satisfactory method of analysis is by the chromatographic separation of the spin isomers, and numerous such spin separations have been reported since the original work of Moore and Ward[1], and Van Hook and Emmett[2]. The best separations have been obtained with glass capillary columns operating at 77°K (ref. 3), or at lower temperatures[4,5]. However, glass capillary columns have a number of drawbacks. They require special etching before they are suited to this particular application, and they seem to turn into relatively expensive and complex systems.

An inherently simple technique involves the use of a copper column filled with activated alumina and operated in a bath of liquid nitrogen. Le Roy and co-workers[6–8] have used such a column with n-H_2 as the carrier. They have obtained partial separations of deuterium and were able to detect the excess *para*-hydrogen in a sample. A generally similar alumina system has been reported by Van Urk and Lindner[9] who used n-H_2 as the carrier gas for a partial separation of deuterium and a Ne–He mixture as the carrier gas for a complete separation of hydrogen. Quickert and Le Roy[7] have given a detailed analysis of the characteristics of their chromatographic separation system. A typical retention time for deuterium was approximately 7 min, and the separation factor for the partially resolved p-$^2H_2/o$-$^2H_2$ peaks was about 1.1. Such alumina columns have a relatively high pressure drop (about 12 p.s.i.) at relatively low flow-rates (50 ml/min) and require a stable gas flow.

During the experiments reported here, an attempt was made to reproduce the previous results for alumina columns[6,9] using different mesh sizes of commercial chromatographic grade alumina. Only partial success was obtained and the separations (of *para*- and *ortho*-hydrogen, for example, using a He–Ne carrier) seemed to be dependent on individual samples of alumina as if random impurities were present in different batches of the alumina. The results were characterized by incomplete separations, shifting baselines, and long separation times. (A variety of other commercially available columns[8] packing was also tried, such as etched glass microspheres, with no success.)

A batch of 28–32 mesh alumina was prepared using as starting material irregular hunks (3–4 mesh) of commercial alumina. Particular care was taken to avoid the presence of ferromagnetic impurities which might act as a catalyst on the spin

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isomers. The final alumina powder was poured into a new tube of soft copper, 4 m × 5/32 in. I.D. The copper tube was then wound into a column and baked for 48 h at 475°C with nitrogen (99.9% purity) flowing through the alumina.

Before an experiment on hydrogen or deuterium separation, the activated column was flushed at room temperature with the particular carrier gas to be employed and then immersed in a bath of liquid nitrogen. Signal detection was by a thermal conductivity detector (consisting of a bridge of four Gow-Mac tungsten filaments; bridge current 250 mA) which was also immersed in the liquid nitrogen bath.

Excellent separations of deuterium were obtained using hydrogen as a carrier, and of hydrogen using deuterium as a carrier; typical recorder traces are shown in Fig. 1a and b. The observed separation factors were 1.23 for o-H_2/p-H_2 (n-$^2H_2$ carrier) and 1.55 for p-$^2H_2$/o-$^2H_2$ (n-H_2 carrier). These separation factors compare favourably with those observed using glass capillary columns at lower temperatures[3,4]. In the present experiments the carrier gas flow-rate was set in the range of 12 to 14 ml/sec, which required a pressure drop of 4 to 5 p.s.i. across the column. The total time of analysis was about 150 sec, much faster than previously reported.

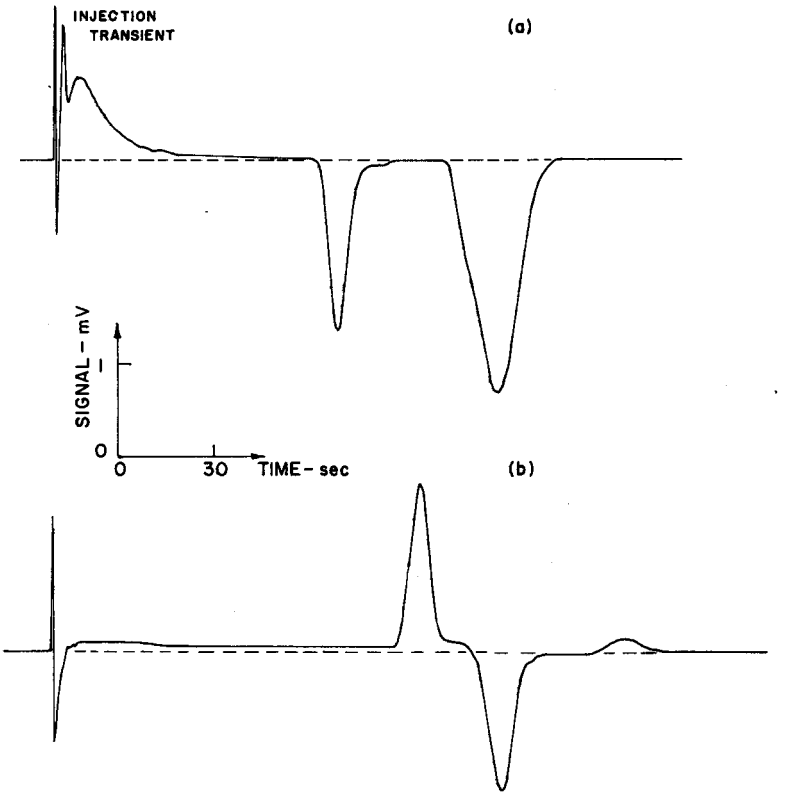

Fig. 1. (a), Separation at 77°K of n-$2H_2$ in an alumina column using n-H_2 as the carrier gas. (b), Separation at 77°K of n-H_2 in the same column as for (a), using n-$^2H_2$ as the carrier gas. In both cases the thermal conductivity detector was at 77°K.

Separations were also attempted using He and a Ne–He mixture as carrier gases. The helium carrier provided a separation for hydrogen (but not for deuterium), but with highly distorted hydrogen peaks. The Ne–He carrier gas gave a clear separation for hydrogen, with some tailing of the peaks, but none for deuterium. Both of these carrier gases required a much higher pressure drop across the column, even at lower flow-rates, and it seemed to be the relatively high pressures which obviated the deuterium separation.

There did not seem to be any obvious characteristic of the various aluminas which made them suitable for this application. However, successful columns were easily identified by the large amount of hydrogen (or deuterium) carrier adsorbed during cooldown, as noticed previously by Quickert and Le Roy[7]. The cooldown adsorption was characteristic of a good column whatever the grain size of the alumina; the best overall results, however, were obtained when the column was able to operate at high carrier flow-rates and with small pressure drops.

It may seem surprising to use deuterium gas as the carrier in a chromatographic experiment, but the net expense was about the same as that for the Ne–He carrier. The column reported here was also suited for the detection of excess p-H_2, using n-H_2 as the carrier (after Schultz and Le Roy[6]), but the sensitivity was reduced and there seemed to be no particular advantage to the technique. The immersion of the detector in the bath of liquid nitrogen gives an important improvement in sensitivity. The column response becomes non-linear with respect to relative spin isomer concentration, and calibration is required. Immersion in a bath of ethanol and dry ice also gives excellent sensitivity, but an undesirable baseline drift which arises apparently from gradual changes in the temperature of the bath. The column was also tested in an external magnetic field of about 1 kG, with no noticeable change in its performance.

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Note

Assay for conjugated estrogens in tablets using fused-silica capillary gas chromatography

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Pharmaceutical products containing conjugated estrogens, especially tablets, have been in use since 1942 for the treatment of estrogen deficiencies in post-menopausal women. The official compendial tests for the analysis of these products include a colorimetric assay for strength along with estimates of estrone and equilin content[1]. In addition, there is a gas chromatographic (GC) test that separates ten of the major estrogen components and provides a qualitative "identity" test. This GC procedure has also been applied to the quantitative analysis of conjugated estrogens in dosage forms[2]. However, it has not been adopted as an official compendial assay since collaborative studies have shown that the column is difficult to reproducibly prepare and a packing cannot be purchased which provides the necessary resolution of the ten estrogens. Also, the method involves retention times of up to one hour for a complete run.

In order to overcome these deficiencies in the packed column GC method and the shortcomings of the USP colorimetric methods, the use of glass capillary GC has been explored. Two recent papers have dealt with the resolution of estrogens using capillary column GC. Zweig *et al.*[3] used this procedure for separating the trimethylsilyl derivatives of the estrogens in pregnant mares' urine. However, the separations described did not resolve β-estradiol from α-dihydroequilin and did not show improved separation of the other estrogens as compared to the packed column procedure. Pillai and McErlane's approach[4] involves formation of a dual derivative (oxime–trimethylsilyl). Excellent separation of the estrogens is achieved. However, the reaction conditions used are quite strong, and the formation of *syn* and *anti* isomers due to the methoxime derivatives is a potential problem.

With the advent of fused-silica capillary column technology and the increased efficiency associated with this type of column, an investigation was undertaken to resolve the estrogen mixture using this recent development. The results are reported in this paper and the following advantages are noted: (1) separation of all ten estrogens, including β-estradiol from α-dihydroequilin; (2) formation of a single trimethylsilyl derivative at room temperature; (3) no need for temperature programming; (4) 28-min run time; (5) commercially available column; (6) acceptable accuracy and precision data on tablet samples; (7) column adaptability and ruggedness.

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EXPERIMENTAL

Apparatus

The instrument used was a Packard Model 421 gas chromatograph with a factory-equipped inlet splitter and flame-ionization detector assembly for capillary column use (Packard, Downers Grove, IL, U.S.A.). The column was a 10 m × 0.24 mm I.D.) fused-silica wall-coated open tubular column coated with SP-1000. It was a commercial column purchased from Quádrex (New Haven, CT, U.S.A.). Column temperature was 225°C. Inlet and detector temperatures were 280°C. Carrier gas was helium with a column pressure of 17 p.s.i. resulting in a flow-rate of about 1 ml/min. The splitting ratio was about 20 to 1. The detector purge rate was 50 ml helium/min and the injection size was 0.2 μl with an electrometer setting of $1 \cdot 10^{-10}$ A f.s. A Hewlett-Packard 3354B lab automation system was used for the data collection.

Method

The same sample and standard preparations are used as the previously published packed-column method[2] except that 1 ml of a 100 μg/ml solution of 1-methylestrone (Steraloids, Wilton, NH, U.S.A.) in methanol is substituted for testerosterone as the internal standard. This, in addition to the GC conditions, are the only changes which have been made.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of a standard mixture of the trimethylsilyl derivatives of the ten estrogens found in a typical Premarin® tablet (Ayerst Labs., New York, NY, U.S.A.) using the fused-silica column. Fig. 2 shows an actual tablet analysis (1.25-mg tablet). Compared to Fig. 3, which is the same tablet preparation using the DEGS packed column procedure, two distinct advantages are evident: (1) the capillary column decreases assay time from 69 to 28 min; (2) resolution is enhanced using the capillary column, especially in the diol region and estrone/equilin region of the chromatogram. These advantages are important when large numbers of samples are routinely assayed. Sample throughput is increased and quantitation is made easier due to the improved peak separation.

The efficiency of the SP-1000 column has been very acceptable considering the type of compounds involved. Estrogens are notoriously adsorptive in nature and extremely inert columns and connections are required to minimize tailing and band broadening. Using equilin as a reference peak, these columns have consistently yielded about 25,000 theoretical plates or about 2500 plates per meter. Greater efficiencies could be obtained by injecting smaller amounts of the estrogens since the amounts used in the method now are on the verge of overloading the column. However, smaller amounts mean higher sensitivities and the baseline at the solvent peak would be affected and make quantitation more difficult in the diol region of the chromatogram.

In addition, the columns have been found to be very stable, despite the fact that analyses are performed at the theoretical temperature limit of the liquid phase (225°C). It is very important that precautions are taken to exclude oxygen from the carrier gas during use and during storage. Carrier gas lines equipped with oxygen

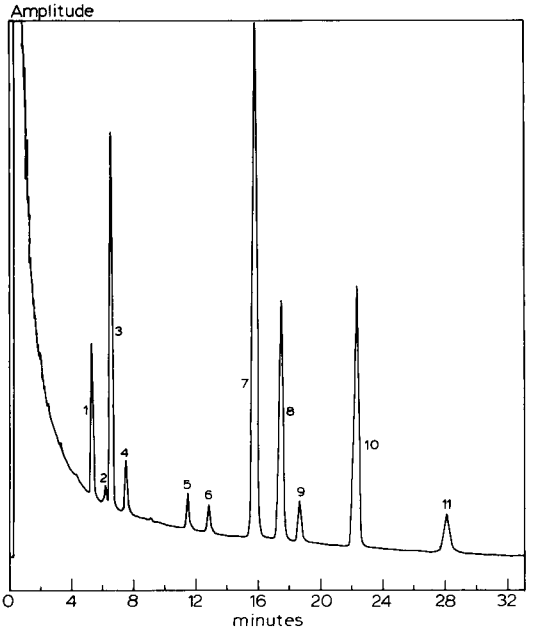

Fig. 1. Chromatogram of the ten estrogen standard mixture on the SP-1000 fused-silica column. 1 = α-Estradiol, 2 = β-estradiol, 3 = α-dihydroequilin, 4 = β-dihydroequilin, 5 = α-dihydroequilenin, 6 = β-dihydroequilenin, 7 = estrone, 8 = equilin, 9 = $\Delta^{8,9}$-dehydroestrone, 10 = 1-methylestrone, 11 = equilenin. (All estrogens present as the trimethylsilyl ethers).

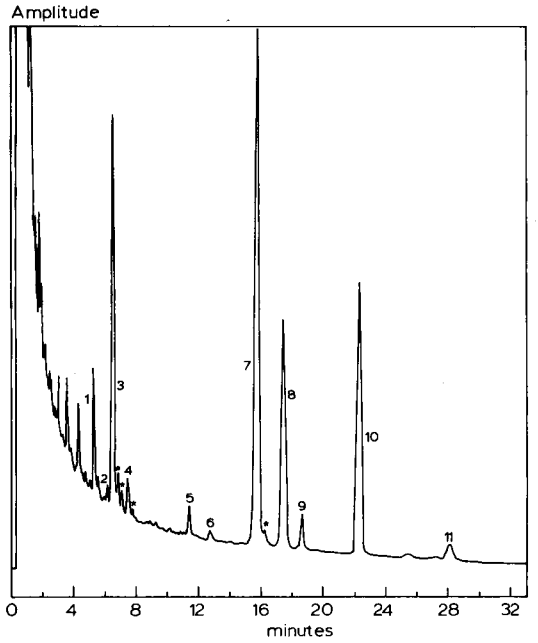

Fig. 2. Chromatogram of a typical Premarin tablet extract on the SP-1000 fused-silica column.

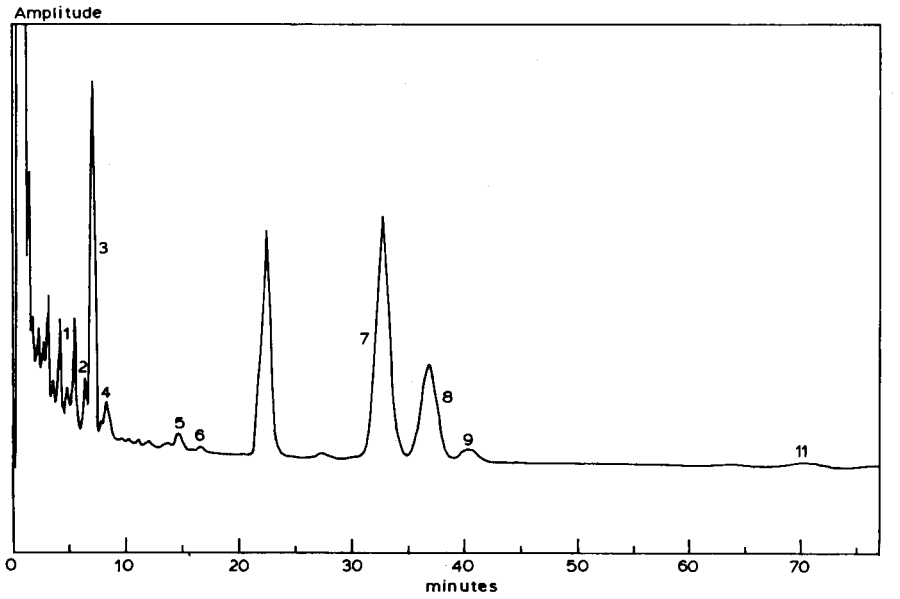

Fig. 3. Chromatogram of a typical Premarin tablet extract using the DEGS packed-column procedure. Column temperature = 200°C.

scrubbers are a necessity. During periods of storage outside the instrument, columns should be connected to an inert gas flow.

The ruggedness of these columns is also a feature which contributes to the efficiency of the system. Due to a fused-silica column's high resistance to breakage and its narrow O.D., the end of the column can actually be placed inside the flame tip, thus minimizing any dead volume in the detector connections. Also, the same column can be switched to different gas chromatographs without any modifications since the fused-silica material can be curved to almost any shape without breakage.

Precision experiments were performed to document the method. A batch of conjugated estrogen tablets* (1.25 mg) was analyzed five times on each of two days. Table I shows the results obtained from these experiments. The interday and intraday variation is considered very acceptable. The major estrogens such as estrone, equilin and α-dihydroequilin show very good coefficients of variation. As the amounts decrease for the other estrogens, the variation increases, which is to be expected. These compounds are present at only 8 to 40 μg per tablet.

Table II shows the comparison of the capillary column results with results obtained on the same tablet pool using the packed-column procedure. Overall, the results agree quite well. Some results, however, tend to be slightly lower by the capillary assay and can be explained by the increased resolution. α-Dihydroequilin, β-dihydroequilin and estrone have been separated from nearby components (indicated by asterisks). These peaks are included in the packed-column assay since they are either masked by the main component or are present as unresolved shoulders on the parent peaks. The retention times of these peaks do not correspond to a large number

* Premarin tablets, Ayerst Labs.

TABLE I

PRECISION OF ASSAY OF THE CAPILLARY COLUMN METHOD

Five tablet pools assayed on each of 2 days. C.V. = Coefficient of variations.

*Component**	*Day 1*		*Day 2*		*Overall*
	μg/tablet (average of 5 runs)	*C.V. (%)*	*μg/tablet (average of 5 runs)*	*C.V. (%)*	*C.V. (%)*
α-Estradiol	54	1.7	52	2.1	2.5
β-Estradiol	8.0	7.0	7.6	12.1	9.4
α-Dihydroequilin	195	1.6	191	1.7	1.9
β-Dihydroequilin	21	6.2	18	11.5	12.4
α-Dihydroequilenin	22	6.9	25	3.6	6.9
β-Dihydroequilenin	10	8.4	8.0	3.7	14.9
Estrone	710	1.5	688	1.1	2.1
Equilin	330	1.0	319	1.8	2.3
$\Delta^{8,9}$-Dehydroestrone	56	5.7	54	9.2	9.9
Equilenin	40	7.3	37	6.2	7.2

* Estrogens present as the sodium sulfate salts.

of typical estrogenic steroids for which standards are available[5]. The other component, for which some variation exists, is equilenin. This can be attributed to the nature of the peak itself. The packed column produces a broad flat peak which is extremely difficult to integrate accurately whereas the capillary column produces a sharp, easily quantitated peak.

The capillary method has revealed a peak pattern which might explain some of the small unknown peaks. α-Estradiol, α-dihydroequilin and β-dihydroequilin are all followed by a smaller peak. This repeating dual peak pattern is suggestive of the

TABLE II

COMPARISON PACKED-COLUMN AND CAPILLARY COLUMN ASSAY RESULTS ON A TABLET SAMPLE

*Component**	*μg/tablet*	
	Capillary column	*Packed column*
α-Estradiol	53	53
β-Estradiol	7.7	Not quantitated
α-Dihydroequilin	193	198
β-Dihydroequilin	19	32
α-Dihydroequilenin	24	19
β-Dihydroequilenin	9.0	8.0
Estrone	699	720
Equilin	324	330
$\Delta^{8,9}$-Dehydroestrone	55	51
Equilenin	39	31

* Estrogens present as the sodium sulfate salts.

presence of double bond isomers or possible different *cis–trans* ring junctions within the molecule. A number of isomers of α-dihydroequilin[6] and equilin[7] have already been identified. $\Delta^{8,9}$-Dehydroestrone, an isomer of equilin[8], and present in these tablets, is an example of this pattern. There is also a peak resolved from estrone. It is probably not an isomer of estrone and amounts to only about 1% of the total estrogenic content of the tablets.

The accuracy of the method was determined by spiking a placebo tablet pool with a known amount of sodium estrone sulfate. The equivalent of one tablet was spiked with 1 ml of an 837.3 μg per ml solution of sodium estrone sulfate in water and then carried through the procedure and compared to a known estrone standard workup. Duplicate workups yielded results of 831.1 and 829.0 μg sodium estrone sulfate for an average of 99.2% recovery.

CONCLUSION

A fused-silica capillary column GC method has been developed for the determination of conjugated estrogens in tablets. It offers several advantages as compared to the packed-column method, the primary ones being speed of analysis and increased resolution. The method, atlhough applied in this report only to tablets, should also be easily adapted to the assay of other pharmaceutical formulations such as injectables, creams and raw materials.

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Note

Analysis of piperazine estrone sulfate in tablets by ion-pair high-performance liquid chromatography

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Piperazine estrone sulfate is a synthetic water-soluble estrogen conjugate which is more effectively absorbed than the other estrogens. It is used in the treatment of menopausal and postmenopausal symptoms.

The U.S.P. XX[1] introduced a reversed-phase high-performance liquid chromatography (HPLC) procedure for free estrone and the assay of piperazine estrone sulfate in raw material only. As piperazine estrone sulfate is a salt of the strongly acidic sulfate ester of estrone, it is an ideal candidate for ion-pair chromatography[2,3]. The addition of quaternary alkylammonium ions to the mobile phase increased the affinity of the estrone sulfate salt for the lipophilic stationary phase. Moreover, this offered an additional way to improved resolution by careful selection of the length of the alkyl chain of the counter ion as well as its concentration[4].

EXPERIMENTAL

Apparatus

A modular HPLC system was used, consisting of a pump (Constametric II, Laboratory Data Control, Riviera Beach, FL, U.S.A.) operated at 2.0 ml/min., a variable wavelength UV detector (Schoeffel Model SF770, Westwood, NJ, U.S.A.), set at 225 nm and loop injector adjusted for 7000 p.s.i. (equipped with a 10 μl loop) (Rheodyne Model 7120, Berkeley, CA, U.S.A.). The column (250 × 4.6 mm I.D.) was packed with totally porous irregularly shaped micro-silica particles to which an octadecyl group had been chemically bonded (RP-18; Brownlee Labs., Santa Clara, CA, U.S.A.).

Peak retention times and areas were obtained by the use of a reporting integrator (Automation System 3385A, Hewlett-Packard, Avondale, PA, U.S.A.).

Reagents

Piperazine estrone sulfate, estrone and 17β-estradiol were USP reference standards.

17α-Estradiol, methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben (Sigma, St. Louis, MO, U.S.A.) and biphenyl (Aldrich, Milwaukee, WI, U.S.A.) were reagent-grade and used without further purification.

Sodium salts of equilin sulfate, 17α-estradiol sulfate and 17β-estradiol sulfate were buffered mixtures (Ayerst Labs, Montreal, Canada).

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Cetyltrimethyl ammonium bromide (CTMABr; Aldrich) was recrystalized from methanol–diethyl ether. Acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.) was HPLC grade. Water was double-distilled in glass.

Mobile phase

CTMABr (0.003 *M*) in a 55% acetonitrile in phosphate buffer solution (pH 5.0, 0.02 *M*) filtered through a 0.2 μm membrane (F.H.O.2 μ; Millipore, Bedford, MA, U.S.A.) and degassed, was used.

Internal standard solution

A solution of biphenyl in mobile phase was prepared at a concentration of 0.1 mg per ml.

Standard preparations

Piperazine estrone sulfate standard solution was prepared in internal standard solution at a concentration of 0.3 mg per ml.

Estrone standard solution was prepared in internal standard solution at a concentration of·6 μg per ml.

Sample preparation

Not less than 20 tablets were weighed and finely powdered. An amount of powder equivalent to one tablet (3 mg of piperazine estrone sulfate) was accurately weighed into a 15-ml centrifuge tube equipped with a PTFE-lined cap. Internal standard solution (10.0 ml) was added. The tube was capped and vigorously shaken for 30 min. The tube was then centrifuged to obtain a clear solution.

Procedure

A 10 μl aliquot of piperazine estrone sulfate standard preparation, estrone standard preparation, and sample preparation were successively injected into the chromatograph. The peak area ratios of piperazine estrone sulfate and of estrone to the internal standard were calculated. The quantity of piperazine estrone sulfate and of free estrone per tablet were calculated using their respective formulas:

Piperazine estrone sulfate:

$$C_{u_2} = 10\, C_{s_2} \cdot \frac{R_{u_2}}{R_{s_2}} \cdot \frac{W_t}{W_u}$$

where C_{s_2} = concentration of piperazine estrone sulfate in standard preparation in mg/ml; R_{u_2} = area ratio of piperazine estrone sulfate in sample preparation; R_{s_2} = area ratio of piperazine estrone sulfate in standard preparation; W_t = average weight per tablet; W_u = weight of sample taken.

Free estrone:

$$C_{u_1} = 333\, C_{s_1} \cdot \frac{R_{u_1}}{R_{s_1}} \cdot \frac{W_t}{W_u}$$

where C_{u_1} = percent of free estrone; C_{s_1} = concentration of estrone in standard

preparation in mcg/ml; R_{u_1} = area ratio of estrone in sample preparation; R_{s_1} = area ratio of estrone in standard preparation; W_t = average weight per tablet; W_u = weight of sample taken.

RESULTS AND DISCUSSION

Complete resolution was achieved between free estrone, piperazine estrone sulfate, internal standard, preservatives (methylparaben and *n*-propylparaben) and coloring agent used in tablet formulation (Table I).

TABLE I

CAPACITY FACTORS OF PIPERAZINE ESTRONE SULFATE AND OF OTHER COMPOUNDS OF INTEREST

$k' = (t - t_0)/t_0$, where t = retention time of peak and t_0 = time for elution of a non-retained peak.

Compound	*k'*
Piperazine estrone sulfate	8.06
Sodium equilin sulfate	7.63
Sodium α-estradiol sulfate	6.45
Sodium β-estradiol sulfate	5.30
Estrone	2.78
α-Estradiol	2.16
β-Estradiol	1.95
Biphenyl	11.04
Methylparaben	0.85
Propylparaben	1.86
Coloring agent	4.54

Retention of the piperazine estrone sulfate ion-pair increased with ion-pair concentration while that of neutral compounds remained unchanged. Resolution of these latter was affected by acetonitrile concentration only. So, for a particular column, resolution could be adjusted by varying these two factors.

Although there was significant mobile phase light absorption (maximum at 212 nm, 1.4 a.u., against water) at 225 nm, the sensitivity was still better than at other wavelengths (269 nm and 280 nm for piperazine estrone sulfate and estrone, for UV maximum absorption, respectively). Amounts of estrone as low as 0.01 μg were easily detected and quantified.

Sample preparations were relatively stable, no significant changes being detected when solutions were re-assayed 24 h later.

Linear response *versus* concentration was determined for piperazine estrone sulfate (0–5 μg) and estrone (0–0.06 μg). Within the ranges studied, the standard curves passed close to the origin and their correlation coefficients were 0.9998 and 0.986, respectively.

Quantitative analysis of a commercial formulation gave results comparable to the U.S.P. procedure for piperazine estrone sulfate (98.2%, coefficient of variation

(C.V.) 0.46%, and 98.5%, C.V. 0.52%, respectively). The free estrone content (2.7%, C.V. 3.8%) was found to be slightly above compendial limit for raw material (2.0%); however, no limit exists for tablets. Good reproducibility was obtained as shown by the C.V. Single tablet analysis showed there was no content uniformity problem with the product.

The use of separate estrone standard solutions allowed the direct estimation of free estrone in the sample without any correction for the contribution of free estrone from piperazine estrone sulfate reference standard.

CONCLUSION

This HPLC procedure is fast and accurate. It has been specially designed for single dosage form analysis as required in U.S.P. content uniformity test.

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Note

Comparison between straight and reversed phases in the high-performance liquid chromatographic fractionation of retinol isomers

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The importance of the analysis of retinol and retinyl esters and their geometric isomers, in order to evaluate their activity, has been recognized by many workers in food science and various biological fields.

Many studies of these compounds have been carried out by means of high-performance liquid chromatography (HPLC). McKenzie *et al.*[1] and Halley and Nelson[2] fractionated and identified eleven geometric isomers of methyl retinoates. As far as retinol isomers are concerned, Tsukida *et al.*[3], Paanakker and Groenendijk[4] and Bridges *et al.*[5] separated four geometric isomers, *i.e.*, all-*trans*-, 9-*cis*-, 13-*cis*- and 11-*cis*-retinol. However, the complete and rapid resolution of all possible isomers of retinol still remains a problem.

In this paper a rapid separation of five common isomers of retinol (all-*trans*-, 9-*cis*-, 9,13-di-*cis*-, 13-*cis*- and 11,13-di-*cis*-retinol) is described. In addition HPLC separation of two di-*cis*-isomers of retinol is reported for the first time. 2-Nitrofluorene was used as internal standard[6]. Two different modes (straight and reversed phase) of HPLC have been compared, in order to determine the best system for solving this analytical problem.

EXPERIMENTAL

Apparatus

The liquid chromatographic apparatus consisted of a Series 3 pumping system (Perkin-Elmer, Norwalk, CT, U.S.A.) connected to a column oven (P.E. LC 100). The spectrophotometric detector (P.E. variable-wavelength LC 55B) was equipped with a digital scanner (P.E. LC 55S), which permitted the stop flow recording of the UV-visible spectra of the eluted peaks. Spectra and chromatograms were recorded with a P.E. Model 56 recorder.

Materials and methods

The columns used were 5-μm Si 60 (250 × 4 mm) (E. Merck, Darmstadt, G.F.R.) for straight-phase and 5-μm Supelcosil LC 18 (150 × 4.6 mm) (Supelco, Bellefonte, PA, U.S.A.) for reversed-phase chromatography. A Supelco precolumn filled with 40-μm pellicular packing (ODS or silica) was always used in order to protect the analytical columns.

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All-*trans*-retinol (puriss.) and 2-nitrofluorene (purum) were purchased from Fluka (Buchs, Switzerland). 9-*cis*-Retinal was obtained from Sigma (St. Louis, MO, U.S.A.).

Silver nitrate and all the solvents (*n*-hexane, *n*-heptane, dioxan, ethyl methyl ketone, methanol and ethanol) were of analytical reagent grade (E. Merck) and were used without further purification.

Test mixture

In order to compare the two selected chromatographic modes, a mixture of retinol isomers, starting from the two available pure standards of all-*trans*-retinol and 9-*cis*-retinal, was prepared as described below.

All-*trans*-retinol in heptane was submitted to controlled photoisomerization as suggested by Halley and Nelson[7]. The obtained solution of retinol isomers was evaporated under a gentle stream of nitrogen and the retinols then redissolved in ethanol.

The starting solution of 9-*cis*-retinal had previously been tested by HPLC and found to be contaminated (about 10%, w/w) with 9,13-di-*cis*-retinal. Thus, the corresponding retinol solution, obtained by reduction with sodium borohydride according to Hubbard *et al.*[8], contained 9-*cis*- and a minor quantity of 9,13-di-*cis*-retinol. This solution was added to that obtained by photoisomerization. After standing, an ethanol solution containing five major retinol isomers and three other isomers in small quantity was obtained. This test solution was stored under nitrogen in the dark at −20°C. Test solutions remained stable for over 2 months under such conditions.

RESULTS AND DISCUSSION

Isomer identification

Isomer identification was achieved as follows.

Spectral data. The spectrum in the region of 300–350 nm was recorded for each major eluting peak. The spectral shapes and wavelengths of the absorbance maxima are as expected[8,9] (Table I). There is practically no shift of the absorbance maxima of the retinols nor of the spectral shapes in the different solvent systems used (water–methanol or hexane–dioxan). However, a shift of λ_{max} and a change in shape were observed for 2-nitrofluorene.

Internal standard. Coelution of the test mixture with the two known pure isomers all-*trans*- and 9-*cis*-retinol was performed. All-*trans*- and 9-*cis*-retinols are the

TABLE I

UV SPECTRAL DATA FOR RETINOL ISOMERS

Compound	λ_{max} *(nm)*		
	In ethanol[8]	*In hexane–dioxan (75:25) at 20°C*	*In water–methanol (20:80) at 20°C*
11,13-Di-*cis*-retinol	312	312	311.5
13-*cis*-Retinol	328	328.5	328
9,13-Di-*cis*-retinol	324	324.5	–
9-*cis*-Retinol	323	323.5	–
All-*trans*-retinol	325	326	325
2-Nitrofluorene	–	323.5	330

last to be eluted and their separation is difficult. Our 9-*cis*-compound was slightly contaminated with 9,11-di-*cis*-retinol, and its coelution with the test mixture permitted the positive identification of the 9-*cis*- and 9,13-di-*cis*-peaks.

Comparison of relative retention times. The elution order of retinol compounds is already known. Methyl retinoates follow the order reported by Halley and Nelson[2]. Retinols follow the same order but, as pointed out by Paanakker and Groenendijk[4], the order of the 13-*cis*- and 11-*cis*-isomers is inverted compared to the correspondent esters or aldehydes. However, 11-*cis*-retinol (as well as the more hindered 7-*cis*-retinol) was not expected to be present in detectable amounts in our test mixture, owing to the conditions used in the photoisomerization[3,7]. Therefore, in our sample solution, the elution order was expected[2-4] to be as follows: 2-nitrofluorene (internal standard); 11,13-di-*cis*-; 13-*cis*-; 9,13-di-*cis*; 9-*cis*-; all-*trans*-retinol. Three other minor peaks were observed. According to Halley and Nelson[7] one would expect to find in such a mixture of photoisomerized retinols also the 9,11,13-tri-*cis*- and the 13-*cis*-(5 → 10)-photocyclized-retinol. However, we did not try to identify these minor peaks.

Reversed-phase HPLC

This chromatographic mode has previously been used for resolving all-*trans*- and 13-*cis*-retinol[10]. The same eluent system of water–methanol mixtures was adopted, several different percentages of methanol (from 65 to 95%, v/v) being investigated. The best chromatogram obtained in this mode is presented in Fig. 1. Efforts to improve the resolution in this elution mode included the use of silver ions. Silver nitrate ($58.9 \cdot 10^{-3}$ *M*) dissolved in water used in the mobile phase had no appreciable effect on the chromatogram. De Ruyter and De Leenheer[11] observed an increase in selectivity of the reversed-phase system for retinyl esters when using silver ions. They also noticed no effect of silver ions on the retention times of retinyl esters with a saturated fatty acid chain, although the retinyl moiety contains five double bonds; they used the all-*trans*-retinyl moiety. This lack of any effect of silver ions in the case of retinol isomers was confirmed.

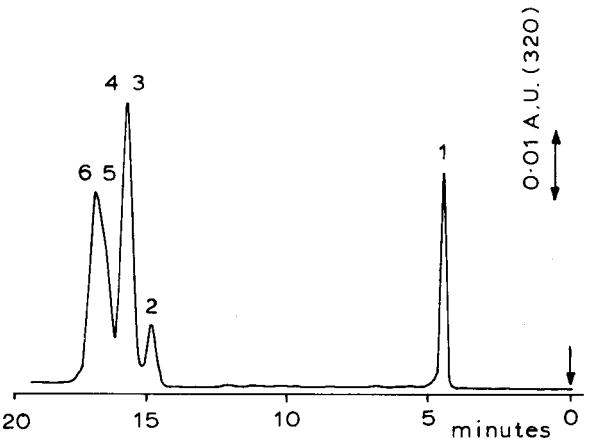

Fig. 1. Chromatogram of a text mixture of five *cis-trans*-isomers of retinol. Column: Supelcosil LC 18 (150 × 4.6 mm). Mobile phase: water–methanol (20:80); pressure 3.8 MPa; flow-rate 1 ml/min. Temperature 40°C. Wavelength: 320 nm. Peaks: 1 = 2-nitrofluorene (internal standard); 2 = 11,13-di-*cis*-retinol; 3 = 13-*cis*-retinol; 4 = 9,13-di.*cis*-retinol; 5 = 9-*cis*-retinol; 6 = all-*trans*-retinol.

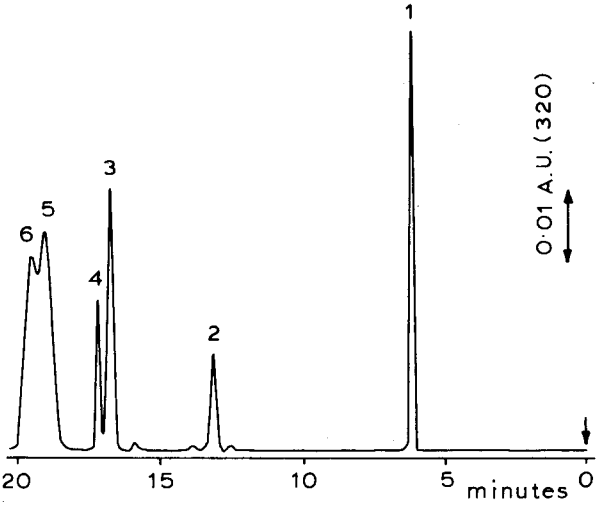

Fig. 2. Chromatogram of a test mixture of five *cis-trans*-isomers of retinol. Column: Merck Si 60 (250 × 4 mm). Mobile phase;*n*-hexane–dioxan (92:8); pressure 4.1 MPa; flow-rate 1 ml/min. Other details as in Fig. 1.

Straight-phase adsorption HPLC

Adsorption HPLC has been applied to retinol isomers using hexane-dioxan as eluent. The results are presented in Fig. 2. Comparing the chromatograms of Figs. 1 and 2, it is clear that adsorption chromatography gives better resolution of retinol isomers than the reversed-phase system. Several different percentages of the dioxan

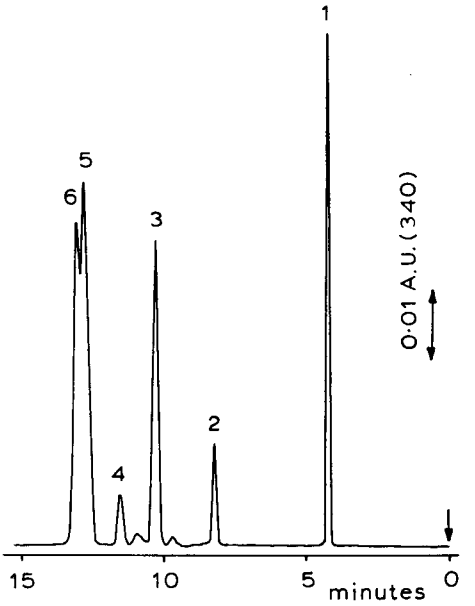

Fig. 3. Chromatogram of a test mixture of five *cis-trans*-isomers of retinol. Column as in Fig. 2. Mobile phase: *n*-hexane–methyl ethyl ketone (85:15); pressure 3.8 MPa; flow-rate 1 ml/min. Temperature: 40°C. Wavelength: 340 nm. Peaks as in Fig. 1.

modifier (from 6 to 15%, v/v) have been tried in order to improve resolution. Peak 4 (Fig. 2) shifted quickly from the cluster of peaks 5 and 6 towards peak 3 on decreasing the percentage of dioxan. However, with this elution system it has not been possible to obtain a resolution factor greater than about 0.4 between the 9-*cis*- and the all-*trans*-isomers.

Methyl ethyl ketone was also adopted as a modifier. The UV cut-off of this compound occurs at 330 nm, therefore the detection of the eluting peaks was shifted to 340 nm. This resulted in a loss of sensitivity of about 20%. There are two advantages in choosing methyl ethyl ketone: the low viscosity [0.30 *versus* 1.54 cP (20°C) of dioxan] allows better mass transfer; the low adsorptive energy (0.51 *versus* 0.56 $E°Al_2O_3$, where $E°Al_2O_3$ is the adsorptive energy of the considered eluent referred to Al_2O_3) allows the use of higher and a wider range of percentages of the modifier and a more reproducible pumping system. Fig. 3 shows a chromatogram obtained using methyl ethyl ketone–hexane as mobile phase. A better resolution between peaks 3 and 4 was obtained with this system, and there was no shift of relative retention times.

It is concluded that straight-phase HPLC, relative to reversed-phase HPLC, enables a rapid and better resolution of retinol isomers.

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Note

Reversed-phase high-performance liquid chromatography of pesticides

VI*. Separation and quantitative determination of some rice-field herbicides

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Herbicides of different chemical natures can be exploited to control several rice infesting weeds, *e.g.*, Bentazon (I), 2,4-D (II), MCPA (III), Propanil (IV), Molinate (V) and Drepamon (VI) attack weeds such as *Scirpus mucronatus*, different *Alisma (plantago* and *lanceolata)*, *Echinochloa (crusgalli* and *colonum)* and *Cyperus flavescens*[6]. Although other herbicides can be used, the above mentioned are the most effective under our environmental conditions[7].

These herbicides have been determined by several analytical procedures[8]; recently some high-performance liquid chromatographic (HPLC) analyses have been reported[9]. However, almost all of these methods have been developed for standards and not for environmental samples or for mixtures involving more than two herbicides. Furthermore, only normal phase liquid chromatography has been used.

Since the compounds I–VI are water pollutants, we thought that reversed-phase liquid chromatographic (RPLC) columns should provide the best tool for their analysis. Therefore we have developed a procedure which allows the direct simultaneous separation and determination on a RP-8 column of these herbicides together with widely used insecticides such as Phentoate (VII) in rice-field waters.

EXPERIMENTAL

Chemicals

Propanil (3′,4′-dichloropropianilide), MCPA [(4-chloro-*o*-tolyloxy)ethanoic acid] and 2,4-D [(2,4-dichlorophenoxy)ethanoic acid] were AnalGrade standards (Pestanol®, $\geqslant 99.0\%$; Hoechst, Milan, Italy). Bentazon [3-isopropyl-(1H)-benzo-2,1,3-thiadiazin-4-one-2,2-dioxide], $\geqslant 99.0\%$, Molinate [S-ethyl N,N-hexamethylenethiocarbamate], $\geqslant 97.0\%$, Drepamon [S-benzyl di-*sec.*-butylthiocarbamate],

* For parts I–V; see refs. 1–5.

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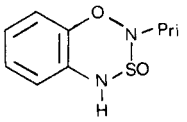

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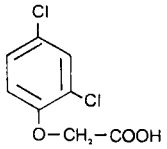

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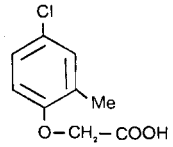

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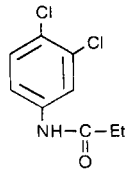

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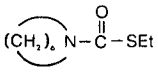

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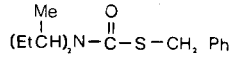

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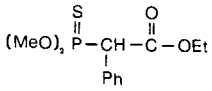

VII

Et = Ethyl
Me = Methyl
Ph = Phenyl
Pri = Isopropyl

$\geqslant 98.5\%$, and Phentoate [S-α-ethoxycarbonylbenzyl O,O′-dimethyl phosphorodithioate], $\geqslant 99.0\%$, were Analytical Standards kindly donated by BASF Agritalia, SIPCAM and Montedison respectively. Acetonitrile and methanol were HPLC grade solvents purchased from E. Merck (Darmstadt, G.F.R.) and Carlo Erba (Milan, Italy). Water was twice distilled and filtered through a Millipore apparatus before use. Ethyl acetate employed for extractions from water was Anal-Grade (E. Merck).

TABLE I

UV SPECTRA OF THE HERBICIDES IN ACETONITRILE

Herbicide	λ_1 *(nm)*	$\varepsilon \cdot 10^{-3}$	λ_2 *(nm)*	$\varepsilon_2 \cdot 10^{-3}$	ε_{220}
1 Bentazon	221.5	30.7	230.0	11.0	15.4
2 2,4-D	200.0	46.8	227.0	11.2	10.4
3 MCPA	198.0	42.4	227.0	11.2	9.0
4 Propanil	210.0	30.8	250.0	20.2	10.2
5 Molinate	198.0	12.7	–	–	7.6
6 Phentoate	197.0	38.5	220.0	13.8	13.8
7 Drepamon	195.0	30.7	–	–	11.7

TABLE II

RETENTION TIMES OF PESTICIDES UNDER DIFFERENT CONDITIONS

Herbicide	*Water–methanol*						*Water–acetonitrile*					
	RP-2		*RP-18*		*RP-8*		*RP-2*		*RP-18*		*RP-8*	
	0:100	*15:85*	*60:40*	*30:70*	*90:10*	*30:70*	*10:90*	*30:70*	*40:60*	*30:70*	*40:60*	*30:70*
1 Bentazon	1.74	2.07	1.45	1.44	2.85	1.54	1.45	1.70	1.35	1.33	1.45	1.44
2 2,4-D	2.67	2.07		1.44	3.96	1.54	2.26	1.70	1.35	1.33	1.68	1.78
3 MCPA	2.67	2.07	>40.00	6.90	5.64	1.54	2.48	1.70	1.35	3.81	1.75	1.86
4 Propanil	2.67	3.64	>40.00	6.90	>40.00	5.97	2.68	3.65	5.33	3.81	4.88	3.75
5 Molinate	2.80	4.00	>40.00	8.68	>40.00	7.73	2.95	5.52	7.77	5.31	8.67	5.22
6 Drepamon	3.03	5.07	>40.00	18.41	>40.00	20.17	3.52	8.41	>40.00	15.25	17.47	8.44
7 Phentoate	2.80	3.84	>40.00	10.40	>40.00	6.46	2.95	4.29	11.60	5.99	5.88	4.42

Apparatus

A Varian 5020 liquid chromatograph equipped with a Valco AH 20 automatic injector (loop 50 μl), UV/visible Varichrom detector, CDS 111 L data system and Varian 9176 recorder (1 mV/full scale) was employed. From the UV spectra of the pesticides (Table I), the best wavelength for their simultaneous determination was found to be 220 nm.

Chromatography

Merck columns Hibar® RP-2, RP-8 and RP-18 (250 × 4.2 mm I.D., 10 μm) were employed. The mobile phases were water–methanol, water–acetonitrile and mixtures of 0.2 *M* acetic acid–sodium acetate, pH 3.0, 3.5 or 4.0, and 0.067 *M* phosphate pH 5.0, 6.0 or 7.0, buffers in acetonitrile in different ratios.

RESULTS AND DISCUSSION

A preliminary investigation carried out on RP-18, RP-2 and RP-8 columns and under isocratic elution conditions showed that the herbicides could be divided into two main groups, depending on their retention times. While Bentazon, 2,4-D and MCPA (group A) were quickly eluted, Propanil, Molinate and Drepamon (group B) had longer retention times. This behaviour was observed reproducible and was found to be independent of the eluting mixtures and columns (see Table II).

A separation of the pesticides was obtained with water–acetonitrile (40:60 to 30:70) on a RP-8 column, and an improved selectivity was achieved using buffers instead of water in the eluting mixtures. With buffers of pH 7.0, 6.0 or 5.0 no significant changes in the separation of the group A peaks were observed; however, with buffers of pH 4.0 a good separation was obtained and carboxylic acids showed sharper peaks (according to their pK_a values[8]).

A further 5% increase in buffer content of the eluting mixture resulted in optimum separations. Significant changes were observed on decreasing the buffer pH values (see Table III) from pH 4.00 to pH 3.50; below the latter value the chromatogram remained virtually unchanged (see Fig. 1).

The detection limits for injection of a 50-μl non-concentrated water sample (0.004 AU) ranged between 0.01 and 0.03 ppm (see Table IV).

TABLE III

INFLUENCE OF pH ON RETENTION TIMES ON A RP-8 COLUMN

Herbicide	*Retention time (min)*		
	Buffer, pH 4 (35:65)	*Buffer, pH 3.5 (35:65)*	*Buffer, pH 3 (35:65)*
Bentazon	1.75	1.77	2.19
2,4-D	1.92	2.07	2.66
MCPA	2.01	2.26	2.87
Propanil	4.14	4.12	4.16
Molinate	4.86	4.82	4.89
Phentoate	6.26	6.22	6.31
Drepamon	10.99	10.97	11.17

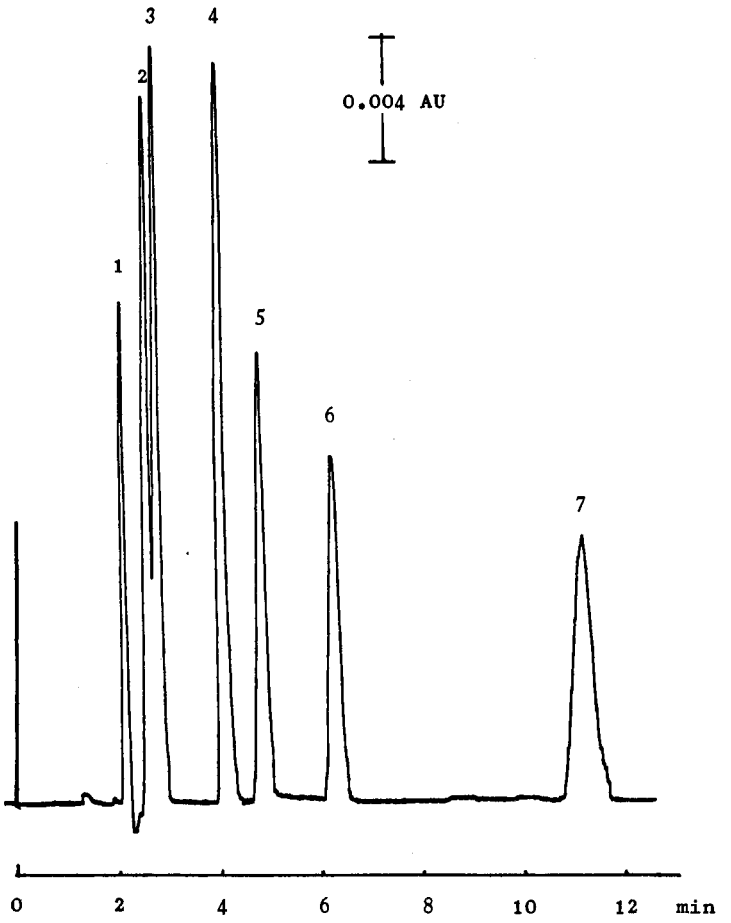

Fig. 1. Chromatogram of heribicides (see Table I) on a RP-8 column. Eluent: buffer, pH 3–acetonitrile (35:65); flow-rate 1 ml/min. Detector: UV at 220 nm. Peaks: 1 = Bentazon; 2 = 2,4-D; 3 = MCPA; 4 = Propanil; 5 = Molinate; 6 = Phentoate; 7 = Drepamon.

TABLE IV

DETECTION LIMITS AT 220 nm

Pesticide	*Detection limit (ppm)*
Bentazon	0.01
2,4-D	0.03
MCPA	0.03
Propanil	0.03
Molinate	0.03
Phentoate	0.03
Drepamon	0.03

ACKNOWLEDGEMENTS

We are indebited to BASF Agritalia (Milan, Italy), SIPCAM (Milan, Italy) and Montedison (Milan, Italy) for gifts of herbicides, and to Dr. Lai and the Consorzio di Bonifica del Campidano di Oristano for their cooperation. We also thank Dr. Franco Cozzi for helpful discussion. This work was made possible by the financial support of Regione Autonoma della Sardegna and has been carried out within the Oriented Project No. 8 of CNR (Italian Research Council).

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Note

Extraction of glucuronide metabolites of Δ^9-tetrahydrocannabinol by diethyl ether

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Conjugation of drugs and their metabolites converts them into highly polar compounds that are excreted rapidly by the kidney. This increase in polarity is exploited analytically for studying the metabolism of drugs as it is generally assumed that solvents such as ether extract the relatively non-polar free compounds leaving the polar conjugates in the aqueous phase. Thus diethyl ether extraction of conjugated metabolites is thought to occur only after hydrolysis to their free form[1].

In contrast, we have found that conjugated neutral metabolites and conjugated weakly polar acids including Δ^9-tetrahydrocannabinol-11-oic (THC-11-oic) acid, the major urinary metabolite of THC, are extracted along with unconjugated metabolites from acidified, unhydrolyzed human urine with anydrous diethyl ether. This result challenges the generally held assumption that conjugated metabolites are not extracted by ether, and suggests that previous work on the metabolism of THC, especially those using radioactivity or gas chromatography–mass spectrometry (GC–MS) as the analytical detection technique, be reevaluated.

EXPERIMENTAL

Extraction of conjugated Δ^9-THC-11-oic acid from acidified, unhydrolyzed urine

A volume of urine containing metabolites of THC was concentrated by evaporation to 10 ml, adjusted to pH 8 and shaken with anydrous ethyl ether (ether), 3 × 15 ml, to extract unconjugated THC-11-oic acid[2,3]. The combined ether extracts were evaporated to approximately 15 ml in a stream of nitrogen with heat (not over 50°C), washed once with 5% $NaCHO_3$ and dried with anhydrous, granular Na_2SO_4. The dried ether was evaporated and the residue chromatographed as described below (Fig. 1, chromatogram A).

The aqueous phase was adjusted to pH 4 and extracted with ether as above. The extracts were combined and the ether evaporated. The dried residue was dissolved in 10 ml of absolute ethanol and divided in two. The ethanol was evaporated and each residue was mixed with 10 ml of a blank urine adjusted to pH 5.5. Both were incubated at 55–60°C for 45 min, one without enzyme (Fig. 1, chromatogram B), the other with 0.1 ml of Boehringer-Mannheim β-glucuronidase–arylsulphatase (Fig. 1, chromatogram C). Both were extracted with ether and the ether extract of each was washed twice with $NaCHO_3$, then processed as previously described.

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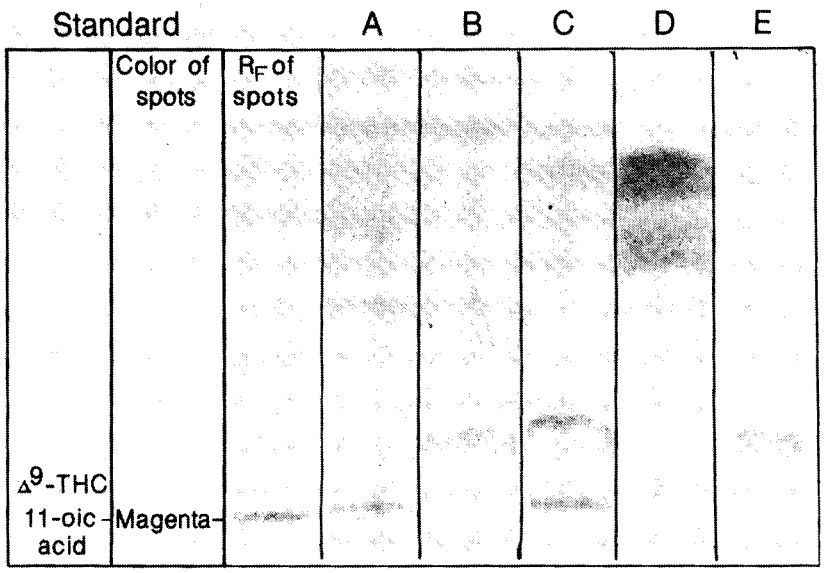

Fig. 1. Extraction of conjugated Δ^9-THC-11-oic acid by ether from acidified, unhydrolyzed urine. Chromatograms of a single aliquot of post-THC urine extracted with ether sequentially. A, unconjugated Δ^9-THC-11-oic acid extracted in ether from unhydrolyzed urine at pH 8. B and C, aqueous phase treated as described. B, incubated without enzyme, C, with enzyme. Only C shows reaction to THC-11-oic acid. D, extract of aqueous phase hydrolyzed at pH 5.5. Absence of reaction at R_F corresponding to THC-11-oic acid indicates all TH-11-oic acid had been extracted during the previous treatment. E, extract of hydrolyzed blank urine. Color of spots due to reaction with FBSB. Chromatographic solvent systems: acetone–chloroform–triethylamine (80:20:1), first pass; light petroleum–diethyl ether–glacial acetic acid (50:50:1.5), second pass. See text for more detail. Spots not identified by color are not characteristic of reaction between cannabinoids and FBSB.

To ensure that most, if not all, the conjugated THC-11-oic acid was extracted from the unhydrolyzed urine, at pH 4, the aqueous phase remaining was adjusted to pH 5.5, incubated with 0.1 ml enzyme at 55–60°C for 45 min, then extracted with ether. The ether extract, after washing twice with $NaCHO_3$, was processed as previously described. A 10-ml amount of the blank urine was also analyzed to ensure that it did not contribute contamination of interference (Fig. 1, chromatograms D and E).

Ethanolic solutions of the residues of the ether extracts were quantitatively transferred in a streak to an Analtech pre-coated 250-μm silica gel G thin-layer plate and chromatographed 10 cm in two saturated tanks using two solvent mixtures; the first was acetone–chloroform–triethylamine (80:20:1) and the second was light petroleum (b.p. 35–60°C)–diethyl ether–glacial acetic acid (50:50:1.5). Between solvent passes, the thin-layer plate was placed in a fume hood for 5 min. Approximately 5 min after the second pass, the thin-layer plate was sprayed with a cold solution of freshly prepared 0.1 % Fast Blue Salt B (FBSB) in 2 *N* NaOH. A reference standard of THC-11-oic acid was chromatographed at the same time[4].

Extraction of conjugated neutral metabolites of Δ^9-THC by ether from acidified, unhydrolyzed urine

Four 10-ml aliquots of a concentrate of a urine containing neutral metabolites

were adjusted to pH 2.5, 2.5, 7.0 and 12.4, respectively, and extracted with ether, 3 × 15 ml. The combined ether extracts of each aliquot were evaporated and the dry residues were mixed with 10 ml of the blank urine at pH 5.5. All were incubated at 55–60°C for 45 min, one, of the two that were extracted at pH 2.5, without enzyme, all the others with 0.1 ml of enzyme. After cooling, all were adjusted to pH 12.4 and extracted with ether as above[5]. The combined ether extracts of each were evaporated and the residues chromatographed as described below. The aqueous phases that remained after the initial ether extraction were adjusted to pH 5.5 and incubated at 55–60°C for 45 min, one as before, without enzyme, the others with enzyme. After cooling, all were adjusted to pH 12.4 and processed as described. These eight extracts were chromatographed sequentially as previously reported[5] with three minor modifications; only one pass was made with the first solvent system, glacial acetic acid was eliminated from the latter and FBSB was prepared in 2 *N* NaOH.

RESULTS

THC-11-oic acid

The extraction of unhydrolyzed urine at pH 8 removed all but a minimum amount of the unconjugated THC-11-oic acid (Fig. 1, chromatograms A and B). The latter plus the conjugated THC-11-oic acid were extracted at pH 4 (Fig. 1, chromatograms B and C). The intense reaction in chromatogram C corresponding to THC-11-oic acid is due to the reaction between hydrolyzed conjugated THC-11-oic acid and FBSB. The faint reaction in chromatogram B at the same R_F value indicates very little FBSB-reactive THC-11-oic acid. This faint reaction was most likely due to a small amount of unconjugated THC-11-oic acid that was not initially extracted at pH 8. The absence of a reaction at the R_F value of THC-11-oic acid in chromatogram D shows that all the THC-11-oic acid had been extracted from the urine by the previous treatment. The data from this chromatogram and chromatogram C prove that conjugated THC-11-oic acid is extractable from acidified, unhydrolyzed urine with ether.

Neutral metabolites

All the chromatograms of Fig. 2 are of ether extracts prepared at pH 12.4. Each pair, A and B, etc., is from one aliquot. A, C, E and G are of residues of unhydrolyzed urine initially extracted at pH 2.5, 2.5, 7.0 and 12.4, respectively, then mixed with blank urine at pH 5.5 and incubated, A, without enzyme and C, E and G with enzyme. B, D, F and H are of the aqueous phases remaining after the initial extractions, incubated at pH 5.5, B, without enzyme D, F, and H with enzyme. Thus, any magenta colored spots in chromatograms A and B are due to unconjugated metabolites of THC and similarly colored spots in the other chromatograms are due to unconjugated and hydrolyzed conjugated metabolites. Chromatograms A and B show that only a minimum amount of the neutral metabolites are excreted unconjugated and that all of them were extracted at pH 2.5. Chromatogram C, except for hydrolysis, is equivalent to chromatogram A. It shows numerous and intensely colored spots due to the reaction between both unconjugated and hydrolyzed conjugated metabolites and FBSB. This clearly shows that conjugated neutral metabolites are extracted by ether from acidified, unhydrolyzed urine. Chromatogram D, except for hydrolysis, is equivalent to chromatogram B. It shows some colored spots due to the

reaction between hydrolyzed conjugated metabolites and FBSB. This indicates that not all of the conjugated metabolites were extracted from the unhydrolyzed urine at pH 2.5. Chromatograms E and G, except that the extractions of the unhydrolyzed urine were at pH 7.0 and 12.4, respectively, instead of 2.5, are equivalent to chromatogram C. Each shows a single spot. In chromatogram E the spot may be due to a combination of unconjugated and hydrolyzed conjugated metabolites. In chromatogram G the spot is probably due to unconjugated metabolite only. Chromatograms F and H, except that the extractions of the unhydrolyzed urine were at pH 7.0 and 12.4, respectively, instead of 2.5, are equivalent to chromatogram D. One of the more polar spots in F and H is not present in D. The metabolite(s) responsible for this spot was completely extracted from the acidified, unhydrolyzed urine (chromatogram C). Another difference among these chromatograms is the greater intensity of the spots in F. Between F and D the difference appears to be quantitative. The intensity of the spots in D are less because much of the metabolites were extracted at pH 2.5 from the unhydrolyzed urine. Between F and H the difference appears to be a loss of FBSB reacting material perhaps due to the longer exposure to the alkaline pH. The data in these chromatograms show that the neutral urinary metabolites of THC are excreted as conjugates, primarily, and that these conjugated metabolites are extractable with ether from acidified, unhydrolyzed urine.

Extraction of conjugated THC-11-oic acid and conjugated neutral metabolites

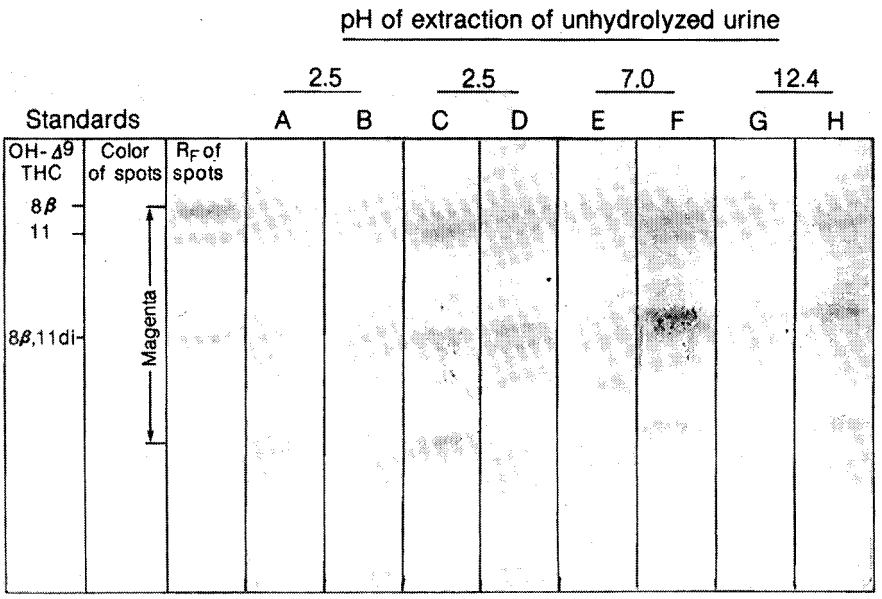

Fig. 2. Extraction of conjugated neutral metabolites of Δ^9-THC by ether from urine. Chromatograms of ether extracts prepared at pH 12.4 of four aliquots of a single post-THC urine. See text for details. Magenta colored spot in A is due to unconjugated metabolites; in C, to unconjugated as well as hydrolyzed, conjugated metabolites; in E and G to unconjugated metabolites; and in D, F and H to hydrolyzed, conjugated metabolites. Absence of spots in B indicates no FBSB reacting metabolites. Numerous and intensely colored spots in C demonstrate that conjugated, neutral metabolites of THC are extractable by ether from acidified, unhydrolyzed urine. Spots not identified by color are not characteristic of reaction between cannabinoids and FBSB.

from acidified, unhydrolyzed urine by ether was not due to hydrolysis. If it was, stronger reactions would have occurred at the appropriate R_F values in chromatograms B of Fig. 1 and A of Fig. 2.

DISCUSSION

Our results differ from those who found that conjugated and unconjugated metabolites of THC could be separated by ether extraction, the conjugated metabolites remaining in the aqueous phase[6]. This difference may be due to our using raw urine and their using Amberlite XAD-2 purified urine. Others, using raw urine as we have, interpreted the data of their studies of the metabolism of THC assuming that ether extracted only unconjugated metabolites of THC from acidified, unhydrolyzed urine[7–9]. Since our results yield a different interpretation to such data, our work may affect the understanding of the metabolism of THC.

The extraction of conjugated metabolites of THC along with the unconjugated metabolites from acidified, unhydrolyzed urine explains differently the data of Agurell *et al.*[7]. It explains the manifold increase in the amount of metabolites of THC extracted from unhydrolyzed urine at pH 3.8 compared to pH 7.8. At pH 7.8, the amount of conjugated metabolites extracted from unhydrolyzed urine by ether is minimal while the amount of unconjugated metabolites is almost complete. At pH 3.8, conjugated as well as unconjugated metabolites are almost completely extracted into the ether from unhydrolyzed urine. Thus our results do not support (1) the interpretation that only a small amount of labile conjugates (of THC and its metabolites) are present in rabbit urine after the administration of THC, and (2) the suggestion of the introduction of an acidic group in Δ^1-THC-^{3}H not affected by glucuronidase. Furthermore, interpreting their data using our results yields a ratio of conjugated to unconjugated metabolites of approximately 4:1 rather than the very small difference they evaluated.

Our results suggest reevaluating the interpretation of the results of Melikian *et al.*[8] and Green[9] as their conclusions of the effect of pH on the ether extraction of metabolites of THC from unhydrolyzed urine do not take into account the extraction of conjugated metabolites without hydrolysis. Their conclusions were based on radioactive and GC–MS methods, respectively. The former did not differentiate different forms of metabolites and the latter was so programmed that it would detect only the unconjugated form of THC-11-oic acid.

The conjugates of THC and its metabolites have been presumptively identified as glucuronides and/or sulphates based on measurement after treatment with glucuronidase and sulphatase in man, rabbit and rat[6,7,10]. Recently two groups have definitely identified glucuronides by MS. One, an O-glucuronide of THC, from rabbit urine, the other, an ester linked glucuronide of THC-11-oic acid from human urine[11,12]. Thus, we feel reasonably certain that the metabolites extracted by ether were truly glucuronides.

Residues of extracts were mixed with a blank urine instead of aqueous buffer because our previous experience indicates that the solute content of the aqueous phase significantly affects the extraction of THC-11-oic acid by ether[3].

A procedure for separating unconjugated and conjugated THC-11-oic acid by liquid–liquid extraction of raw urine will be presented separately.

ACKNOWLEDGEMENTS

Tetrahydrocannabinol, Δ^9-tetrahydrocannabinol-11-oic acid, 8β-hydroxy-, 11-hydroxy- and 8β,11-dihydroxytetrahydrocannabinol were made available by the National Institute on Drug Abuse, Public Health Service, NIMH. This study was supported in part by grant No. DA-00424 and the Research Services of the Veterans Administration.

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Note

Separation of dichloro- and chlorofluorobenzophenone isomers by high-performance liquid chromatography

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A variety of chromatographic techniques has been used for the separation and characterization of benzophenones and related compounds. Vessman and co-workers[1–8] reported extensive work on the characteristics of benzophenones by gas–liquid chromatography (GLC) with electron-capture detection. Abou-Donia[9] studied four liquid phases on Chromosorb W in combination with an electron-capture detector for the GLC analysis of 25 DDT-type compounds including 4,4′-dichlorobenzophenone and 4-chlorobenzophenone. Benezra[10] separated mixtures of aromatic ketones at the sub-nanogram range by plasma chromatography. Reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection was used to separate and detect 2,4′- and 4,4′-dichlorobenzophenones, the degradation products of the corresponding 2,4′- and 4,4′-Dicofol[11]. Chlorine-selective detection for liquid chromatography with a Coulson electrolytic conductivity detector (CElCD) showed high selectivity to organochlorine compounds relative to hydrocarbons, with a linear range of 10^5. However, the CElCD/UV_{254} response for dichlorobenzophenone was 0.01 thus favoring the UV detection[12].

Relatively little work has been published on the isomeric determination of dihalogenated benzophenones. Determination of the isomer ratios for compounds related to dichlorobenzophenone, di(chlorophenyl)methane and di(chlorophenyl)-ethane by infrared, ^1H nuclear magnetic resonance and mass spectroscopy were found to be of a limited use[13–17]. Abraham *et al.*[17] separated a mixture of dichlorobenzophenones by GLC. However, in our hands the 2,3′- and 2,4′-dichloro-isomers were not completely resolved. Recently[18] we reported the complete separation of 2,2′-, 2,3′- and 2,4′-dichlorobenzophenones by thin-layer chromatography (TLC). Both the GLC[17] and TLC[18] techniques require a long time for resolving the isomeric mixture.

The objective of this work was to find suitable HPLC conditions which allow the resolution of dichloro- and chlorofluorobenzophenones in a short time and the application of such a system in the routine identification of the various isomers.

EXPERIMENTAL

The system used for the HPLC work included a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.). The detector was a Varian variable-wave-

length Vari-chrom unit (Varian, Palo Alto, CA, U.S.A.) operated at 254 nm and fitted with an 8-μl flow cell. Data were collected with a Hewlett-Packard 1000 series 2117F computer (Hewlett-Packard, Palo Alto, CA, U.S.A.) at a sampling rate of 0.33 Hz. Post-run plots of the chromatograms were generated on a Hewlett-Packard Model HP-72215 plotter. A Rheodyne Model 70-10 liquid sampling valve (Rheodyne, Berkeley, CA, U.S.A.) with a 20-μl sample loop was attached to a 25 × 0.46 cm DuPont Zorbax®-ODS column. The column was maintained at room temperature. However, the column was wrapped with an insulating material to prevent large fluctuations in temperature. The mobile phase consisted of "Milli-Q" water–tetrahydrofuran (55:45). This solution was degassed and kept magnetically stirred. Because of the viscosity of the eluting solvent, a system equilibration time of about 30 min was required to reach a stable baseline. The mobile phase was pumped at a flow-rate of 1.0 ml/min through the column, producing a column head pressure of approximately 3000 p.s.i. The benzophenone standards were dissolved at a concentration of about 0.08 mg/ml of the eluting solvent. The isomers of interest were available from our Process Research Laboratory (see Acknowledgement).

RESULTS AND DISCUSSION

To the best of our knowledge this is the first HPLC system reported to the literature for the separation of dihalogenated benzophenones. The capacity ratios of the seven benzophenones of interest to us are given in Table I. For the monosubstituted rings (compounds 1–3) the order of increasing retention time follows the sequence: 2,2′ < 2,3′ < 2,4′. A similar trend was reported by Abraham *et al.*[17] in the GLC work on dichlorobenzophenones. We found that in every case, the retention times of the chlorofluorobenzophenones were less than the corresponding dichlorobenzophenones. The 2,4-disubstituted ring (compound 4) showed the longest elution time. Fig. 1 shows the baseline resolution of the isomers.

TABLE I

CAPACITY RATIOS OF SOME DIHALOGENATED BENZOPHENONES

No.	*Isomer*	*Capacity ratio*
Dichlorobenzophenones		
1	2,2′	4.5
2	2,3′	6.6
3	2,4′	8.1
4	2,4	9.0
Chlorofluorobenzophenones		
5	2,2′	3.7
6	2,3′	5.3
7	2,4′	5.8

In conclusion, this HPLC system allowed us to separate the positional isomers of the chlorofluorobenzophenone series and the dichlorobenzophenone series in less than 16 min, thus assuring a rapid identification of the isomeric content per sample.

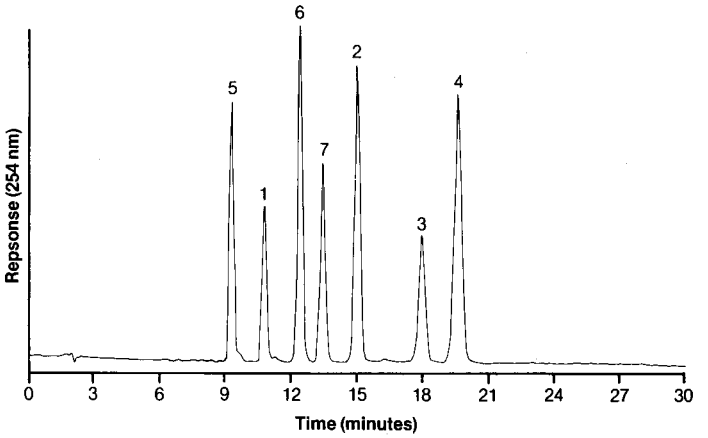

Fig. 1. HPLC of some dihalogenated benzophenones. Peak numbers correspond to Table I.

This isocratic HPLC procedure complements the TLC procedure reported before[18]. The detailed quantitation of the isomeric content and other related material in 2,4′-dichlorobenzophenone raw material will be the subject of another manuscript.

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Note

Use of DEAE-cellulose paper in the paper chromatographic separation of uronic acids

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In the course of our studies on polysaccharides we have had occasion to identify by paper chromatography the uronic acids present in polysaccharide hydrolysates. While using the solvent system ethyl acetate–acetic acid–water, (3:1:1), it was found that glucuronic acid could be clearly separated from galacturonic acid and most of the common reducing sugars on DEAE-cellulose paper (Whatman DE-81), whereas these separations could not be achieved by us with Whatman No. 1 paper, a most commonly used cellulose paper, or with Whatman No. 20, a very close grained cellulose paper.

EXPERIMENTAL AND RESULTS

Aliquots (5 μl) of aqueous solutions containing uronic acids and reducing sugars, 5 mg/ml, were applied to the line of origin of the paper chromatograms and eluted descendingly for 24–48 h or over the weekend with the solvent system described above. This system was convenient to use because it was monophasic, did not contain pyridine or other solvents with more objectionable odors, or cause the paper to deteriorate as systems containing strong acids tend to do. The uronic acids and reducing sugars were then located by dipping the DEAE paper in 4% ethanolic aniline malonate, and subsequent air drying and heating as described in Caldes and Prescott[1]. It was necessary to use a higher concentration of aniline malonate than the 2% used for ordinary cellulose papers, otherwise the heating had to be greatly prolonged. The color of the galacturonic acid spot was a deeper color than that of the glucuronic spot, although they were both of orange-brown hue.

Figs. 1 and 2 show typical chromatograms obtained with Whatman No. 1 and Whatman DE-81 paper, respectively. The uronic acids migrated faster on the Whatman No. 1 paper, but glucuronic acid did not clearly separate from galacturonic acid,

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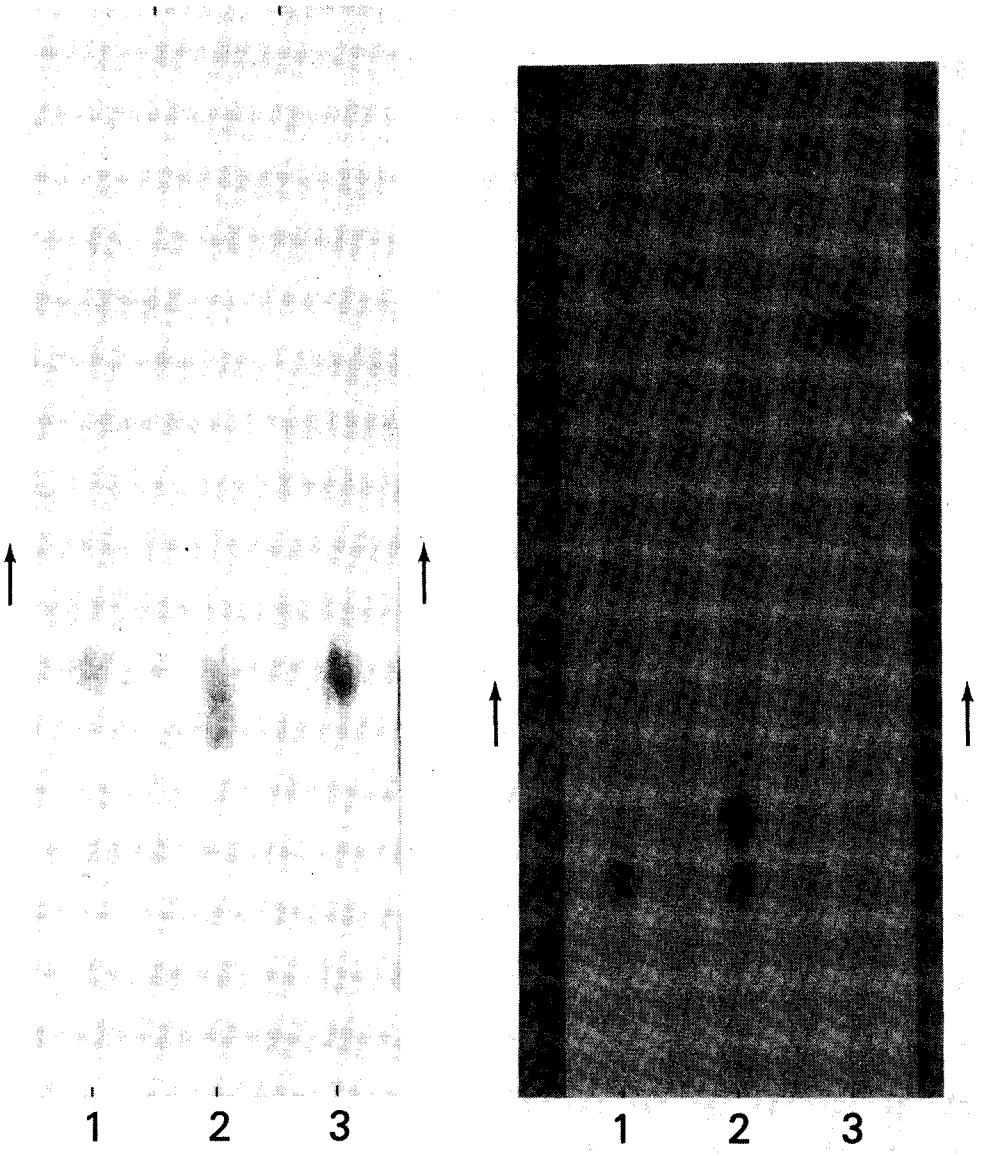

Fig. 1. Descending chromatography of uronic acids and glucose, 50 μg each, on Whatman No. 1 paper for 24 h. with the solvent ethyl acetate–acetic acid–water (3:1:1). Glucuronic acid was applied to spot 1 on the line of origin at the left. A mixture of glucuronic and galacturonic acids was applied to the center spot (2), and glucose was applied to the spot at the right (3).

Fig. 2. As Fig. 1, except that Whatman DE-81 paper was substituted for the Whatman No. 1 paper, and the paper was eluted for 30 h.

although these uronic acids could be distinguished by the difference in color and the separation of the spot nuclei. Increasing the time of elution beyond 24 h did not improve the separations on Whatman No. 1 paper. Other solvent systems tried with Whatman

No. 1 paper in an effort to separate glucuronic from galacturonic acid were ethyl acetate–acetic acid–water, (3:1:3)[2], and butanol–acetic acid–water (4:1:5)[3]. Neither of these solvent systems in our hands completely separated glucuronic from galacturonic acid. Another advantage of using the DE-81 paper for uronic acid identification was that glucose and galactose, reducing sugars very commonly found in polysaccharide hydrolysates, migrated much faster than the uronic acids, whereas on Whatman No. 1 paper they migrated at about the same speed as the uronic acids. Man-

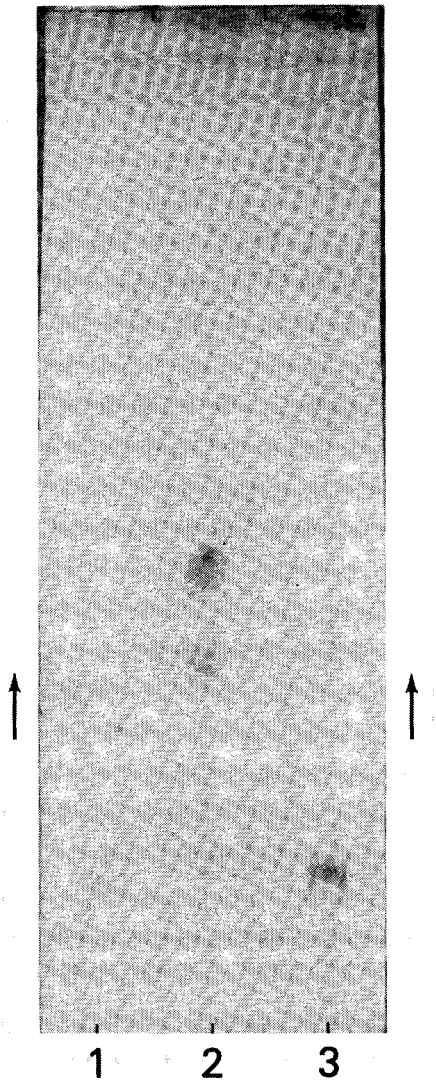

Fig. 3. Descending chromatography of a dilute acid hydrolysate of Type III pneumococcal polysaccharide and standards on Whatman DE-81 paper for 48 h. An amount of hydrolysate equivalent to 125 μg of polysaccharide was applied to spot 1 at the left, 250 μg at the right (spot 3), and 50 μg each of glucuronic, galacturonic, and synthetically prepared cellobiouronic acid at the center spot (2).

nose, arabinose, xylose, and rhamnose also were separated from the uronic acids on DEAE paper, but they could also be separated on Whatman No. 1 paper.

Cellobiuronic acid could be clearly separated from glucuronic and galacturonic acid on DEAE paper, and this property was used to help identify cellobiuronic acid in a hydrolysate of Type III pneumococcal polysaccharide (Fig. 3). Prolonged heating, 3 h at 100°C, was necessary to bring out the color of the cellobiuronic acid spots, even with 4% aniline malonate solution. Increasing the concentration of malonate to 10% speeded the color development.

Mannuronic acid could not be completely separated from galacturonic acid, using the 3:1:1 solvent system, although it did migrate a little faster. It could, however, be easily separated from glucuronic acid. There was one other difference between Whatman DE-81 and Whatman No. 1 paper: on DEAE paper, galacturonic acid migrated faster, whereas on cellulose paper glucuronic acid migrated faster.

Aminoethanol paper (Whatman AE-81) and silica gel paper (Whatman SG-81) were also tried but did not separate the uronic acids with the 3:1:1 solvent system.

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CHROM. 14,301

Letter to the Editor

Gas chromatographic–mass spectrometric analysis of [^{13}C]carbon monoxide

Sir,

In an article recently published in this journal[1], we described a method for the gas chromatographic–mass spectrometric analysis of [^{13}C]carbon monoxide (CO) in air. We described the use of Hopcalite to effect a post-column catalytic conversion of the eluent CO to carbon dioxide (CO_2) in order to take advantage of certain properties of the mass spectrometer background. Our observations suggested that the CO carbon moiety is conserved throughout the process and that the accuracy of the assay is not corrupted by the Hopcalite.

In a recent attempt to assay similarly samples of air containing [^{18}O]carbon monoxide, we encountered substantial and irreproducible isotopic dilution and memory effects. The following was typical of our results. No ^{18}O was observed in samples known to contain 1–5 atom % ^{18}O when a fresh charge of Hopcalite was used in the post-column reactor. When a neat sample of $C^{18}O$ is assayed, the ^{18}O content of the CO_2 product tended to increase from an initial value of 10–15 atom % to larger values approaching 90% in replicate runs. Subsequent to the exposure of the Hopcalite to a single sample of neat $C^{18}O$ unenriched CO would react with the catalyst to produce CO_2 with variable amounts of incorporated ^{18}O.

We conclude that the oxygen moiety of CO is not conserved as it exchanges freely with the Hopcalite oxygen pool and that therefore this method is inappropriate to the isotopic assay of [^{18}O]carbon monoxide.

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1 A. R. Swanson and M. W. Anders, *J. Chromatogr.*, 207 (1981) 365–372.

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journal of chromatography news section

SYMPOSIUM PROGRAM

EIGHTEENTH INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY
"CHROMATOGRAPHY '82"

The Eighteenth International Symposium on Advances in Chromatography will be held April 15–17, 1982, at the City Center Hall in Tokyo, Japan.

A total of 45 papers and 57 posters will be presented at the Symposium; Japanese and English will be the languages used at the meeting. A special feature of this meeting will be an exposition of the latest instrumentation and books.

Registration should be made in advance. The programs and registration forms can be obtained from:

Professor Albert Zlatkis
Chemistry Department
University of Houston
Houston, TX 77004, U.S.A.
Tel. (713) 749-2623

The detailed program of the Symposium is given below.

THURSDAY, APRIL 15, 1982

L.S. Ettre, presiding

9:00 Welcome to Symposium
9:15 Presentation of the Tswett Chromatography Medal

CONTEMPORARY CHROMATOGRAPHY

S. Tsuge and G. Schill, presiding

9:30 G. Muto (Saitama Institute of Technology, Tokyo) – Development of chromatography in Japan.
9:50 E. Jellum and A.K. Thorsrud (University of Oslo, Oslo, Norway) – Multicomponent analyses of human tissues and body fluids using capillary GC–MS and high resolution two-dimensional electrophoresis.
10:20 F. Morishita, H. Murakita, Y. Takemura and T. Kojima (Kyoto University, Kyoto) – Prediction of molecular structures of thiols and sulfides by retention indices.
10:40 Intermission
10:50 A. Karmen, S.K. Lam and G. Malikin (Albert Einstein College of Medicine, Bronx, NY, U.S.A.) – Therapeutic drug monitoring by chromatography.

11:20 N. Ôi, T. Doi, H. Kitahara and Y. Inda (Sumitomo Chemical Co., Ltd., Hyogo-ken) – Gas chromatographic determination of optical isomers of some carboxylic acids and amines with optically active stationary phases.

11:40 R.M. Barkely, S. Singhawangcha, M.A. Wizner, R.S. Hutte, M.K. Conditt, E.J. Williams and R.E. Sievers (CIRES, University of Colorado, Boulder, CO, U.S.A.) – New gas chromatographic techniques applied to environmental problems.

Thursday Afternoon

CAPILLARY GAS CHROMATOGRAPHY

N. Ôi and R.E. Kaiser, presiding

2:00 S. Tsuge, Y. Matsushita, N. Watanabe, M. Yanagisawa, K. Kitagawa and Y. Hoshika (Nagoya University, Nagoyashi) – Rapid determination of polynuclear hydrocarbons in airborne particulates by automated thermal extraction–gas chromatographic system.

2:20 J.A. Rijks and C. Tilburg (Eindhoven University of Technology, Eindhoven, The Netherlands) P. Sandra and M. Roelenbosch (University of Ghent, Belgium) and G. Schomburg and H. Usmann (Max-Planck-Institut für Kohlenforschung, Mülheim/Ruhr, G.F.R.) – Testing of capillary column performance. Part I. Introduction and results of a round-robin experiment.

2:50 H. Hatano and S.Z. Lian (Kyoto University, Kyoto), T. Miki (Tokyo University of Agriculture, Tokyo), H. Wada (Shinwa Kako Co., Kyoto) and Y. Hinaga (Electron Scientific Co., Tokyo) – High resolution whisker glass capillary column GC– and LC–mass spectrometries for organic and biochemical compounds.

3:10 F.W. Karasek, R.E. Clement and A.C. Viau (Guelph-Waterloo Centre for Graduate Work in Chemistry, Waterloo, Canada) – Distribution of PCDD and other toxic compounds generated on fly ash particulates in municipal incinerators.

3:40 Intermission

R.P.W. Scott and T. Kojima, presiding

3:50 H. Saito (Shimadzu Corporation, Kyoto) – A study on high purity fused-silica capillary columns for gas chromatography.

4:10 S.R. Lipsky and W.J. McMurray (Yale University, New Haven, CT, U.S.A.) – Cross linking of silicone stationary phases on fused-silica glass capillary columns in gas chromatography: a preliminary assessment of techniques.

4:40 K. Watabe, T. Hobo and S. Suzuki (Tokyo Metropolitan University, Tokyo) – Adsorption phenomenon under DC electric field applied across the liquid crystal column in gas chromatography.

FRIDAY, APRIL 16, 1982

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

N. Suzuki and E. Gil-Av, presiding

9:00 H. Hatano (Kyoto University, Kyoto) – Progress of modern liquid chromatography in Japan.

9:20 E.sz. Kováts and F. Riedo (École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland) – Adsorption from liquid mixtures and liquid chromatography.

9:50 K. Seta, M. Washitake and H. Yoshizawa (Taisho Pharmaceutical, Ltd., Saitama), N. Takai (University of Tokyo, Tokyo) and T. Okuyama (Tokyo Metropolitan University, Tokyo) – High-performance liquid chromatography of body fluids using macroreticular anion-exchange and cation-exchange resins.

10:10 E. Katz, K. Ogan and R.P.W. Scott (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.) – Dispersion in packed beds: Fact and theory.

10:40 Intermission

A. Tsuji and E. Jellum, presiding

10:50 T. Tsuda, I. Tanaka and G. Nakagawa (Nagoya Institute of Technology, Nagoya-shi) – Packed microcapillary liquid chromatography with reduced I.D. columns.

11:10 K. Klotter, T. Schlabach, R. Cunico and R.E. Majors (Varian Associates, Walnut Creek, CA, U.S.A.) – Improved post-column detection of nitrogenous-based compounds in HPLC.

11:40 S. Terabe, K. Yamamoto and T. Ando (Kyoto University, Kyoto) – Application of the streaming current detector for high-performance liquid chromatography to the analysis of individual bile acids.

Friday Morning

GENERAL AND BIOMEDICAL CHROMATOGRAPHY

T. Okuyama and F.W. Karasek, presiding

9:00 H. Miyazaki, M. Ishibashi, Y. Hashimoto, G. Idzu and Y. Furuta (Nippon Kayaku Co., Tokyo) – Simultaneous determination of glyceryl trinitrate and its principal metabolites of 1,2- and 1,3-glyceryl dinitrates in plasma by gas chromatography–negative ion chemical ionization–selected ion monitoring.

9:20 J.K. Haken and J.A. Obita (University of New South Wales, Kensington, Australia) – The chromatographic analysis of aromatic polyhydrazides, oxalyl arylene polyhydrazides and aromatic poly(amidehydrazides) after alkali fusion.

9:50 S. Honda, M. Takahashi, G. Matsuda and K. Kakehi (Kinki University, Higashi-Osaka), A. Honda (Koshien University), T. Takai (Yodogawa Christian Hospital) and S. Ganno (Hitachi, Ltd., Tokyo) – Sensitive high-performance anion-exchange chromatography of carbohydrate borate complexes using a new resin and a new reagent for post-column labelling.

10:10 T. Hanai and J. Hubert (Université de Montreal, Montreal, Canada) – Hydrophobicity and chromatographic behaviour of urinary compounds.

10:30 J.C. Giddings, M.N. Myers, F.-S. Yang, J.-P. Chang and K.D. Caldwell (The University of Utah, Salt Lake City, UT, U.S.A.) – A study of the transition from normal to steric field-flow fractionation.

Friday Afternoon

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. Hatano and R.E. Sievers, presiding

2:00 M. Goto, E. Sakurai and D. Ishii (Nagoya University, Nagoya-shi) – Dual electrochemical detector for micro high-performance liquid chromatography and its application to the determination of catecholamines.

2:20 T. Crispin and I. Halász (Universität des Saarlandes, Saarbrücken, G.F.R.) – Determination of the pore size distribution, by exclusion chromatography, of ion exchange polymers which swell in water.

2:50 N. Takai, H. Takahashi, K. Takahashi and T. Magara (Institute of Industrial Science, University of Tokyo, Tokyo) and A. Takeuchi, T. Dohi and H. Hunakubo (Tokyo University, Tokyo) – High-performance liquid chromatography of multi-wave detector.

3:10 E. Gil-Av (The Weizmann Institute of Science, Rehovot, Israel) – Approaches to the chromatographic resolution of optical isomers.

3:40 Intermission

M. Novotny and T. Nambara, presiding

3:50 Y. Hanaoka, T. Murayama, S. Muramoto, T. Matsuura and A. Nanba (Yokokawa Electric Works Ltd., Musashinoshi) – Ion chromatography with an ion exchange membrane suppressor.

4:10 C.J.W. Brooks, W.J. Cole, J.H. Borthwick and G.M. Brown (University of Glasgow, Glasgow, United Kingdom) – Characterization of dihydroarenediols and related compounds by GC–MS: Comparison of derivatives.

4:40 I. Hara, K. Shiraishi and M. Okazaki (Tokyo Medical and Dental University, Chiba) – High-performance liquid chromatography of human serum lipoprotein.

Friday Afternoon

GENERAL AND BIOMEDICAL CHROMATOGRAPHY

A. Karmen and K. Kanoda, presiding

2:00 S. Mori (Mie University, Tsu) – Size-exclusion chromatography of copolymers: Problems and solutions.

2:20 R.E. Kaiser (Institute of Chromatography, Bad Dürkheim, G.F.R.) – Computer graphics for improved chromatogram evaluation.

2:50 J. Goto, N. Goto and T. Nambara (Tohoku University, Sendai) – Separation and determination of naproxen enantiomers in serum by high-performance liquid chromatography.

3:10 K.W.M. Siu and W. Aue (Dalhousie University, Halifax, Canada) – Studies on the mechanism of the electron-capture detector.

3:40 Intermission

S. Mori and J.A. Rijks, presiding

3:50 K. Kojima, T. Manabe and T. Okuyama (Tokyo Metropolitan University) and T. Tomono, T. Suzuki and E. Tokunga (Japan Red Cross Central Blood Center, Tokyo) – A two-dimensional separation system for analysis of proteins employing isoelectric focusing and high-performance liquid chromatography.

4:10 R. Segura and X. Navarro (Autonomous University of Barcelona, Bellaterra, Spain) – Determination of lipid classes using submicroliter amounts of plasma by thin-layer chromatography and "*in situ*" spectrofluorometry.

SATURDAY, APRIL 17, 1982

Saturday Morning (9:00 - 12:00 a.m.)

POSTER SESSIONS : GAS CHROMATOGRAPHY

1. Y. Nagayanagi and Y. Hayashi (Shimadzu Corporation, Kyoto) – "Moving precolumn" injector and its application.
2. S. Yasui (Shimadzu Corporation, Kyoto) – Sensitivity characteristics of FTD using carrier gas containing hydrocarbon.
3. T. Murata, S. Takahashi, S. Ohnishi and K. Hosoi (Shimadzu Corporation, Kyoto) and T. Nakashima, Y. Ban and K. Kuriyama (Kyoto Prefectural University of Medicine, Kyoto) – Characterization of bile acid methylester acetate derivatives of rat bile using solventless glass capillary gas–liquid chromatography, electron impact and ammonia chemical ionization mass spectrometry.
4. S. Takatsuto, B. Ying, M. Morisaki and N. Ikekawa (Tokyo Institute of Technology, Tokyo) – Microanalysis of brassinolide and its analogs by gas chromatography and GC–MS.
5. Y. Matsuki, T. Ito, K. Fukuhara, T. Nakamura and H. Ono (Food and Drug Safety Center, Hadano) and T. Nambara (Tohoku University, Sendai) – A new method for simultaneous determination of 1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline(captopril) and its disulphide in biological fluids.
6. S. Honda, N. Yamauchi, M. Nagata, S. Suzuki, K. Maeda, M. Teramae and K. Kakehi (Kinki University, Higashi-Osaka) – Capillary gas–liquid chromatography of carbohydrates as dithioacetal derivatives.

7. H. Miyazaki, M. Ishibashi, K. Yamashita and I. Ohguchi (Nippon Kayaku Co.) and H. Saito, H. Kurono and M. Shimono (Shimadzu Corporation, Kyoto) – Microdetermination of prostaglandins and thromboxane B_2 by gas chromatography using electron-capture detector.
8. T. Yamaguchi, Y. Morimoto, Y. Sekine and M. Hashimoto (Dainippon Pharmaceutical Co., Ltd., Osaka) – Determination of β-adrenergic blocking drugs as cyclic boronates by gas chromatography with nitrogen-selective detection.
9. N. Kashihira, K. Makino, K. Kirita and Y. Watanabe (Training Institute for Environmental Pollution Control, Tokorozawa) – Chemiluminescent nitrogen detector–gas chromatography and its application to measurement of atmospheric ammonia and amines.
10. E.F. Dawes (Scientific Glass Engineering, Pty. Ltd., Melbourne, Australia) – A simple solution to on-column injection into capillaries.
11. L. Zhou (Dalian Institute of Chemical Physics, Dalian, China) and H. Shanfield and A. Zlatkis (University of Houston, Houston, TX, U.S.A.) – Quantitative determination of lecithin and sphingomyelin at nanogram levels by HPTLC using fluorescence.
12. S. Honda, Y. Fukuhara, Y. Takai, Y. Ohkaru, K. Takeda, K. Mukai and K. Kakehi (Kinki University, Higashi-Osaka) – Studies of the structures of carbohydrate chains by gas–liquid chromatography of periodate oxidation products.
13. S. Noda and T. Morishita (Hikari Kogyo Co. Ltd., Tokyo) and S. Ohgoshi, T. Takahashi and T. Sato (Government Industrial Development Laboratory, Sapporo) – Simplified analysis of heavy water by gas chromatography.

LIQUID CHROMATOGRAPHY

14. S. Matsushita, Y. Tada and T. Ikushige (Toyo Soda Mfg. Co., Ltd., Yamaguchi) – Separation of large anions using a new anion separator column.
15. T. Yamabe (Kanagawa University), N. Takai (University of Tokyo, Tokyo) and S. Ganno and K. Tsukada (Hitachi, Ltd., Tokyo) – High-performance liquid chromatography of silanized silica gel.
16. A. Nakamoto, A. Seido, T. Sato, S. Takimoto and I. Kohsaka (Shimadzu Corporation, Kyoto) – Performance of a new microprocessor-controlled LC system and its applications.
17. K. Jinoo, C. Fujimoto and D. Ishii (Toyohashi University of Technology, Toyohashi) – A buffer memory technique for the combination of micro high-performance liquid chromatography and infrared spectroscopy.
18. A. Wada, K. Hibi, S. Yagi, T. Shimizu, M. Saito, M. Sugawara and K. Hayashi (Japan Spectroscopic Company Ltd., Tokyo) – New gradient elution and temperature programming methods by a micro-processor-controlled HPLC system, Jasco Tri Rotar-III.
19. Y. Mito and H. Iwasaki (Shimadzu Corporation, Kyoto) – Photodiode array detector for HPLC.
20. T. Ohno, H. Kobayashi and S. Mizusawa (Chiba University, Chiba) – Simultaneous determination of ionic species and UV-absorptive substances in solutions by ion-exchange chromatography with conductometric and UV-absorptiometric detector.
21. T. Takeuchi and D. Ishii (Nagoya University, Nagoya-shi) – High back-pressure liquid chromatography. II. Development of post-column controlled flow system.
22. S. Mori (University of Tokyo, Tokyo) – A rapid flow monitoring system of radioactive organic/amino acids.
23. K. Nunogaki, Y. Nunogaki and W. Murayama (Sanki Engineering Ltd., Kyoto) – Centrifugal partition chromatograph and its application.
24. S. Ishiguro, Y. Inoue and T. Hosogane (Instrument Products Research Laboratory, Tokyo) – High speed, high resolution gel permeation chromatography for small molecules and oligomers.
25. H. Hatano (Kyoto University, Kyoto) and K. Sumizu (Kyoto Blood Research Institute, Kyoto) – New packings (polyvinylalcoholic porous gel), for high-performance gel filtration of organic and biochemical compounds.
26. K. Saitoh, E. Ozaki and N. Suzuki (Tohoku University, Miyagi) – Solvent dependence of gel chromatographic retention of low-molecular-weight compounds on polystyrene-divinylbenzene gel.
27. S. Aoyagi, K. Hirayanagi and T. Ishikawa (Chiba University, Chiba) – Preparative separation of some phosphate esters on non-ionic gel columns.
28. T. Yamada, Y. Sato, K. Mizukoshi, T. Hosogane and S. Nakamura (Showa Denko K.K., Tokyo) – New automated high-speed aqueous GPC preparative system.

29. N. Nimura, A. Toyama, Y. Kasahara, T. Suzuki and T. Kinoshita (Kitasato University, Tokyo) – Reversed-phase high-performance liquid chromatographic resolution of amino acid enantiomers.
30. S. Hara, Y. Dobashi and K. Oka (Tokyo College of Pharmacy, Tokyo) – Silica gel liquid–liquid chromatography using aqueous phase systems.
31. S. Hara (Tokyo College of Pharmacy, Tokyo), K. Kunihiro and H. Yamaguchi (Wakunaga Pharmaceutical Co., Hiroshima) and E. Soczewinski (Medical Academy, Lublin, Poland) – Ternary solvent system design for liquid–solid chromatography.
32. S. Ohnishi, M. Murase and T. Yoneya (Kanebo Cosmetics Laboratory, Kanagawa) and S. Hara (Tokyo College of Pharmacy, Tokyo) – Column selectivity in normal phase liquid chromatography.
33. R. Horikawa, H. Sakamoto and T. Tanimura (Toyama Medical and Pharmaceutical University, Toyama) – Separation of α-hydroxy carboxylic acid enantiomers by high-performance liquid chromatography using chiral eluent.
34. T. Nakagawa, A. Shibukawa and T. Uno (Kyoto University, Kyoto) – Liquid chromatography with crown ether-containing mobile phases. II. Retention behaviour of β-lactam antibiotics on a hydrophobic stationary phase.
35. M. Kimura and Y. Itokawa (Kyoto University, Kyoto) – High sensitive determination of total blood thiamine content and erythrocyte transketolase by high-performance liquid chromatography.
36. K. Kuwata, M. Uebori and Y. Yamazaki (Environmental Pollution Control Center, Osaka City) – Liquid chromatographic determination of alkylthiols in polluted air via derivatization with 5,5'-dithiobis(2-nitrobenzoic acid).
37. J. Kawase, A. Nakae, H. Ueno and K. Tsuji (Kao Soap Co., Ltd., Tochigi) – High-performance liquid chromatography of urea and related compounds with post-column derivatization.
38. T. Ui, M. Mitsunaga, T. Tanaka and M. Horiguchi (Jikei University School of Medicine, Tokyo) – Simultaneous determination of predisone and prednisolone in human serum by high-pressure liquid chromatography.
39. K. Mibe (Tokyo Hospital of the Printing Bureau, Tokyo) – A correlation of retention behavior with molecular structure of 16α-hydroxyprednisolone acetal derivatives and related compounds by chemical bonded reversed-phase liquid chromatography.
40. T. Sakai, S. Yanagihara and K. Ushio (Tokyo Labor Accident Hospital, Tokyo) – Determination of 5'-nucleotidase activity in human erythrocytes and plasma using high-pressure liquid chromatography.
41. A. Nakagawa and K. Nakamura (Sankyo Co. Ltd., Tokyo) and T. Ishizaki and K. Chiba (National Medical Center Hospital, Tokyo) – An automated HPLC method for the determination of antipyrine and its metabolites in urine: Some preliminary results obtained from smokers and non-smokers.
42. Y. Watanabe and K. Imai (University of Tokyo, Tokyo) – High-performance liquid chromatography of amino acids derivatized with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole.
43. Y. Kawahara, T. Yamazaki and T. Morioka (Sankyo Co. Ltd., Tokyo) – High-performance liquid chromatographic analysis of glutathione, cysteine and captopril in biological samples.
44. K. Matsubara, N. Hagi and H. Watanabe (Toyo Soda Manufacturing Co., Ltd., Kanagawa-ken) and T. Nakanishi, H. Okuda and H. Obata (Tokyo Women's Medical College, Tokyo) – Reversed-phase high-performance liquid chromatographic separation and enzymatic determination of individual bile acids: free, glycine- and taurine-conjugated bile acids.
45. K. Mori (National Institute of Industrial Health, Kawasaki) – Automated measurement of tryptophan and metabolites in urine, blood and tissue by high-performance liquid chromatography with fluorometric detection.
46. H. Miyagi, J. Miura, M. Taki, Y. Takata, S. Kamitake and S. Ganno (Hitachi Ltd., Ibaraki) and Y. Yamagata (Hitachi General Hospital, Ibaraki) – Analyses of body functions using a clinical liquid chromatograph.
47. T. Okano, Y. Sakurai, K. Kataoka, I. Gonoi, T. Eguchi, H. Obata, S. Akimoto, H. Okuda and I. Ohi (Tokyo Women's Medical College, Tokyo), H. Nakamura and M. Deai (Toyo Soda Manufacturing Co., Ltd., Tokyo) and S. Takai and M. Senou (University of Tokyo, Tokyo) – Correlation matrix method for urine analysis by HPLC and its application to cancer diagnosis.
48. S. Chikui (Kobe Municipal Technical College, Kobe) – The quantitative determination of nucleosides (and nucleotides) by "chromoelectrography" using papers (CM, DEAE, ECTEOLA) or thin layers (cellulose, alumina, polyamid, HPTLC) and separation.

49. M. Igawa, H. Takagi, M. Tanaka and T. Yamabe (Kanagawa University, Yokohama) – Ion chromatography on silica-coated polyamide crown resin.
50. I. Matsumoto, Y. Ito and N. Seno (Ochanomizu University, Tokyo) – New carrier for affinity adsorbents.
51. Y. Hashimoto, K. Kawanishi, H. Tomita and M. Moriyasu (Kobe Women's College of Pharmacy, Kobe) – Histochemical chromatography and its application.
52. M. Okamoto and F. Yamada (Gifu Prefectural Institute of Public Health, Gifu) and T. Ohmori (Kanto-Merck Chemical Co., Inc., Tokyo) – Preparation and evaluation of NH_2-treated plates for the high-performance thin-layer chromatographic analysis of some sugar compounds.
53. T. Mizutani and T. Narihara (Nagoya City University, Nagoya) – Adsorption chromatography of proteins on siliconized porous glass.
54. Y. Kato, K. Nakamura, T. Kitamura and T. Hashimoto (Toyo Soda Manufacturing Co., Ltd., Yamaguchi) – Dependence of resolution on operational variables in medium-performance ion-exchange chromatography of proteins.
55. S. Tsunasawa and K. Narita (Osaka University, Osaka) – Micro-identification method for amino terminal acetylamino acids in proteins using HPLC.
56. U. Matsumoto, Y. Shibusawa and M. Ban (Tokyo College of Pharmacy, Tokyo) – Surface affinity chromatographic separation of human blood cells.
57. E. Isohata, Y. Saito and M. Uchiyama (National Institute of Hygienic Sciences, Tokyo) – High-performance liquid chromatographic determination of sterigmatocystin in foodstuffs and animal tissues by derivative formation.

Saturday Afternoon

NEW DEVELOPMENTS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

D. Ishii and E. sz. Kováts, presiding

2:00 G. Schill (University of Uppsala, Uppsala, Sweden) – Reversed-phase HPLC with detection by ion-pairing agent.

2:30 K. Saitoh, M. Kobayashi and N. Suzuki (Tohoku University, Miyagi) – High-performance liquid chromatography of metal tetraphenylporphyrin chelates with C_{18}-bonded stationary phase.

2:50 M. Novotny and V.L. McGuffin (Indiana University, Bloomington, IN, U.S.A.) – Element-specific thermionic detection in micro-HPLC.

3:20 N. Tanaka, Y. Tokuda, K. Iwaguchi and M. Araki (Kyoto Technical University, Kyoto) – The effect of the stationary phase structure on the retention and selectivity in reversed-phase liquid chromatography.

3:40 Intermission

N. Tanaka and R.E. Majors, presiding

3:50 E. Grushka, I. Atamna and C. Gilon (Hebrew University, Jerusalem, Israel) – Separation and detection of N-methylated amino acids using reversed-phase HPLC.

4:10 A. Tsuji, M. Maeda and S. Kamada (Showa University), Y. Umezawa (Tokyo University) and T. Kurahashi (Yanagimoto Seisakusho, Tokyo) – Separation and determination of bile acids by HPLC equipped with 3-α-hydroxysteroid dehydrogenase column and electrochemical detector.

4:30 V.V. Berry and R.E. Shansky (Polaroid Corporation, Cambridge, MA, U.S.A.) – Universal liquid chromatography methods. III. New techniques for sensitive, low-wave wavelength, full-gradient runs.

5:00 Closing of Symposium

NEW BOOKS

Electron capture – Theory and practice in chromatography (Journal of Chromatography Library, Vol. 20), edited by A. Zlatkis and C.F. Poole, Elsevier, Amsterdam, Oxford, New York, 1981, XII + 429 pp., price Dfl. 180.00, US$76.50, ISBN 0-444-41954-3.

Recent developments in mass spectrometry in biochemistry, medicine and environmental research, 7 *(Proc. 7th Int. Symp., Milan, June 16–18, 1980)* (Analytical Chemistry Symposia Series, Vol. 7), edited by A. Frigerio, Elsevier, Amsterdam, Oxford, New York, 1981, IX + 360 pp., price Dfl. 170.00, US$72.25, ISBN 0-444-42029-0.

Affinity chromatography and related techniques – Theoretical aspects/Industrial and biomedical applications *(Proc. 4th Int. Symp., Veldhoven, June 22–26, 1981)* (Analytical Chemistry Symposia Series, Vol. 9), edited by T.C.J. Gribnau, J. Visser and R.J.F. Nivard, Elsevier, Amsterdam, Oxford, New York, 1982, XVIII + 584 pp., price Dfl. 195.00, US$83.00, ISBN 0-444-42031-2.

Wilson and Wilson's Comprehensive analytical chemistry, Vol. XIII, Analysis of complex hydrocarbon mixtures, edited by G. Svehla, Elsevier, Amsterdam, Oxford, New York, 1981; **Part A, Separation methods,** *ca.* 382 pp., price Dfl. 225.00, US$95.75, ISBN 0-444-99736-9; **Part B, Group analysis and detailed analysis,** *ca.* 400 pp., price Dfl. 225.00, US$95.75, ISBN 0-444-99735-0.

Applications of mass spectrometry to trace analysis *(Lectures of a course, Ispra, Sept. 29–Oct. 3, 1980)*, edited by S. Facchetti, Elsevier, Amsterdam, Oxford, New York, 1982, X + 322 pp., price Dfl. 185.00, US$78.75, ISBN 0-444-42042-8.

Advances in steroid analysis *(Proc. Symp., Eger, May 20–22, 1981)* (Analytical Chemistry Symposia Series, Vol. 10), edited by S. Görög, Elsevier, Amsterdam, Oxford, New York, 1982, 464 pp., price Dfl. 225.00, US$95.75, ISBN 0-444-99711-3.

Food Flavours, Part A, Introduction, edited by I.D. Morton and A.J. MacLeod, Elsevier, Amsterdam, Oxford, New York, 1981, VIII + 472 pp., price Dfl. 275.00, US$117.00, ISBN 0-444-41857-1.

1982 DAL NOGARE AWARD

MARCEL GOLAY WINS DAL NOGARE AWARD

Marcel J.E. Golay is the recipient of the 1982 Dal Nogare Award sponsored by the Chromatography Forum of the Delaware Valley. The award will be presented during the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Tuesday afternoon, March 9, 1982, in Atlantic City, NJ, U.S.A., as part of a special award symposium.

Dr. Golay was born in Neuchatel, Switzerland, on May 3, 1902. He received the licentiate in electrical engineering on the Eidgenossische Technical School in Zurich in 1924. From 1924 through 1928, he was on the staff of Bell Telephone Laboratories in New Jersey. In 1928, he entered the University of Chicago, where in 1931, he received a Ph.D. in atomic physics. Golay then joined the U.S. Signal Corps Engineering Laboratories, where he remained until 1955. He has been a consultant to both the Philco Corporation and Perkin-Elmer Corporation. Since 1963, he has been a Senior Scientist at Perkin-Elmer.

Dr. Golay has received numerous awards, including the Harry Diamond Award of the Institute of Electric and Electronic Engineers, the American Chemical Society Award in Chemical Instrumentation, the Distinguished Achievement Award of the Instrument Society of America, the M.S. Tswett Chromatography Medal, and the ACS Chromatography Award. He has an honorary Doctor of Science degree from Ecole Polytechnique Federale of Lausanne, Switzerland.

His accomplishments cover the fields of analytical chemistry, engineering, and mathematics. In 1955, he predicted the development of the open tubular column for gas chromatography, and in 1958, demonstrated its utility. He has also contributed to the theory of liquid chromatography.

His address at the Dal Nogare Award Symposium will be, "The Transport of Solutes Along Boundary Surfaces by the Combined Action of Fluid Shear and Diffusion".

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH		J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography		234/1 234/2 235/1 235/2	236/1 236/2	The publication schedule for further issues will be published later.									
Chromatographic Reviews													
Biomedical Applications		227/1	227/2										

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

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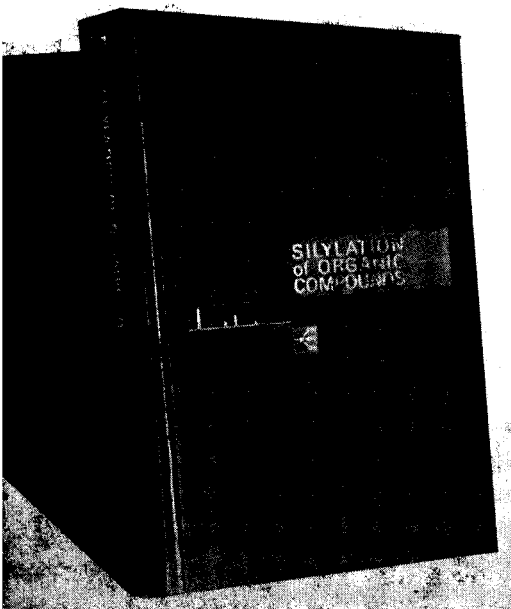

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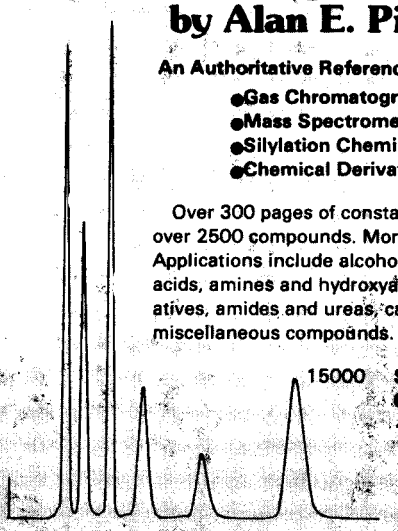

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