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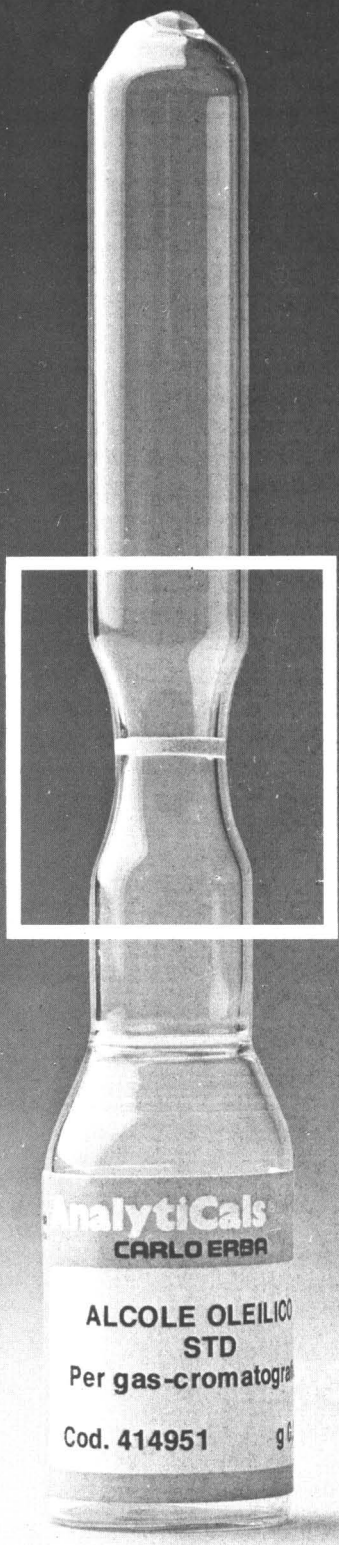

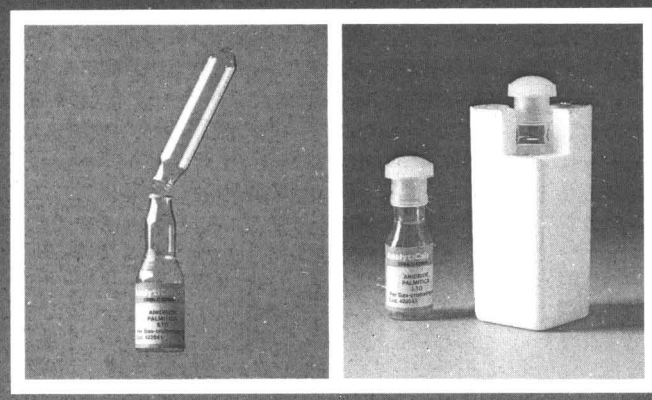

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EFFECT OF MOLECULAR STRUCTURE AND CONFORMATIONAL CHANGE OF PROLINE-CONTAINING DIPEPTIDES IN REVERSED-PHASE CHROMATOGRAPHY

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(Received July 29th, 1981)

SUMMARY

Peptides that contain proline residues may yield multiple peaks in high-performance liquid chromatography if the proline is not at the N-terminus. The phenomenon is caused by slow kinetics of isomerization that are on the same time-scale as the chromatographic separation with such peptides. In this study, the shape and number of peaks are examined qualitatively in view of the influence of isomerization kinetics on the bandspreading, and as functions of pH, temperature and flow velocity. These effects in the chromatography of alanylproline are shown in detail. Single peaks are obtained for each peptide investigated by proper adjustment of these variables in light of their effect on the pertinent rate and equilibrium constants.

INTRODUCTION

The appearance of more peaks in a chromatogram than expected from the number of sample components is usually attributed to impurities. It is known, however, that a given substance can yield multiple peaks for a variety of physical reasons, including poor sample introduction, maldistribution of eluent flow[1] and non-uniform radial temperature profile in the column[2]. In the present work we examine peak splitting that occurs as a consequence of a conformation change of an eluite at a rate that is commensurable with that of the chromatographic distribution process between the mobile and stationary phases. Dipeptides that contain L-proline have been chosen as model substances because their pertinent properties are well documented in the literature. Thus, we can relate the effect of operational conditions on the observed unusual behavior to the molecular structure of the sample components and the underlying secondary equilibria. Similar phenomena have been observed in thin-layer chromatography (TLC)[3] and in high-performance liquid chromatography (HPLC) with other substances[4,5]. As early as 1960, band-spreading, due to concomitant migration and reaction, was treated quantitatively by Giddings[6,7]. Recently the significance of slow kinetics in determining column efficiency in HPLC has been pointed out in a general fashion by Horváth and Lin[8]. Results presented here are expected to facilitate

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recognition of such secondary equilibria in practice. A qualitative understanding of the relationship between operating conditions and separation efficiency can lead to optimization of separation, particularly in the case of biological substances.

All peptides can be in either the *cis* or *trans* conformation with respect to the amide bond, although most of them exist exclusively in *trans* form[9,10]. However, certain peptides linked via the imino nitrogen of proline are peculiar because they can be present both in *cis* and *trans* forms under usual conditions[10–21]. Furthermore, the rate at which a new equilibrium composition of their mixture is attained upon slight change in conditions is relatively slow[10,13,14,17,18]. In some cases relaxation times are of the order of minutes, *i.e.*, on the time-scale of chromatographic runs in HPLC. If the retention factors of the *cis* and *trans* form are not identical, peak splitting, or at least excessive band-spreading, can be expected as a result of the relatively slow kinetics of the conformation change.

Examination of appropriate molecular models of dipeptides containing proline reveals that the hydrophobic surface area that affects the strength of eluite binding to the stationary phase[22,23] is different for the two conformers. This can be seen from the space-filling models for *cis*- and *trans*-alanylproline shown in Fig. 1. In the *cis* conformer a plane through the two α-carbons separates non-polar residues from the polar amino and carboxyl groups. No such plane that separates the polar from the non-polar residues can be found for the *trans* form of alanylproline, however. Consequently the solvophobic theory[22–24] predicts that in reversed-phase chromatography the retention of the *cis* conformer, which can have a larger contact area upon binding to the hydrocarbonaceous ligates of the stationary phase, will be greater than that of the *trans* form, which has a smaller hydrophobic surface area.

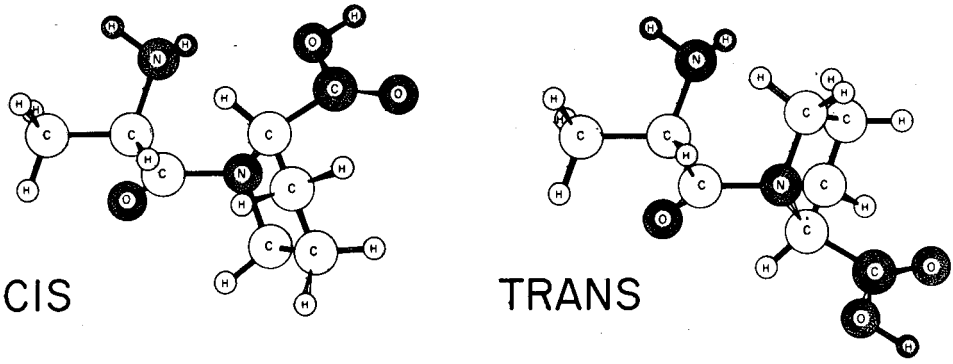

Fig. 1. Perspective molecular structures of *cis*- and *trans*-L-alanyl-L-proline with the polar functions shaded. Whereas polar and non-polar residues in the *cis* conformation can be placed on opposite sites of a hypothetical plane, no such plane exists for *trans* conformation. Consequently, retention of the *cis* conformer in reversed-phase chromatography is expected to be greater than that of the *trans* conformer.

EXPERIMENTAL

The apparatus consisted of a Model 100A pump (Altex, Berkeley, CA, U.S.A.), a Model 70-10 sampling valve (Rheodyne, Berkeley, CA, U.S.A.) a Model LC-55 variable-wavelength spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Model 123 recorder (Perkin-Elmer). The precolumn heat exchanger and the

column were thermostatted by using a Model K-2/R circulating water bath (Messgeraetewerke, Lauda, G.F.R.). The column effluent was monitored at 210 nm detector setting.

The 10-μm LiChrosorb RP-18 column (250 × 4.6 mm I.D.) was obtained from Knauer (Berlin, G.F.R.). Reagent phosphoric acid, NaH_2PO_4 and Na_2HPO_4 were obtained from Fisher (Pittsburgh, PA, U.S.A.). The peptides were purchased from Sigma (St. Louis, MO, U.S.A.).

Perspective drawings of L-alanyl-L-proline and L-prolyl-L-alanine molecules were prepared by using the PROPHET system.

RESULTS AND DISCUSSION

Chromatograms of L-prolyl-L-alanine and L-alanyl-L-proline obtained on octadecyl-silica with plain aqueous buffer, pH 6, as the eluent at 25°C are depicted in Fig. 2. The second peptide, in which proline is the C-terminal residue, yielded a broad "peak" with fairly sharp spikes on the leading and trailing edges, whereas more normal peak shape has been observed for the first peptide. Similar results were obtained with the dipeptide pairs L-valyl-L-proline and L-prolyl-L-valine, as well as L-prolyl-L-phenylalanine and L-phenylalanyl-L-proline. In each case, the recorder response of the dipeptide with a C-terminal proline residue, which we will call a *proline peptide*, yielded "peaks" that consisted of two spikes and an intermediary plateau region. In contradistinction the tracing of the corresponding peptide isomer with proline at the N-terminus, which we will call *prolyl peptide*, showed only one sharp

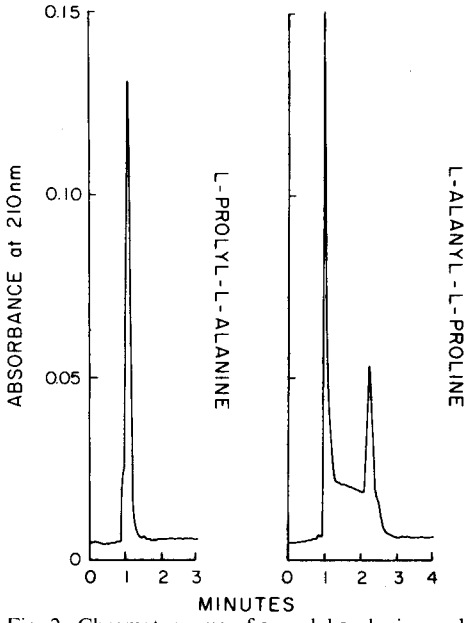

Fig. 2. Chromatograms of L-prolyl-L-alanine and L-alanyl-L-proline. The data were obtained with neat aqueous 0.05 *M* phosphate buffer, pH 6.0, at 3 ml/min flow-rate he column (250 × 4.6 mm I.D.) was packed with 10-μm LiChrosorb RP-18. The temperature was 25°C.

peak in all cases. In some other cases the effect was less dramatic; *e.g.*, glycylproline did not yield split peaks but only a trailing shoulder under similar chromatographic conditions. Results shown in Fig. 2 can be interpreted on the basis of the above-mentioned facts that (i) the prolyl peptide exists only in *trans* form, (ii) the proline peptide can be present both in *cis* and *trans* forms, and (iii) owing to its smaller hydrophobic surface area, the *trans* conformer elutes faster than the corresponding *cis* conformer. Indeed the first spike of the reaction "peak" of a proline peptide has the same retention as the single peak obtained when the corresponding prolyl peptide is chromatographed. Whereas with the proline peptides the concentrations of the *cis* and *trans* conformers are commensurate, according to the literature[13,14,16,20,25] the prolyl peptides exist only in *trans* form under the conditions investigated here.

The observation that chromatographic peak splitting does not occur with peptides having N-terminal proline supports the proposition that the behavior described here is not due to the presence of proline *per se* in the peptide but only occurs when proline is not at the N-terminus of the peptide. Peak splitting can be readily explained by the slow kinetics of isomerization that are expected on the basis of literature data when proline is not at the N-terminus of the peptides. In some experiments we collected fractions of the column effluent corresponding to various portions of the "reaction peak" obtained with proline peptides. In each case the original chromatogram was reproduced upon rechromatographing these fractions, and this finding suggests that the effects reported here were not caused by sample impurities.

The effect of flow-rate on the shape of alanylproline "peak" is shown in Fig. 3. The results were obtained at two flow-rates, 1.0 and 9.0 ml/min, under conditions otherwise identical with those stated in Fig. 2. The "reaction peak" obtained at the higher flow-rate of 9.0 ml/min is more disperse and bimodal than that obtained at 1.0 ml/min, or even than that recorded at a flow-rate of 3.0 ml/min, and is shown in Fig. 2. It is seen that with decreasing flow-rate the proline peptide elutes as an increasingly monodisperse broad peak. This is consistent with the hypothesis that peak splitting is the consequence of slow kinetics of isomerization. Deviations from equilibrium concentrations develop in the mobile phase as the *cis* conformer of the proline peptide is preferentially bound by the stationary phase. The respective concentrations of the two eluite forms in the mobile phase will relax toward the equilibrium concentrations. When the isomerization rate is much less than the rate for the equilibration of the less retained component between the mobile and stationary phase, the two conformational isomers migrate down the column nearly independently and consequently two recorder peaks or, to be more precise, a bimodal reaction peak is observed. As the rate of isomerization becomes greater, however, the peptide appears as a single peak and, in the limit of infinitely rapid isomerization, only a monodisperse peak with no excessive broadening due to kinetic effects will be observed.

The effect of temperature on the retention and the peak shape of alanylproline is shown in Fig. 4. The chromatograms were run under the conditions given in Fig. 2 except that the column temperature was kept at 25, 40 or 55°C. It is seen that with increase of temperature the tracing becomes increasingly monodisperse until, in this case at 55°C, only somewhat excessive peak broadening hints at the peak splitting found at lower temperatures. This result is consistent with the dominant role of kinetic effects in the determination of peak shape. As the temperature increases the rate of isomerization will increase, because the activation energy for the isomerization

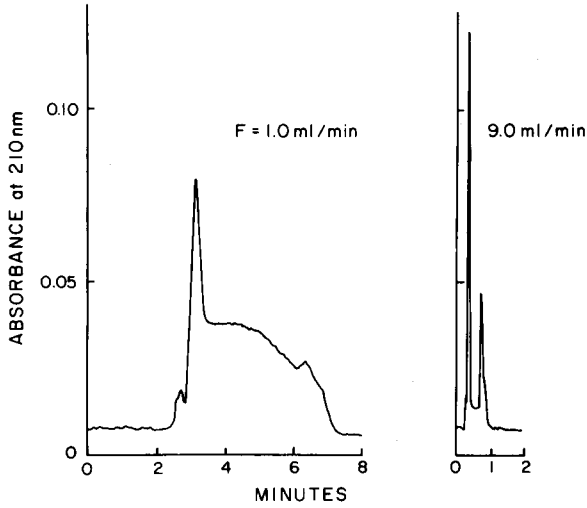

Fig. 3. Effect of flow-rate on the shape of the L-alanyl-L-proline peaks. Chromatographic conditions were those stated in Fig. 2 except the flow-rate, F, which was 1.0 or 9.0 ml/min.

is typically *ca.* 20 kcal/mol[10,14,17,20,26,27]. On the other hand the effect of temperature on retention is less dramatic. The enthalpy of the *cis–trans* equilibrium is nearly zero in aqueous solutions[17,18]. As a result the equilibrium composition will not change palpably with temperature and therefore no change in retention factor is anticipated

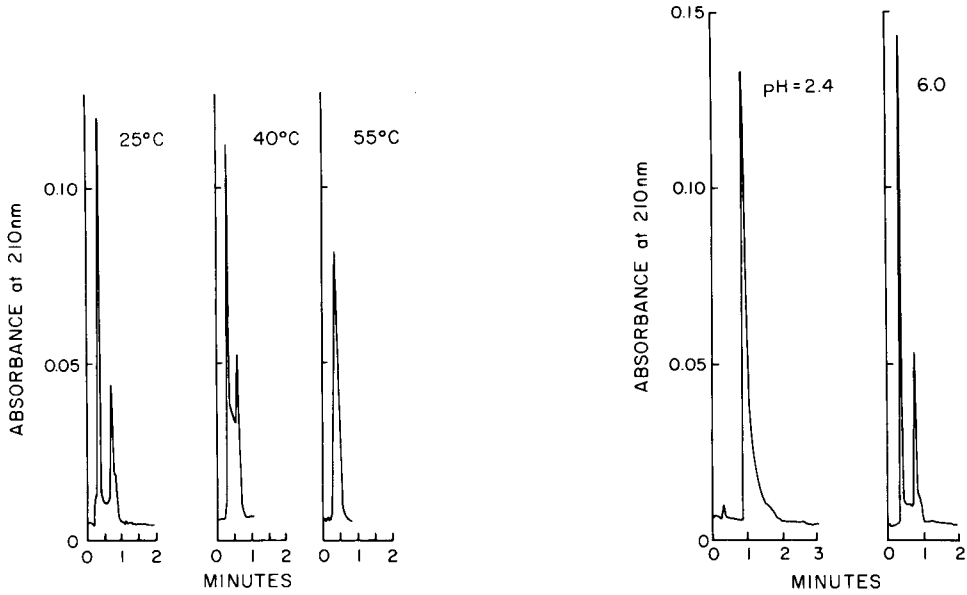

Fig. 4. Effect of column temperature on the elution of L-alanyl-L-proline. The temperature was 25, 40, or 55°C, and the flow-rate was 9.0 ml/min. Other conditions as in Fig. 2.

Fig. 5. Effect of pH on the elution of L-alanyl-L-proline. The mobile phase was 0.05 M phosphate buffer, pH 2.4 or 6.0. The flow-rate was 9.0 ml/min. Other conditions as in Fig. 2.

from that source, although some decrease in retention is expected because the retention enthalpies in reversed-phase chromatography are usually negative[24].

The effect of pH on peak shape of alanylproline is illustrated in Fig. 5. The single peak obtained at the lower pH is somewhat better retained than the split reaction peak of the peptide obtained at higher pH. Such an effect is to be expected if the peak splitting is a consequence of (i) the presence of the *cis* and *trans* forms in approximately equal amounts and (ii) the relatively slow kinetics of the interconversion.

The equilibrium composition contains *ca.* 50% of the *cis* conformer under zwitterionic conditions and decreases to *ca.* 10% under cationic conditions[13-16]. Furthermore, at pH 2.4, the relaxation rate for isomerization is much larger than that found at pH 6.0[14,18]. These effects both reduce the axial dispersion of the peptide peak at low pH values. The observation that the retention appears to increase with decrease of pH conforms with the general experience that the magnitude of the retention factors for the zwitterionic form of amino acids or peptides is lower than that of the corresponding cationic form[29] under otherwise identical chromatographic conditions.

Indeed, decreasing pH and increasing temperature can be employed simultaneously to improve peak shapes so that the chromatogram is suitable for extraction of quantitative data without elaborate computer analysis. The combined effect of relatively low eluent pH and high column temperature is seen on the chromatogram

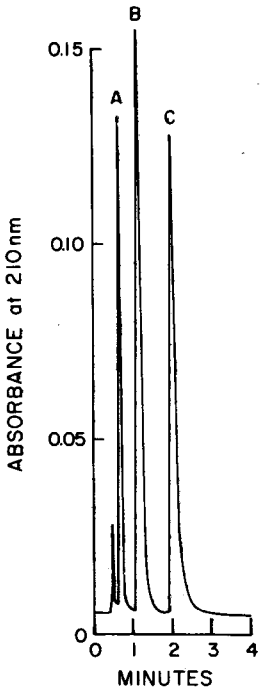

Fig. 6. Chromatogram of L-prolyl-L-glycine (A), L-alanyl-L-proline (B) and L-prolyl-L-valine (C) obtained at low eluent pH and elevated temperature in order to reduce excessive bandspreading of alanylproline. Conditions as in Fig. 2, except the eluent pH, column temperature and flow-rate were 2.4, 55° and 5 ml/min, respectively.

in Fig. 6, where the peak exhibits neither bimodal character nor excesssive band-spreading.

CONCLUSIONS

The data presented here indicate that molecules that exist in two or more distinguishable conformations can display anomalously broad and/or split chromatographic peaks. Knowledge of the effects of operating conditions, such as temperature, pH, mobile phase composition, and salts, on the equilibria and kinetics, however, can be used to reduce the influence of such phenomena on separation efficiency. In the cases examined here, decrease of eluent pH and increase of column temperature both decrease the relaxation time for isomerization with concomitant improvement in the peak shape, as illustrated in Figs. 4–6.

The results are consistent with the interpretation that multiple peaks observed in the chromatography of proline-containing peptides arise from the presence of the peptide in two slowly interconvertible *cis* and *trans* conformers. By extension, multiple peaks should be anticipated in the chromatography of other molecules when the rates of conformational change are slow on the time-scale of the separation by HPLC. Such a situation is likely to occur when free rotation about a bond in the eluite molecule is hindered. Indeed, multiple spots in TLC of cyclic peptides, such as D-methylalanine[1]-tentoxin[28], and multiple peaks in HPLC of the peptides gramicidin and 2-melanotropin[22] were explained by assuming the existence of different conformers of the peptides.

The phenomena investigated here are the consequence of relatively slow kinetics of conformational change in the mobile phase. An understanding of the underlying chemistry facilitates optimization of the chromatographic system as far as the separation efficiency is concerned. In turn, it also seems likely that study of the chromatographic system may facilitate understanding of the physical chemistry of the system insofar as the peak shape contains information on the pertinent equilibrium and rate constants. Extraction of these physico-chemical parameters from chromatographic data appears to be an intriguing and challenging task and is currently under investigation in our laboratory.

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PREDICTIONS OF GAS CHROMATOGRAPHIC RETENTION CHARACTERISTICS OF BARBITURATES FROM MOLECULAR STRUCTURE

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(Received May 11th, 1981)

SUMMARY

Gas chromatographic retention indices of 23 5,5-disubstituted barbituric acid derivatives have been related to the numbers of carbon atoms, molecular weights and molecular connectivity values of the substituent groups. Correlations are low when all compounds are considered, but are excellent when the barbiturates are divided into chemically similar sub-groups. Retention data can be predicted with great accuracy. Overall correlations can be improved either by combination of selected connectivity terms or by modification of existing rules for their calculation. General relationships with very high predictive power are described and their applications discussed.

INTRODUCTION

The use of chromatographic methods for the confirmation of identity relies on the availability of reference data or authentic samples of compounds for comparative purposes. If neither data nor sample are at hand, as is often the case in forensic analyses, the problem of identification is particularly acute. Chromatographic discrimination of barbiturates has recently been examined, and an effective approach to the separation of these closely related compounds has been proposed[1]. However, the usefulness of these data is necessarily restricted to those compounds which have been studied. The wide range of barbiturates means that a sample of any particular one may not always be immediately available for an analysis. It is therefore desirable that accurate predictions of the chromatographic behaviour of uncommon barbiturates can be made so that such barbiturates can be excluded as possible identities in qualitative analyses.

Relationships have been demonstrated between retention properties and a variety of physico-chemical parameters (*e.g.* boiling point, heat of solution). All of these parameters must be determined experimentally, although calculation of some is possible using substituent constants. In many cases, procedures for obtaining a particular value are complex and impractical in routine use. In the following work the gas chromatographic (GC) retention data of barbiturates differing only in the nature of their substituent groups have been related to parameters based on molecular structure (*e.g.* carbon number, molecular weight, connectivity (χ) terms[2]), and which do

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not need to be experimentally determined. The study concentrates on predictions based on the fundamental and simple GC system, since correlations between GC and high-performance liquid chromatographic (HPLC) retention data have been demonstrated[1]. The work is further restricted to a series of 23 5,5-disubstituted barbiturates.

METHODS

GC retention indices for 23 5,5-disubstituted barbiturates, abstracted from data presented by Gill *et al.*[1], were obtained using a 2 m × 4 mm I.D. glass column packed with 3% SE-30 on Chromosorb G HP (80–100 mesh) at a column temperature of 200°C and a nitrogen flow-rate of 45–50 ml/min.

The number of carbon atoms and the combined molecular weight of the two substituents at the C-5 position were calculated.

Molecular connectivity indices (χ), characteristic of the combined topological structure of the two substituent groups at the C-5 position, were also calculated. Indices of this type are derived from the size of, and numerical degree of branching in, molecular skeletons. A discussion of molecular connectivity and an introduction to the determination of χ values are given by Kier and Hall[2]. The quidelines they presented have since been modified (*e.g.* ref. 3), and other workers have used or interpreted them in different ways (*e.g.* refs. 4 and 5). There appears to be no single unified set of principles for calculating the connectivity index of a complicated structure. In the present work, first-order substituent connectivity indices ($\Delta^1\chi^o$) and valence connectivity indices ($\Delta^1\chi^v$) were calculated using the rules of Kier and Hall[2] (see i and ii below). Valence connectivity indices following our own empirical modification of conventional rules to suit the barbiturate group ($\Delta^1\chi^v_N$) were also calculated (see (iii) below). Calculations were performed as follows:

(i) $\Delta^1\chi^o$ — a skeleton structure of the substituent groups is drawn (including the carbon atom at the 5-position of the barbiturate ring). Each atom is assigned a number (δ), which is the number of atoms other than hydrogen attached to it. Each bond is assigned a value which, for a bond between atoms i and j, is $(\delta_i\delta_j)^{-\frac{1}{2}}$. The sum of these values for all bonds in the substituent groups, *i.e.* $\Sigma(\delta_i\delta_j)^{-\frac{1}{2}}$, is the substituent connectivity index ($\Delta^1\chi^o$). In calculating $\Delta^1\chi^o$ in the above way, double and triple bonds are considered as single bonds.

(ii) $\Delta^1\chi^v$ — as for $\Delta^1\chi^o$ above, except that δ^v values are used instead of δ values. Each atom is assigned a valence value, δ^v, being the difference between the number of valence electrons of an atom (Z^v) and the number of hydrogen atoms (h) bonded to it, *i.e.* $\delta^v = Z^v - h$. Double and triple bonds are thus accounted for. The bromine atom (in brallobarbitone, ibomal and sigmodal) takes an empirical δ^v value of 0.254. $\Delta^1\chi^v$ is equal to $\Sigma(\delta^v_i\delta^v_j)^{-\frac{1}{2}}$.

(iii) $\Delta^1\chi^v_N$ — as for $\Delta^1\chi^v$ above, except that δ^v_N values are used instead of δ^v values. δ^v_N values are obtained as follows. Where a carbon atom is involved in a double bond within a chain, 1 is substracted from its δ^v value for each substituent group attached to the atom. Where double bonds are at the end of a chain, 1 is subtracted from the δ^v value of the terminal carbon atom. In addition, 0 is subtracted from the δ^v value of the penultimate carbon if no substituent group other than the rest of the chain is bonded to it; 2 is subtracted if a substituent group, as well as the chain, is attached. Where a ring is involved it is opened at the C-1 position to give the

equivalent chain isomer; the broken bond should be considered to be still attached to the distal carbon atom. The number of valence electrons (Z^v) for the C-1 carbon atom is therefore reduced to three. If a double bond occurs at the C-1 position and it is the only one in the ring, then the ring should be opened at that bond. δ_N^v values are designated as described above and the ring is rejoined before the calculation of $^1\chi_N^v$. If there is a double bond at C-1 then 1 is subtracted from the δ^v value of that carbon atom (*cf.* subtracting 1 for a substituent on a double bond in a chain, see above). $^1\chi_N^v$ is equal to $\Sigma(\delta_{i(N)}^v \delta_{j(N)\cdot}^v)^{-\frac{1}{2}}$.

In all the above calculations no changes were made in the rules for the extra bond of a ring structure relative to the equivalent chain isomer (*cf.* ref. 6), and the connectivity index of the core barbiturate structure was not added.

RESULTS AND DISCUSSION

The relationship between GC properties and the structures of molecules in a congeneric series is well known (*e.g.* the carbon numbers of straight-chain hydrocarbons are used as the basis for retention index calculations). In the present work, correlations have been made between retention indices of a series of 23 5,5-disubstituted barbiturates and the carbon number, molecular weight, and molecular connectivity terms ($^1\chi^o$ and $^1\chi^v$) of the two substituents at the C-5 position. Retention data and structural parameters are given in Table I. Poor overall correlations are observed (Table II) and thus predictions of retention characteristics are poor. However, if the barbiturates are separated on the basis of their substituents into chemically similar sub-groups (*e.g.* dialkyl or alkyl-allyl derivatives, where the barbiturate core-structure remains constant throughout), correlation coefficients increase markedly (Table II), and hence the accuracy of predictions within a sub-group improves considerably. The situation is reflected in the regression lines calculated for the different sub-group relationships; the lines are separate but parallel in all cases (*e.g.* retention indices *vs.* molecular weight, Fig. 1). Since retention characteristics are dependent on non-topological characteristics (not described by carbon number, molecular weight or connectivity) as well as structural differences, similar parallel lines are to be expected for all sub-groups (including those not examined in the present work).

The more rigorous and sophisticated definition of the structure of a molecule provided by connectivity terms has been widely used for correlating chromatographic retention data of various types of compounds[5,7–9]. In the present work, $^1\chi^o$ values of compounds in chemically similar sub-groups are shown to provide more accurate predictions than do either carbon number or molecular weight, although for practical purposes correlations between retention characteristics and the latter two parameters are as good. Relationships using either carbon number or molecular weight are disadvantageous however, since neither can adequately represent complex molecular structures. Further, they cannot be modified in order to combine the different parallel regression lines associated with derivatives with different substituent or functional groups (*e.g.* Fig. 1). Molecular connectivity indices can be modified in this way. More complicated terms (*e.g.* higher order connectivity indices accounting for more than one bond in the substituent group[2], valence δ values (*e.g.* $^1\chi^v$, accounting in part for electrostatic forces in a substituent group[5,8], and combination of the above-mentioned parameters and introduction of interactive terms[5] have all been reported to

TABLE I

SOME STRUCTURAL PARAMETERS, CALCULATED FOR THE SUBSTITUENTS AT THE C-5 POSITION* AND GAS CHROMATOGRAPHIC RETENTION DATA FOR 23 BARBITURATES

Name	*Number of carbon atoms*	*Molecular weight*	$^1\chi^o$	$^1\chi^v$	$^1\chi^v_N$	*Retention Index***
Allobarbitone	6	82.15	3.121	2.340	2.678	1586
Amylobarbitone	7	100.21	3.477	3.477	3.477	1700
Aprobarbitone	6	84.16	3.004	2.613	2.783	1600
Barbitone	4	58.12	2.121	2.121	2.121	1482
Brallobarbitone***	–	161.04	3.477	3.228	4.303	1842
Butalbital	7	98.19	3.477	3.087	3.256	1658
Butobarbitone	6	86.18	3.121	3.121	3.121	1645
Cyclobarbitone	8	110.20	4.166	3.861	4.753	1945
Cyclopentobarbitone	8	108.18	4.166	3.442	4.193	1858
Heptabarbitone	9	124.23	4.666	4.361	5.253	2035
Hexethal	8	114.23	4.121	4.121	4.121	1835
Ibomal***	–	163.06	3.360	3.496	4.407	1866
Idobutal	7	98.19	3.621	3.231	3.400	1698
Nealbarbitone	8	112.22	3.768	3.377	3.546	1720
Pentobarbitone	7	100.21	3.542	3.542	3.542	1733
Phenobarbitone	8	106.17	4.166	3.221	4.975	1934
Phenylmethylbarbituric acid	7	92.14	3.605	2.661	4.414	1875
Probarbitone	5	72.15	2.504	2.504	2.504	1550
Quinalbarbitone	8	112.22	4.042	3.651	3.821	1770
Secbutobarbitone	8	86.18	3.042	3.042	3.042	1650
Sigmodal***	–	191.11	4.398	4.534	5.445	2031
Talbutal	7	98.19	3.542	3.151	3.321	1704
Vinbarbitone	7	98.19	3.542	3.215	3.828	1755

* Connectivity indices also include the carbon atom at the 5 position.
** Obtained using a 3% SE-30 column, see Methods section.
*** Barbiturates which contain a bromine atom in the substituent group.

TABLE II

LINEAR CORRELATION COEFFICIENTS FOR THE COMBINATION OF STRUCTURAL PARAMETERS* AND GAS CHROMATOGRAPHIC RETENTION DATA

Combination	*Overall (n = 23)***	*Alkyl-alkyl derivatives (n = 7)*	*Alkyl-allyl derivatives (n = 6)*
Number of carbon atoms	0.884	0.991	0.913
Molecular weight	0.747	0.991	0.913
$^1\chi^o$	0.887	0.997	0.981
$^1\chi^v$	0.801	0.997	0.980
$A\,{}^1\chi^o + B\,{}^1\chi^v$	0.891	0.997	0.981
$^1\chi^v_N$	0.996	0.997	0.981

* Calculated for the substituents at the C-5 position.
** Except for combination with carbon number where bromoallyl derivatives are not included; $n = 20$ in this case.

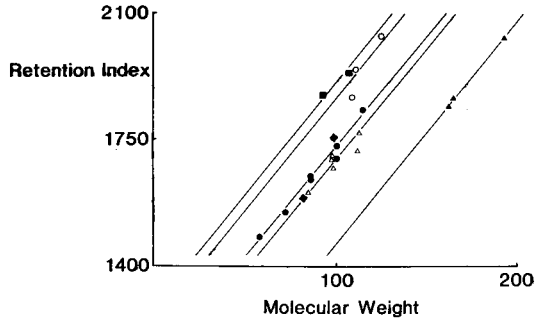

Fig. 1. Correlation of GLC retention data (SE-30 stationary phase) for 23 barbiturates with the molecular weight of the substituents at the C-5 position. ● = 5,5-Dialkylbarbituric acids; △ = 5-alkyl-5-allylbarbituric acids; ▲ = 5-alkyl-5-bromoallylbarbituric acids; ■ = 5-alkyl-5-phenylbarbituric acids; ○ = other cyclic barbiturates; ◆ = others.

improve overall correlations with retention data by drawing the parallel lines together.

The more complicated higher-order connectivity indices are relatively difficult to calculate and are therefore of limited practical value. The separate parallel lines remain when $^1\chi^v$ values alone are used (Fig. 2), and consequently overall correlations are not improved. In all cases where parallel regression lines exist, the retention properties of a barbiturate can only be predicted if at least one compound within the same chemical sub-group has already been characterised such that a regression line parallel to those relating to other sub-groups can be drawn. Use of substituent interactive terms[5], accounting for the differences between related molecules and designed to combine a family of parallel regression lines, is undesirable for the same reason. Thus, at least one compound in the same sub-group must again be characterised before an interactive term can be applied. Correlations are improved by combination of the simple connectivity terms $^1\chi^o$ and $^1\chi^v$ discussed individually above (*e.g.* with $^1\chi^v$, $r = 0.801$ and $n = 23$, whereas with a combination of $^1\chi^o$ and $^1\chi^v$, $r = 0.891$ and $n = 23$). Obviously the form of the best-fit equations provided by such

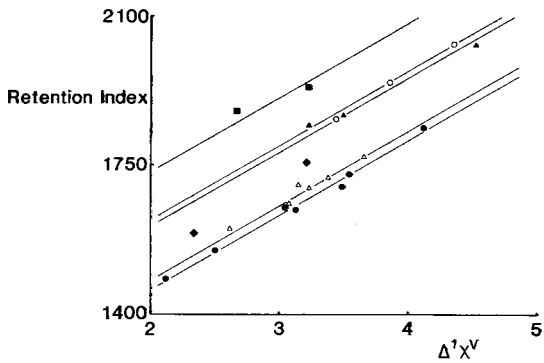

Fig. 2. Correlation of GLC retention data (SE-30 stationary phase) for 23 barbiturates with the molecular valence connectivity index of the substituents at the C-5 position ($^1\chi^v$). ● = 5,5-Dialkylbarbituric acids; △ = 5-alkyl-5-allylbarbituric acids; ▲ = 5-alkyl-5-bromoallylbarbituric acids; ■ 5-alkyl-5-phenylbarbituric acids; ○ other cyclic barbiturates; ◆ = others.

combinations is regulated by the available data, and the usefulness of the equations is generally limited because extrapolations cannot always be performed with confidence.

In contrast, very highly correlated relationships are obtained by simple modification of the conventional rules for calculating connectivity indices. The modifications performed, described in full in the Methods section, account for all the differences in structure of the 23 barbiturates examined. They consider the whole series of compounds rather than just a sub-group (*cf.* interactive terms). This suggests that use of such modifications has a wider application than does either use of interactive terms or combination of connectivity indices. Recalculation of substituent connectivity indices following empirical modification of conventional rules, to give $^{4}\chi^{v}_{N}$ values, allows the separate parallel regression lines associated with each chemical sub-group (*e.g.* Fig. 2) to be drawn as a single line (Fig. 3). This line, accounting for all the available data, has a very high correlation coefficient ($r = 0.996$). The retention indices of four barbiturates not included in the original group of derivatives have been predicted from this regression line following calculation of their $^{4}\chi^{v}_{N}$ values. It can be seen from the data in Table III that reliable extrapolations can be made as predictions are excellent. Further, the fact that such good predictions can be made for vinylbitone and reposal, barbiturates which represent previously unexamined sub-groups (alkyl-vinyl- and alkyl-bicyclo-oct-2-enyl-barbiturates, respectively), indicates the more general applicability of connectivity indices calculated using the modified rules.

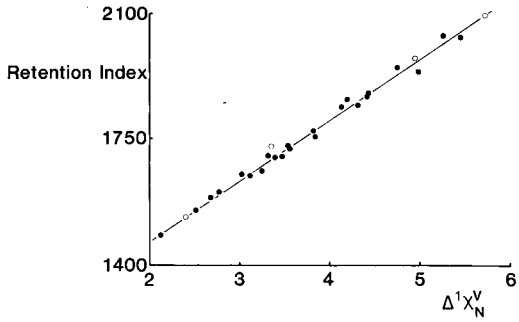

Fig. 3. Correlation of GLC retention data (SE-30 stationary phase) for 23 barbiturates with the molecular connectivity index of the substituents at the C-5 position following modification of rules for their calculation ($^{4}\chi^{v}_{N}$). Correlation coefficient, $r = 0.996$, $n = 23$. Barbiturates not in the original group (○, see Table III), are also included to compare them with the regression line.

While accurate overall predictions are possible with best-fit combinations of connectivity indices or recalculated values, there are disadvantages associated with their use. Thus, neither approach can be applied to chromatographic systems other than the one for which it was originally designed, as both are derived for a specific set of experimental data. However, use of combinations, and particularly of modified rules, has an immediate appeal because both approaches account for a far wider range of barbiturate types than does sub-group regression analysis. Simple connectivity terms, carbon numbers and molecular weights can only be used for accurate prediction of retention data after division of the compounds into chemically similar sub-groups. Compared with these three parameters (*i.e.* simple connectivity terms,

TABLE III

OBSERVED AND PREDICTED* RETENTION INDICES FOR BARBITURATES IN DIFFERENT CHEMICAL SUB-GROUPS**

	$^{A}{}^{1}\chi_{N}^{v}$	*Retention index*	
		Observed	*Predicted*
Ethyl-allylbarbituric acid (alkyl-allyl)	2.400	1532***	1532
Vinylbitone (alkyl-vinyl)	3.347	1730 §	1691
Butallylonal (alkyl-bromoallyl)	4.945	1969***	1960
Reposal (alkyl-bicyclo-oct-2-enyl)	5.720	2092 §	2090

* Using modified connectivity rules.

** The individual compounds are not included in the original group of 23 barbiturates examined; examples of the sub-groups alkyl-vinyl and alkyl-bicyclo-oct-2-enyl barbituric acids are not included in the original group.

*** Estimated from data in Machata and Battista[10].

§ Estimated from data in Menez *et al.*[11], and Stead *et al.*[12].

carbon number, and molecular weight) there is little difference in sub-group correlations when simple connectivity indices are combined or recalculated following modification of conventional rules (Table II).

The present work demonstrates the versatility of molecular connectivity terms in providing relationships which allow accurate prediction of chromatographic retention indices of disubstituted barbiturates. Predictive relationships of this type can be used to advantage in determining the retention characteristics of new and related compounds (*i.e.* those which may be produced illicitly and for which no authentic specimen may be available). Further, they can be used to determine whether a derivative whose retention characteristics are unknown is likely to interfere in an analysis for a specific barbiturate. This has obvious potential for aiding the forensic toxicologist in analyses of the wide range of barbiturates available.

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DEPENDENCE OF RETENTION INDICES OF ALKYLBENZENES ON THEIR MOLECULAR STRUCTURE

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SUMMARY

An open tubular column coated with squalane was used for the measurement of the retention of approximately 70 alkyl derivatives of benzene and naphthalene at two temperatures. Steric factors were shown to have a decisive effect on the elution sequence of isomers and on the retention index increment when the same alkyl groups were introduced at different positions of the hydrocarbon molecule. The increase in the Kováts index was higher when the alkyl groups were introduced in the ring than in a side chain. Proportionality factors proposed for calculating differences in the retention indices of aromatic isomers on the basis of differences in their boiling points varied in the range 3.9–4.5.

INTRODUCTION

The gas chromatographic (GC) retention parameters of hydrocarbons are usually determined in particular by intermolecular Van der Waals forces and by dispersion forces, if the separation occurs in a column containing a non-polar liquid stationary phase. The fact that the interaction forces are additive led Kováts[1] to suggest a retention index, which in its quantitative form reflects the incremental contributions to the free energy of interaction from the individual structural groups of a molecule. Thus, the value of this retention index is determined by the structural characteristics of the compounds analyzed, by the types and positions of the functional groups and the positions of the double bonds and by steric effects[2–20]. There is a linear correlation between the Kováts index and the free energy of dissolution, as well as other thermodynamic functions of dissolution[2–26]. Attention has been focused on the study of the laws of retention of aromatic hydrocarbons characterized by particular molecular structures.

The measurement of Kováts' indices for aromatic hydrocarbons and the prob-

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lems of qualitative evaluation of actual commercial mixtures have been the subject of a great number of studies[27–36]. The dependence of the retention indices and their increments on column temperature, structure and physicochemical properties of alkylbenzenes have been reported[32,36–39].

In the present study Kováts' indices were measured for aromatic hydrocarbons in chemical products of thermal treatment of lignite and bituminous coal. On the basis of the data obtained, the relation of the retention indices and their increments to the structural characteristics of the aromatic hydrocarbons was investigated.

EXPERIMENTAL

The measurements were made on a Chrom 41 chromatograph (Laboratory Instruments, Prague, Czechoslovakia) equipped with a flame ionization detector. Conditions: column, stainless-steel coated open capillary (50 m × 0.25 mm); stationary phase, squalane; temperatures, column 86,96°C, evaporation cell 260°C; carrier gas (nitrogen) inlet pressure, 103,950 Pa; splitting ratio, 1:100; flow-rates, hydrogen 30 ml/min, air 350 ml/min; sample volume, $0.1 \cdot 10^{-3}$–$0.2 \cdot 10^{-3}$ ml; column efficiency, 71,500 theoretical plates (with respect to *m*-xylene).

The hydrocarbons studied were injected by means of a Terumo (Japan) microsyringe in the form of mixtures of five to seven compounds. The retention times of the hydrocarbons were obtained at a known chart speed (20 mm/min) from at least three measurements for each compound. After correction for the retention time of methane, the values were employed for the calculation of the Kováts indices using a special program and a Hewlett-Packard calculator 9830A. The experimental results and variations in the retention indices per °C, $\partial I/\partial T$, are listed in Table I.

RESULTS AND DISCUSSION

The values of $\partial I/\partial T$ for the aromatic hydrocarbons studied and their isomers vary over a fairly wide range: monoalkylbenzenes, 0.21–0.49; dialkylbenzenes, 0.15–0.33; trialkylbenzenes, 0.17–0.27; tetramethylbenzenes, 0.31–0.35; naphthalene and its derivatives, 0.32–0.58. It is to be noted that the corresponding values for many cycloparaffins and cycloolefins[9] are comparable with the values of some aromatic hydrocarbons, which complicates group identification in multi-component mixtures.

The investigation of intermolecular interactions in squalane, which lacks a dipole moment, induction forces and hydrogen bonding, led to the conclusion that these interactions are determined by dispersion forces, the distinguishing feature of which is their additivity. The extent and the importance of the dispersion forces depends on the shapes and volumes of the molecules of the substances separated, and on the stereochemistry of the substituents in the aromatic ring.

The retention indices (Table I) vary with the boiling points of alkylbenzenes, which in turn are a function of vapour pressure. Fig. 1 shows the linear relationship of the retention indices and logarithms of vapour pressure of alkylbenzenes. Similar relationships were found for numerous isomers, the elution sequence for the particular group of hydrocarbons being dependent on the structure and mutual position of the substituents in the benzene ring (Table II).

The replacement of one methyl group of dimethylbenzene with an arbitrary

TABLE I

RETENTION INDICES OF ALKYLBENZENES ON SQUALANE AND THEIR TEMPERATURE COEFFICIENTS

Compound	*Temperature (°C)*		$\partial I/\partial T \cdot 10$
	86	*96*	
Benzene	648.0	650.22	2.2
Methylbenzene	755.0	757.1	2.1
Phenylacetylene	830.4	834.1	3.7
Ethylbenzene	845.3	847.7	2.4
p-Dimethylbenzene	859.0	861.2	2.3
m-Dimethylbenzene	861.0	863.2	2.2
o-Dimethylbenzene	880.8	883.0	2.2
Isopropylbenzene	904.1	906.7	2.6
Allylbenzene	917.8	920.0	2.2
n-Propylbenzene	932.7	935.1	2.4
Styrene	872.0	874.5	2.5
α-Methylstyrene	957.8	960.0	2.2
2-Methylstyrene	972.1	974.2	2.1
3-Methylstyrene	976.3	978.7	2.4
4-Methylstyrene	978.6	980.3	1.7
1-Methyl-3-ethylbenzene	945.9	947.4	1.5
1-Methyl-4-ethylbenzene	948.2	950.4	2.2
1-Methyl-2-ethylbenzene	961.1	963.4	2.3
1,3,5-Trimethylbenzene	965.0	967.1	2.1
1,2,4-Trimethylbenzene	982.0	985.3	2.5
1,2,3-Trimethylbenzene	1007.8	1010.5	2.7
tert.-Butylbenzene	969.9	972.5	2.6
Isobutylbenzene	985.5	988.3	2.8
sec.-Butylbenzene	986.4	989.1	2.7
n-Butylbenzene	1032.3	1034.9	2.6
1-Methyl-3-isopropylbenzene	1000.3	1001.8	1.5
1-Methyl-4-isopropylbenzene	1007.7	1009.9	2.2
1-Methyl-2-isopropylbenzene	1013.5	1015.8	2.3
2,3-Dihydroindene	1011.2	1014.5	3.3
Indene	1011.8	1015.7	3.9
2-Methylindene	1054.1	1057.7	3.6
1,2-Dimethylhydrindene	1124.7	1128.8	4.1
5-Ethylindene	–	1203.6	–
1,3-Diethylbenzene	1025.7	1028.1	2.4
1,2-Diethylbenzene	1034.8	1038.1	3.3
1,4-Diethylbenzene	1036.8	1039.3	2.5
1-Methyl-3-*n*-propylbenzene	1030.4	1032.8	2.4
1-Methyl-4-*n*-propylbenzene	1035.9	1038.8	2.9
1-Methyl-2-*n*-propylbenzene	1042.0	1045.4	3.4
trans-Decahydronaphthalene	1064.7	1069.3	4.6
cis-Decahydronaphthalene	1101.4	1106.2	4.8
tert.-Pentylbenzene	1065.9	1069.5	3.6
sec.-Pentylbenzene	1074.7	1077.8	3.1
Isopentylbenzene	1094.9	1098.4	3.5
n-Pentylbenzene	1131.3	1136.2	4.9
1-Methyl-3-*tert.*-butylbenzene	1053.6	1056.1	2.6
1-Methyl-4-*tert.*-butylbenzene	1072.3	1074.7	2.4

(Continued on p. 288)

TABLE I *(continued)*

Compound	*Temperature (°C)*		*∂I/∂T·10*
	86	*96*	
1-Methyl-2-*tert.*-butylbenzene	1088.4	1090.9	2.5
2,4-Dimethylstyrene	1080.9	1084.2	3.3
1-Methyl-4-*sec.*-butylbenzene	1088.7	1091.0	2.3
1-Ethyl-3-isopropylbenzene	1074.3	1076.5	2.2
1-Ethyl-2-isopropylbenzene	1076.9	1079.8	2.9
1-Ethyl-4-isopropylbenzene	1094.5	1097.3	2.8
1,2,4,5-Tetramethylbenzene	1102.8	1105.9	3.1
1,2,3,5-Tetramethylbenzene	1108.4	1111.4	3.0
1,2,3,4-Tetramethylbenzene	1129.2	1132.7	3.5
1-Ethyl-3-*n*-propylbenzene	1108.4	1111.1	2.7
1-Ethyl-2-*n*-propylbenzene	1116.7	1119.2	2.5
1-Ethyl-4-*n*-propylbenzene	1121.9	1125.2	3.3
1,3-Diisopropylbenzene	1116.3	1118.8	2.5
1,2-Diisopropylbenzene	1118.1	1120.3	2.2
1,4-Diisopropylbenzene	1151.2	1153.4	2.2
1,2-Dihydronaphthalene	1127.9	1133.2	5.3
1,2,3,4-Tetrahydronaphthalene	1133.2	1137.6	4.4
Naphthalene	1146.0	1151.8	5.8
1-Ethyl-4-*tert.*-butylbenzene	1158.5	1161.0	2.5
1,4-Dimethyl-2-*n*-butylbenzene	1165.3	1167.1	1.8
1,3,5-Triethylbenzene	1189.1	1190.8	1.7
1,2,4-Triethylbenzene	1206.3	1208.0	1.7
n-Hexylbenzene	1225.4	1228.8	3.4
Pentamethylbenzene	1253.9	1258.6	4.7
1-Methylnaphthalene	1264.7	1267.9	3.2
2-Methylnaphthalene	1265.5	1271.1	5.6
1,3,5-Triisopropylbenzene	1285.7	1287.5	1.8

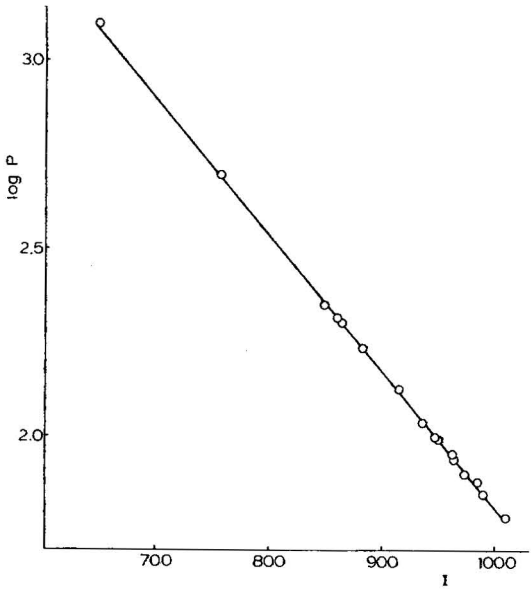

Fig. 1. Dependence of the retention indices on the logarithms of the vapour pressure of alkylbenzenes at 96°C.

TABLE II

ELUTION ORDER OF DIALKYLBENZENES ON SQUALANE IMPREGNATED OPEN TUBULAR COLUMN

No. of hydrocarbon group	*Group constituents*	*Elution sequence*
I	Dimethylbenzenes	1 *para* 2 *meta* 3 *ortho*
II	Methylethylbenzenes Methylpropylbenzenes Methylisopropylbenzenes Methylbutylbenzenes Methylpentylbenzenes	1 *meta* 2 *para* 3 *ortho*
III	Ethylpropylbenzenes Ethylisopropylbenzenes Diethylbenzenes Diisopropylbenzenes Ethylbutylbenzenes	1 *meta* 2 *ortho* 3 *para*

alkyl radical C_2–C_5 results in enhanced elution of the *meta* isomer as compared with that of the *para* isomer (Fig. 2a). Replacement of the other methyl group with an alkyl radical C_2–C_3 further retards the *para* isomer, which in this case leaves the column after the *meta* and *ortho* isomers (Fig. 2b). In Fig. 3 both these effects are shown within one homologous series of alkylbenzenes.

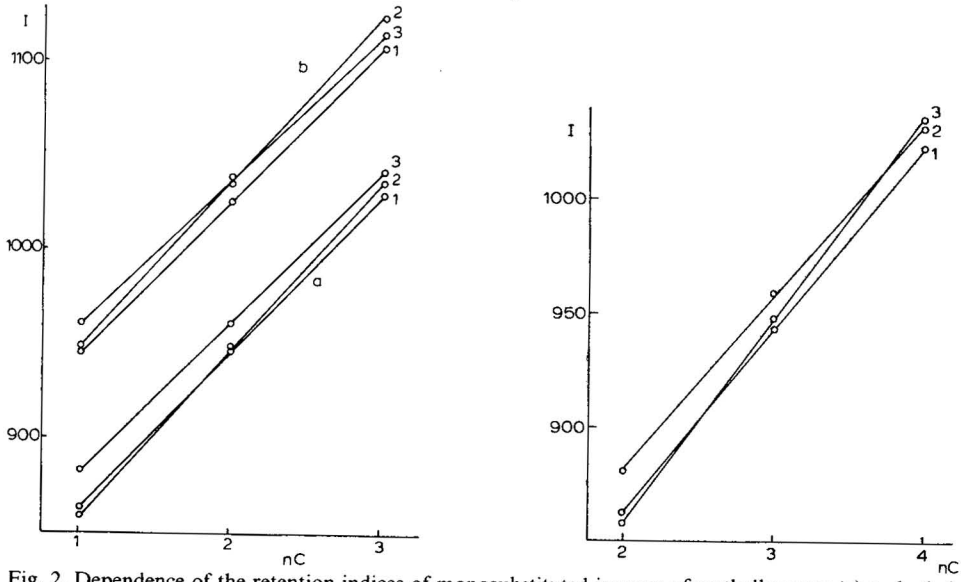

Fig. 2. Dependence of the retention indices of monosubstituted isomers of methylbenzene (a) and ethylbenzene (b) on the number of carbon atoms of the substituents. 1 = *meta*; 2 = *para*; 3 = *ortho* isomers.

Fig. 3. Dependence of the retention indices and elution sequences of isomers on the number of carbon atoms of the alkyl group of C_8–C_{10} alkylbenzenes. 1 = *meta*; 2 = *ortho*; 3 = *para* isomers.

The explanation of these relationships is to be found in the structural characteristics and the electron density distribution of the molecules studied. The introduction of substituents affects the electron distribution and density of the delocalized π-orbital of benzene. The presence of two alkyl groups produces effects dependent on their character and mutual position. The greatest asymmetry of the *o*-dimethylbenzene in group I of the isomers causes the maximum retention due to the hindered rotation of this isomer in the squalane lattice. In addition, the *ortho* isomers in the group of dialkylbenzenes are characterized by a more compact arrangement of atoms, which is confirmed by their higher density, dielectric constant, coefficient of refraction and boiling point. As a result of the very near mutual position of the alkyl groups in the *ortho* isomers, the σ/π conjugation of the C–H bond of a carbon atom α to the aromatic ring is disturbed, leading to a decrease in the electron mobility of *ortho* isomers compared with other isomers. It is obvious that the observed relationships have a decisive influence on the elution sequence of isomers of groups I and II, one alkyl group of which is methyl. The elution order of isomers of group III, the alkyl constituents of which contain two or more carbon atoms, is in agreement with that of group II as regards the *meta* isomer, and has nothing in common with group I.

Since the alkyl substituents have practically equal "polarities", the above features can be accounted for in terms of the respective alkyl chain lengths. Indeed, extension of the substituent chain results in an increase in the number of sites in the molecules which are capable of mutual attraction. The degree of attraction, and consequently of the dispersion forces, is a maximum, provided the linear alkyl radical can form a "bent" or zigzag configuration, thus assisting in intimate contact of the dialkylbenzene molecules with the stationary phase. This interaction mechanism is confirmed by the relative retention data of the isomers studied (Table III). The relative retention of *p*-dialkylbenzenes (Table III), the lowest value of which is characteristic of methylbenzenes, increases with increasing alkyl chain length, and exceeds the retention of *m*-dialkylbenzenes by 0.8% (group II) and that of *o*-dialkylbenzenes by 2.3% (group III). The stronger retention of *para* isomers is directly connected with the length of the end alkyl groups which have two micropoles in contrast to the *meta* and *ortho* isomers characterized by a common dipole moment. It is to be noted that in this particular case the attractive forces of the alkyl groups predominate over the steric factors, *p*-diisopropylbenzene exhibiting the greatest retention relative to the *ortho* isomers of the hydrocarbons in group III.

If the retention of the *para* isomers of groups II and III is determined by the interaction of the alkyl groups with the molecules of the stationary phase, then the

TABLE III

MEAN VALUES OF RETENTION INDEX DIFFERENCES IN COMPARISON WITH BENZENE (%)

Dialkylbenzenes	*Group of isomers*		
	I	*II*	*III*
para Isomers	32.5	53.8	69.9
meta Isomers	32.8	53.0	66.9
ortho Isomers	35.9	55.2	67.6

TABLE IV

STRUCTURAL INCREMENTS OF ALKYLBENZENES ON SQUALANE

Compound	*Temperature (°C)*	
	86	*96*
Toluene	55.0	57.1
1,4-Dimethylbenzene	59.0	61.2
1,3-Dimethylbenzene	61.0	63.2
1,2-Dimethylbenzene	80.8	83.0
1,3,5-Trimethylbenzene	65.0	67.1
1,2,4-Trimethylbenzene	82.8	85.3
1,2,3-Trimethylbenzene	107.8	110.5
1,2,4,5-Tetramethylbenzene	102.8	105.9
1,2,3,5-Tetramethylbenzene	108.4	111.4
1,2,3,4-Tetramethylbenzene	129.2	132.7
Pentamethylbenzene	153.9	158.6
Ethylbenzene	45.3	47.7
1,3-Diethylbenzene	25.7	28.1
1,2-Diethylbenzene	34.8	38.1
1,4-Diethylbenzene	36.8	39.3
1,3,5-Triethylbenzene	−11.9	−9.2
1,2,4-Triethylbenzene	6.3	8.0
n-Propylbenzene	32.7	35.1
Isopropylbenzene	4.1	6.7
1,3-Diisopropylbenzene	−83.7	−81.2
1,2-Diisopropylbenzene	−81.9	−79.7
1,4-Diisopropylbenzene	−48.8	−46.6
1,3,5-Triisopropylbenzene	−214.3	−212.5
tert.-Butylbenzene	−30.1	−27.5
Isobutylbenzene	−14.5	−11.7
sec.-Butylbenzene	−13.6	−10.9
n-Butylbenzene	32.3	34.9
tert.-Pentylbenzene	−34.1	−30.5
sec.-Pentylbenzene	−25.3	−22.2
Isopentylbenzene	−5.1	−1.6
n-Pentylbenzene	31.3	36.2
n-Hexylbenzene	25.4	28.8
1-Methyl-3-ethylbenzene	45.9	47.4
1-Methyl-4-ethylbenzene	48.2	50.4
1-Methyl-2-ethylbenzene	61.1	63.4
1-Methyl-3-*n*-propylbenzene	30.4	32.8
1-Methyl-4-*n*-propylbenzene	35.9	38.8
1-Methyl-2-*n*-propylbenzene	42.0	45.4
1-Methyl-3-isopropylbenzene	0.3	1.8
1-Methyl-4-isopropylbenzene	7.7	9.9
1-Methyl-2-isopropylbenzene	13.5	15.8
1-Methyl-4-*sec.*-butylbenzene	−11.3	−9.0
1-Methyl-3-*tert.*-butylbenzene	−46.4	−43.9
1-Methyl-4-*tert.*-butylbenzene	−27.7	−25.3
1-Methyl-2-*tert.*-butylbenzene	−11.6	−9.1
1-Ethyl-3-*n*-propylbenzene	8.4	11.1
1-Ethyl-2-*n*-propylbenzene	16.7	19.2
1-Ethyl-4-*n*-propylbenzene	21.9	25.2
1-Ethyl-3-isopropylbenzene	−25.7	−23.5
1-Ethyl-2-isopropylbenzene	−23.1	−20.2
1-Ethyl-4-isopropylbenzene	−5.5	−2.7
1-Ethyl-4-*tert.*-butylbenzene	−41.5	−39.0

elution order of the other isomers is closely associated with the steric effects. Thus, increasing of one of the two alkyls in dialkylbenzene results in an increase in steric hindrances, weakening the dispersion interaction of *meta*- compared with *para* isomers by approximately 1.0%, and enhancing the elution of *meta* isomers with respect to the dialkylbenzenes of group II (Table III). Increasing chain length of the other alkyl substituent causes an additional intensification of the steric hindrance, resulting in elution of the *ortho* before the *para* isomers of the hydrocarbons of group III.

The experimental values of the retention indices can be used to calculate values of the homomorphous factor, H

$$H = I_{ab} - I_p$$

where I_{ab} and I_p are the retention indices of an alkylbenzene and of an *n*-alkane with the same number of carbon atoms, respectively. The values obtained, which permit an evaluation of the effect of the molecular structure of the alkylbenzene on the retention index, are given in Table IV.

The homomorphous factor increases with increasing methyl substitution from methylbenzene to pentamethylbenzene. The introduction of one methyl group in the benzene ring results in an increase in H on average by 25–30%. In all the other cases the values of H decrease with the increasing number of carbon atoms in a molecule, including the introduction of a methyl group in the side chain of a ring. A sudden decrease in H occurs from *n*-alkylbenzenes to their respective *iso* derivatives (*n*-propylbenzene–isopropylbenzene, *n*-butyl- and *n*-pentylbenzenes and their isomeres) and in a number of derivatives of isopropylbenzene. In a homologous series of *n*-alkylbenzenes the decrease in H is insignificant. It is to be noted that the H value for *n*-propylbenzene does not fit the approximately linear dependence (line 1 in Fig. 4) of the homomorphous factor on the number of carbon atoms. It is possible to deduce from this figure another dependence involving the points corresponding to C_9 and C_{11} alkylbenzenes (broken line in Fig. 4). The anomalously low retention indices of *n*-propylbenzene and its dialkyl derivatives has been treated in detail by Soják and co-workers[39,40].

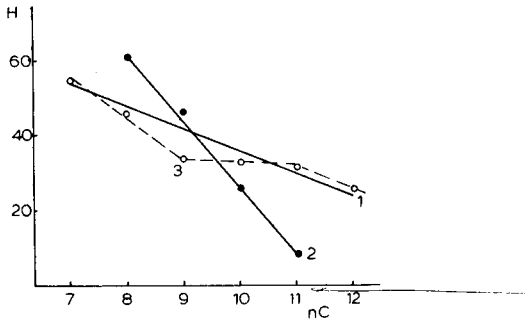

Fig. 4. Dependence of H on the number of carbon atoms for homologous series of alkylbenzenes at 86°C. 1 = Monosubstituted *n*-alkylbenzenes; 2 = 1,3-dialkylbenzenes; 3 = a different interpretation of dependence 1.

TABLE V

CONTRIBUTION OF ONE METHYLENE GROUP TO THE RETENTION INDICES OF HOMOLOGOUS SERIES OF ALKYLBENZENES

B = Benzene; Me = methyl; Et = ethyl; Pr = propyl; Bu = butyl; Pe = pentyl; Hex = hexyl; Hep = heptyl; Oct = octyl; Non = nonyl; *i* = *iso*; *s* = *sec*; *t* = *tert*.

Series and compounds		*No. of carbon atoms*	*Temperature (°C)*	
Basic compound	*CH_2 group incorporated*		*86*	*96*
B	MeB	C_6–C_7	107.0	106.9
MeB	1,2-Di-MeB	C_7–C_8	125.8	125.9
EtB	1-Me-2-EtB	C_8–C_9	115.8	115.7
n-PrB	1-Me-2-*n*-PrB	C_9–C_{10}	109.3	110.3
i-PrB	1-Me-2-*i*-PrB	C_9–C_{10}	109.4	109.1
n-BuB	1-Me-2-*n*-BuB	C_{10}–C_{11}	–	106.5*
n-PeB	1-Me-2-*n*-PeB	C_{11}–C_{12}	–	102.3*
n-HexB	1-Me-2-*n*-HexB	C_{12}–C_{13}	–	104.1*
n-HepB	1-Me-2-*n*-HepB	C_{13}–C_{14}	–	103.6*
n-OctB	1-Me-2-*n*-OctB	C_{14}–C_{15}	–	103.2*
n-NonB	1-Me-2-*n*-NonB	C_{15}–C_{16}	–	102.6*
MeB	1,4-Di-Meb	C_7–C_8	104.0	104.2
EtB	1-Me-4-EtB	C_8–C_9	102.9	102.7
n-PrB	1-Me-4-*n*-PrB	C_9–C_{10}	103.2	103.7
i-PrB	1-Me-4-*i*-PrB	C_9–C_{10}	103.6	103.2
n-BuB	1-Me-4-*n*-BuB	C_{10}–C_{11}	103.1	102.3**
t-BuB	1-Me-4-*t*-BuB	C_{10}–C_{11}	102.4	102.2
s-BuB	1-Me-4-*s*-BuB	C_{10}–C_{11}	102.3	101.9
n-PeB	1-Me-4-*n*-PeB	C_{11}–C_{12}	103.2	103.3**
n-HexB	1-Me-4-*n*-HexB	C_{12}–C_{13}	101.3	102.1**
MeB	1,3-Di-MeB	C_7–C_8	106.0	106.1
EtB	1-Me-3-EtB	C_8–C_9	100.6	99.7
n-PrB	1-Me-3-*n*-PrB	C_9–C_{10}	97.7	97.7
n-BuB	1-Me-3-*n*-BuB	C_{10}–C_{11}	–	93.0***
n-PeB	1-Me-3-*n*-PeB	C_{11}–C_{12}	–	91.3***
1-Me-3-EtB	1,2-Di-Me-3-EtB	C_9–C_{10}	138.3	139.2*
1-Me-4-EtB	1,2-Di-Me-4-EtB	C_9–C_{10}	119.8	120.0*
1-Me-4-EtB	1,3-Di-Me-4-EtB	C_9–C_{10}	115.2	115.3*
1-Me-2-EtB	1,3-Di-Me-2-EtB	C_9–C_{10}	106.8	107.1*
1-Me-2-EtB	1,4-Di-Me-2-EtB	C_9–C_{10}	95.8	95.6*
i-PrB	1-Me-3-*i*-PrB	C_9–C_{10}	96.2	95.1
t-BuB	1-Me-3-*t*-BuB	C_{10}–C_{11}	83.7	83.6
1,3-Di-MeB	1,2,3-Tri-MeB	C_8–C_9	146.8	147.3
1,2-Di-MeB	1,2,3-Tri-MeB	C_8–C_9	127.0	127.5
1,4-Di-MeB	1,2,4-Tri-MeB	C_8–C_9	123.8	124.0
1,3-Di-MeB	1,3,5-Tri-MeB	C_8–C_9	104.0	103.9
1,2-Di-MeB	1,2,4-Tri-MeB	C_8–C_9	102.0	102.3
1,2,4-Tri-MeB	1,2,3,4-Tetra-MeB	C_9–C_{10}	146.4	147.4
1,3,5-Tri-MeB	1,2,3,5-Tetra-MeB	C_9–C_{10}	143.4	144.3
1,2,3-Tri-MeB	1,2,3,4-Tetra-MeB	C_9–C_{10}	121.4	122.2
1,2,4-Tri-MeB	1,2,4,5-Tetra-MeB	C_9–C_{10}	120.0	120.2
1,2,3-Tri-MeB	1,2,3,5-Tetra-MeB	C_9–C_{10}	100.6	100.9

(Continued on p. 294)

TABLE V *(continued)*

Series and compounds		*No. of carbon atoms*	*Temperature (°C)*	
Basic compound	*CH_2 group incorporated*		*86*	*96*
1,2,4,5-Tetra-MeB	1,2,3,4,5-Penta-MeB	C_{10}–C_{11}	151.1	152.7
1,2,3,5-Tetra-MeB	1,2,3,4,5-Penta-MeB	C_{10}–C_{11}	145.5	147.2
1,2,3,4-Tetra-MeB	1,2,3,4,5-Penta-MeB	C_{10}–C_{11}	124.7	125.9
Penta-MeB	Hexa-MeB	C_{11}–C_{12}	152.1	154.1**
Styrene	4-Me-Styrene	C_8 –C_9	106.6	105.8
Styrene	3-Me-Styrene	C_8 –C_9	104.3	104.2
Styrene	2-Me-Styrene	C_8 –C_9	100.1	99.7
Styrene	α-Me-Styrene	C_8 –C_9	85.0	85.5
Indene	2-Me-Indene	C_9 –C_{10}	42.3	42.0
2-Me-Styrene	2,4-Di-Me-Styrene	C_9 –C_{10}	108.8	110.0
4-Me-Styrene	2,4-Di-Me-Styrene	C_9 –C_{10}	102.3	103.9
Naphthalene	2-Me-Naphthalene	C_{10}–C_{11}	119.5	119.3
Naphthalene	1-Me-Naphthalene	C_{10}–C_{11}	118.7	116.1
Introduction of CH_2 into side chain				
MeB	EtB	C_7 –C_8	90.0	90.6
EtB	*n*-PrB	C_8 –C_9	87.4	87.4
n-PrB	*n*-BuB	C_9 –C_{10}	99.6	99.8
n-BuB	*n*-PeB	C_{10}–C_{11}	99.0	101.3
n-PeB	*n*-HexB	C_{11}–C_{12}	94.1	92.6
1,4-Di-MeB	1-Me-4-EtB	C_8 –C_9	89.2	89.1
1-Me-4-EtB	1-Me-4-*n*-PrB	C_9 –C_{10}	87.7	88.4
1-Me-4-*n*-PrB	1-Me-4-*n*-BuB	C_{10}–C_{11}	99.6	98.9**
1-Me-4-*n*-BuB	1-Me-4-*n*-PeB	C_{11}–C_{12}	96.4	97.2**
1-Me-4-*n*-PeB	1-Me-4-*n*-HexB	C_{12}–C_{13}	95.8	96.7**
1-Me-4-*n*-HexB	1-Me-4-*n*-HepB	C_{13}–C_{14}	103.0	101.2**
1-Me-4-*i*-PrB	1-Et-4-*i*-PrB	C_{10}–C_{11}	86.8	87.4
1-Et-4-*i*-PrB	1-*n*-Pr-4-*i*-PrB	C_{11}–C_{12}	83.8	83.6**
1-Me-4-EtB	1,4-Di-EtB	C_9 –C_{10}	88.6	88.9
1,4-Di-EtB	1-Et-4-*n*-PrB	C_{10}–C_{11}	85.1	85.9
1-Et-4-*n*-PrB	1-Et-4-*n*-BuB	C_{11}–C_{12}	100.1	100.5**
1-Et-4-*n*-BuB	1-Et-4-*n*-PeB	C_{12}–C_{13}	95.2	95.1**
1-Et-4-*n*-PeB	1-Et-4-*n*-HexB	C_{13}–C_{14}	101.3	99.6**
1-Me-4-*n*-PrB	1-Et-4-*n*-PrB	C_{10}–C_{11}	86.0	86.4
1-Et-4-*n*-PrB	1,4-Di-*n*-PrB	C_{11}–C_{12}	85.1	85.8**
1,4-Di-*n*-PrB	1-*n*-Pr-4-*n*-BuB	C_{12}–C_{13}	99.1	98.7**
1-*n*-Pr-4-*n*-BuB	1-*n*-Pr-4-*n*-PeB	C_{13}–C_{14}	110.4	108.6**
1-Me-4-*n*-BuB	1-Et-4-*n*-BuB	C_{11}–C_{12}	86.6	87.8**
1-Et-4-*n*-BuB	1-*n*-Pr-4-*n*-BuB	C_{12}–C_{13}	84.1	84.0**
1-*n*-Pr-4-*n*-BuB	1,4-Di-*n*-BuB	C_{13}–C_{14}	101.3	100.2**
1-Me-4-*t*-BuB	1-Et-4-*t*-BuB	C_{11}–C_{12}	86.2	86.3**

TABLE V *(continued)*

Series and compounds		*No. of carbon atoms*	*Temperature (°C)*	
Basic compound	*CH_2 group incorporated*		*86*	*96*
1,3-Di-MeB	1-Me-3-EtB	C_8–C_9	84.9	84.2
1-Me-3-EtB	1-Me-3-*n*-PrB	C_9–C_{10}	84.5	85.4
1,3-Di-EtB	1-Et-3-*n*-PrB	C_{10}–C_{11}	82.7	83.0
1-Et-3-*n*-PrB	1,3-Di-*n*-PrB	C_{11}–C_{12}	83.0	83.2**
1-Me-3-EtB	1,3-Di-EtB	C_9–C_{10}	79.8	80.7
1-Me-3-*n*-PrB	1-Et-3-*n*-PrB	C_{10}–C_{11}	78.0	78.3
1-Me-3-*i*-PrB	1-Et-3-*i*-PrB	C_{10}–C_{11}	74.0	74.7
1,2-Di-MeB	1-Me-2-EtB	C_8–C_9	80.3	80.4
1-Me-2-EtB	1-Me-2-*n*-PrB	C_9–C_{10}	80.9	82.0
1-Me-2-*n*-PrB	1-Me-2-*n*-BuB	C_{10}–C_{11}	–	96.3*
1-Me-2-*n*-BuB	1-Me-2-*n*-PeB	C_{11}–C_{12}	–	95.0*
1-Me-2-*n*-PeB	1-Me-2-*n*-HexB	C_{12}–C_{13}	–	96.8*
1-Me-2-*n*-HexB	1-Me-2-*n*-HepB	C_{13}–C_{14}	–	99.2*
1-Me-2-*i*-PrB	1-Et-2-*i*-PrB	C_{10}–C_{11}	63.4	64.0
1-Me-2-EtB	1,2-Di-EtB	C_9–C_{10}	73.7	74.7
1,2-Di-EtB	1-Et-2-*n*-PrB	C_{10}–C_{11}	81.9	81.1
1-Et-2-*n*-PrB	1-Et-2-*n*-BuB	C_{11}–C_{12}	–	93.2*
1-Et-2-*n*-BuB	1-Et-2-*n*-PeB	C_{12}–C_{13}	–	94.2*
1-Et-2-*n*-PeB	1-Et-2-*n*-HexB	C_{13}–C_{14}	–	97.0*
1-Et-2-*n*-HexB	1-Et-2-*n*-HepB	C_{14}–C_{15}	–	97.3*
1-Et-2-*n*-HepB	1-Et-2-*n*-OctB	C_{15}–C_{16}	–	98.6*
1-Me-2-*n*-PrB	1-Et-2-*n*-PrB	C_{10}–C_{11}	74.7	73.8
1-Et-2-*n*-PrB	1,2-Di-*n*-PrB	C_{11}–C_{12}	–	72.8*
1,2-Di-*n*-PrB	1-*n*-Pr-2-*n*-BuB	C_{12}–C_{13}	–	91.8*
1-*n*-Pr-2-*n*-BuB	1-*n*-Pr-2-*n*-PeB	C_{13}–C_{14}	–	92.6*
1-*n*-Pr-2-*n*-PeB	1-*n*-Pr-2-*n*-HexB	C_{14}–C_{15}	–	94.9*
1-*n*-Pr-2-*n*-HexB	1-*n*-Pr-2-*n*-HepB	C_{15}–C_{16}	–	97.5*
1-Me-4-*n*-BuB	1-Et-2-*n*-BuB	C_{11}–C_{12}	–	67.6*
1-Et-2-*n*-BuB	1-*n*-Pr-2-*n*-BuB	C_{12}–C_{13}	–	71.4*
1-*n*-Pr-2-*n*-BuB	1,2-Di-*n*-BuB	C_{13}–C_{14}	–	90.7*

* According to measurements by Soják *et al.*[39].
** According to measurements by Engewald and Wennrich[38].
*** According to measurements by Döring *et al.*[32].

The overall effect of the number and position of the alkyl groups in the benzene ring was evaluated by means of the increment δI

$$\delta I = I_2 - I_1$$

where I_1 and I_2 are the retention indices of alkylbenzenes differing by one or several structural groups, respectively. The values of the increments δI_{CH_2} are listed in Table V. This increment is dependent primarily on the site of introduction of the structural element. If an alkylbenzene molecule is extended by a $-CH_2-$ group, the value of δI

usually exceeds 100 *I*-units, if substitution occurs in the benzene ring. If, however, the CH_2 group is introduced in the side chain of the ring, the δI value is lower than 100.

Introduction of a methyl group in the ring

The values of δI_{CH_2} for isomeric dialkylbenzenes increase in the order: *meta* < *para* < *ortho*. The δI_{CH_2} values for *m*- anc *p*-dimethyl benzenes, being mutually comparable, slightly differ from the δI_{CH_2} value characterizing the introduction of a CH_2 group in an unsubstituted benzene ring, which shows there are no obstacles to the introduction of the methyl group in the *meta* and *para* positions. The *ortho* position is associated with the greatest electron density, consequently the introduction of a methyl group results in an increase in δI_{CH_2} compared with the other isomers. The anomalous retention behaviour of various *ortho*-substituted compounds has been dealt with in many papers[41–44]. One study[42] indicates that in the *ortho* position the free rotation of the methyl groups is made more difficult and is changed to rotational–vibrational motion. Increasing the length of the alkyl chain counterbalances these effects, and for C_{11}–C_{12} *o*-dialkylbenzenes the values of δI_{CH_2} decrease to 102–106 units, comparable with the values for *p*-dialkylbenzenes (Fig. 5).

The lowest δI_{CH_2} values (< 100 units) are characteristic for *m*-dialkylbenzenes, which permits to consider the differences in the retention indices (or δI_{CH_2}) between the extreme substituents of the corresponding isomers (in this case *ortho* and *meta*) as a quantitative measure of the screening effect of the substituents.

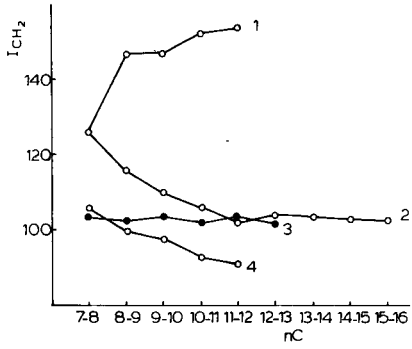

Fig. 5. Dependence of δI_{CH_2} on the number of carbon atoms for homologous series of alkylbenzenes at 96°C. 1 = Methyl-substituted benzenes; 2 = 1-methyl-2-alkylbenzenes; 3 = 1-methyl-4-alkylbenzenes; 4 = 1-methyl-3-alkylbenzenes.

The decisive influence of the methyl group on the size of the retention index increment can be well demonstrated by trialkylbenzenes (Table V). The increase in δI_{CH_2} for isomeric dimethylbenzenes is directly associated with the mutual position of the methyl groups in the ring: 1,4 < 1,3 < 1,2.

Increasing the number of methyl groups in the benzene rings increases the degree of its screening. The highest δI_{CH_2} value is achieved when a methylene group is introduced in the *ortho* position with respect to methyl groups already occupying *ortho* positions with respect to each other (a double *ortho*-effect): 1,2,4,5-tetramethylbenzene–1,2,3,4,5-pentamethylbenzene, and pentamethylbenzene–hexamethylbenzene (Fig. 5).

It is interesting that for styrols with a methyl substituent and for indenes the lowest δI_{CH_2} value is that for the *ortho*-isomers. The anomalies observed for *n*-propylbenzene and its derivatives, in the case of side chains of the aromatic ring[39], do not appear when the CH_2 group is introduced in a ring containing a propyl group (*e.g.*, in *meta* position to the *n*-propyl).

Introduction of a methylene group in the side chain

In this case the values of δI_{CH_2} for isomeric alkylbenzenes increase in the sequence *ortho* < *meta* < *para*. In each group of isomers the value of δI_{CH_2} with increasing length of side chains approaches 100, this value being attained by *para* isomers with a lower number of carbon atoms in comparison with other isomers.

The increments δI_{CH_2} in the case of *ortho* isomers are higher for introduction of the CH_2 group into the longer side chain: with increasing length of the longer chain the increments δI_{CH_2} for the introduction in the shorter side chain decrease, and *vice versa* —the extension of the shorter side chain produces a decrease in the δI_{CH_2} value for the introduction of an alkyl group in the longer side chain.

For *p*-dialkylbenzenes such a distinct pattern does not exist (Table VI). In each of the homologous series studied there are differences with respect to the general dependences and trends, which defy interpretation. The value δI_{CH_2} is determined by the length, mutual position and the structure of the alkyls. In a homologous series with a branched alkyl group, δI_{CH_2} is smaller than in a series with an analogous *n*-alkyl group (*i.e.*, alkylpropylbenzenes–alkylisopropylbenzenes). The influence of the isoalkyl group (branched alkyls) increases with increasing length of the other alkyl group, and with decreasing distance between the side chain alkyls. The minimum value of δI_{CH_2} was found for *ortho* isomers with one isoalkyl group and increased in the sequence: *ortho* < *meta* < *para*. The dependence discussed here results from all the steric hindrances in the molecule.

TABLE VI

δI_{CH_2} VALUES FOR VARIOUS ALKYL CHAIN LENGTHS IN *p*-DIALKYLBENZENES

No. of C atoms		*1-X-4-MeB to 1-X-4-EtB*	*1-X-4-EtB to 1-X-4-PrB*	*1-X-4-PrB to 1-X-4-BuB*	*1-X-4-BuB to 1-X-4-PeB*	*1-X-4-PeB to 1-X-4-HexB*
Basic aromatic	$+CH_2$ *group*					
7	8	90.0				
8	9	89.2	87.4			
9	10	88.6	87.7	99.6		
10	11	86.0	85.1	99.6	99.0	
11	12*	86.6	85.1	100.1	96.4	94.1
12	13*	85.4	84.1	99.1	95.2	95.8
13	14*	90.9	99.3	101.3	110.4	101.3

* Data from ref. 38.

Introduction of alkyl groups containing more than one carbon atom (C_2–C_5)

The changes in retention index due to the introduction of alkyl groups with two and more carbon atoms are characterized by systematic deviations from the

TABLE VII

ALKYL GROUP INCREMENTS FOR HOMOLOGOUS SERIES OF ALKYLBENZENES

Series and compounds		*No. of C atoms*	*Range of δI*
Basic compound	*Alkylene incorporated*		
Introduction of C_2H_4 into ring			
B	EtB	C_6 –C_8	197
n-Alkyl-B	1-Et-2-*n*-Alkyl-B	C_7 –C_{15}	206–169
n-Alkyl-B	1-Et-4-*n*-Alkyl-B	C_7 –C_{14}	193–190
n-Alkyl-B	1-Et-3-*n*-Alkyl-B	C_7 –C_{11}	190–170
Di-EtB	Tri-EtB	C_{10}–C_{12}	170–163
Introduction of n-C_2H_4 into side chain			
n-Alkyl-B*		C_7 –C_{14}	178–199
1,4-Di-MeB*	1-Me-4-*n*-Alkyl-B	C_8 –C_{14}	177–199
1,2-Di-MeB*	1-Me-2-*n*-Alkyl-B	C_8 –C_{15}	161–198
Introduction of n-C_3H_6 into ring			
B	*n*-PrB	C_6 –C_9	285
n-Alkyl-B*	1-*n*-Pr-2-*n*-Alkyl-B	C_7 –C_{14}	288–240
n-Alkyl-B*	1-*n*-Pr-4-*n*-Alkyl-B	C_7 –C_{12}	281–274
n-Alkyl-B*	1-*n*-Pr-3-*n*-Alkyl-B	C_7 –C_{11}	276–263
Introduction of n-C_3H_6 into side chain			
n-Alkyl-B		C_7–C_{15}	277–299
1,4-Di-MeB	1-Me-4-*n*-Alkyl-B	C_8–C_{14}	276–295
1,2-Di-MeB	1-Me-2-*n*-Alkyl-B	C_8–C_{15}	259–295
Introduction of i-C_3H_6 into ring			
B	*i*-PrB	C_6 –C_9	256
n-Alkyl-B*	1-*i*-Pr-2-*n*-Alkyl-B	C_7 –C_{12}	259–214
n-Alkyl-B*	1-*i*-Pr-4-*n*-Alkyl-B	C_7 –C_{12}	253–244
n-Alkyl-B*	1-*i*-Pr-3-*n*-Alkyl-B	C_7 –C_{11}	245–229
1,3-Di-*i*-PrB	1,3,5-Tri-*i*-PrB	C_{12}–C_{15}	169
Introduction of i-C_3H_6 into side chain			
MeB	*i*-BuB	C_7 –C_{10}	231
Introduction of n-C_4H_8 into ring			
B	*n*-BuB	C_6 –C_{10}	385
n-Alkyl-B*	1-*n*-Bu-4-*n*-Alkyl-B	C_7 –C_{14}	384–374**
n-Alkyl-B*	1-*n*-Bu-2-*n*-Alkyl-B	C_7 –C_{15}	385–327**
Introduction of n-C_4H_8 into side chain			
n-Alkyl-B*		C_7 –C_{15}	376–394***

TABLE VII *(continued)*

Series and compounds		*No. of C atoms*	*Range of δI*
Basic compound	*Alkylene incorporated*		
Introduction of n-C_5H_{10} into ring			
B	*n*-PeB	C_6 –C_{11}	485
n-Alkyl-B*	1-*n*-Pe-4-*n*-Alkyl-B	C_7 –C_{14}	476–483**
n-Alkyl-B*	1-*n*-Pe-2-*n*-Alkyl-B	C_7 –C_{16}	480–418***
Introduction of n-C_5H_{10} into side chain			
n-Alkyl-B*		C_7 –C_{15}	470–493***

* The values are valid for a group of homologues and the compounds under the heading *Alkylene incorporated* are the final products of incorporation in the range of molecule sizes as determined by the number of C-atoms (indicated on the second place in the column *No. of C-atoms*). For example: by incorporation of the *i*-C_3H_6 group into the benzene ring of *n*-alkyl-B in the *ortho* position results in 1-*i*-Pr-2-*n*-alkyl-B with the molecule size from C_{12-3} to C_{12}. The first datum under the heading *No. of C-atoms* is C_7, this means that the lowest member of the homologous series used as basic compound was in this case methylbenzene and the δI_{CH_2}-values decreased from 259 *I*-units (incorporation of *i*-Pr into methylbenzene) to 214 (incorporation of *i*-Pr into $C_{12-3} = C_9$ *n*-alkylbenzene, *i.e.* *n*-propylbenzene, what gives 1-*i*-Pr-2-*n*-Pr-B.

** According to measurements by Engewald and Wennrich[38].

*** According to measurements by Soják *et al.*[39].

additivity principle. Independently of the site of substitution, the Kováts retention index increment is smaller than the value to be expected on the additivity principle, *i.e.*, for the group C_2H_4, $\delta I < 200$, for the group C_3H_6, $\delta I < 300$, etc. (Table VII).

For these groups the value of δI decreases as a result of introduction of the C_2–C_5 groups in the aromatic ring, but increases when the incorporation takes place in the side chain. However, the difference in retention indices due to the introduction of the same C_2–C_5 alkyl group in the aromatic ring and in the side chain is small, equal to 10–20 *I*-units, in contrast to the case of methylene groups which produce a difference of 30–40 *I*-units. Thus, the increase in alkyl chain length reduces the influence on δI of the position of incorporation.

The rôle of the steric factor is important especially in the systems 1,3-diisopropylbenzene–1,3,5-triisopropylbenzene where the introduction of the third isopropyl group produces an increment of only 169 *I* units (instead of the 300 units expected).

As has been reported by many authors[44,45], the Kováts rule

$$\delta I \approx 5 \cdot \hat{c}\, T_b$$

(where T_b = boiling point) for two isomers is not completely valid in all cases, *i.e.*, the proportionality factor, K_p, varies in a wider interval. From Table VIII it follows that the values of the proportionality factor calculated on the basis of experimental and theoretical data are smaller than 5. The proportionality factors for the other isomers are: trimethylbenzenes, 3.7–4.0; tetramethylbenzenes, 3.1–4.3; triethylbenzenes, 8.2; butylbenzenes, 3.9–4.6; pentylbenzenes, 3.4–5.8.

TABLE VIII

PROPORTIONALITY FACTORS FOR ISOMERS OF DIALKYLBENZENES

Dialkylbenzenes	*86°C*			*96°C*		
	meta–ortho	*meta–para*	*para–ortho*	*meta–ortho*	*meta–para*	*para–ortho*
Dimethylbenzenes	3.7	2.9	3.6	3.7	2.9	3.6
Dimethylbenzenes[27,28]	3.7	3.1	3.7	3.7	2.9	3.7
Methylethylbenzenes	3.9	3.3	4.0	4.1	4.3	4.1
Methylethylbenzenes[27,28]	4.0	3.4	4.1	4.1	2.9	4.4
Methylpropylbenzenes	3.9	3.2	4.7	4.2	3.5	5.1
Methylpropylbenzenes[27,28]	3.8	–	–	4.0	3.5	4.6
Methylisopropylbenzenes	4.2	3.6	5.3	4.4	4.0	5.4
Methylisopropylbenzenes[27]	–	–	–	4.1	3.9	4.8
Ethylpropylbenzenes	4.2	3.9	3.5	4.1	4.0	4.0
Ethylisopropylbenzenes	1.7	3.7	4.4	2.2	3.8	4.4
Diethylbenzenes	4.0	4.2	5.0	4.4	4.2	3.0

The comparison of the experimental results obtained (Table VIII) with the data in Table IX, which gives the average values of factors K_p in a temperature interval 80–115°C calculated on the basis of literature data[27,28,33–35,38], shows a satisfactory agreement. The proportionality factors for *para* and *ortho* isomers are somewhat higher than for the other pairs of isomers which have approximately identical values of K_p. The statistical analysis of the deviations in the case of alkylbenzenes showed that the numerous proportionality factors K_p calculated for particular groups and positions yielded no substantial improvement of accuracy. Therefore, the following values were deduced on the basis of literature data[27,28,33–35,38] for the average proportionality factors of the alkylbenzenes studied: dialkyl- and trimethylbenzenes, 3.9; tetramethylbenzenes, 3.6; butylbenzenes, 4.4; pentylbenzenes, 4.5.

The boiling points and retention indices of alkylbenzenes, calculated by means of the values indicated here, are in good agreement with corresponding literature and experimental data. The maximum differences found do not exceed 1°C and 2.5–3.0 *I* units.

TABLE IX

AVERAGE VALUES OF PROPORTIONALITY FACTORS, K_p, FOR DIALKYLBENZENES

Dialkylbenzenes	*Meta–ortho*	*Meta–para*	*Para–ortho*
Dimethylbenzenes	3.8	2.8	3.7
Methylethylbenzenes	4.1	3.5	4.1
Methylpropylbenzenes	4.1	3.5	4.8
Methylisopropylbenzenes	4.2	3.8	5.2
Diethylbenzenes	4.0	4.8	2.8
Diisopropylbenzenes	–	4.6	4.1

CONCLUSIONS

The elution sequence of isomeric alkylbenzenes using a squalane coated open capillary column is determined by the length and by the mutual distribution and position of the alkyl groups. Thus, increase in the alkyl chain length increases the dispersion interaction of *para* isomers with the stationary phase. This leads to an increase in retention in the sequence: dimethyl-, methylalkyl- and ethyl-alkylbenzenes. In contrast, corresponding decreases occur for the retention sequence of *ortho* isomers.

The retention index variations studied show that the δI_{CH_2} value is determined primarily by the character of the site of introduction of the CH_2 group. For introduction into the ring of dialkylbenzenes the value δI for the first member of a homologous series is lower than 100 I units, and with increasing alkyl chain length it approaches 100. The presence of an isopropyl group in alkylbenzenes produces a decrease in δI_{CH_2}. The difference in retention indices corresponding to the introduction of a methylene group in the ring and in the side chain is 30–40 I units. This difference diminishes with increasing length of the group incorporated; in the case of C_2–C_5 alkyl groups it is equal to 10–20 I units.

For the isomers investigated here, the proportionality factor in the equation

$$\delta I \approx K_p \cdot \partial T_b$$

varies from 1.7 to 8.2. Average values of K_p calculated on the basis of both literature and experimental data enables in most cases a good correlation between physicochemical and chromatographic data of individual alkylbenzenes.

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GAS CHROMATOGRAPHIC DETERMINATION OF MOLECULAR POLARITY AND QUANTUM CHEMICAL CALCULATION OF DIPOLE MOMENTS IN A GROUP OF SUBSTITUTED PHENOLS

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SUMMARY

A chromatographic polarity parameter, $I - b \cdot \mathrm{MR}$, has been calculated, where I is a gas–liquid chromatographic retention index, MR is the molar refractivity of the compound analysed and b is the stationary phase polarizability coefficient. To determine b, retention data are obtained on two phases of different polarities. The polarity parameter has been shown to correlate well with quantum chemically calculated dipole moments in the case of phenols without space-consuming substituents.

INTRODUCTION

During the past decade chromatography has become a powerful tool for the determination of molecular structural data employed in studies of quantitative structure–activity relationships (QSARs)[1,2].

To apply the linear free energy relationship based on Hansch's approach in QSAR, quantitative data are required concerning partitioning, electronic and steric characteristics of the compounds considered. Of the so-called electronic data, the most commonly used are individual Hammett's substituent constants (or their various modifications), dipole moments, pK_a values, spectroscopic data and quantum chemical parameters. The capacity of a drug for dipole–dipole interaction with the biological receptor is often assumed to be of importance for its bioactivity. As determinations of dipole moments are rather tedious there is a need to develop a convenient method for evaluation of dipole moments or a quantity related to them. The recently reported[2] gas chromatographic (GC) polarity parameters are used here with substituted phenols. The polarity parameters are compared to dipole moments calculated by a quantum chemical method.

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THEORETICAL

Gas–liquid chromatographic GLC retention indices of fatty acid methyl esters[3] on SE-30 and SILAR 5 CP stationary phases are related by the empirical equation

$$I = K_1 N + K_2 \chi + K_3 \tag{1}$$

where N is a parameter related to the molecular polarity of the solutes, χ (connectivity index[4,5]) characterizes the ability of the solutes to undergo dispersive interactions with stationary phases and K_1–K_3 are constants. The dispersion effect is expected to be important even in the interactions between molecules with large dipole moments.

The connectivity index, χ, is a convenient parameter for the characterization of molecular polarizability. It is easily calculated for hydrocarbons and organic compounds containing nitrogen and oxygen. However, in the case of other atoms, *e.g.*, halogens, χ values are uncertain and generally the molecular refractivity, MR, seems to be the more reliable parameter for the description of molecular polarizability. Thus, our starting relation has the form

$$I = a\mu^2 + b \cdot \mathrm{MR} + c \tag{2}$$

where a can be considered as a measure of the stationary phase polarity and b as a measure of its polarizability. The equation is similar to that recently proposed by Gassiot-Matas and Firpo-Pamies[6]. Rearranging eqn. 2 one gets:

$$I - b \cdot \mathrm{MR} = a\mu^2 + c \tag{3}$$

Thus, if eqn. 2 holds, the quantity $I - b \cdot \mathrm{MR}$ should be linearly related to μ^2 and describe the polarity of the solute. The molecular refractivity, MR, can easily be calculated for any given molecule as a sum of substituent[7] or bond[8] refractivities. Thus, if the retention indices can be determined and the value of b is known, we have a convenient, quantitative measure for the polarity of the solutes.

The constant b is calculated as follows. If one has retention indices for any given compound on two phases of different polarity then one can write

$$I_{\mathrm{P}} = a_1\mu^2 + b_1 \cdot \mathrm{MR} + c_1 \tag{4}$$

$$I_{\mathrm{NP}} = a_2\mu^2 + b_2 \cdot \mathrm{MR} + c_2 \tag{5}$$

where I_{P} and I_{NP} are retention indices on polar and non-polar phases, respectively. Taking μ^2 from eqn. 5

$$\mu^2 = (I_{\mathrm{NP}} - b_2 \cdot \mathrm{MR} - c_2)/a_2 \tag{6}$$

eqn. 4 can be rewritten as:

$$I_{\mathrm{P}} = a_1 (I_{\mathrm{NP}} - b_2 \cdot \mathrm{MR} - c_2)/a_2 + b_1 \cdot \mathrm{MR} + c_1 \tag{7}$$

After rearrangement one gets

$$I_P = \frac{a_1}{a_2} \cdot I_{NP} - \left(\frac{a_1}{a_2} \cdot b_2 - b_1\right) MR + k_3 \quad (8)$$

or

$$I_P = k_1 I_{NP} - k_2 \cdot MR + k_3 \quad (9)$$

where k_1–k_3 are constants. These can be determined and statistically evaluated from regression analysis of experimental retention indices and molecular refractivity data for a sufficiently large set of compounds. A statistically significant relation of the type 9 proves the validity of the assumptions expressed by eqn. 2. With knowledge of k_1 and k_2 one can then undertake to calculate b.

Let us first consider the situation when $b_1 = b_2$, *i.e.*, the two phases, polar and non-polar, are of equal polarizability. To a first approximation one can assume this to be the case when the two phases have similar molecular weights. Then:

$$b_1 = b_2 = b = k_2/(k_1 - 1) \quad (10)$$

After calculation of b one can use eqn. 3 to determine the chromatographic polarity parameter, $I - b \cdot MR$.

If the polarizabilities of the phases, b_1 and b_2, differ significantly, the polarizability of one of the two phases must be known. Then:

$$b_1 = k_1 b_2 - k_2 \quad \text{or} \quad b_2 = (k_2 + b_1)/k_1 \quad (11)$$

A possible way to obtain data related to the polarizability of a chosen standard phase would be to find a relation between the molecular refractivity and retention indices for a group of non-polar compounds. The phase chosen should be as non-polar as possible. Alternatively different values of b calculated from eqn. 11 can be compared with those obtained from eqn. 2. In order to get a statistically significant relationship for an equation of the type 2 a certain number of retention indices, dipole moments and molecular refractivities is required. Substituted phenols offer a group of compounds for which quite a few such data are available in the literature. Additionally, these compounds exhibit polar interactions with stationary phases. Retention indices for 43 phenols on three stationary phases of different polarities have recently been reported by Grzybowski *et al.*[9]. For 20 of them, dipole moment values have been determined experimentally in benzene[10–16].

It seemed interesting to compare the chromatographic polarity parameter, $I - b \cdot MR$, with quantum chemically calculated dipole moments for these 43 phenols. The dipole moments were calculated using the CNDO/2-MO method[17,18], which for this purpose is well established. The results are shown in Table I. In order to obtain reliable values, comparable to those in the literature for solutions in benzene[10–16], we first calculated the most stable conformations of all the phenols. In cases where more than one energetically favoured conformer exists, the value μ_{calc} represents the arithmetic mean of the μ values of the corresponding conformers. For the molecular geometries of the phenols, standard values were used[19].

TABLE I

RETENTION INDICES, MOLAR REFRACTIVITIES, EXPERIMENTAL AND CALCULATED DIPOLE MOMENTS AND CHROMATOGRAPHIC POLARITY PARAMETERS FOR A GROUP OF SUBSTITUTED PHENOLS

No.	*Phenol*	*Kováts retention indices**			*Molar refractivity, MR***	*Dipole moment values*		*Chromatographic polarity parameters,* $I_{NGA} - 21.3825\ MR$
		SE-30	*OV-225*	*NGA*		μ_{obs}***	μ_{calc} §	
1	2-CH_3	1035	1587	1742	32.83	1.45[10]	1.72	1040
2	4-CH_3	1059	1654	1813	32.83	1.61[10]	1.87	1111
3	3-CH_3	1065	1648	1782	32.83	1.58[10]	1.79	1080
4	2,6-$(CH_3)_2$	1098	1593	1716	37.45	1.38 §§[11]	1.69	915
5	2,4-$(CH_3)_2$	1134	1660	1825	37.45	1.39 §§[11]	1.77	1024
6	3-C_2H_5	1160	1742	1898	37.48	–	1.60	1096
7	4-C_2H_5	1162	1746	1890	37.48	–	1.85	1088
8	3,5-$(CH_3)_2$	1163	1706	1877	37.45	1.55 §§[11]	1.82	1076
9	2,3-$(CH_3)_2$	1169	1693	1857	37.45	1.25 §§[11]	1.74	1056
10	2,4-Cl_2	1183	1708	1877	38.21	1.59[12]	2.12	1060
11	4-Cl	1192	1922	2058	33.21	2.19[13]	2.36	1348
12	3-Cl	1194	1911	2061	33.21	2.14[13]	2.03	1351
13	2,4,6-$(CH_3)_3$	1204	1621	1778	42.07	1.40[13]	1.78	878
14	2,6-Cl_2	1206	1727	1871	38.21	–	3.20	1054
15	4-OCH_3	1210	1930	2050	35.05	1.92[13]	2.21	1300
16	3-OCH_3	1211	1940	2083	35.05	–	2.30	1334
17	2,3,5-$(CH_3)_3$	1260	1823	1960	42.07	–	1.78	1060
18	4-Br	1274	2054	2191	36.06	2.19[13]	2.70	1420
19	3-CH_3-4-Cl	1283	2025	2135	37.84	–	2.02	1326
20	4-NH_2	1314	2154	2277	32.60	–	2.50	1580
21	4-OH	1334	2330	2515	28.03	1.40[14]	1.61 (3.21)	1916

22	3-NH_2	1335	2219	2352	32.60	1.83[16]	2.53 (2.61)	1655
23	2,4,6-Cl_3	1349	1928	2067	43.21	1.62[15]	1.67	1143
24	2,4,5-Cl_3	1362	2039	2158	43.21	–	1.99	1234
25	3-OH	1368	2371	2576	28.03	2.07[14]	3.49	1977
26	3,5-Cl_2	1391	2217	2343	38.21	2.18[13]	2.17	1526
27	4-I	1398	2230	2348	41.02	2.13[13]	–	1471
28	4-CO_2CH_3	1500	2376	2461	40.05	–	2.71	1604
29	4-$COCH_3$	1578	2478	2529	38.36	–	3.15	1709
30	2-NH_2	1242	2039	2196	32.60	–	2.85	1499
31	3-Br	1270	2069	2214	36.06	–	2.34	1443
32	2-*iso*-C_3H_7-5-CH_3	1271	1776	1932	45.75	–	1.82	954
33	2,6-(*tert.*-C_4H_9)$_2$-4-CH_3	1494	1782	1830	70.01	–	1.75	333
34	2-OCH_3	1095	1544	1627	35.05	–	2.12	878
35	2-NO_2	1149	1556	1703	34.54	3.10[16]	3.82	964
36	2,6-(OCH_3)$_2$	1347	1936	2014	41.89	–	3.00	1118
37	2-OCH_3-4-C_3H_7	1392	1810	1884	48.98	–	2.28	837
38	2-OCH_3-4-CHO	1447	2199	2235	40.90	–	3.39	1360
39	2,6-(OCH_3)$_2$-4-CH_3	1473	2076	2106	46.51	–	3.07	1112
40	2-OCH_3-4-$COCH_3$	1531	2283	2326	38.36	–	2.89	1506
41	2,6-(OCH_3)$_2$-4-C_3H_7	1624	2254	2256	55.82	–	3.20	1062
42	2,6-(OCH_3)$_2$-4-$COCH_3$	1849	2685	2683	52.04	–	4.49	1570
43	2-OCH_3-4-CH_2-CH = CH_2	1367	1848	1923	48.51	–	2.18	886

* Data from ref. 9.

** Calculated according to Hansch *et al.*[7].

*** Determined in benzene solution at 25°C if not specified otherwise.

§ Arithmetic mean of the μ values for the energetically favourable conformers.

§§ Determined in benzene at 20°C.

RESULTS AND DISCUSSION

Correlations between retention indices on polar and non-polar phases

In Table I the Kovát's retention indices are shown for 43 phenols on dimethylpolysiloxane (SE-30), 3-cyanopropylmethylpolysiloxane (OV-225) and polyneopentyl glycol adipate (NGA) coated on Chromosorb W HMDS (80–100 mesh)[9]. Molar refractivites have been calculated as a sum of fragmental refractivites according to Hansch *et al.*[7]. The dipole moment values μ_{obs} were taken from the literature[10–16], and are for benzene solutions at 25°C (or at 20°C in four cases). The data are from several sources, but seem to be reliable as the values obtained for the same compounds by independent authors are very similar. Besides, the data are in accord with the calculated values.

The relationship between retention indices on OV-225 and SE-30 has the form

$$I_{OV\text{-}225} = 1.95\ (\pm\ 0.16)\ I_{SE\text{-}30} - 22.08\ (\pm\ 3.56)\ MR + 285.76$$
$$n = 43,\ R = 0.9691,\ s = 71 \qquad (12)$$

where n is a number of compounds considered, R is the multiple correlation coefficient and s is the standard deviation from the regression equation. The numbers in parentheses are 95% confidence limits. All the equations presented are statistically significant at least at the 99.9% significance level, as are the variables used. The corresponding equation in the case of the phases NGA and SE-30 has the form:

$$I_{NGA} = 1.81\ (\pm\ 0.20)\ I_{SE\text{-}30} - 24.50\ (\pm\ 4.36)\ MR + 682.59$$
$$n = 43,\ R = 0.9472,\ s = 87 \qquad (13)$$

Determination of the stationary phase polarizability coefficients

After determination of eqns. 12 and 13 we undertook to calculate the chromatographic polarity parameter, $I - b \cdot MR$, as defined by eqn. 3.

We have found experimental dipole moments for a group of 20 compounds. For four compounds, dipole moments determined in benzene at room conditions did not fit eqn. 2. These are 4-OH, 3-OH and 3-NH_2 substituted phenols for which the experimental dipole moments are significantly lower than expected from eqn. 2, and

TABLE II

CORRELATION COEFFICIENTS, R, AND STANDARD DEVIATIONS, s, FOR LINEAR EQUATIONS $I = \alpha X + \beta$ RELATING RETENTION INDEX, I, TO DIPOLE MOMENTS OR MOLAR REFRACTIVITIES, X, FOR A GROUP OF PHENOLS

α and β are constants.

I	X	R	s
$I_{SE\text{-}30}$	μ^2_{obs}	0.6065	95
$I_{OV\text{-}225}$	μ^2_{obs}	0.8692	114
I_{NGA}	μ^2_{obs}	0.8641	110
$I_{SE\text{-}30}$	MR	0.6479	91
$I_{OV\text{-}225}$	MR	0.2330	225
I_{NGA}	MR	0.2463	213

2-nitrophenol, the experimental dipole moment of which is to high to fit eqn. 2. The cause of the much higher polarity of the first three compounds in comparison with their polarity in benzene solutions is probably partial ionization at high-temperature chromatographic conditions. As far as 2-nitrophenol is concerned its low polarity under chromatographic conditions may be due to an increase in intramolecular bonding. The dipole moments of the remaining 16 compounds under chromatographic conditions are (to a first approximation) linearly related to the experimental ones. Bearing in mind all the limitations, the following equations can be considered as supporting the hypothesis:

$$I_{\text{SE-30}} = 66.67\ (\pm\ 14.42)\ \mu^2_{\text{obs}} + 25.34\ (\pm\ 5.22)\text{MR} + 57.53$$
$$n = 16,\ R = 0.9679,\ s = 31 \qquad (14)$$

$$I_{\text{V-225}} = 164.72\ (\pm\ 36.61)\ \mu^2_{\text{obs}} + 24.48\ (\pm\ 12.90)\ \text{MR} + 408.33$$
$$n = 16,\ R = 0.9472,\ s = 77 \qquad (15)$$

$$I_{\text{NGA}} = 155.69\ (\pm\ 33.63)\ \mu^2_{\text{obs}} + 23.99\ (\pm\ 12.19)\ \text{MR} + 599.81$$
$$n = 16,\ R = 0.9477,\ 2 = 73 \qquad (16)$$

The two-parameter eqns. 14–16 are highly statistically significant. The correlation coefficients for the corresponding one-parameter equations relating the retention indices to μ^2_{obs} and MR are much lower or of no statistical value (Table II).

Next it was of interest to compare b values obtained from eqns. 14–16 with those calculated from the coefficients in eqns. 12 and 13. For calculation of b_1 by eqn. 11, the value $b_2 = 25.3412$ has been assumed as obtained from eqn. 14. Then, from eqn. 12, b for the OV-225 phase was calculated as 27.3510. The corresponding value for the NGA phase calculated from eqn. 13 is 21.3825.

Relationship between the chromatographic polarity parameter and the calculated dipole moments

As can be concluded from reports by Karger *et al.*[20] and Scott[21], the polar term in equations of the type 1 reflects more than just the charge distribution in the molecule. It includes a steric component and reflects the ability to form hydrogen

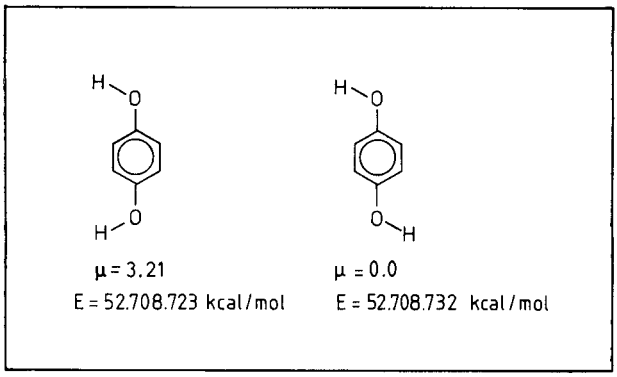

Fig. 1. Dipole moments and conformational energies (E) for energetically favoured conformations of hydroquinone.

bonds between the solutes and the stationary phases. Therefore a chromatographic polarity parameter might be able to simulate the capacity of a molecule to approach an unspecific binding site; this is of great importance in drug–receptor interactions.

The chromatographic polarity parameter, being dynamic in nature, reflects the actual dipole moment of the solute under chromatographic conditions. On the other hand, calculated dipole moments represent the molecular conformations in the gas phase which may well differ from those in the adsorbed state. This can be demonstrat-

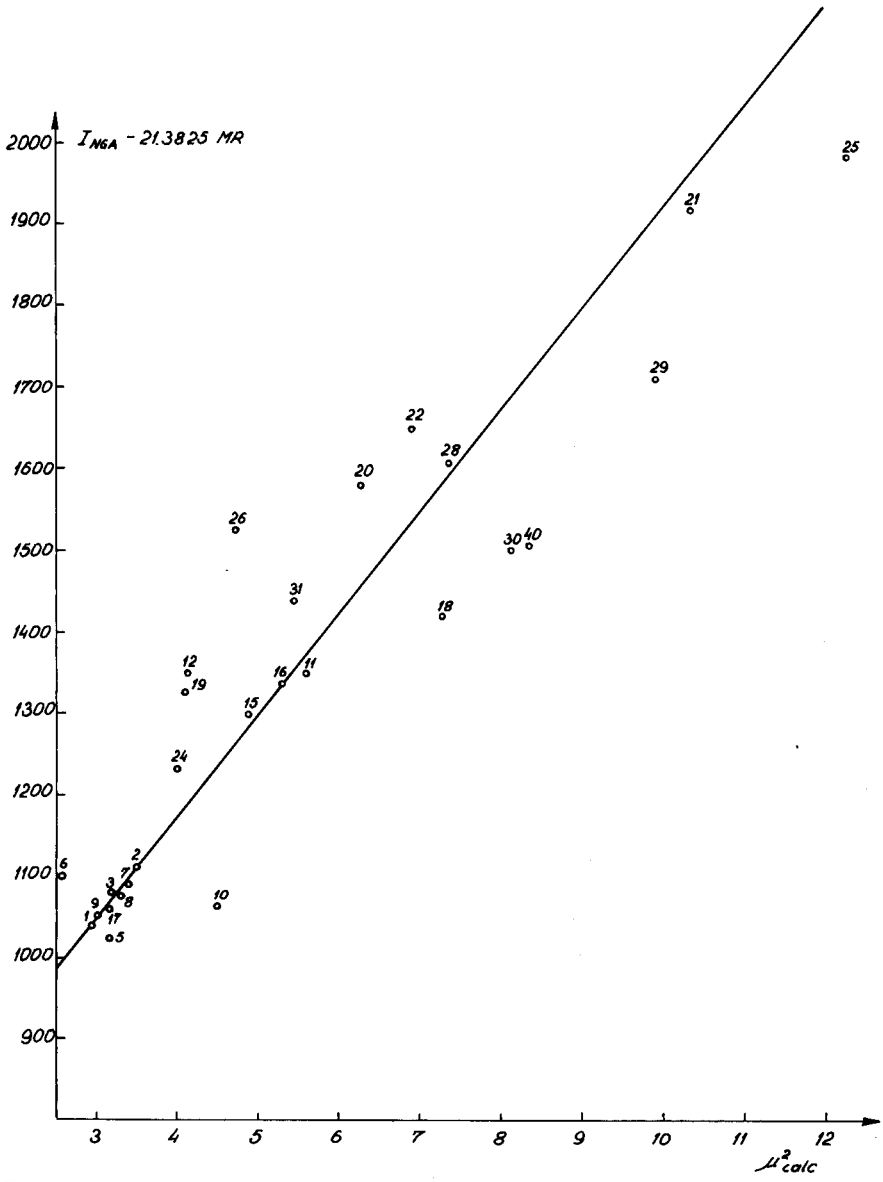

Fig. 2. Correlation between the chromatographic polarity parameter, $I - b \cdot MR$, and calculated dipole moments for phenols without space-consuming substituents.

ed with 4-hydroxyphenol. For this compound μ_{obs} and μ_{calc} are in a very good agreement but there is no correlation with the chromatographic polarity parameter; μ_{calc} is the arithmetic mean for the two conformers (Fig. 1). If one takes the μ_{calc} of only one conformer ($\mu_{calc} = 3.21$) there is a very good correlation with the polarity parameter. This means that in the gas phase and benzene solution we have a mixture of two conformers, whereas at the stationary phase only one conformer is bound. A similar situation can be expected at the surface of a biological receptor.

The idea that the chromatographic polarity parameter reflects more than the charge distribution in the molecule seems to be supported by the correlation shown in Fig. 2:

$$I_{NGA} - 21.3825\ MR = 98.85\ \mu^2_{calc} + 810.62$$
$$n = 27,\ R = 0.919,\ s = 8.47 \qquad (17)$$

The good correlation between the chromatographic polarity parameter and μ_{calc} described by eqn. 17 was obtained after elimination of all phenols with space-consuming substituents (33,37,41,43) as well as 2,6-disubstitution (4,13,14,23,33,36,39,41,42), also 34 (steric hindrance and hydrogen bonding), 35 (hydrogen bonding) and 38 (thermal reactions?), leaving 27 phenols. This procedure seems to be reasonable because in the remaining 27 phenols the interaction between the phenol and the stationary phase is dominated by the electronic properties of the molecule (dipole moments), whereas in all the excluded cases additional factors play a significant rôle.

The easily determined chromatographic polarity parameter seems to be a convenient numerical measure of the molecular polarity and might be of importance in medicinal chemistry. Application of it to QSAR will be published elsewhere[22].

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FLUORESCENCE AS AN AID IN UNDERSTANDING GAS CHROMATOGRAPHY

A COLOUR FILM OF A GAS CHROMATOGRAPHIC PROCESS AND THE OBSERVATION OF ADSORPTION

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SUMMARY

By using fluorescent compounds, gas chromatographic processes can be made visible and can even be filmed. Some pictures from such a film are shown. In the system used, adsorption of a compound appeared to be an important phenomenon. Consequences of the use of fluorescence in characterizing the events in a gas chromatographic apparatus are discussed.

INTRODUCTION

For various reasons it is interesting to visualize the processes taking place in a gas chromatograph. Foremost we were interested in adsorption phenomena, because one of the problems in practice is the adsorption of injected compounds to the system. Two kinds of adsorption can be distinguished: irreversible adsorption, whereby part of the injected sample molecules do not elute from the system, and reversible adsorption, which manifests itself by tailing of the end of a peak. Though to some extent all sorts of substances may adsorb in a gas chromatograph, adsorption manifests itself most clearly with polar compounds. For such compounds it is of importance to quantitate and subsequently redress adsorption. In collecting the desired information about adsorption two guidelines should be followed. Firstly the entire gas chromatographic (GC) system, *i.e.* from injection device to detector, should be considered in relation to adsorption; secondly, for measuring small amounts of a compound left behind, a sensitive analytical method should be used. The application of radioactive compounds and measurements fulfils these conditions. Two such studies are mentioned in the literature[1,2]. We studied the behaviour of the radioactive anti-epileptic drug [^{14}C]phenytoin in a GC system equipped with micropacked columns[2]. One of the

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conclusions of this study was that the amount of irreversible adsorption was considerable: 10–20%. This amount could not be predicted from the standard curves of cold phenytoin, because the limit of sensitivity of cold phenytoin was much less than the amount of radioactivity lost. An explanation of this phenomenon might be that the total amount of molecules injected will appear at the end of the system; however, the outgoing molecules need not necessarily be the injected ones. If active places in the system are interpreted as "drug receptors", it is conceivable that receptor hopping takes place; *i.e.* incoming drug molecules are exchanged for already adsorbed ones. In this light, adsorption is a dynamic process that might be an important co-factor in plug elongation and in the realization of retention time.

The study of GC processes with radioactive compounds is practicable though not convenient. Besides radioactive substances, fluorescent compounds can also be used for sensitive measurements of amounts adsorbed and left behind in the system. Moreover, fluorescence visualizes what happens in the process. A fluorescent pyrromethene pigment was used in this way for two purposes[3]: in the first place it served as a tool for rapid global screening of adsorption by eye; secondly, by means of a fluorimeter, adsorption of fluorescence in small amounts, not visible to the eye, could be measured. This gives a sensitive and objective method to compare deactivation procedures. The fluorescence quantum efficiency of pyrromethene pigments being very high[4], it appeared possible to film the separation process of two such pigments in the oven of a GC apparatus*. In this way we could record dynamically the observed phenomena in a glass injection liner and glass capillary column. This article describes the techniques used and comments upon some of the film pictures.

MATERIALS AND METHODS

A Packard Becker 420 gas chromatograph was used. A heat-resistant box with two glass windows was mounted on the oven top (Fig. 1). One of the windows was used for letting in radiation, the other for observing the process. The light source was a 200-W mercury lamp. With appropriate filters, the excitation wavelength had a maximum of 360 nm and a bandwidth at half transmission of λ_{max} of *ca.* 50 nm[3]. The columns used had special dimensions to facilitate observations (Fig. 2). In columns of the usual type the rings overlap each other. Moreover, the rings move out of the beam of light and focus of the camera.

The column was a glass Duran 50, *ca.* 4 m × 0.7 mm I.D. The column ends were stretched before coating. The column was coated dynamically with 10-μm tullanox particles and afterwards with a solution of 3% OV 275[5]. A thick inside layer was pursued to intensify fluorescence of compounds on the column. The injection system was a falling glass needle system[6], mounted in such a way that injection with a long needle took place in the oven. Thus the injection process also became visible. By this procedure, however, no separate heating of the injection could be used. The carrier gas was helium, at a flow-rate of 10 ml/min. For flame ionization detection, air and

* The 15-min 16-mm colour film with optic sound track can be ordered for only the cost of copying, if used for instruction or for scientific purposes. The title is: "Fluorescence as an aid in understanding gas chromatography". For information contact the authors. The film was presented at the *Fourth International Symposium on Capillary Chromatography, Hindelang, 3–7 May, 1981.*

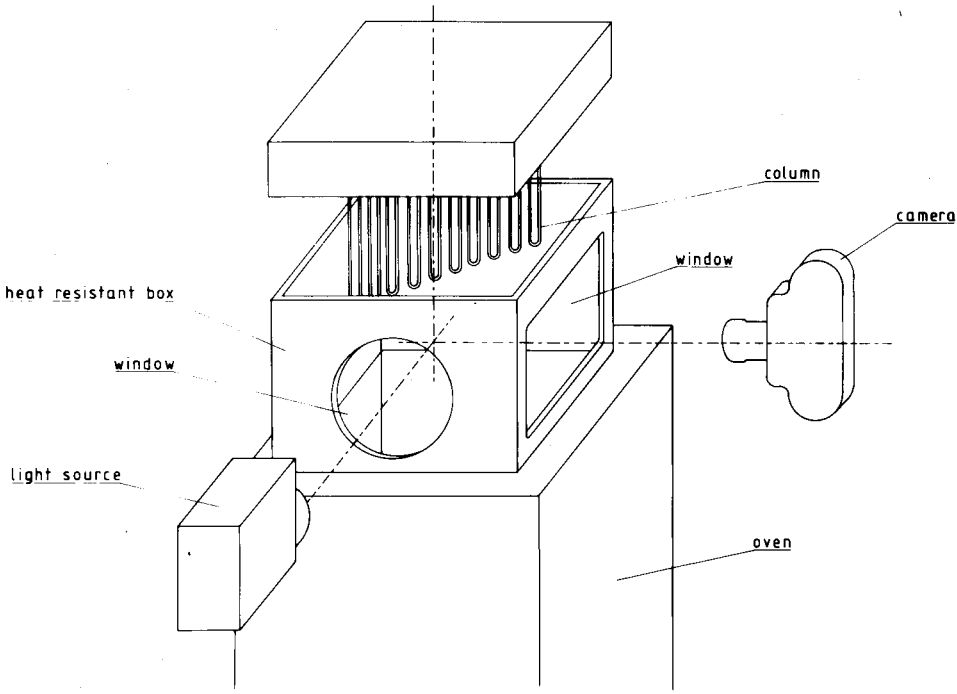


Fig. 1. Technical equipment used to film GC process in oven.

hydrogen were used at flow-rates of 270 ml/min and 20 ml/min, respectively. The oven temperature was fixed at 200°C. The detection temperature was 250°C. The oven fan was switched off to avoid movement of the column during filming.

Two pyrromethene pigments were chromatographed (Fig. 3). The left-hand one had a green fluorescence and a shorter retention time than the right-hand one, which fluoresced yellow. The syntheses and properties of these compounds have been described[4,7,8]. Injections were done with *ca.* 2 μg.

The GC process was filmed with Fuji colour film, 500 ASA, at 8 of 16 frames

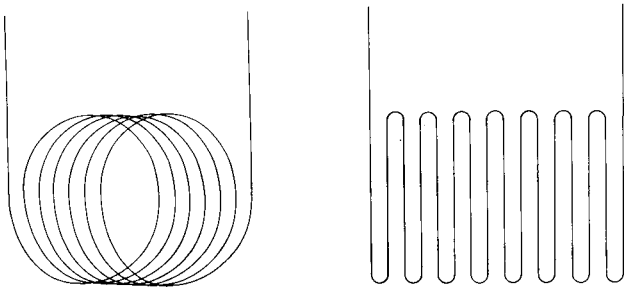


Fig. 2. (A) Conventional type of circular column. (B) Column used in experiment with fluorescent compounds, called a two-dimensional or radiator type column.

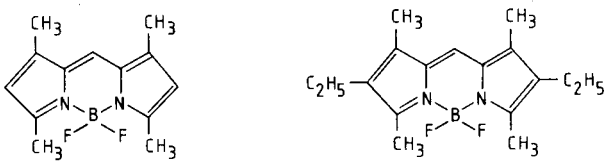


Fig. 3. The two fluorescing pyrromethene pigments.

per second. During development the film was upgraded to 4000 ASA. The Figures in this paper were taken from this film.

RESULTS

Injection

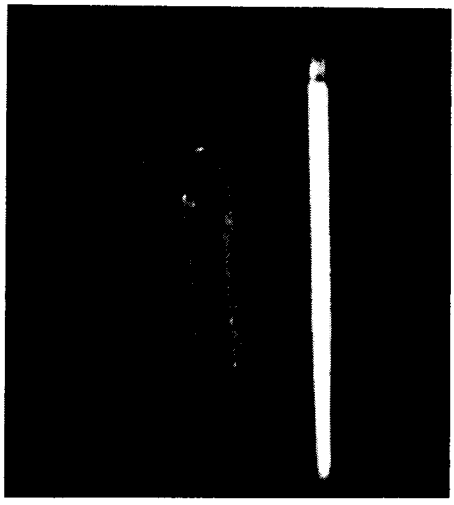
Fig. 4 shows the two compounds flowing from the needle tip, at the top of the picture. The outside of the liner is just visible. The fluorescence in the liner is mirrored by the first column sections. Injection took place in 10 sec. Using the falling needle system no back flush was seen.

Separation

In the first ascending part of the column the two fluorescent compounds were already almost separated (Fig. 5). A later stage of the separation is shown in Fig. 6. Note the elongation of the plugs, illustrating peak broadening.

Uniformity of coating

Fig. 7 shows that the thick inside layer of tullanox OV 275 was not smooth but



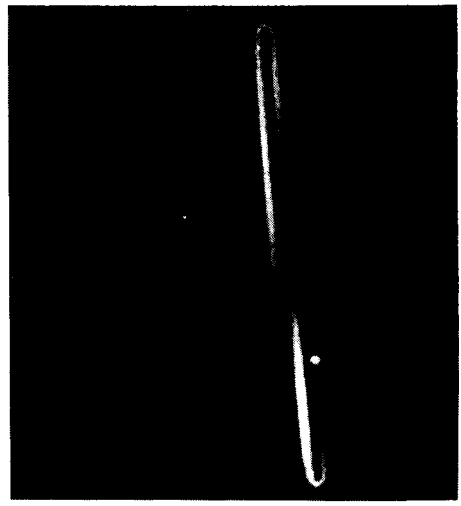


Fig. 4. Injection of the two pyrromethene pigments.

Fig. 5. Separation process; early phase.

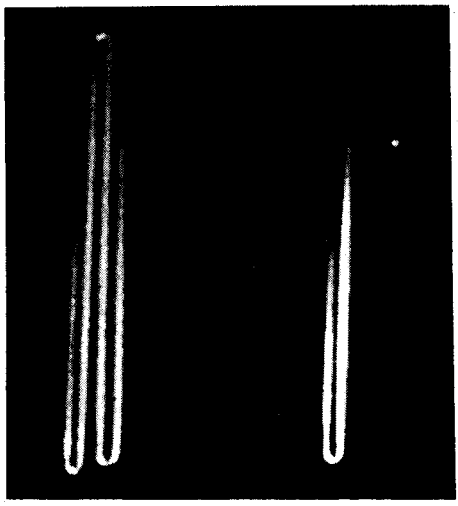


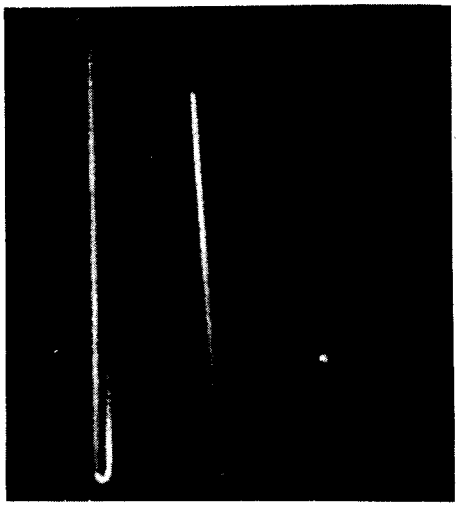
Fig. 6. Separation process; late phase.

Fig. 7. Inequalities in coating, owing to the thick tullanox layer used.

rugged. By destroying the coating by flame it appeared that in this part of the column the compounds were not retained (Fig. 8).

Adsorption

Adsorption in the uncoated liner of the injection port did not become visible. However, in daylight the presence of a faint red colour indicated that the compound must have got lost there. It may not have shown up under UV light because in a



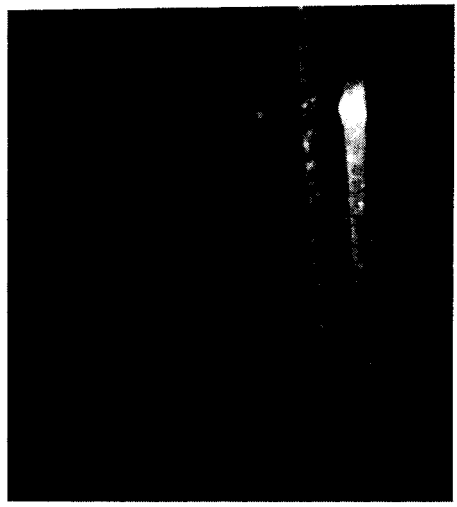


Fig. 8. Overheated coated column section. Between the two glowing column parts, fluorescence was absent or only very faint.

Fig. 9. Close-up of the remaining adsorption at the column inlet.

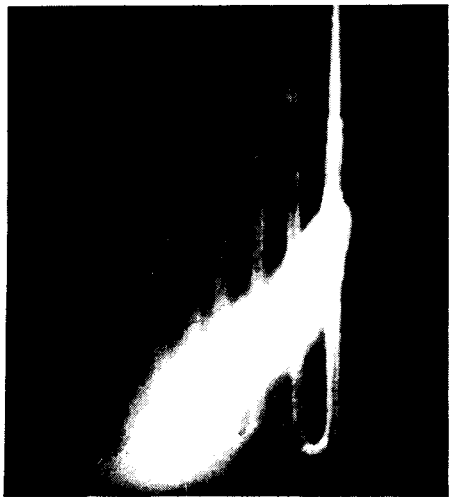


Fig. 10. Leak in shrink PTFE.

crystalline configuration compounds do not fluoresce because of self-quenching. At the transition of liner to column (shrink PTFE), and in the first part of the column, a huge remaining adsorption was noted (Fig. 9). This can also be seen in Figs. 6–8. The PTFE was shrunk adequately and there was no leakage. A leak is demonstrated in Fig. 10.

Exchange of molecules

That molecules can easily be forced from "receptors" was shown by the follow-

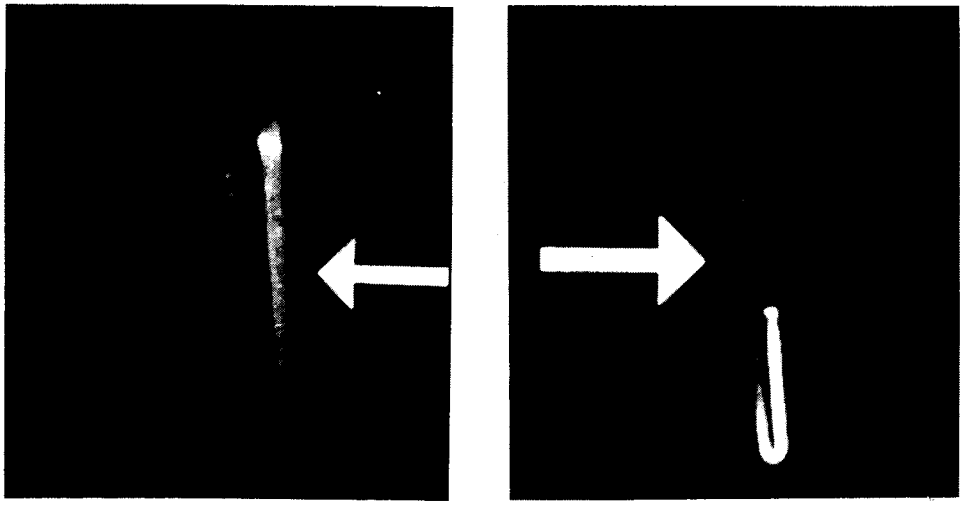


Fig. 11. Generation of a "ghost" peak. No fluorescence is streaming from above, because an empty needle was injected, but from the column inlet a small plug emerges.

Fig. 12. Injection of the yellow compound forced a green plug from the column inlet, running before the yellow one.

ing experiments. After two injections of only the green compound, we made three injections with an empty needle. Hence these were blank injections. From Fig. 11 (and other pictures not shown here) it can be seen that nevertheless a small plug is generated from the zone with much adsorption at the beginning of the column. The three small "ghost" peaks also showed up on the recorder. Immediately after these three blank injections only the yellow compound was injected. Now a more substantial green plug was removed from the initial zone with maximum adsorption, as shown in Fig. 12. This might indicate that the two similar compounds compete for the same sort of "receptors" at the beginning of the column.

DISCUSSION

Techniques used

The equipment required to visualize GC was relatively simple. We think that with some adroitness one can readily observe in this way what happens in columns and liners. Though in the pictures shown the dynamic dimension as well as some of the brightness and sharpness of the film images got lost, the importance of the method for fundamental study and teaching of chromatography is evident. By using not only optical but also electronic instrumentation, GC processes can be studied in more detail. Pyrromethene pigments appear to be useful in this approach. However, the techniques can also be used without them, as was demonstrated in the study of the behaviour of fluorescent pollutants[9].

Film images

From the film it became clear that with the techniques used one can study the contribution of separate steps in GC such as injection, column performance, and coupling devices on the result of the whole GC process. A first estimation of what these elements contribute to plug elongation can already be made from the film. A more rigorous tackling of the problem awaits continuous electronic scanning of fluorescent plug length throughout the system. In the assemblage shown the column did not behave as an entity but showed parts functioning rather differently.

Adsorption, exchange of molecules and ageing of column

A striking aspect of the pictures is the huge adsorption centred around the column inlet. This observation is not new. Similar observations were made by fluorimetric measurement of pyrromethene remnants on DMCS-treated columns and OV 275 SCOT columns[3]. At the injection site adsorption was always greater than in the middle sections of the column. Using another compound, [^{14}C]phenytoin, and another type of column (micropacked column with a mixture of OV 17 and OV 225) the same sort of results were obtained[2]. Twelve years ago, predominant adsorption of derivatized [^{14}C]glucose at the inlet of a packed Carbowax column had already been demonstrated[1]. Abundant adsorption at the inlet of columns, therefore, has been reported for several substances, in various systems, using different measuring methods. This leads to two conclusions: firstly the phenomenon of inlet adsorption might be more ubiquitous than one may realize; secondly the behaviour of the pyrromethene pigments in this respect is not exceptional and hence these compounds might be tried as probes for adsorption. Some reservations about the observed inlet adsorption

may be more inert at the injection site. However, this situation does not need to remain throughout the life of the column. Column ageing processes can be studied by means of fluorescence.

The concept of polar compounds doing "receptor hopping", as put forward in the Introduction, was strengthened by the way "ghost" peaks emerge following blank injections or an injection of a similar substance. The suggestion of exchange of molecules at active column sites, every time a wave of molecules passes, seems plausible, but requires more experimental evidence. It also has to be determined for what sort of molecules and supports the concept is valuable. It is clear that, whereever attraction between compound and active sites on the support is prominent, GC cannot be described solely as partition chromatography.

Prospects

The study of GC by the use of fluorescence seems promising, especially in the fields of the design of injection techniques and the estimation of column performance and column preparation. The role to be played by pyrromethenes will depend on their suitability as universal GC probes. By attaching distinctive polar groups to these compounds, a fluorescent "polarity mixture" may be created. In this way the affinity of some molecular structures for active sites in the system can be observed more easily. By a more fundamental understanding of the events in the entire GC instrument, especially by evaluating the influence of extra-column factors and of adsorption on the results, theoretical models of the GC process will also become more complete.

CONCLUSION

The use of fluorescing compounds such as pyrromethene pigments is a valuable addition to the range of methods used in studying the dynamics of GC. Its application will lead to a better understanding of GC processes and improved design of columns and instruments.

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TEMPERATURE AND ELUENT EFFECTS ON THE SELECTIVITY OF SOME NITROAROMATIC BONDED PHASES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Nucleosil·10 NH_2 has been converted into 2,4-dinitroanilino, bis(3-nitrophenyl) sulphone and 2,4,7-trinitrofluorenimine silicas. Net retention volumes per gram of sorbent have been measured for a number of monosubstituted benzenes and unsubstituted polycyclic aromatic hydrocarbons, using *n*-hexane and dichloromethane–hexane (35:65) as eluent at 10, 25 and 40°C. The influences of temperature and of eluent strength on the bonded phase properties are described and related to the structure of the phases. At equilibrium, net retention data can be described in terms of the adsorption model, developed by Snyder for bare adsorbents.

INTRODUCTION

Tetranitrofluorenone (TENF), either physically adsorbed[1] or chemically bound to porous silica[2–5], and 3-(2,4-dinitroanilino)propylsilyl silica[6,7] have been the subject of several chromatographic investigations. The complexing ability of nitrated aromatic compounds towards potential electron donors is well-known[8], as is the fact that such complexing sites on a silica carrier can give rise to an improved selectivity. Hemetsberger *et al.*[5] and Nondek *et al.*[7] related retention data on this type of bonded phases to complex association constants determined by means of suitable spectroscopic methods. Although a correlation was found, it is not evident whether high-performance liquid chromatography (HPLC) can yield well-defined complexation constants. In this respect, the relatively large concentration of ligands on the silica surface seems to hamper a straightforward interpretation of the results.

In this work, attention is focused on the effects of temperature and eluent strength on solute retention, and an attempt is made to relate these effects to the structure of the bonded phase. Although the proposed approach has a limited scope, the obtained results may form a basis for further investigations in this field. This work is part of a project, the aim of which is to examine the applicability of Snyder's adsorption model[9] (for bare adsorbents) to bonded phases with a moderate surface coverage of monomers. Previously reported retention data on octadecylsilyl-[10,11], N-

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2-cyanoethyl-N-methylamino-[12] and aminobutylsilica[13] could be described reasonably well in terms of the parameters of this model. Provided that the chromatographic conditions obey the underlying assumptions of the model, it has proved to be a valuable tool for understanding the retention behaviour of simple solutes on these phases. Encouraged by these results, we proceeded to complexing phases with a relatively high amount of bound organic material.

THEORETICAL

In this work, only the most simple and basic concepts of Snyder's adsorption model will be considered. For a more extended account the reader is referred to Snyder's book[9] and previous papers on this subject[11–13].

The net retention volume per gram of adsorbent in the column is given by

$$\log (V_N/W) = \log V_A + \alpha (S^0 - \varepsilon^0 A_S) \quad (1)$$

where α is the adsorbent activity, S^0 a measure of the Lewis basicity (or acidity) of the solute, ε^0 the (mean) eluent strength and A_S the (effective) surface area of the adsorbate molecule (expressed in units of 8.5 Å^2, the surface area of a –CH= group in benzene). V_A is related to the specific surface area of the adsorbent. The eluent strength scale was arbitrarily fixed with $\varepsilon^0 = 0$ for *n*-hexane. If S^0 values on the bonded phase are equal (or proportional) to those on bare silica, the adsorbent parameters α and log V_A are readily obtained from plots of log (V_N/W) data of monosubstituted benzenes in hexane *versus* their S^0 values on silica. Of course the magnitude of α is related to $\alpha = 0.83$ of a (wide-pore) silica[9]. In order to obtain A_S values from eqn. 1, it is useful to apply ε^0 values which hold on bare silica. This holds true even when complex formation contributes to solute retention[13]. It was shown that, on silica and on bonded phases, A_S values of monosubstituted benzenes are given by

$$A_S = A_S \text{ (calc.)} + \gamma_i \Delta a_i \text{ (SiO}_2\text{)} \quad (2)$$

where A_S (calc.) = A_S when no solute and eluent localization (due to adsorbent heterogeneity) occurs. $\Delta a_i(SiO_2)$ is the difference between A_S and A_S (calc.) on a deactivated silica. As these cases are rather rare, a correction term is usually required. The localization parameter, γ_i can readily be evaluated with A_S (calc.) and Δa_i (SiO$_2$) values given by Snyder[9]. On bare silica and on the previously examined bonded phases, γ_i did not significantly depend on temperature, nor on the substituent *i*.

The S^0 values of unsubstituted polycyclic aromatic hydrocarbons are readily obtained by applying the estimated α and log V_A in eqn. 1. They can be described by

$$S^0 = n\, Q^0_{-C=} - \zeta\, (n - 6) \quad (3)$$

where n is the number of aromatic carbon atoms of the arene and $Q^0_{-C=}$ the contribution of a –CH= moiety in benzene (a non-delocalized solute) to its S^0 value. The second term accounts for delocalization effects. On a wide-pore bare silica, $\zeta = 0.14$ (ref. 14), but owing to complex formation of the arenes with bonded phases ζ can decrease and even become negative[13].

Finally, the A_S values of the arenes can be compared to those on bare silica given by

$$A_S = 6 + 0.8\,(h - 6) + 0.25\,(c - h) \quad (4)$$

where c and h are the numbers of carbon atoms and protons in the arenes, respectively.

If both the solute and eluent molecules can form a complex with bonded phase sites, the effective eluent strength will be larger than that on silica. Hence, the term $\varepsilon^0 A_S$ in eqn. 1 (and thus the obtained A_S values) will be anomalously large[13]. If $\alpha\zeta$ is small or even negative (which points to complex formation by the solute), but the A_S values are similar to those on silica, the eluent molecules apparently have a similar affinity to bonded phase sites and silanol groups. These rules of thumb are consistent with the adsorption model and will be applied in the following.

EXPERIMENTAL

Chemicals and sorbent characterization

All solutes and reagents (Fluka, Buchs, Switzerland) were of the highest available purity and were used as received. Dichloromethane and *n*-hexane were supplied by Baker (Deventer, The Netherlands) and were dried with molecular sieve 5A.

Nucleosil 10 NH_2, particle diameter 10 μm (Macherey, Nagel & Co., Düren, G.F.R.), from the same batch as used in a previous investigation[13] was the starting material for the first two modifications described below. From elemental analysis (4.25% C and 1.24% N) and its BET surface area (388 $\pm$ 2 m^2/g) it was concluded that the monomers are aminobutyl groups, that the amounts of C, N and aminobutylsilyl (ABS) per gram of bare silica are 0.0473, 0.0138 and 0.1133 g, respectively, that the surface concentration of ABS groups is 2.54 $\pm$ 0.02 μmole/m^2. It was assumed that the silane had reacted bifunctionally and that the remaining reactive group was hydrolysed.

Preparation of 2,4-dinitroanilino(DNA)-silica

A suspension of 3 g Nucleosil 10 NH_2 in 25 ml of 0.7 *M* $NaHCO_3$ was degassed and homogenized by sonication. To this mixture a solution of 1.6 g 2,4-dinitrofluorobenzene in 20 ml ethanol was added dropwise while the contents of the flask were gently swirled. The mixture was allowed to react for 20 h at room temperature. Thereafter, the yellow product was neutralized, rinsed with distilled water, methanol and acetone and dried at 60°C and 1 mmHg for 2 h. Elemental analysis showed the presence of 9.14% C. Combination of this result with those for the starting ABS-silica yields an amount of 0.1586 g of bound 2,4-dinitrophenyl (DNP) per gram of bare silica, which corresponds to a surface coverage of 2.45 μmole dinitrophenyl per m^2 and 0.09 μmole ABS per m^2. A similar large yield of DNP groups was reported by Nondek and Málek[6].

Preparation of bis(3-nitrophenyl) sulphone (DNPS)-silica

A 3-g amount of Nucleosil 10 NH_2 was suspended in 130 ml of 0.025 *M*

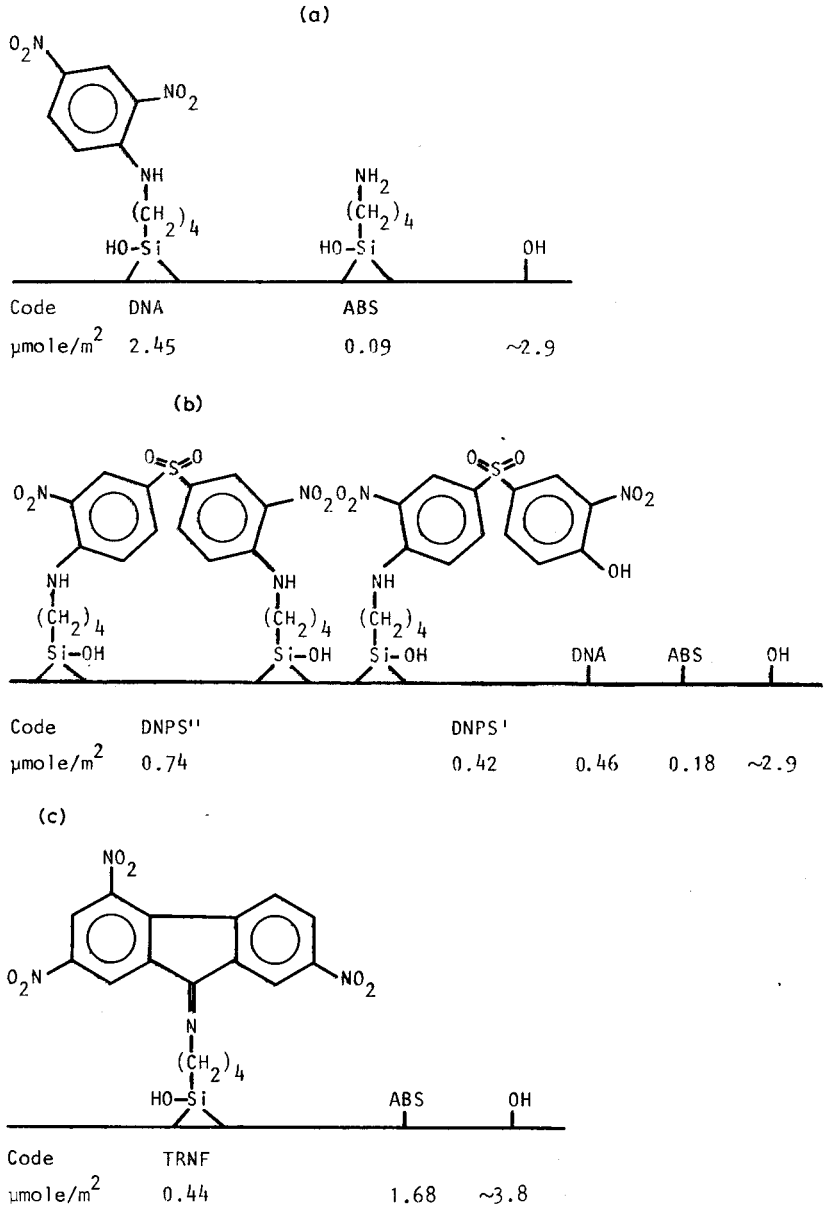

Fig. 1. Structure, code and surface concentration of bound groups on DNA-silica (a), DNPS-silica (b) and TRNF-silica (c).

$NaHCO_3$ in water–dimethylformamide (DMF) (18:42) in a three-necked flask. After degassing and homogenizing, a solution of 0.45 g bis(4-fluoro-3-nitrophenyl) sulphone in 10 ml ethanol was added dropwise while the contents of the flask were swirled. The number of moles of reagent is half the number of moles of ABS groups in order to promote a bifunctional reaction. The reaction mixture was kept at 60°C under

gentle reflux. During the reaction the pH was monitored and maintained below a pH meter reading of pH = 10 (at which silica is stable in this medium) by bubbling CO_2 through the suspension. The reaction was stopped after 120 h when its rate had become very low (as followed by spectrophotometric analyses of the supernatant at 420 nm). The yellow product was neutralized, rinsed and dried as described above. Elemental analysis gave 9.03% C and 2.08% N. From these results and those for ABS-silica, it can be estimated that the weight of bound sulphone per gram of bare silica and the surface coverage of sulphone are 0.135 ± 0.003 g and 1.14 ± 0.01 μmole/m^2 (bifunctional reaction), or 0.145 ± 0.003 g and 1.16 ± 0.01 μmole/m^2 respectively (monofunctional reaction). This indicates a 83% conversion of the sulphone. In order to estimate the extent of bifunctional reaction, 200 mg of the product were suspended in 10 ml of 0.01 *N* HCl and degassed. After 12 h the hydrochloric acid was washed out and titrated with a CO_2-free borax solution using methyl red–bromocresol green as indicator. The surface coverage of free ABS groups appeared to be 0.64 μmole/m^2. It can now easily be estimated that 64% of the bound sulphone reacted bifunctionally.

Finally, the product was treated with 2,4-dinitrofluorobenzene (as described above) in order to reduce the number of ABS groups. Elemental analysis showed 9.86% C. From this result it can be estimated that the final product contains 0.46 μmole 2,4-dinitrophenyl per m^2 and that 0.18 μmole ABS per m^2 are left on the silica surface.

Preparation of 2,4,7-trinitrofluorenimine (TRNF)-silica

A 3-g amount of Nucleosil 10 NH_2 (surface coverage 2.12 μmole ABS per m^2) was suspended in a solution of 1.2 g 2,4,7-trinitrofluorenone in 60 ml dry tetrahydrofuran (THF). The mixture was degassed and homogenized and was allowed to react for 4 days in the dark at room temperature. The brown product was thoroughly washed with THF and dichloromethane and dried at 50°C and 1 mmHg. Elemental analysis showed 5.81% C and 1.75% N. It was concluded that the surface concentration of TRNF groups is about 0.44 μmole/m^2. A similar low yield was obtained for TENF-silica, prepared with 2,4,5,7-tetranitrofluorenone. These surface concentrations are roughly in accord with those reported by Lochmüller *et al.*[4]

The stability of the DNA- and the DNPS-phase was good. The fluorenimine phases, however, showed a slow decomposition in dichloromethane, as judged from an increased background of the UV-detector after storage of the columns for several months. In particular, the TENF-phase was troublesome and only plate height measurements will be reported for this bonded phase. TRNF-silica was examined more closely, and apart from its surface concentration, the results seem reliable enough to characterize its sorbent properties.

Apparatus and procedure

The columns (precision-bore stainless steel, 25 cm × 2.1 mm I.D.) were packed by means of a slurry technique. The slurries (10 wt. % sorbent in dry tetrachloromethane) were degassed and homogenized by sonication and forced into the columns with 200 ml *n*-hexane at 350 atm. Finally, 300 ml dry dichloromethane were flushed through the columns. The weights of the sorbents in the columns were 0.46 g (DNA-), 0.43 g (DNPS-) and 0.40 g (TRNF-silica).

The apparatus and the measuring technique have been described previously[11]. The sample size was less than 25 μg. The reproducibility of triplicate V_N measurements was about 10 μl or 2% for strongly sorbed solutes. The column temperature was regulated by means of a water-jacket around the column and a heat exchanger. By means of some valves, a rapid switch to a second water thermostat could be performed. This arragement made possible a 15°-change of the column temperature within a few minutes. These temperature jumps were applied in order to measure desorption entropies and enthalpies at a fixed sorbent state, as will be outlined below.

The following eluents were used: *n*-hexane (H, $\varepsilon^0 = 0$) and *n*-hexane–dichloromethane (65:35) (B, $\varepsilon^0 = 0.22$ according to Snyder[15]). Measurements were made at 10, 15 and 40°C.

RESULTS AND DISCUSSION

Effects of sorbent state on solute retention and dispersion

In a preliminary investigation on the solute retention, some interesting phenomena were observed.

(1) Temperature effects. An abrupt jump of the eluent temperature from 25 to 40°C caused an instantaneous decrease of V_N, as expected. Thereafter, however, V_N appeared to increase steadily to a constant value. A reversed jump from 40 to 25°C had an analogous, but opposite, effect on the retention behaviour. These experiments were performed at a small pressure drop across the columns (50 atm) and low flow-rates (< 0.5 ml/min). The solute sorption–desorption equilibrium is fast (as usual), as a three-fold increase of the flow-rate did not affect the magnitude of V_N. After a temperature jump, constant V_N data were obtained after about 5.5 h (DNA-silica), 3 h (DNPS-silica) and 2 h (TRNF-silica) of pumping. These equilibration times were roughly the same for both 15°-jumps (positive or negative) and did not clearly depend on the original temperature (10, 25 or 40°C). In the binary eluent B the equilibration times were shorter (1.5 h).

Obviously, a temperature change causes a reversible alteration of the sorptive properties of the bound layers. As the mean distance between adjacent ABS groups is about 9.5 Å and the conversion of these groups into DNA and DNPS groups is nearly complete, the mutual distance of the latter groups must also be small. Further, the total area of these groups is about equal to the silica surface area underneath. Therefore, it seems justified to assume that they are arranged in a rather dense layer on top of more or less upright-standing spacer groups. With this picture in mind, the following equilibria in the bound layers can be expected

$$S + A \rightleftarrows AS \tag{5}$$

$$S + AA \rightleftarrows AA\cdots S \tag{6}$$

$$A + A \rightleftarrows AA \tag{7}$$

where S and A denote the solute and the DNA or DNPS groups, respectively. As the TRNF content is rather low, but ABS–TRNF (donor–acceptor) interaction is feasible, the following equilibria will be of prime importance

$$S + D \rightleftarrows DS \tag{8}$$

$$S + A \rightleftarrows AS \tag{9}$$

$$S + AD \rightleftarrows AD \cdots S \tag{10}$$

$$A + D \rightleftarrows AD \tag{11}$$

where D denotes a "free" ABS group. It is noted that the amino groups on ABS-silica are largely adsorbed to silanol groups in the weak eluents used[12]. Finally, it has been shown that (free) ABS groups can form complexes with dichloromethane[13]:

$$D + CH_2Cl_2 \rightleftarrows D \cdots CH_2Cl_2 \tag{12}$$

As a result of a positive temperature jump all equilibria will shift to the left, but due to the restricted mobility of the anchored groups, the equilibria 7 and 11 will shift much more slowly than the equilibria 5,6,8,9 and 10. Hence, after a sharp drop, V_N will increase slowly to a constant value. This explanation is based on the plausible assumption that AS complexes are far more stronger than $AA \cdots S$ (or $AD \cdots S$) interactions.

From the foregoing it is evident that the influence of the temperature on solute desorption can only be evaluated unambiguously at a fixed sorbent state. This can be achieved by extrapolation of net retention volumes after abrupt jumps from 25 to 10°C and 25 to 40°C to zero equilibration time, denoted by (A25, E10) and (A25, E40). Of course, the (A25, E25) datum can also be used. Analogously, the data (A10, E25) and (A40, E25) can be combined with (A10, E40) and (A40, E40), respectively. The change of the chemical potential of the solute on desorption is given by

$$\Delta\mu^0_{d,E} = RT \ln (V_N/W\beta_E) \tag{13}$$

where β_E is the effective volume of the sorbing phase per gram sorbent. From $\ln (V_N/W)$ data at different temperatures (but at the same sorbent state), $\Delta\bar{S}^0_d - R \ln \beta$ and $\Delta\bar{H}^0_d$ values can be estimated in 15°-intervals. Results for acetophenone and benzonitrile on DNA- and DNPS-silica in both eluents are given in Table I. In Fig. 2, $\Delta\bar{H}^0_{d,E}$ is plotted *versus* $\Delta\bar{S}^0_{d,E} - R \ln \beta_E$. A pair of parallel lines (slope 360°K) is obtained for the eluents. Hence, apart from a temperature effect on both thermodynamic quantities which gives rise to a displacement of the data points alongside the lines, there is at least one mechanism which occurs only in one of the eluents. As the slopes of the lines which connect corresponding data points on the two lines (450 ± 20°K and 520 ± 30°K at 290.7°K and 305.7°K, respectively) only depend on temperature, and the estimated errors in the $\Delta\bar{H}^0_d$ and $\Delta\bar{S}^0_d$ values are rather small (see Table I), it seems justified to conclude that the occurrence of the two lines in Fig. 2 be attributed to a single mechanism[16] and a small second-order temperature effect (on this mechanism, probably). The occurrence of two lines cannot merely be due to the trival term $-R \ln (\beta_B/\beta_H)$ because by sorption of dichloromethane in the bond layers, $\beta_B/\beta_H > 1$. Hence, at equal values of $\Delta\bar{H}^0_{d,B}$ and $\Delta\bar{H}^0_{d,H}$, $\Delta\bar{S}^0_{d,B} - \Delta\bar{S}^0_{d,H} > 2.8$ cal/mole·°K. However, it is conceivable that the solute molecules can to some extent

TABLE I

$\Delta\mu_d^0 + RT \ln \beta_E$, $\Delta\bar{S}_d^0 - R \ln \beta_E$ AND $\Delta\bar{H}_d^0$ VALUES OF BENZONITRILE AND ACETOPHENONE ON DNA- AND DNPS-SILICA

Data are given for the sorbent at 10, 25 and 40°C in hexane (E = H) and in the binary eluent (E = B). Errors in the three quantities are about 7 cal/mole, 0.7 cal/mole · K and 0.2 kcal/mole, respectively.

		DNA-silica							*DNPS-silica*						
		A10	*A25*	*A40*		*A10*	*A25*	*A40*		*A10*	*A25*	*A40*		*A10*	*A25*	*A40*
$\Delta\mu_d^0 + RT \ln \beta_E$ *(cal/mole)*																
Benzonitrile	H,10	1814	2009		B	246	337		H,10	1802	2087		B	324	428	
	25	1577	1747	1897		150	205	259	25	1597	1842	2018		246	286	355
	40		1562	1691			115	143	40		1605	1777			158	215
Acetophenone	H,10	2087	2359		B	505	622		H,10	2139	2540		B	674	804	
	25	1801	2033	2265		368	478	546	25	1856	2238	2538		546	628	737
	40		1820	1992			358	416	40		1935	2193			473	559
$\Delta\bar{S}_d^0 - R \ln \beta_E$ *(cal/mole · °K)*																
Benzonitrile	H,17.5	15.8	17.5		B	6.4	8.8		H,17.5	13.7	20.2		B	5.2	9.5	
	32.5		12.3	13.7			6.0	7.7	32.5		15.8	16.1			8.5	9.3
Acetophenone	H,17.5	19.1	21.7		B	9.1	9.6		H,17.5	18.9	20.1		B	8.5	11.7	
	32.5		17.5	18.2			8.0	8.7	32.5		16.3	23.0			10.3	11.9
$\Delta\bar{H}_d^0$ *(kcal/mole)*																
Benzonitrile	H,17.5	6.3	7.0		B	2.1	2.8		H,17.5	5.7	8.0		B	1.8	3.1	
	32.5		5.4	·6.0			2.0	2.6	32.5		6.6	6.8			2.8	3.1
Acetophenone	H,17.5	7.5	8.5		B	3.1	3.3		H,17.5	7.5	8.2		B	3.1	4.1	
	32.5		7.3	7.7			2.9	3.1	32.5		7.0	9.4			3.7	4.3

enter the solvated ligand layers in the binary solvent, whereas in hexane, adsorption on top of the bound layers prevails. As a result of the solvation of the ligands (and solute molecules), the complex stability (and hence $\Delta\bar{H}_d^0$) will be diminished. But due to the constraint of the motions of the solute molecules within the dense ligand layers, $\Delta\bar{S}_d^0$ will not decrease proportionally (*i.e.*, according to the slope 360°K), but less. Unfortunately, it is not possible to establish whether the observed solvent effect on $\Delta\bar{H}_d^0$ and $\Delta\bar{S}_d^0$ is typical for dense ligand layers, because corresponding data in dilute solutions are scarce and generally not very reliable[8].

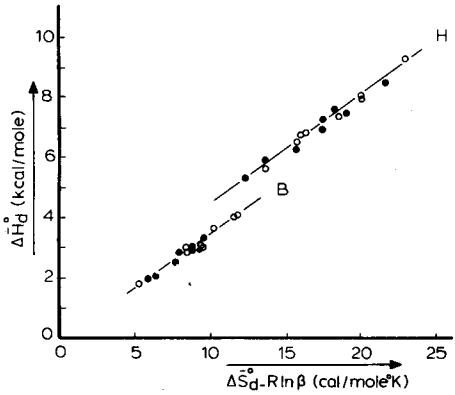

Fig. 2. Plots of $\Delta\bar{H}_d^0$ *versus* $\Delta\bar{S}_d^0 - R\ln\beta$ for benzonitrile and acetophenone on DNA-silica (○) and DNPS-silica (●) at 17.5 and 32.5°C in *n*-hexane (H) and in the binary eluent (B).

(2) Effects of eluent change. After a change from H to B, equilibrium is attained by flushing about 50 ml of B through the columns (*i.e.*, 3 h of pumping at 50 atm) (Fig. 3a). The same time is required to reach apparent equilibrium on DNPS-silica after a change from B to H. On DNA- and TRNF-silica this time is bout 16 h. In all instances the column temperature was 40°C. The thermostat kept on overnight, but the pump was stopped. Next day, retention measurements were made at small time intervals, starting immediately after actuation of the pump. During a period of about 20–30 min the retention volumes decreased by about 40%, but then increased steadily to their final values on the day before (Fig. 3c and d). In the following days the retention "dents" grew smaller and smaller and after 42 h of pumping (700 ml) *true* equilibrium is achieved on DNPS-silica; on DNA- and TRNF-silica about 940 ml hexane are required (*i.e.*, 56 h of pumping). On DNA-silica, the pump was stopped, before apparent equilibrium was reached. Next day, the V_N values seemed to increase from the level reached the day before (Fig. 3b).

The following explanation is proposed. Before the change from B to H the bonded phases are in the solvated ("swollen") state. When apparent equilibrium is attained in hexane, most but not all the dichloromethane is removed from the "shrunken" sorbing phases. The remaining dichloromethane diffuses very slowly into the mobile phase. When the flow is stopped for a sufficiently long period, the dichloromethane concentration in the hexane can increase to such an extent that partial reswelling occurs. When the hexane starts flowing again two phenomena occur with opposite effects on solute retention. On the one hand (part of) the dichloromethane diffuses rapidly out of the "reswollen" sorbing phase into the hexane. This solvates the

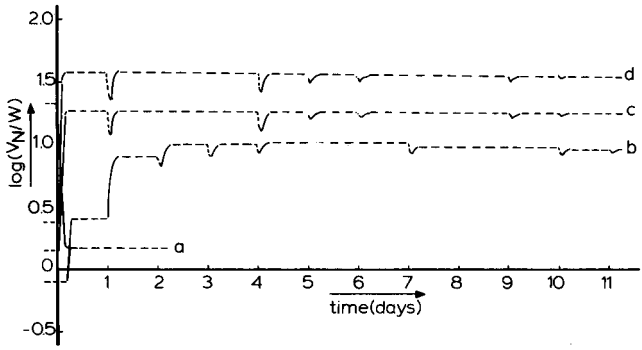

Fig. 3. Plots of log (V_N/W) data at 40°C *versus* time after the change from eluent H to B for chrysene on DNPS-silica (a), and after the change from B to H on DNA-silica for methyl benzoate (b), and on DNPS-silica for benzonitrile (c) and acetophenone (d).

sorbing phase as it moves through the column and causes a decrease in retention. On the other hand, an increasing amount of the sorbing phase comes into contact with hexane. This causes an increase in retention, which is slowed down by the slow self-association of the ligand groups. The two effects counterbalance after 20–30 min. The retention volumes become constant when equilibrium 7 or 11 has been re-established and the sorbent is again in its stable "shrunken" state.

This explanation is supported by gas chromatographic analyses of the eluate during the first retention "dent" observed on the DNPS column. During the first 4 min of pumping the dichloromethane concentration increased sharply. Thereafter it decreased steadily until it became constant (about 0.4‰, v/v) after 2 h (*i.e.*, when the sorbent has reached its "shrunken" state).

The above mentioned equilibration phenomena are also apparent on a silica with a moderate coverage of bound pyridylethylsilyl groups. Obviously they are commonly encountered on this type of aromatic polar bonded phases.

(3) Peak dispersion. Strongly retained solutes (capacity ratio, $k' > 12$) showed skewed peaks, in accord with the results of Lochmüller and Amoss[3]. As the net retention volumes are independent of the flow-rate, peak skewness cannot be attributed to the slow rate of complex dissociation. In the foregoing it is proposed that di- and monomeric complexing sites with different sorptive strengths are present in the bound layer. Thus, apart from the possibility that residual silanol groups contribute to the retention, non-linear isotherms can be expected which explains peak skewing on this type of bonded phases. In the following, only symmetrical peaks are examined.

According to Done and Knox[17], reduced plate heights ($h \equiv H/d_p$) are related to reduced linear flow-rates ($v \equiv vd_p/D_M$) by

$$h \approx h_{extra} + 1.4/v + v^{0.33} + Cv \tag{14}$$

where h_{extra} is the trivial contribution of peak broadening outside the column, d_p is the particle diameter, v the linear flow-rate and D_M the solute diffusion coefficient in the mobile phase. D_M values can be estimated with the Wilke–Chang equation[18].

In Fig. 4, $h - (1.4/v + v^{0.33})$ data for nitrobenzene and benzonitrile are plotted *versus* v. The slope of the plots, although dependent on v on DNPS-silica, represents

the mass transfer parameter C. On DNA-silica (and the fluorenimine phases) $C \approx 0.2$, *i.e.*, rather close to the values obtained on wide-pore silicas where $C \lesssim 0.06$ (ref. 19) (wall effects[20] probably contribute to C in our narrow-bore columns). In the binary eluent B similar plate height data were obtained. On DNPS-silica however, $C \approx 2.5$ and in addition C depends strongly on the eluent strength.

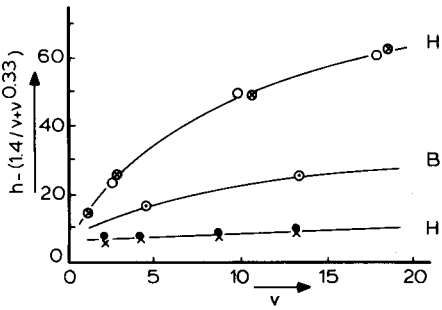

Fig. 4. The influence of slow mass transfer at 25°C in the bound layers of DNA-silica [nitrobenzene, $k' = 3.97$ (●); benzonitrile, $k' = 9.96$ (×)] and of DNPS-silica [nitrobenzene, $k' = 5.08$ (○) and benzonitrile, $k' = 10.46$ (⊗) [in *n*-hexane (H) and of DNPS-silica [nitrobenzene, $k' = 0.43$ (⊙)] in the binary eluent (B).

In view of the slow diffusion of dichloromethane through the "shrunken" bound layers, it is concluded from the rapid mass transfer on DNA-silica (and the fluorenimine phases) that the solutes are mainly adsorbed on top of the bound layer of these phases. On DNPS-silica the solutes are probably partly sorbed within the polar part of the bound layer. In the binary eluent "swelling" occurs and molecular diffusion is less severely restricted.

Snyder's adsorption model

In view of the proposed picture of the bonded phases derived from the rather intricate solute retention behaviour, an attempt to apply a monolayer adsorption model may seem a precarious undertaking. However, it will be shown below that this model describes the retention data surprisingly well, although caution should be excercised in attributing a physical meaning to the obtained parameters. In the following only true equilibrium retention data will be considered.

(1) Adsorbent activity. Experimental log (V_N/W) data for some monosubstituted benzenes on DNA-, DNPS- and TRNF-silica in *n*-hexane are given in Table II. Linear regression analysis of these data on S^0 values, given by Snyder[9] for bare silica, yields the α and log V_A values presented in Table II (see eqn. 1). The data for anisole, methyl benzoate and acetophenone were excluded because they appear to deviate significantly from a plot of log (V_N/W) data on the present sorbents *versus* those on ODS-silica. Similar deviations were observed on ABS-silica and can be ascribed to steric effects on complexation[13]. The α values on DNA- and DNPS-silica appear to be about equal to those on ODS-silica[11], where $\alpha = 0.51 \pm 0.01$ (25°C) and 0.48 ± 0.01 (43.5°C). Obviously, these bonded phases have approximately equal effective strengths towards monosubstituted benzenes as free silanol groups. The deviations from the regression line are largely systematic and indicate that the S^0 values on bare silica deviate slightly from the effective values on the bonded phases. An interpretation of the log V_A data in terms of a monolayer adsorption model is not feasible.

TABLE II

log (V_N/W) DATA FOR MONOSUBSTITUTED BENZENES ON DNA-, DNPS- AND TRNF-SILICA IN HEXANE, AND α log V_A VALUES, THEIR ERRORS AND THE STANDARD ERROR OF THE FIT (s_f)

i	S^0	*DNA-silica*			*DNPS-silica*			*TRNF-silica*	
		10°C	*25°C*	*40°C*	*10°C*	*25°C*	*40°C*	*25°C*	*40°C*
H	1.50	−0.49	−0.52	−0.55	−0.43	−0.47	−0.55	−0.35	−0.40
F	1.35	−0.40	−0.43	−0.46	−0.29	−0.42	−0.51	−0.31	−0.37
Cl	1.30	−0.34	−0.36	−0.38	−0.26	−0.39	−0.47	−0.25	−0.33
Br	1.33	−0.28	−0.30	−0.34	−0.22	−0.30	−0.38	−0.17	−0.25
CH_3	1.61	−0.49	−0.52	−0.55	−0.37	−0.48	−0.58	−0.33	−0.42
SCH_3	2.79	0.19	0.14	0.06	0.20	0.17	0.08	0.27	0.20
OCH_3*	3.33	0.25	0.19	0.12	0.28	0.26	0.22	0.35	0.31
NO_2	4.27	0.99	0.88	0.79	0.97	0.92	0.83	0.86	0.78
CO_2CH_3*	4.95	1.15	1.05	0.96	1.17	1.18	1.09	1.00	0.92
CN	4.83	1.40	1.28	1.18	1.39	1.35	1.24	1.15	1.06
$COCH_3$*	6.19	1.61	1.49	1.39	1.65	1.64	1.53	1.46	1.36
α		0.50	0.48	0.45	0.47	0.49	0.49	0.41	0.40
s		0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.02
log V_A		−1.12	−1.11	−1.11	−0.99	−1.11	−1.19	−0.86	−0.92
s		0.14	0.10	0.10	0.10	0.09	0.09	0.07	0.06
s_f		0.14	0.14	0.14	0.13	0.12	0.12	0.10	0.09

* Excluded from regression analyses (see text).

TABLE III

log (V_N/W) DATA FOR SOME ARENES ON DNA-, DNPS- AND TRNF-SILICA IN HEXANE, AND ζ VALUES FOR FUSED ARENES AND POLYPHENYLS, THEIR STANDARD ERRORS AND THE STANDARD ERROR OF THE FIT (s_f)

Solute	n	*DNA-silica*			*DNPS-silica*			*TRNF-silica*	
		10°C	*25°C*	*40°C*	*10°C*	*25°C*	*40°C*	*25°C*	*40°C*
Benzene	6	−0.49	−0.52	−0.55	−0.43	−0.47	−0.55	−0.35	−0.40
Naphthalene	10	0.22	0.15	0.06	0.20	0.13	0.04	0.23	0.15
Acenaphthene*	10	0.38	0.29	0.21	0.35	0.28	0.18	0.34	0.29
Fluorene	12	0.55	0.45	0.36	0.52	0.46	0.35	0.56	0.49
Anthracene	14	0.90	0.78	0.66	0.87	0.79	0.66	0.85	0.76
Phenanthrene	14	0.91	0.79	0.68	0.88	0.80	0.67	0.86	0.76
Fluoranthene	16	1.28	1.13	1.01	1.24	1.14	1.00	1.21	1.09
Pyrene	16	1.28	1.15	1.01	1.25	1.14	1.00	1.20	1.12
Chrysene	18	1.62	1.46	1.31	1.59	1.47	1.32	1.56	1.43
Benz[*a*]pyrene	20	2.00	1.85	1.66	1.97	1.85	1.66	1.98	1.88
Perylene	20	2.11	1.92	1.76	2.04	1.92	1.74	2.02	1.92
Bibenzyl*	12	0.06	−0.01	−0.09	0.07	0.00	−0.08	0.23	0.16
Biphenyl	12	0.22	0.13	0.08	0.18	0.14	0.03	0.27	0.21
p-Terphenyl	18	0.80	0.67	0.61	0.75	0.70	0.57	0.83	0.81
p,p'-Quaterphenyl	24	1.38	1.22	1.18	1.32	1.22	1.07	1.35	1.23
Fused arenes ζ		−0.158	−0.151	−0.154	−0.166	−0.143	−0.128	−0.206	−0.209
s		0.007	0.007	0.008	0.009	0.007	0.006	0.012	0.013
Polyphenyls ζ		−0.002	0.006	−0.007	0.001	0.010	0.018	−0.025	−0.027
s		0.009	0.006	0.005	0.004	0.009	0.008	0.007	0.014
s_f		0.09	0.08	0.09	0.10	0.08	0.07	0.12	0.18

* Outlier, excluded from regression analyses (see text).

Both α and log V_A values on TRNF-silica are close to those on ABS-silica[13] where $\alpha = 0.39 \pm 0.01$ and log $V_A = -0.79 \pm 0.04$ at 25°C. This result is expected since the surface concentration of the ABS groups is rather large on this mixed-phase sorbent. The low activity of ABS-silica in weak eluents is due to the adsorption of the amino groups to free silanol groups left on the silica surface[12]. It is interesting that α increases when the adsorption of the bulky DNP and DNPS groups is hampered.

(2) Solute–sorbent interactions. Experimental log (V_N/W) data on polycyclic aromatic hydrocarbons in hexane are given in Table III. Apart from acenaphthene and bibenzyl*, the S^0 values (obtained form eqn. 1) can be described accurately by means of eqn. 3 with different ζ values for the fused arenes and the polyphenyls. These ζ values are given in Table III. According to eqn. 3, the selectivity of the bonded phase j towards arenes can be evaluated from

$$\log (V_{N,n+1}/V_{N,n})_j = \alpha_j (Q^0_{-C=} - \zeta_j) \tag{15}$$

where $Q^0_{-C=} = 0.204 \pm 0.004$. At 25°C the following data (for fused arenes and polyphenyls, respectively) are obtained: DNPS- $\approx$ DNA- (0.170; 0.095) $\gtrsim$ TRNF- (0.168; 0.092) > ABS- (0.112; 0.089) > bare silica (0.091; –). We conclude:

(i) Despite their different structures, TRNF-, DNA- and DNPS-silica form complexes with effectively equal stability

(ii) Obviously, arenes can form complexes with electron acceptor and electron donor ligands, but with the nitroaromatic phases the interactions are stronger than with ABS-silica

(iii) On all complexing phases, a polyphenyl shows weaker interaction than is expected for a fused arene with the same number of aromatic carbon atoms. This is due to the small extent of π-conjugation in the slightly twisted polyphenyl molecules, which limits the stability of the charge-transfer complex[13]

(iv) The sharp increase of the selectivity caused by only a small amount of TRNF groups points to a strong complexing ability of these electron acceptor sites Recently, Lochmüller *et al.*[4] showed that the retention of arenes substantially increases with the degree of nitration of the fluorenimine nucleus.

(3) Primary eluent effects. Experimental log (V_N/W) data for some monosubstituted benzenes and arenes in the binary eluent B are given in Tables IV and V, respectively. Combination of these data with those in hexane gives A_S values (eqn. 1), from which γ_i values for the benzenes can be calculated by means of eqn. 2. An analysis of the variance[21] of the γ_i values revealed that they depend on the substituent i and on the bonded phase, but the influence of temperature appeared to be insignificant. The A_S values of the arenes are only slightly temperature-dependent. Therefore it suffices to consider the mean γ_i and A_S values presented in Tables IV and V, respectively.

The A_S values of the monosubstituted benzenes and arenes on DNA- and DNPS-silica are about 15% smaller than those on ODS-silica[11]. The similarity of the relative deviations for both solute classes points to a change of the sorbent properties in the binary eluent: the small A_S (and γ_i) values on DNA- and DNPS-silica can be attributed to a small but significant increase of the sorbent activity α_B

* The rather small S^0 value of bibenzyl may be related to the poor stabilization of the π,π-complex. Acenaphthene shows rather large S^0 values, presumably due to inductive effects by its methylene groups.

TABLE IV

log (V_N/W) DATA FOR SOME MONOSUBSTITUTED BENZENES ON DNA-, DNPS- AND TRNF-SILICA IN THE BINARY ELUENT B, Σa_i (calc.), Δa_i (SiO_2) AND MEAN γ_i VALUES

i	log (V_N/W)								Σa_i (calc.)[9]	Δa_i ($Si0_2$)[9]	$\bar{\gamma}_i \pm s$		
	DNA-silica			DNPS-silica			TRNF-silica				DNA-	DNPS-	TRNF-
	10°C	25°C	40°C	10°C	25°C	40°C	25°C	40°C					
NO_2	−0.02	−0.07	−0.15	0.01	−0.04	−0.11	−0.20	−0.27	7.3	6.2	0.31 ± 0.03	0.27 ± 0.04	0.68 ± 0.02
CO_2CH_3	0.01	−0.03	−0.09	0.14	0.10	0.02	−0.08	−0.14	8.3	5.8	0.36 ± 0.03	0.29 ± 0.01	0.64 ± 0.02
CN	0.19	0.15	0.10	0.25	0.21	0.15	−0.01	−0.07	6.6	7.8	0.55 ± 0.01	0.51 ± 0.04	0.81 ± 0.01
$COCH_3$	0.39	0.35	0.29	0.52	0.46	0.39	0.27	0.20	7.5	7.7	0.45 ± 0.02	0.43 ± 0.02	0.73 ± 0.01

TABLE V

log (V_N/W) DATA FOR SOME ARENES ON DNA-, DNPS- AND TRNF-SILICA IN THE BINARY ELUENT B, AND CALCULATED AND (MEAN) EXPERIMENTAL A_S VALUES

Solute	log (V_N/W)								A_S (calc.)	$\bar{A}_S \pm s$		
	DNA-silica			DNPS-silica			TRNF-silica			DNA-	DNPS-	TRNF-
	10°C	25°C	40°C	10°C	25°C	40°C	25°C	40°C				
Anthracene	−0.12	−0.19	−0.26	−0.11	−0.16	−0.28	−0.14	−0.24	10.2	9.3 ± 0.1	9.0 ± 0.3	11.2 ± 0.2
Phenanthrene	−0.08	−0.16	−0.23	−0.05	−0.16	−0.26	−0.12	−0.21	10.2	9.1 ± 0.1	8.8 ± 0.2	11.0 ± 0.1
Fluoranthene	0.21	0.13	0.04	0.21	0.11	0.02	0.14	0.08	10.7	9.7 ± 0.1	9.5 ± 0.4	10.7 ± 0.2
Pyrene	0.31	0.21	0.10	0.29	0.19	0.07	0.20	0.13	10.7	9.0 ± 0.2	8.9 ± 0.3	11.3 ± 0.1
Chrysene	0.40	0.30	0.21	0.39	0.28	0.17	0.42	0.33	12.3	11.1 ± 0.1	11.1 ± 0.4	12.6 ± 0.1
Benz[*a*]pyrene	0.81	0.68	0.55	0.77	0.63	0.51	0.86	0.74	12.8	11.0 ± 0.2	11.2 ± 0.4	12.7 ± 0.3
Perylene	0.92	0.78	0.66	0.88	0.73	0.59	0.93	0.80	12.8	10.9 ± 0.1	11.0 ± 0.2	12.4 ± 0.3
p-Terphenyl				−0.39	−0.46	−0.58	−0.47	−0.47	13.4		10.8 ± 0.2	14.1 ± 0.2
p,p'-Quaterphenyl	−0.22	−0.27	−0.34	−0.27	−0.30	−0.42	−0.14	−0.21	17.1	14.7 ± 0.5	14.4 ± 0.7	16.4 ± 0.1

(0.04 ± 0.01) and the sorbent volume (12 ± 7%) in the binary eluent due to "swelling" of the sorbing phase. The γ_i and A_S values on TRNF-silica are larger than those on the DNA-phase. Obviously, residual ABS groups in this phase are involved which can cause a substantial increase of A_S values[13].

CONCLUSIONS

In bonded phases with a sufficiently large coverage of (polar) ligands on flexible spacer chains, self-association can occur to some extent in the presence of apolar or weakly polar solvents. After a perturbation of the association equilibrium by a sudden change of the temperature, the new sorbent state is established only after a few hours. The slow rate of this type of relaxation process facilitates the determination of solute desorption enthalpies and entropies at a fixed sorbent state.

An analysis of the desorption enthalpies and entropies for benzonitrile and acetophenone on DNA- and DNPS-silica shows that the sorption mechanisms in a hexane and in a dichloromethane–hexane eluent are different. In the former, adsorption on top of the bound layer prevails, whereas in the latter the solute molecules can to some extent enter the solvated ligand layers.

An eluent change from 35% dichloromethane in hexane to pure hexane requires a 40–60 h flush of hexane before reproducible retention data are obtained. The slow transfer of the dichloromethane to the mobile phase is due to the difficulty in permeating the associated layer of ligand groups, which is formed in the hexane eluent. A reversed eluent change requires only a few hours flush of the binary eluent through the columns, because of the solvation and "swelling" of the bound layers in this eluent.

On DNPS-silica an enormous contribution to the plate height occurs as a result of partial penetration of the solutes into the bound layer and of restricted solute diffusion in this layer.

Equilibrium net retention data on these bonded phases can be described very well with the basic equations of Snyder's adsorption model. The sorbent activity of DNA- and of DNPS-silica is close to that of free silanol groups. TRNF-silica has an activity close to ABS-silica due to the large amount of ABS groups in this mixed bonded phase. The adsorption model is a useful tool for comparing the complexing abilities of bonded phases. The complex stability appears to depend on the extent of π-conjugation in the solute molecules.

Due to diminished self-association of the ligands and "swelling" of the bound layer in the binary eluent, the eluent effect is slightly smaller than predicted by Snyder's model.

From a practical point of view, the fluorenimine phases are not recommended because of their poor chemical stability. Under isothermal and isocratic conditions, DNA-silica is quite useful.

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CORRELATION OF TWO CRITERIA OF POLARITY FOR STATIONARY PHASES IN GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Linear relationships between the partial molar excess Gibbs function of a solute methylene group, $\Delta\bar{G}^{E}(CH_2)$, and the average McReynolds constant, ΔI, and between $\Delta\bar{G}^{E}(CH_2)$ and the individual McReynolds constants were determined for a set of 55 liquid stationary phases. All these relationships were identical at the given level of statistical significance. An analysis of the relationships indicates that $\Delta\bar{G}^{E}(CH_2)$ and ΔI are equivalent criteria of polarity for liquid stationary phases and that polarity can adequately be characterized by a single criterion.

INTRODUCTION

McReynolds[1] showed that the selectivity and/or polarity of a stationary phase in gas–liquid chromatography (GLC) can be characterized by differential Kováts' retention indices, ΔI, measured for several deliberately chosen solutes on the given stationary phase and on squalane as a non-polar reference; he evaluated 226 stationary phases in this way. Since then, several algebraic methods have been applied to his set of data in an attempt to systematize the variations in the ΔI values and establish a standard set of stationary phases for GLC. For instance, the Euclidian distance between a given stationary phase and squalane was calculated[2,3] and "principal component" analysis[4,5] as well as factor analysis[6] were applied to the published ΔI data. It was found[7] that a substantial part of the variations in the ΔI values could be attributed to a single factor called "polarity". However, the termodynamic meaning of this factor is somewhat vague[4].

In the initial McReynolds paper[1], stationary phases were grouped according to increasing polarity, which was arbitrarily defined as the arithmetic mean of the ΔI values determined for benzene, 1-butanol, 2-pentanone, 1-nitropropane and pyridine. In this work, the McReynolds constants for 55 GLC stationary phases are correlated with the corresponding values of the partial excess Gibbs function of one mole of solute methylene, $\Delta\bar{G}^{E}(CH_2)$. The latter quantity is a measure of the deviation of the solution of methylene in a given solvent from an ideal solution and can therefore be considered as a thermodynamically defined criterion of polarity for liquid stationary phases[8–15].

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THEORETICAL

McReynolds constants, ΔI_i

Let us consider a monofunctional solute i of the type $(CH_3)_M(CH_2)_NX$ where X is a functional group and N is the only variable in a given homologous series. Provided that the standard molar Gibbs function of sorption for solute i, $\Delta G^0_{sp}(i)$, comprises the sum of the standard Gibbs function contributions corresponding to the individual groups of the solute[16], *i.e.*

$$\Delta G^0_{sp}(i) = M\Delta G^0_{sp}(CH_3) + N\Delta G^0_{sp}(CH_2) + \Delta G^0_{sp}(X) \quad (1)$$

and that $\Delta G^0_{sp}(CH_3) \approx \Delta G^0_{sp}(CH_2)$, the Kováts' retention index of solute i on a given stationary phase, I_i, can be expressed as[17]

$$I_i = 100[n_i + \Delta G^0_{sp}(X)/\Delta G^0_{sp}(CH_2)] \quad (2)$$

where n_i is the number of carbon atoms in the solute molecule. The difference between the retention indices of solute i on stationary phase y and on squalane (sq) is then given by:

$$\Delta I_i = 100[\Delta G^0_{sp}(X)/\Delta G^0_{sp}(CH_2)]_y - 100[\Delta G^0_{sp}(X)/\Delta G^0_{sp}(CH_2)]_{sq} \quad (3)$$

The retention of solute i on squalane is due to dispersive solute–solvent intermolecular interactions. If the functional group of the solute has a sufficiently large dipole moment, induction forces may also play a rôle, but, owing to the relatively low polarizabilities of C–C and C–H (aliphatic) bonds[18], the contribution of such forces is unimportant. However, with other stationary phases (y), orientation, induction and hydrogen-bonding interactions may appreciably contribute to the retention of solute i. The value of the ratio $[\Delta G^0_{sp}(X)/\Delta G^0_{sp}(CH_2)]_y$ in eqn. (3) will increase with increasing significance of these interactions. Hence, the value of ΔI_i is a measure of the extent to which non-dispersive intermolecular forces contribute to the retention of solute i on stationary phase y, and the arithmetic mean of ΔI_i values measured for several solutes, ΔI, can be considered as a plausible criterion of polarity for stationary phases.

Partial excess Gibbs function of one mole of solute methylene, $\Delta G^E(CH_2)$

The concept of the additivity of the contributions of individual groups in the solute molecule to the partial molar excess Gibbs function of solute in a solute–stationary phase system was introduced by Pierotti *et al.*[19]. The partial excess Gibbs function of one mole of solute methylene has been proposed[8–12] and employed[8–15] as a criterion of polarity for chromatographic stationary phases. It can formally be written for a given stationary phase and a given homologous series of compounds $(CH_3)_M(CH_2)_NX$ at a given temperature as

$$\Delta G^E(CH_2) = -RT\,\mathrm{d}\ln(V^0_g P^0)/\mathrm{d}N \quad (4)$$

and for two consecutive members of the series, $i_N = (CH_3)_M(CH_2)_NX$ and $i_{N+1} = (CH_3)_M(CH_2)_{N+1}X$, we obtain:

$$\Delta G^{E}(CH_2) = RT \ln [V_g^0 P^0(i_N)/V_g^0 P^0(i_{N+1})] \quad (5)$$

The reluctance of a stationary phase (solvent) to mix with a solute is reflected in the excess enthalpy of the solute–solvent system, ΔH^{E}. This reluctance is due to different types and intensities of solute–solute and solvent–solvent intermolecular interactions. Since it is the change in the system's potential energy associated with the solution formation that contributes most to the respective enthalpy of mixing, ΔH^{E} can be expressed according to lattice theory[20] as

$$\Delta H^{E} = zN_A \Delta w = zN_A [\tfrac{1}{2}(w_{11} + w_{22}) - w_{12}] \quad (6)$$

where z is the lattice coordination number, N_A is the Avogadro constant and w_{11}, w_{22} and w_{12} are the pairwise potential energies of the solvent–solvent, solute–solute and solvent–solute intermolecular interactions, respectively.

Let us consider the mixing of a given solvent with a paraffinic solute. The solute molecules interact with each other only by means of dispersion forces, whereas the solvent–solvent interactions may involve also interactions by induction and orientation forces and/or by hydrogen bonding, according to the constitution of the solvent molecule. Random mixing of a paraffinic solute with a large excess of solvent to produce an infinitely dilute solution results in the cancellation of all solute–solute interactions and a certain proportion of the solvent–solvent interactions. This destabilization of the system is compensated for by dispersion and, if applicable, inductive solute–solvent interactions. If the solvent is also a paraffin, this compensation is fairly complete, *i.e.*, $(w_{11} + w_{22})/2 \approx w_{12}$. In such a case, for the partial molar enthalpies of two consecutive paraffinic solute homologues, p_N and p_{N+1}, we can write $\Delta H^{E}(p_N) \approx \Delta H^{E}(p_{N+1})$, *i.e.*, $\Delta H^{E}(CH_2) \approx 0$. With non-paraffinic solvents, orientation and/or specific interactions contribute to the value of w_{11}, the importance of these contributions increasing with increasing proportion of groups with large dipole moments in the solvent molecule. The decrease in the intensity of these polar interactions on mixing the solvent with a paraffinic solute is only partially compensated by dispersive and inductive solute–solvent interactions, *i.e.*, $(w_{11} + w_{12})/2 > w_{12}$, and the system will display a positive excess enthalpy. Within a homologous series of solutes $(CH_3)_M(CH_2)_N X$, the partial molar excess enthalpy of a solute in the solute–solvent system will increase with increasing paraffinic portion that the solute molecule introduces into the system, consequently, $\Delta H^{E}(CH_2) > 0$.

Let us suppose that there is a linear relationship between ΔH^{E} and ΔS^{E} (ref. 21) and, consequently, a direct proportionality between $\Delta H^{E}(CH_2)$ and $\Delta S^{E}(CH_2)$ for a given solvent and a given homologous series of solutes $(CH_3)_M(CH_2)_N X$ at a given temperature. Then there is also a direct proportionality between $\Delta H^{E}(CH_2)$ and $\Delta G^{E}(CH_2)$, and the latter quantity actually characterizes the ability of the stationary phase to interact with solutes of the type $(CH_3)_M(CH_2)_N X$ by means of intermolecular forces other than dispersive ones. Hence, the average partial excess Gibbs function per mole of solute methylene, $\Delta \bar{G}^{E}(CH_2)$, determined from data measured for several different types of solutes, on a given stationary phase, can be looked upon as a representative measure of the polarity of the stationary phase[8–15]. This is illustrated in Fig. 1 by the dependences of $-RT \ln (V_g^0 P^0)$ on N for several typical stationary phases and straight-chain alkanols as solutes. In view of the above concepts, it is possible to expect a meaningful correlation between $\Delta \bar{G}^{E}(CH_2)$ and ΔI.

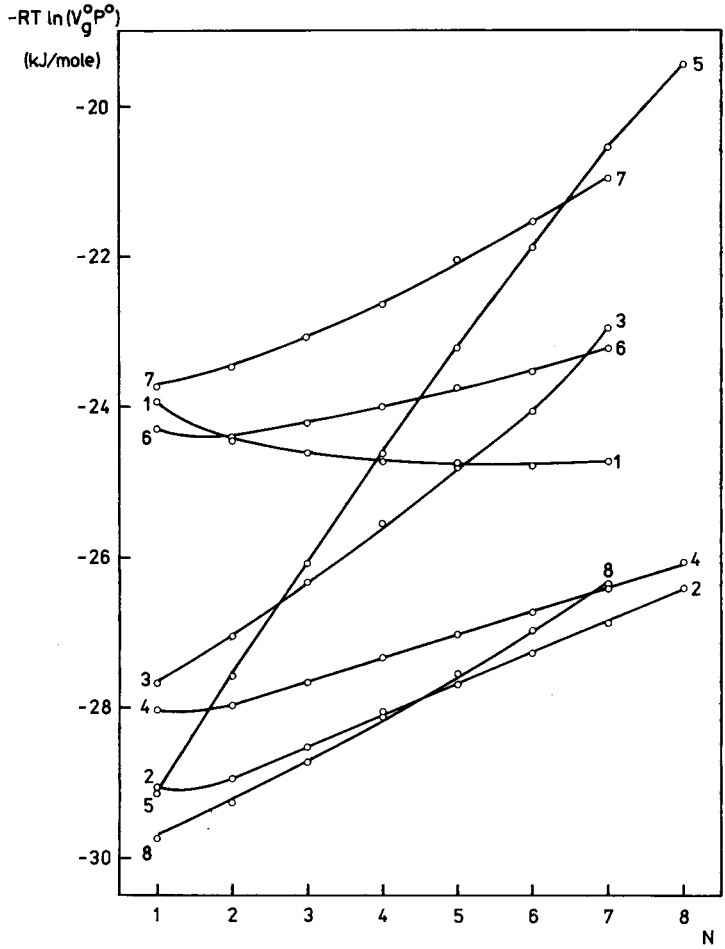

Fig. 1. Plots of $-RT \ln (V_g^0 P^0)$ *versus* methylene number, N for $CH_3(CH_2)_NOH$ alcohols on different stationary phases at 120°C. Phases: 1 = Apiezon L; 2 = Carbowax 20M; 3 = diethylene glycol succinate; 4 = Triton X-305; 5 = diglycerol; 6 = SE-31; 7 = QF-1; 8 = Hyprose SP 80.

RESULTS

The relationships between $\Delta\bar{G}^E(CH_2)$ and the individual ΔI_i values and between $\Delta\bar{G}^E(CH_2)$ and ΔI at 120°C were determined for a set of 55 liquid stationary phases. With all the stationary phases studied, the McReynolds constants and the retention data necessary to calculate the $\Delta\bar{G}^E(CH_2)$ values were taken from the literature. The stationary phases are listed in the legend to Fig. 2. Each $\Delta\bar{G}^E(CH_2)$ value is an arithmetic mean of the $\Delta G^E(CH_2)$ values determined for a given stationary phase from the specific retention volumes[22] and saturation vapour pressures[23] of pairs of homologous straight-chain 1-alkanols, 1-alkanals, 2-alkanones, *n*-alkyl acetates, symmetrical di-*n*-alkyl ethers and alkanes by

$$\Delta G^E(CH_2) = \frac{1}{k} RT \ln \frac{V_g^0 P^0(i_N)}{V_g^0 P^0(i_{N+k})} \qquad (7)$$

TABLE I

SOLUTES USED TO DETERMINE $\Delta\bar{G}^{E}(CH_2)$

Solutes	*k*	*Homologue* i_N	*Homologue* i_{N+k}
1-Alkanols	1	1-Pentanol	1-Hexanol
1-Alkanals	2	1-Pentanal	1-Heptanal
2-Alkanones	1	2-Hexanone	2-Heptanone
n-Alkyl acetates	1	*n*-Pentyl acetate	*n*-Hexyl acetate
Di-*n*-alkyl ethers	2	Di-*n*-butyl ether	Di-*n*-pentyl ether
n-Alkanes	2	*n*-Octane	*n*-Decane

where k is either 1 or 2. The solutes are specified in Table I. Let us recall[12] that the value of $\Delta G^{E}(CH_2)$ is only slightly dependent on the type of solutes employed. The ΔI_i values (i = benzene, 1-butanol, 2-pentanone, pyridine, 1-nitropropane, 2-methyl-2-pentanol and 2-octyne) were taken from the initial McReynolds paper[1]. The parameters of the linear correlations of $\Delta\bar{G}^{E}(CH_2)$ with the individual ΔI_i values are summarized in Table II. In Fig. 2, the $\Delta\bar{G}^{E}(CH_2)$ values are plotted against the corresponding values of ΔI, the latter being the arithmetic means of the individual ΔI_i values exclusive of those of 2-methyl-2-pentanol and 2-octyne. Linear regression of the data in Fig. 2 yielded the relation

$$\Delta\bar{G}^{E}(CH_2) = 1.064\ \Delta I - 18.11$$

the respective correlation coefficient being 0.8591. A comparison of this correlation coefficient and those shown in Table II with the tabulated critical values[24] indicates that there is, with a probability greater than 99%, a linear relationship between $\Delta\bar{G}^{E}(CH_2)$ and ΔI_i as well as between $\Delta\bar{G}^{E}(CH_2)$ and ΔI within the set of stationary phases studied. Further, a statistical evaluation[25] of the parameters of the regression relations shows that with all the relations the intercepts are, with a probability greater than 99%, statistically insignificant. Hence, the dependence of $\Delta\bar{G}^{E}(CH_2)$ on ΔI_i as well as the dependence of $\Delta\bar{G}^{E}(CH_2)$ on ΔI can equally well be represented by a direct proportionality $\Delta\bar{G}^{E}(CH_2) = q\ \Delta I_i$ and/or $\Delta\bar{G}^{E}(CH_2) = q\ \Delta I$, the value of q being significantly different from unity only with i = benzene, 2-pentanone and 2-octyne.

TABLE II

PARAMETERS OF THE CORRELATION OF $\Delta\bar{G}^{E}(CH_2)$ WITH ΔI_i

Solute	*Slope*	*Intercept*	*Correlation coefficient*
Benzene	1.506	16.42	0.8166
1-Butanol	0.886	− 8.37	0.8268
2-Pentanone	1.272	−22.92	0.8798
1-Nitropropane	0.885	−29.84	0.8416
Pyridine	0.900	0.38	0.8725
2-Methyl-2-pentanol	1.165	− 5.07	0.8317
2-Octyne	1.977	51.17	0.7167

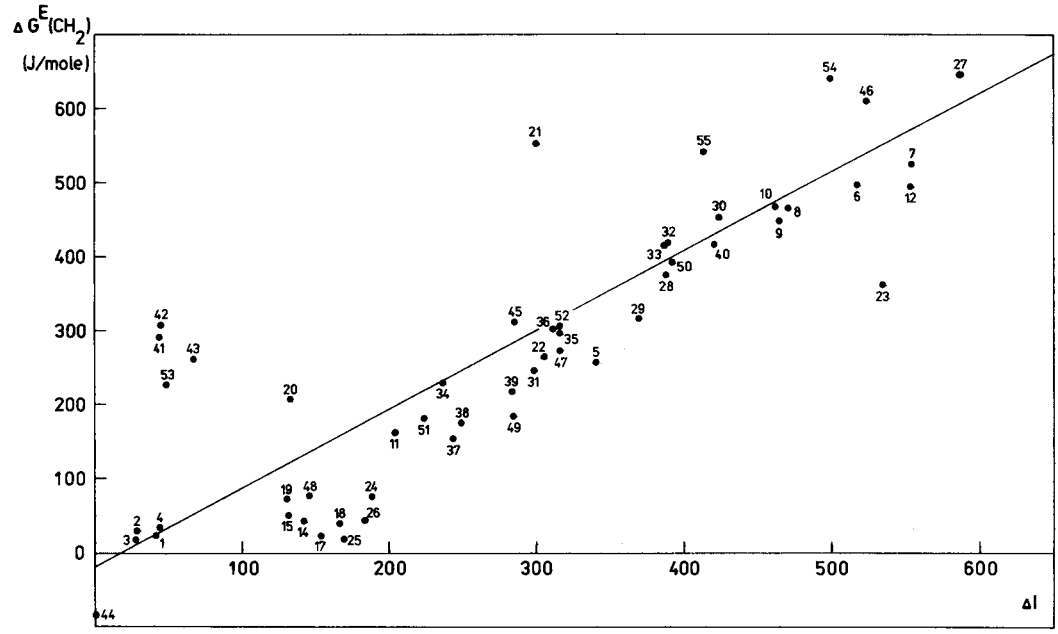

Fig. 2. Correlation of $\Delta\bar{G}^{E}(CH_2)$ with ΔI. Phases: 1 = Apiezon J; 2 = Apiezon L; 3 = Apiezon M; 4 = Apiezon N; 5 = bis(2-ethoxyethyl) phthalate; 6 = Carbowax 1000; 7 = Carbowax 1540; 8 = Carbowax 4000; 9 = Carbowax 6000; 10 = Carbowax 20M; 11 = Castorwax; 12 = diethylene glycol adipate; 13 = diethylene glycol succinate; 14 = di-2-ethylhexyl adipate; 15 = di-2-ethylhexyl sebacate; 16 = diglycerol; 17 = diisodecyl phthalate; 18 = dioctyl phthalate; 19 = dioctyl sebacate; 20 = Dow Corning 550 Fluid; 21 = Dow Corning FS 1265 Fluid (QF-1); 22 = Ethofat 60-25; 23 = ethylene glycol adipate; 24 = Flexol 8N8; 25 = Hallcomid M 18; 26 = Hallcomid M 18 OL; 27 = Hyprose SP 80; 28 = Igepal CO 880; 29 = neopentyl glycol adipate; 30 = neopentyl glycol succinate; 31 = Oronite NIW; 32 = Pluronic F 68; 33 = Pluronic F 88; 34 = Pluronic L 81; 35 = Pluronic P 65; 36 = Pluronic P 85; 37 = poly(phenyl ether), five rings; 38 = poly(phenyl ether), six rings; 39 = Polytergent J 300; 40 = Quadrol; 41 = SE-30; 42 = SE-31; 43 = SE-52; 44 = squalane; 45 = sucrose acetate isobutyrate; 46 = sucrose octaacetate; 47 = Tergitol NPX; 48 = TMP tripelargonate; 49 = tricresyl phosphate; 50 = Triton X-305; 51 = Ucon LB-1715; 52 = Ucon 50 HB-2000; 53 = Versilube F-50; 54 = XF-1150; 55 = Zonyl E 7. Diethylene glycol succinate (13) and diglycerol (16) are not shown on the plot; the coordinates of the respective points are ΔI = 708.6, $\Delta\bar{G}^{E}(CH_2)$ = 707.6 J/mol (13) and ΔI = 657.4, $\Delta\bar{G}^{E}(CH_2)$ = 1071.8 J/mol (16).

DISCUSSION

The data in Fig. 2 show that the stationary phases can be arranged into the following series according to increasing $\Delta\bar{G}^{E}(CH_2)$ values: squalane, Apiezons, amides, esters, polyethers, substances containing highly-acidic hydrogen. However, this is merely a rough classification. The position of a stationary phase of a given chemical type in the $\Delta\bar{G}^{E}(CH_2)$ *versus* ΔI plot depends appreciably on its molecular constitution, the most important factors in this respect being the content of polar groups relative to that of non-polar ones and the steric accessibility of the polar groups. For instance, the significantly larger $\Delta\bar{G}^{E}(CH_2)$ value of sucrose octaacetate (610 J/mol) than that of sucrose diacetate hexaisobutyrate (312 J/mol) is apparently due to the greater steric hindrance of the ether and carbonyl oxygens by the isopropyl groups in the latter compared with that by the methyl groups in the former. Hence,

the $\Delta\bar{H}^{E}(CH_2)$ of sucrose octaacetate is larger than that of sucrose diacetate hexaisobutyrate. In addition, as the diacetate hexaisobutyrate is more bulky than the octaacetate, the entropic component[21] of $\Delta\bar{G}^{E}(CH_2)$, $T\Delta\bar{S}^{E}(CH_2)$, will apparently be larger for the former, thus decreasing the respective $\Delta\bar{G}^{E}(CH_2)$ value. The rôle of the entropic component of $\bar{G}^{E}(CH_2)$ is especially important when comparing stationary phases of markedly different molar volumes. For instance, the difference between the $\Delta\bar{G}^{E}(CH_2)$ values of diglycerol (1071.8 J/mol) and Hyprose SP-80 (646.0 J/mol) is probably only due to the different contributions of $T\Delta\bar{S}^{E}(CH_2)$. In this context, it should be noted that whereas with $\Delta\bar{G}^{E}(CH_2)$ the $T\Delta\bar{S}^{E}(CH_2)$ component will be manifested in full, with ΔI (*cf.*, eqns. 2 and 3) the $T\Delta S_{sp}(X)$ and $T\Delta S_{sp}(CH_2)$ terms may be reduced to some degree in the ratios $\Delta G_{sp}(X)/\Delta G_{sp}(CH_2)$.

With silicone stationary phases the $\Delta\bar{G}^{E}(CH_2)$ values show marked positive deviations from the correlation line. In particular, the $\Delta\bar{G}^{E}(CH_2)$ values of non-polar silicone phases are much larger than one would expect on the basis of their McReynolds constants. Since the courses of the dependences of $-RT\ln(V_g^0P^0)$ on N for homologous solutes chromatographed on silicone stationary phases are as regular as those on the other stationary phases investigated (*cf.*, Fig. 1), this anomaly can hardly be interpreted as being due to non-additivity of the ΔG^{E} values of the individual groups in the solute molecule. A similar anomaly was observed in the retention behaviour of homologous *n*-alkanes chromatographed on silicone stationary phases[26].

CONCLUSIONS

The average McReynolds constant and the partial excess Gibbs function of one mole of methylene group are based on very different concepts and have different thermodynamic meanings. Whereas the first quantity reflects the relative affinity of the stationary phase towards an "average" functional group and a methylene group of solute, the second is a measure of the reluctance of the stationary phase to accommodate a methylene group. In spite of these differences, it can be stated that ΔI and $\Delta\bar{G}^{E}(CH_2)$ are equivalent criteria of polarity for liquid stationary phases; the coordinates of points lying on the regression line in the $\Delta\bar{G}^{E}(CH_2)$ *versus* ΔI plot even have (incidentally) the same numerical values.

Strictly speaking, considerations of the polarity of solvents should be based exclusively on the type and intensity of the intermolecular solute–solvent interactions, *i.e.*, the partial excess enthalpy of one mole of methylene group, $\Delta\bar{H}^{E}(CH_2)$, is a more adequate criterion of polarity. However, the determination of $\Delta\bar{H}^{E}(CH_2)$ requires much more experimental data compared with $\Delta\bar{G}^{E}(CH_2)$ and is much more sensitive to errors incidental to the experimental determination of activity coefficients[21]. A necessary condition for $\Delta\bar{G}^{E}(CH_2)$ to be a representative polarity criterion is the existence of a direct proportionality between the $\Delta G^{E}(CH_2)$ and $\Delta H^{E}(CH_2)$ in the solute–solvent system, which is fulfilled if there is a direct proportionality between $\Delta H^{E}(CH_2)$ and $\Delta S^{E}(CH_2)$. The fact that this condition is not fulfilled with some systems, and the difference in the weights of the entropic effects with $\Delta\bar{G}^{E}(CH_2)$ and with ΔI, are the most likely reasons for the scattering of the points about the regression line in Fig. 2. The equivalence of $\Delta\bar{G}^{E}(CH_2)$ and ΔI as polarity criteria on the one hand and statistically insignificant differences between the correlation of $\Delta\bar{G}^{E}(CH_2)$

versus ΔI and that of $\Delta\bar{G}^{E}(CH_2)$ *versus* ΔI_i on the other indicate that the polarity of a stationary phase can adequately be characterized by a single criterion.

LIST OF SYMBOLS

$\Delta G^0_{sp}(i)$	Standard molar Gibbs function of sorption of solute i
$\Delta G^0_{sp}(X)$	Increment of functional group X to $\Delta G^0_{sp}(i)$
ΔG^E	Excess Gibbs function of the solute–solvent system*
$\Delta G^E(CH_2)$	Partial excess Gibbs function of one mole of solute methylene group
$\Delta\bar{G}^E(CH_2)$	Average of $\Delta G^E(CH_2)$ values of solutes listed in Table I
ΔH^E	Excess enthalpy of the solute–solvent system
$\Delta H^E(CH_2)$	Partial excess enthalpy of one mole of solute methylene group
I_i	Kováts' retention index of solute i on a given stationary phase, y
ΔI_i	Difference $I_i^y - I_i^{squalane}$ (McReynolds constant)
ΔI	Average value of ΔI_i; $\Delta I = \frac{1}{5}\sum_i \Delta I_i$, where i = benzene, 1-butanol, 2-pentanone, 1-nitropropane and pyridine
M	Number of methyl groups in the solute molecule
N	Number of methylene groups in the solute molecule
N_A	Avogadro number
n	Number of carbon atoms in the solute molecule
P^0	Saturation vapour pressure of solute
ΔS^E	Excess entropy of the solute–solvent system
$\Delta S^E(CH_2)$	Partial excess entropy of one mole of solute methylene group
V_g^0	Specific retention volume of solute
w_{ij}	Pairwise potential energy of the interaction of molecules of compounds i and j
Δw	$\frac{1}{2}(w_{11} + w_{22}) - w_{12}$, where subscripts 1 and 2 refer to solvent and solute, respectively
z	Lattice coordination number

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* All excess quantities refer to infinite dilution of solute.

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HOCHLEISTUNGS-GASCHROMATOGRAPHIE AN FLÜSSIGKRISTALL-GLASKAPILLAREN

IV. EINFLUSS DER ALTERNATION AUF DIE TRENNUNG VON ISOMEREN KOHLENWASSERSTOFFEN

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SUMMARY

High-resolution gas chromatography on liquid crystal glass capillaries. IV. Influence of alternation on the separation of isomeric hydrocarbons

The influence of the effect of alternation on separation of isomeric *n*-alkenes C_{10}–C_{15} was investigated on nematic, smectic and cholesteric mesophases. The results were compared with solution behaviour on common stationary phases.

It was found that, as a result of the effect of alternation on liquid crystals, *trans* isomers with an odd number of carbon atoms and the double bond between an odd number of carbon chains and *cis* isomers with an odd number of carbon atoms and the double bond between an even number of carbon chains have a higher retention.

EINLEITUNG

Flüssigkristalle zeigen in homologen Reihen eine Alternation der Klärpunkte[1]. Dabei ist mit zunehmender Kettenlänge ein absteigender Trend zu beobachten. An einer homologen Reihe von Oximestern wurde durch gaschromatographische Messungen eine Alternation der partiellen molaren Lösungsenthalpien und -entropien von verschiedenen Substraten gefunden, die zur Klärpunktsoszillation invers verlief[2].

Die in vorangegangenen Arbeiten studierten Trennungen von isomeren Alkenen an Flüssigkristall-Glaskapillaren ermöglichen die Untersuchung des Einflusses der Alternation auf die Trennung in Abhängigkeit von der Kettenlänge der eingesetzten Substrate[3–5].

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EXPERIMENTELLES

Als Substrate wurden Mischungen aller 66 isomeren *n*-Alkene C_{10}-C_{15} sowie die korrespondierenden *n*-Alkane eingesetzt.

Die Messungen wurden mit einem Gaschromatographen Carlo Erba GI 452 mit Flammenionisationsdetektor durchgeführt. Es wurden Glaskapillaren mit nematischer [4-*n*-Pentylacetophenon-(O-4-*n*-octyloxybenzoyloxim) (OBO)][3], smektischer [5-*n*-Heptyl-2-(4-*n*-nonyloxyl-phenyl)-pyrimidin (NPP)][4] und cholesterinischer [Cholesterylbutyrate (CHOB)][5] Phase verwendet, deren Vorbehandlung, Säulenparameter und Retentionsverhalten bereits beschrieben wurden[3–5].

ERGEBNISSE UND DISKUSSION

Aus Ergebnissen an nematischen und smektischen Flüssigkristallen postulierten wir eine Zunahme der spezifischen Selektivität für die Trennung von benachbarten lageisomeren *n*-Alkenen mit der Verschiebung der Doppelbindung zum Ende des Moleküls[3–5]. Neben diesem wurden auch noch andere Effekte gefunden, die im Folgenden diskutiert werden sollen.

Besonders bemerkenswert war die unerwartete Retention von *trans*-6 nach *trans*-5-Dodecen an nematischer und smektischer Phase. Dabei handelt es sich um den einzigen Fall, dass Lageisomere mit der Doppelbindung in der Mitte des Moleküls nach Isomeren mit der Doppelbindung mehr zum Ende des Moleküls eluiert werden. Eine ähnliche Ausnahme an üblichen stationären Phasen ist die Elution von *trans*-4- vor *trans*-5-Decenen, was jedoch mit dem Einfluss des Propyleffektes erklärt werden kan[6]. Flüssigkristalle behindern den Propyleffekt[3], sodass diese Isomere nicht getrennt werden.

Fig. 1 zeigt den Einfluss der Art der stationären Phase auf die Trennung isomerer *trans*-5- und *trans*-6-Dodecene. Dabei ist zu erkennen, dass an üblichen apolaren (Squalan) und polaren (Carbowax 20M) stationären Phasen die Reihenfolge der Retention *trans*-6 vor *trans*-5 ist. Im Gegensatz zu nematischen und smektischen Phasen wurde an der cholesterinischen Mesophase keine Trennung für *trans*-6- und *trans*-5-Dodecene gefunden (Mesophasetemperatur ist zu hoch). Die Trennung gehört im

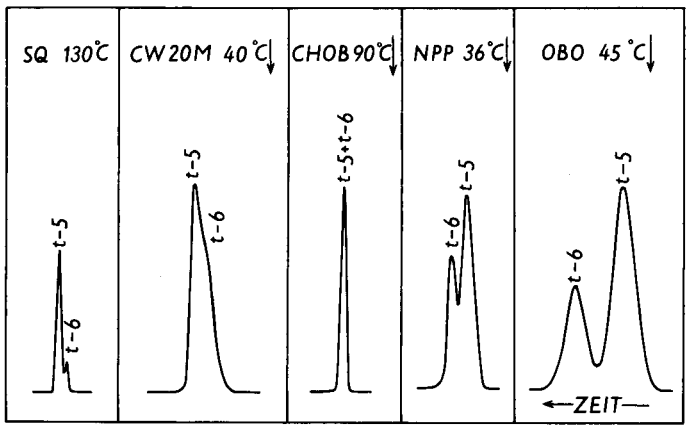

Fig. 1 Trennung der *trans*-5- und *trans*-6-Dodecene an Glaskapillaren mit Squalan (SQ), Carbowax 20M (CW 20M), CHOB, NPP und OBO. ↓ = Kühlung; ↑ = Heizung; *t* = *trans*.

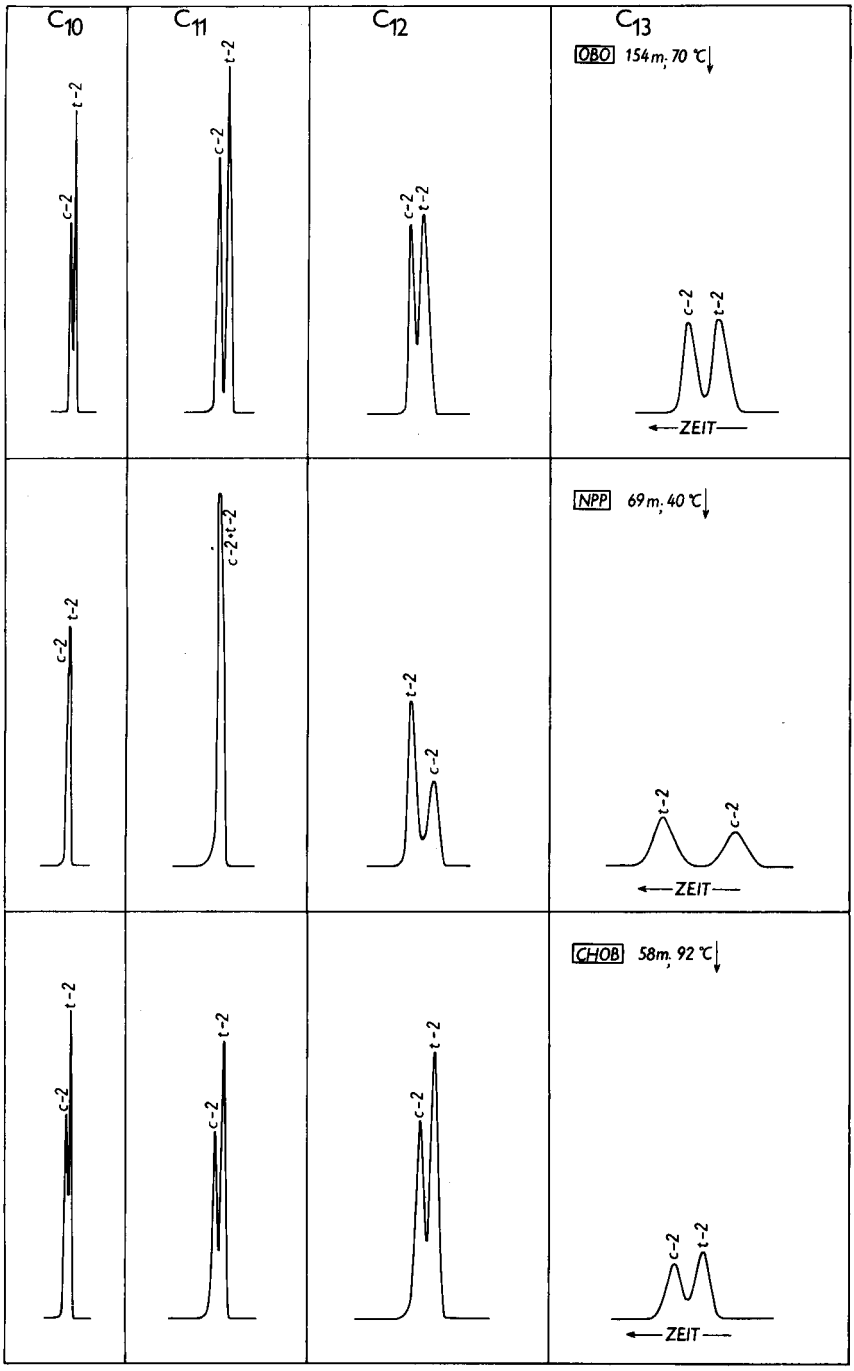

Fig. 2. Trennung von geometrischen C_{10}–C_{13}-2-Alken-Isomeren an OBO, NPP und CHOB. *t* = *trans*; *c* = *cis*.

allgemeinen zu den schwierigsten Trennproblemen und ist bisher an Flüssigkristallen am besten gelungen. Das anomale Verhalten kann auf den Einfluss der Alternation zurückgeführt werden.

Der Effekt der Alternation der Trennung von geometrischen isomeren *cis*-2- und *trans*-2-*n*-Alkenen C_{10}–C_{13} konnte an nematischer Phase (OBO) gezeigt werden[3]. In Fig. 2 wird dieser Effekt mit der Trennung an smektischer (NPP) und cholesterinischer (CHOB) Phase verglichen. Tabelle I zeigt die Trennfaktoren α für diese Trennungen im Vergleich mit Squalan. Dabei ist zu sehen, dass die Werte für Squalan nicht alternieren, was für die smektische und cholesterinische Phase nicht klar erkennbar ist. Zwischen OBO und NPP sind einige Unterschiede in der Selektivität als Folge der umgekehrten Retention dieser Isomere zu erkennen.

TABELLE I

TRENNFAKTOREN α VON GEOMETRISCHEN C_{11}–C_{13}-2-ALKEN-ISOMEREN AN OBO, NPP, CHOB UND SQUALAN

↓ = Kühlung.

cis-2-/*trans*-2-	α OBO 70°C ↓	α NPP 40°C ↓	α CHOB 92°C ↓	α SQ 90°C
n-Undecene	1.016	1.000	1.017	1.037
n-Dodecene	1.009	0.986	1.013	1.036
n-Tridecene	1.010	0.982	1.013	1.035

Tabelle II zeigt die Trennfaktoren α für OBO, NPP und CHOB für lageisomere *trans*- und *cis*-*n*-Alkene C_{11}–C_{13} sowie für Squalan. Während die α-Werte für *trans*- und *cis*-Isomere an Squalan mit zunehmender C-Zahl monoton steigen, zeigen die trans- und cis-Isomere an OBO, NPP und CHOB eine Alternation. Der Vergleich der Trennfaktoren an OBO und Squalan ist in Fig. 3 zu sehen. Es ist auch erkennbar, dass sich der Alternationseffekt im isotropen Bereich verkleinert. Die Temperaturabhängigkeit der Trennfaktoren für *trans*-4-/*trans*-5- und *trans*-5-/*trans*-6-Dodecenen an OBO ist in Fig. 4 dargestellt. Der Alternationseffekt ist auch erkennbar durch Vergleich der α-Werte für korrespondierende geometrische Isomere (Tabelle III).

Aus diesen Ergebnissen folgt, dass alle *trans*-Isomere mit gerader C-Zahl und mit der Doppelbindung zwischen geradzahligen Kohlenstoffketten an Flüssigkristallen selektiver zurückgehalten werden als andere *trans*-Isomere. Der Vergleich der korrespondierenden Strukturen von *trans*-Isomeren zeigt, dass solche Isomere sich der Mesophasenstruktur starker anpassen können (Fig. 5). Ein ähnlicher Effekt wurde für *cis*-Isomere mit gerader C-Zahl und der Doppelbindung zwischen ungeradzahligen Kohlenstoffketten gefunden (Fig. 6).

Aus den Ergebnissen folgt weiter, dass die Koelution von *trans*-4- mit *trans*-5-Decen auf Flüssigkristallen[3] auch durch die Alternationseffekt beeinflusst wird.

Die Klärpunktsoszillation und die parallel dazu verlaufende Oszillation der Aktivitätskoeffizienten an einer homologen Reihe von 4-*n*-Pentylacetophenon-/O-4-*n*-alkoxybenzoyloximen zeigte bei geradzahligen Kettenlängen der Alkoxykette die grösten Werte, was auf eine Alternation in der Polarisierbarkeitsanisotropie der Moleküle und im Ordnungsgrad der Phase schliessen lässt[2]. Die den höheren

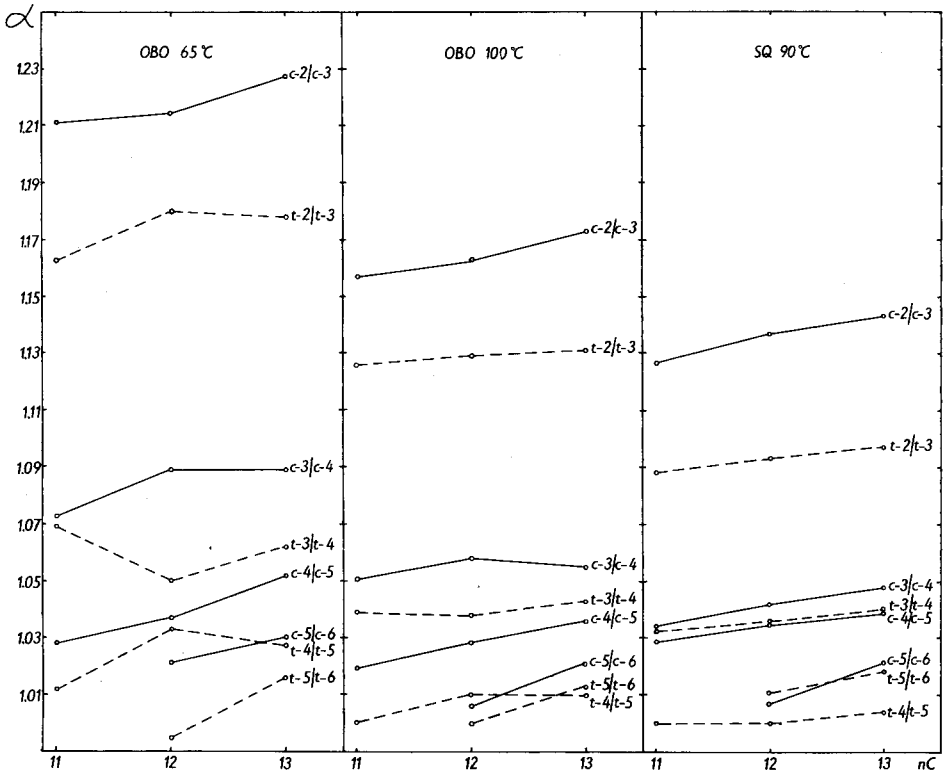

Fig. 3. Trennfaktoren α von C_{11}–C_{13}-*n*-Alkene an OBO und Squalan.

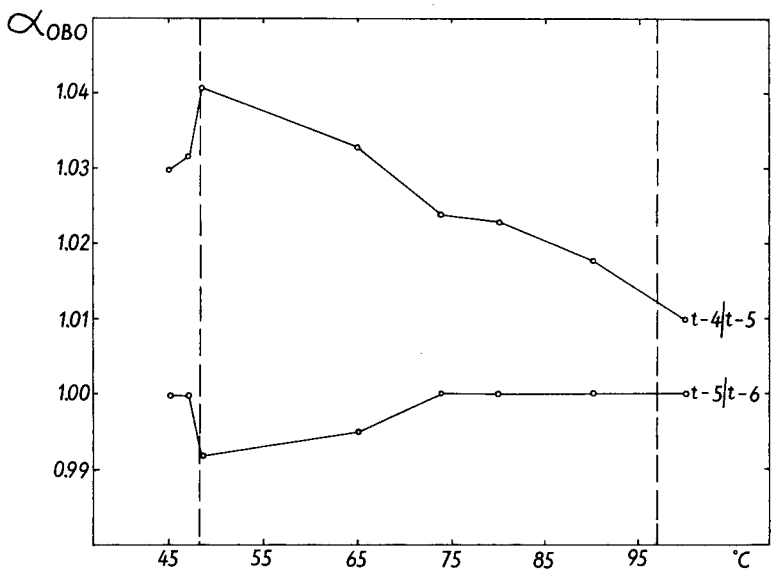

Fig. 4. Temperaturabhängigkeit der Trennfaktoren α für *trans*-4-/*trans*-5- und *trans*-5-/*trans*-6-Dodecene an OBO.

TABELLE II

TRENNFAKTOREN α VON LAGEISOMEREN C_{11}–C_{13} *n*-ALKENEN AN OBO, NPP, CHOB UND SQUALAN (SQ)

	OBO 65°C ↑			*NPP 40°C* ↓			*CHOB 90°C* ↓			*SQ 90°C*		
	C_{11}	C_{12}	C_{13}	C_{11}	C_{12}	C_{13}	C_{11}	C_{12}	C_{13}	C_{11}	C_{12}	C_{13}
t-2-/*t*-3-	1.163	1.180	1.178	1.177	1.205	1.207	1.139	1.143	1.146	1.088	1.093	1.097
t-3-/*t*-4-	1.069	1.050	1.062	1.063	1.058	1.075	1.049	1.046	1.054	1.033	1.036	1.040
t-4-/*t*-5-	1.012	1.033	1.027	1.011	1.042	1.040	1.000	1.022	1.023	1.000	1.000	1.004
t-5-/*t*-6-	–	0.995	1.016	–	0.993	1.021	–	1.000	1.017	–	1.011	1.018
c-2-/*c*-3-	1.211	1.214	1.227	1.218	1.237	1.257	1.182	1.180	1.189	1.127	1.137	1.143
c-3-/*c*-4-	1.073	1.089	1.089	1.072	1.095	1.103	1.066	1.078	1.081	1.034	1.042	1.048
c-4-/*c*-5-	1.028	1.037	1.052	1.028	1.041	1.059	1.024	1.036	1.049	1.029	1.035	1.039
c-5-/*c*-6-	–	1.021	1.030	–	1.015	1.032	–	1.019	1.029	–	1.007	1.021

TABELLE III

TRENNFAKTOREN α VON GEOMETRISCHEN C_{11}–C_{13} *n*-ALKEN-ISOMEREN AUF OBO, NPP UND CHOB

	OBO 65°C ↑			*NPP 40°C* ↓			*CHOB 90°C* ↓		
	C_{11}	C_{12}	C_{13}	C_{11}	C_{12}	C_{13}	C_{11}	C_{12}	C_{13}
cis-2-/*trans*-2-	1.013	1.003	1.006	1.000	0.986	0.982	1.017	1.013	1.013
cis-3-/*trans*-3-	0.972	0.979	0.966	0.966	0.961	0.943	0.984	0.981	0.977
cis-4-/*trans*-4-	0.957	0.939	0.942	0.958	0.928	0.919	0.967	0.954	0.952
cis-5-/*trans*-5-	0.942	0.936	0.919	0.943	0.929	0.902	0.944	0.940	0.929
cis-6-/*trans*-6-	–	0.911	0.906	–	0.909	0.893	–	0.923	0.918

Klärpunkt bewirkende grössere intermolekulare Wechselwirkung der Moleküle der nematischen Phase erschwert den Lösevorgang für Substratmoleküle. Dadurch ist es wahrscheinlich nur für die o.g. *trans*- und *cis*-Isomere möglich, stärker in die Mesophasenstruktur einzudringen.

Messungen der Länge/Breite-Relation ("shape parameter") der *n*-Alken-Moleküle an Kalottenmodellen sind in Tabelle IV zusammengestellt. Sie nehmen in homologer Reihe mit zunehmender C-Zahl zu und sind für *trans*-Isomere grösser als für *cis*-Isomere. Der Unterschied zwischen benachbarten *trans*-Isomeren ist jedoch nicht signifikant. Bei *cis*-Isomeren mit der Verschiebung der Doppelbindung von der Mitte zum Ende des Moleküls wachsen diese Parameter, was in Übereinstimmung steht mit der bereits beschriebenen Selektivität für diese Substrate[3,4]. Die Selektivität für *trans*-Isomere konnte damit jedoch nicht erklärt werden. Die Messgenauigkeit reicht auch nicht aus für die Beschreibung der Alternation, d.h. die gaschromatographische Messung ist für diese Effekte empfindlicher.

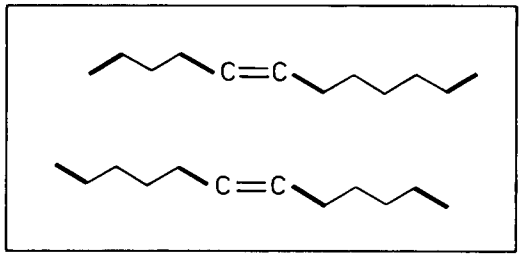

Fig. 5. Strukturschema von *trans*-5- und *trans*-6-Dodecen.

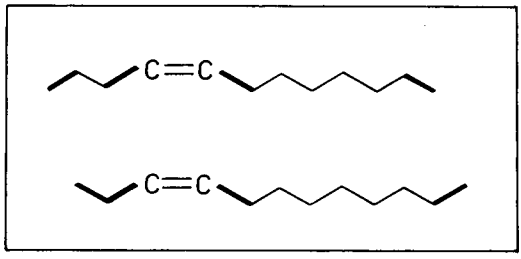

Fig. 6. Strukturschema von *cis*-4- und *cis*-3-Dodecen.

TABELLE IV

LÄNGE/BREITE-VERHÄLTNIS DER C_{10}–C_{13} *n*-ALKEN-MOLEKÜLE

	C_{10}	C_{11}	C_{12}	C_{13}
trans-2-	2.98	3.33	3.59	3.77
trans-3-	3.03	3.29	3.55	3.77
trans-4-	3.03	3.29	3.53	3.77
trans-5-	3.08	3.29	3.55	3.73
trans-6-	–	–	3.53	3.77
cis-2-	2.77	2.90	3.04	3.31
cis-3-	2.63	2.77	2.93	3.29
cis-4-	2.42	2.62	2.73	2.92
cis-5-	2.45	2.56	2.70	2.81
cis-6-	–	–	2.65	2.73
1-	3.08	3.34	3.61	3.88

Untersuchungen des Retentionsverhaltens von Tetradecenen und Pentadecenen wurden an NPP durchgeführt. Der Einfluss der Alternation auf die Trennung von *trans*-5- und *trans*-6-C_{12}-C_{15} *n*-Alkenen ist in Fig. 7 zu sehen. Die Alternation für *trans*-6-Tetradecen zeigt sich deutlich, da *trans*-6- entgegen den Regeln für das Retentionsverhalten[7] aufgrund der Alternation näher zum *trans*-5-Tetradecen eluiert wird (Fig. 8).

Für die praktische Seite der Trennung von isomeren *n*-Alkenen ist der Alternationseffekt sehr wichtig, da er die Trennbedingungen beeinflusst. Lageisomere mit der Doppelbindung in der Mitte des Moleküls sind gewöhnlich die am schwersten zu trennenden Isomerenpaare. Gerade bei Isomeren mit der Doppelbindung zwischen geradzahligen Kohlenstoffketten z.B. bewirkt der Alternationseffekt eine Vergrösserung der Retention für *trans*-6-Dodecen und *trans*-6-Tetradecen. Dadurch lassen sich an Flüssigkristallen bessere Trennungen in kürzeren Zeiten als an üblichen stationären Phasen erzielen. Die negative Beeinflussung der Auflösung benachbarter Lageisomere durch den Alternationseffekt wird in andere Arbeit[8] diskutiert.

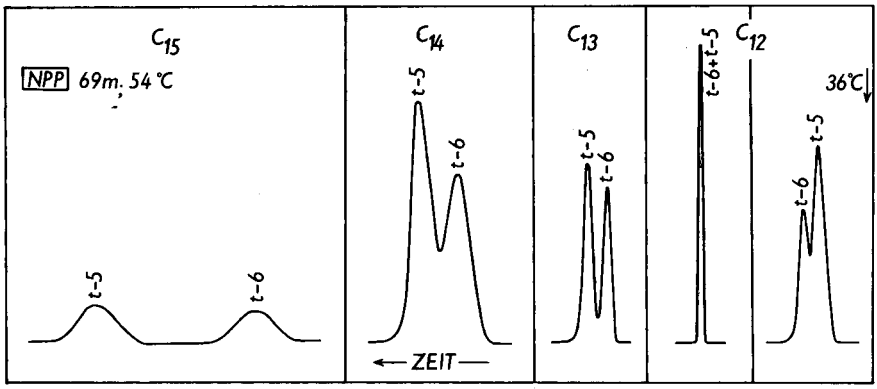

Fig. 7. Trennung der *trans*-5- und *trans*-6-C_{12}–C_{15}-*n*-Alkene an NPP.

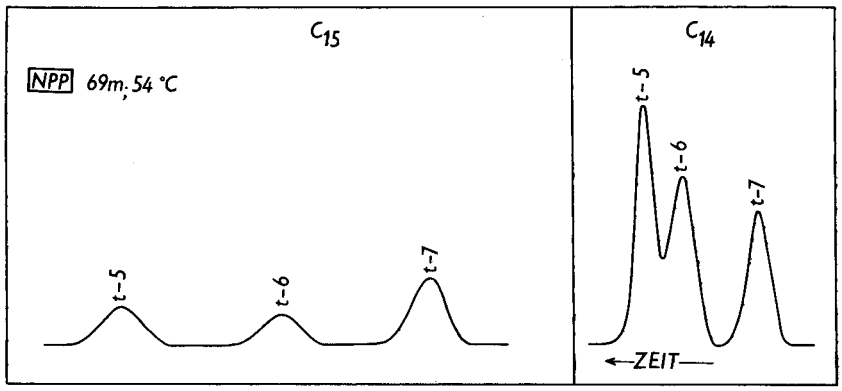

Fig. 8. Trennung der *trans*-5-, *trans*-6- und *trans*-7- C_{14}- und C_{15}-*n*-Alkene an NPP.

SCHLUSSFOLGERUNGEN

Die Leistungsfähigkeit von nematischen, smektischen und cholesterinischen Mesophasen-Trennsystemen konnte an Trennungen von isomeren *n*-Alkenen gezeigt werden. Aufgrund des Alternationseffektes werden *trans*-Isomere mit geradzahliger C-Zahl und der Doppelbindung zwischen geradzahligen Kohlenstoffketten relativ stärker gelöst als andere benachbarte trans-Isomere. Ein ähnlicher Einfluss wurde für *cis*-Isomere mit geradzahliger C-Zahl und der Doppelbindung zwischen ungeradzahligen Kohlenstoffketten gefunden.

Der Einfluss der Alternation auf die Retention ist relativ klein, er ist jedoch bei Verwendung von Hochleistungs-Glaskapillaren signifikant.

Der Alternationseffekt ist sehr wichtig für die schwierige Trennung von isomeren *n*-Alkenen mit der Doppelbindung in der Mitte des Moleküls. Aufgrund der diskutierten Ergebnisse soll seine Anwendung auch für die Trennung von *trans*-7- und *trans*-8-Hexadecenen und *trans*-8 und *trans*-9-Oktadecenen und den entsprechenden *cis*-Isomeren untersucht werden, für welche an üblichen stationären Phasen Trennstufenzahlen von $N = 10^6$–10^7 theoretischen Böden benötigt werden.

ZUSAMMENFASSUNG

Der Einfluss der Alternationseffektes auf die Trennung von isomeren C_{10}–C_{15}-*n*-Alkenen wurde an nematischen, smektischen und cholesterinischen Mesophasen untersucht. Die Ergebnisse wurden mit dem Löseverhalten an üblichen stationären Phasen verglichen.

Der Einfluss der Alternation auf die Retention an Flüssigkristallen wurde gefunden für *trans*-Isomere mit geradzahliger C-Zahl und der Doppelbindung zwischen geradzahligen Kohlenstoffketten sowie für *cis*-Isomere mit geradzahliger C-Zahl und der Doppelbindung zwischen ungeradzahligen Kohlenstoffketten.

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USE OF SMECTIC LIQUID CRYSTALS FOR THE GAS–LIQUID CHROMATOGRAPHIC SEPARATION OF POSITIONAL ISOMERS

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SUMMARY

The smectogenic compounds terephthal-bis-*n*-butylaniline and 4-(4-*n*-hexyloxybenzylideneamino)azobenzene were prepared and used as stationary phases for the gas–liquid chromatographic separation of positional isomers of dibromobenzenes, chloroacetophenones, chloronaphthalenes and methybiphenyls. These solute isomers cannot be sharply separated in the smectic B state whereas a complete separation was achieved in both the smectic A and C phases. It is suggested that the unique selectivity of the smectic A and C states relative to the smectic B state can be explained by the difference in the molecular packing in the layer between the smectic B and the smectic A or C state.

INTRODUCTION

Liquid crystals exhibit interesting solvent properties because of the rod-like shape and the ordered arrangement of their molecules. In addition, liquid crystals are a unique liquid stationary phase in gas–liquid chromatography (GLC) in that they show long-range orientational order in which the rod-like molecules tend towards a mutually parallel alignment[1–3]. It is also well established that the solutes are separated on the basis of molecular shape in GLC using liquid crystals as the stationary phase. Roughly, the more rod-like a solute molecule is, the easier it should fit into the liquid crystal lattice and hence the greater its solubility should be. These unique solvent properties of liquid crystals were applied for the first time to the separation of the positional isomers of various disubstituted benzenes[4]. Recently several reports on the separation of alkylnaphthalenes[5], polycyclic aromatic hydrocarbons[6–9], steroid epimers[10], polychlorinated biphenyls[11], azaheterocyclic compounds[12] and monochlorobiphenyls[13] using liquid crystalline stationary phases have appeared.

Most GLC studies reported so far have been carried out with the use of nematic liquid crystals. Except for a few reports[14–16], another liquid crystalline phase, a smectic liquid crystal, has not been used for the GLC separation of positional isomers. In addition, it has been reported that the separation factor in the temperature range of the stable smectic phase is worse than that in the nematic range in spite of the lower working temperature[1].

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It is well established that the smectic phases can be further subdivided into at least seven modifications, denoted by the letters A–G[17]. From a structural point of view, all of the smectic phases are stratified, the molecules being arranged in layers with a well defined interlayer spacing. Thus, the smectic states are more ordered than the nematic state and always exist at temperatures lower than the nematic state. Hence, it is unlikely that none of the smectic modifications has a selective affinity towards the solute molecules. It seemed interesting to study the GLC separation of various positional isomers in the different smectic states. In this paper we describe a study of the separation of various isomeric disubstituted benzenes, naphthalenes and biphenyls in the smectic A, B and C phases, which are the most frequently observed with smectic liquid crystals.

EXPERIMENTAL

Materials

The two smectogenic compounds used, terephthal-bis-4-*n*-butylaniline (TBBA) and 4-(4-*n*-hexyloxybenzylideneamino)azobenzene (HBAA), were synthesized according to known methods[18,19]. The crude compounds were purified by successive recrystallization from hot ethanol to give constant transition temperatures. The transition temperatures and the purities of the compounds were estimated with a Rigaku differential scanning calorimeter which was calibrated in terms of the temperature and energy. The phase transitions and the liquid crystal textures were determined by using an ordinary polarizing microscope and a Mettler FP microfurnace for sample temperature control. The determined liquid crystalline phases and their transition temperatures are as follows:

TBBA:

$$\mathrm{K} \xrightarrow{113^\circ\mathrm{C}} \mathrm{S(B)} \overset{144^\circ\mathrm{C}}{\leftrightarrow} \mathrm{S(C)} \overset{172^\circ\mathrm{C}}{\leftrightarrow} \mathrm{S(A)} \overset{200^\circ\mathrm{C}}{\leftrightarrow} \mathrm{N} \overset{236^\circ\mathrm{C}}{\leftrightarrow} \mathrm{I}$$

$$\uparrow \quad \updownarrow \; 87^\circ\mathrm{C}$$

$$\mathrm{S(V)} \overset{74^\circ\mathrm{C}}{\leftrightarrow} \mathrm{S(IV)}$$

HBAA:

$$\mathrm{K} \overset{107^\circ\mathrm{C}}{\leftrightarrow} \mathrm{S(B)} \overset{127^\circ\mathrm{C}}{\leftrightarrow} \mathrm{S(A)} \overset{148^\circ\mathrm{C}}{\leftrightarrow} \mathrm{N} \overset{174^\circ\mathrm{C}}{\leftrightarrow} \mathrm{I}$$

in which K denotes the solid phase, S( ) the smectic phase, N the nematic phase and I the isotropic liquid.

The solutes chosen were *m*- and *p*-dibromobenzenes, *m*- and *p*-chloroacetophenones, α- and β-chloronaphthalenes and 3- and 4-methybiphenyls. Individual positional isomer samples which were obtained from Tokyo Kasei (Tokyo, Japan) were GC pure and were used without further purification.

Apparatus and procedure

The support material employed was 100–120-mesh Chromosorb W HP. The

support was coated with the liquid crystalline compounds by using chloroform as solvent, followed by gradual elimination of the solvent by evaporation on a hot water-bath. The concentration of liquid phase in the packing of the coated support was 2.5 % (w/w). The packing was re-sieved to 100–120 mesh and then packed into 1.5 m × 3 mm I.D. glass columns. Prior to use the columns were conditioned for 2 h at 10°C above the nematic–isotropic transition temperature of the liquid stationary phase with the carrier gas flow-rate set at 30 ml/min.

A Hitachi 163 gas chromatograph equipped with a flame-ionization detector and a linear temperature programmer was used. A 1-mV recorder with a chart speed range of 2.5–80 mm/min was used to record chromatograms from which retention times were determined. The flow-rate of the carrier gas (nitrogen) was measured using a soap-bubble flow meter. Methane was used in the determination of the dead time. The solute mixtures to be separated were dissolved in benzene and were injected with a Hamilton 701 10-μl syringe, using the smallest detectable sample volume.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of mixtures of isomeric dibromobenzenes, chloroacetophenones, chloronaphthalenes and methylbiphenyls observed for the smectic B phase on a TBBA column. It should be noted that isomeric pairs of chloroacetophenone and methylbiphenyl can be separated on the smectic B phase whereas no separation of dibromobenzenes or chloronaphthalenes is achieved. In addition, it should be borne in mind that the retention time for the isomer with the more rod-like shape (*p*-isomer) is longer than that for the *m*-isomer. Such a separation behaviour between *meta*- and *para*-positional isomers is essential for GLC using a

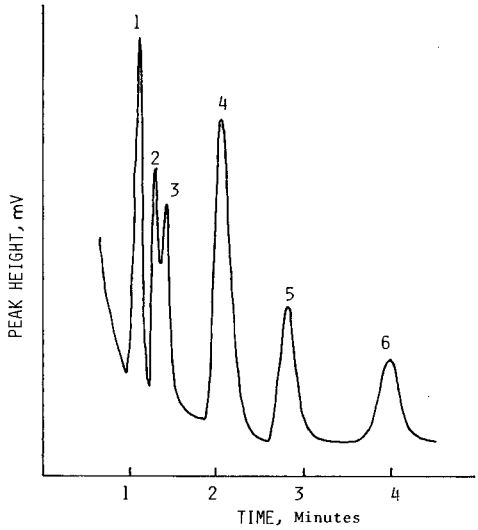

Fig. 1. Gas chromatogram of a mixture of dibromobenzenes, chloroacetophenones, chloronaphthalenes and methylbiphenyls observed in the smectic B phase on a TBBA column. Oven temperature, 125°C; injection temperature, 220°C; flame-ionization detector; nitrogen flow-rate, 25 ml/min. Peaks: 1 = *m*- and *p*-dibromobenzenes; 2 = *m*-chloroacetophenone; 3 = *p*-chloroacetophenone; 4 = α- and β-chloronaphthalenes; 5 = 3-methylbiphenyl; 6 = 4-methylbiphenyl.

liquid crystalline column. It is well recognized that a liquid crystal shows a selective affinity towards linear, rod-like solutes, as these should be able to fit better into its lattice with parallel molecular alignment.

On heating, a drastic increase in the retention volume is observed on passing from the smectic B to the smectic C state. In Fig. 2 a plot is shown of log V_R of dibromobenzene solute molecules *versus* the reciprocal temperature. As shown in Fig. 3, it should be also stressed that all of the solute molecules studied can be separated in this smectic C state, including the isomers of dibromobenzene and chloronaphthalene, which could not be separated in the smectic B state. A sharp increase in the retention volume at the phase transition from the smectic B to the smectic C state indicates that the solubility of the solutes in the smectic C state is much greater than that in the smectic B state. This fact can easily be understood by taking account of the difference in the molecular order in layers between the smectic B and smectic C state. It has been accepted that in the smectic B phase there is high degree of order in a layer and that the molecules form a local three-dimensional hexagonal structure which extends over several smectic layers[20,21]. On the other hand, the molecules in the smectic C state are disordered within the layer but tilted. Such a striking difference in molecular packing in the layer between the smectic B and C states will apparently cause a large change in solubility, and thus a sharp increase in the retention volume occurs on going from the smectic B to the smectic C state.

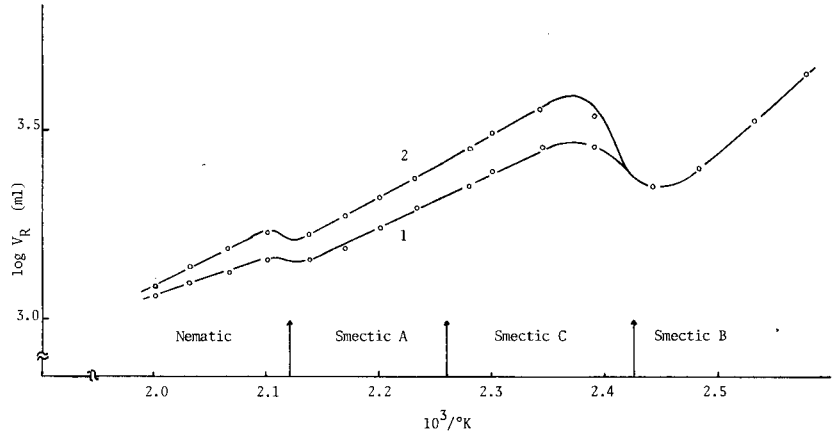

Fig. 2. Relationship between the logarithm of the corrected retention volume (V_R) and the reciprocal of the absolute temperature for *m*-dibromobenzene(1) and *p*-dibromobenzene(2) on a TBBA column.

As shown in Fig. 2, no apparent change in the retention volume can be observed at the smectic C–A phase transition. It is also noteworthy that a substantially continuous change in the retention volume is found at the smectic C–A transition for all of the other solute molecules studied. This behaviour in the change in retention volume with temperature is reasonably expected on the basis of the molecular arrangements of the smectic A and C phases. It is well known that both the smectic C and A phases have a molecular arrangement such that the molecules in each layer are uncorrelated with respect to the centre of mass position. The difference in the molecular alignment between the smectic A and C states consists solely of whether the long

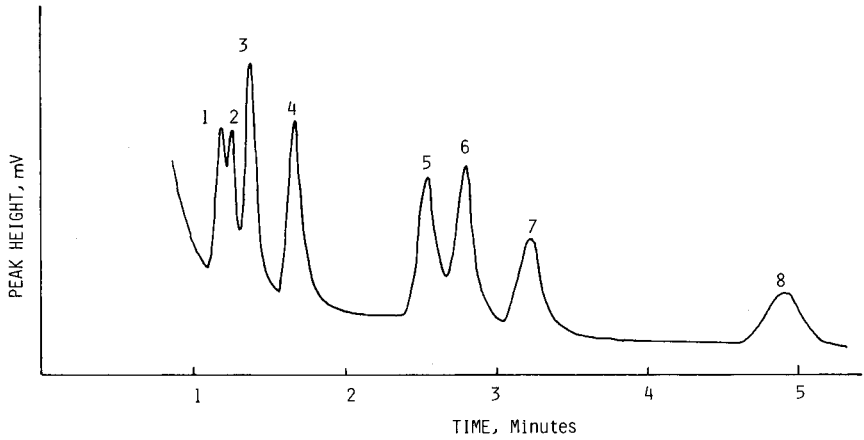

Fig. 3. Gas chromatogram of a mixture of solute molecules observed in the smectic C phase on a TBBA column. Peaks: 1 = *m*-dibromobenzene; 2 = *p*-dibromobenzene; 3 = *m*-chloroacetophenone; 4 = *p*-chloroacetophenone; 5 = α-chloronaphthalene; 6 = β-chloronaphthalene; 7 = 3-methylbiphenyl; 8 = 4-methylbiphenyl.

axis of the molecules lies perpendicular or tilted to the layer. Therefore, the transition energy associated with the smectic A–C phase transition is generally small[22]. It should be noted that log V_R varies nearly linearly with $1/T$ over the mesomorphic range of both the smectic A and C phases, as shown in Fig. 2.

On further heating, the retention volume is gradually changed on passing from the smectic A to the nematic phase. Further, a maximum in the graph of log V_R *vs.* $1/T$ is observed in the vicinity of the phase transition of the smectic A to the nematic phase. A similar maximum in the relationship between the retention volume and the temperature has been found so far for the temperature range of the nematic to isotropic phase transition. Such a characteristic behaviour of the retention volume has been reported by several workers to be typical of all solute species in the nematic and isotropic regions[2,14]. Further, this maximum has been regarded as being caused by a decrease in the free energy of solution of the solute on passing from the nematic to the normal liquid phase. It seems likely that the increase in the retention volume observed at the phase transition of the smectic A to nematic phase can be explained by a similar consideration to that at the nematic–isotropic transition mentioned above. Unfortunately, a more detailed discussion is impossible without other exact thermodynamic data for the solution of a solute in the smectic phase. However, it should be stressed that all of the solute isomers studied can be completely separated in both the smectic A and C states.

Fig. 4 shows the variation of the retention volume of α- and β-chloronaphthalenes with the temperature on an HBAA column. HBAA exhibits two smectic phases, A and C, as well as the nematic phase. The change in retention volume with temperature and the separation behaviour of solute isomers in each liquid crystalline state are similar to those on the TBBA column. The isomers of chloronaphthalenes and dibromobenzenes cannot be separated in the smectic B phase, in analogy with the TBBA column. A large increase in the retention volume is found on going from the smectic B

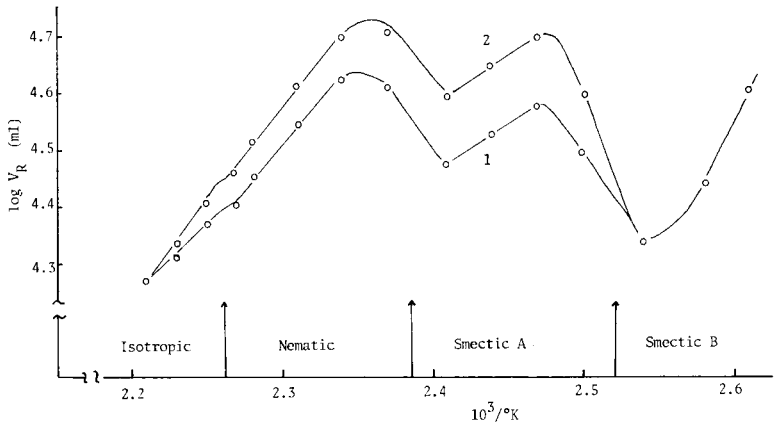

Fig. 4. Relationship between log V_R and 10^3/°K for α-chloronaphthalene (1) and β-chloronaphthalene (2) on an HBAA column.

to the smectic A state, and all of the solutes molecules can be completely separated in the smectic A state.

It has been pointed out so far that the smectic mesophase cannot be regarded as being of high value for practical GLC separations of solutes isomers because the separation efficiency of solutes obtained on the smectic liquid crystal is worse than that in the nematic state. This poor selectivity on the smectic column has been regarded as being caused by the penetration of the solute molecules between the layers in which the typical mesomorphic order does not exist. It seems that this view is in accord with the results obtained for the smectic B phase in our study. Fig. 5 shows the variation of the relative retention (α) of *p*-chloroacetophone (α = 1.0 for the *m*-isomer) with temperature using the TBBA column. It is obvious that the smectic B phase has no selective affinity towards the solutes relative to the other liquid crystalline states. At the phase transition to the smectic C there is a rapid increase in α. As is expected, α continues to decrease on further heating. It must be also stated that the

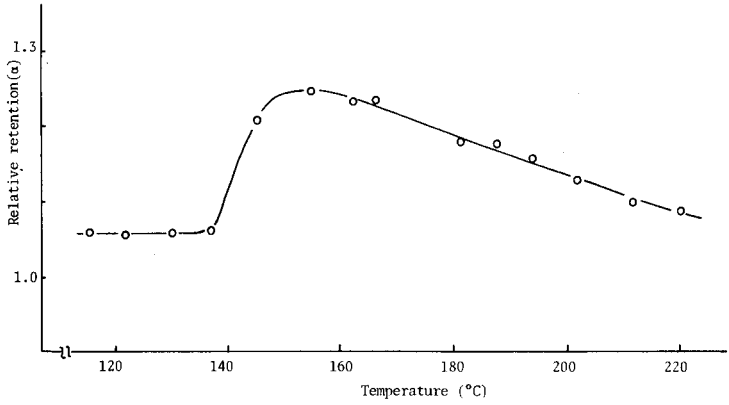

Fig. 5. Variation of the relative retention (α) of *p*-chloroacetophenone (α = 1.0 for the *m*-isomer) with temperature on a TBBA column.

other solute isomers show a variation in relative retention with temperature similar to that shown in Fig. 5. Thus, we conclude that the smectic A or C liquid crystalline phase is useful for the practical separation of positional isomers.

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STUDIES OF CHROMATOGRAPHIC PACKINGS CONSISTING OF POROUS POLYMERS

II. SEPARATION PROPERTIES OF A POROUS STYRENE POLYMER CROSS-LINKED BY DI(METHACRYLOYLOXYMETHYL)NAPHTHALENES

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SUMMARY

A cross-linked porous styrene polymer has been used for chromatographic separation of various classes of organic compounds and its properties have been compared with those of Chromosorb 101 and Porapak Q. This polymer shows higher initial decomposition temperatures than Chromosorb 101 and Porapak Q. Its usefulness as a column packing for gas chromatography has been estimated from polarities, numbers of theoretical plates, resolutions and retention times for various organic mixtures. Besides hydrocarbons, alcohols, carboxylic acids and ketones, the new column packing could be used for separation of amines.

INTRODUCTION

In practical gas chromatography (GC), the use of adsorption chromatography is increasing. This is connected with the development and application of a number of highly effective adsorbents with adequately homogeneous surfaces with respect to adsorption energy but different chemical properties. They are represented by graphitized carbon blac, zeolites, geometrically and chemically modified silica gels and porous glass[1].

Porous polymers are of special interest[2]. They are widely used in analytical and preparative GC owing to such features as their relatively high universality, the possitility of regulating their geometrical and chemical structure in the process of synthesis and sufficiently high thermal stability. Most of the porous polymers known to date were prepared from styrene and various cross-linking reagents, of which the most frequently used is divinylbenzene. In our laboratory, a mixture of di(methacryloyloxymethyl)naphthalenes consisting of the 1,4 and 1,5 isomers, m.p. 110–113°C, was used for the synthesis of porous polymers.

In this paper we present the results of studies of the chromatographic properties of the polymer (ST-DMN), formed from the above cross-linking agent and styrene, under optimal conditions as described previously[3]. The chromatographic

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parameters obtained were compared with those of the known packings Chromosorb 101 (Johns-Manville) and Porapak Q (Waters Assoc.).

EXPERIMENTAL

Synthesis of the porous polymers

In a 50-l glass reactor supplied with a mixer, a back-cooler, a thermometer and a neutral gas (nitrogen) feed, 450 g polyvinyl alcohol were dissolved in 25.5 l distilled water and then heated to 70°C with constant mixing. After the polyalcohol had dissolved, 600 g of a mixture of the isomers 1,4- and 1,5-di(methacryloyloxymethyl)naphthalenes, 600 g styrene, 720 ml *n*-dodecane, 2400 ml toluene and 18 g of α,α'-azoisobutyronitrile as initiator were slowly poured into the reactor. The mixture was constantly stirred and heated at 70°C for 24 h. Suspension polymerization resulted in a copolymer in the form of beads, which, after separation from the post-reaction solution, were washed with distilled water (4×20 dm^3) at 85°C and 30-min intervals. Further washing of the copolymer was conducted at the boiling temperature of the solvent for about 1 h. The product was repeatedly extracted with acetone (11×7 dm^3), methanol (4×7 dm^3), benzene (3×7 l) and then again with methanol (2×7 dm^3). The purified porous copolymer beads were separated into fractions by the wet sieving. The following fractions were obtained: 0.2–0.15 mm, 26 wt.%; 0.15–0.1 mm, 35 wt.%; 0.1–0.088 mm, 14 wt.%; < 0.088 mm, 25 wt.%. These were first dried at 70°C for 2 h under reduced pressure, and then for another 2 h at 150°C under atmospheric pressure.

For chromatographic measurements, 0.15–0.2 mm grains were used the diameters of which approximated those of the graines of Porapak Q (80–100 mesh) and Chromosorb 101 (60–80 mesh).

Measurements of the specific surface area of polymers

These measurements were carried out by the method of thermal desorption of Nelsen and Eggertsen[4], using an apparatus designed and constructed in the Department of Physical Chemistry, M. Curie-Sklodowska University[5]. Nitrogen was used as adsorbate and hydrogen as carrier gas. The specific surface area was calculated by the BET method, assuming that the area of a single nitrogen molecule is 16.2 Å^2. The measurements were preceded by activation of the samples at 200°C for 2 h in a hydrogen stream.

Thermogravimetric (TG) measurements

The measurements were carried out on a derivatograph (MOM, Budapest, Hungary), at a heating rate of 5°C/min in the range from 20 to 1000°C in the air. The initial decomposition temperature was determined from the course of the TG curve.

Chromatographic measurements

The chromatographic measurements were carried out on a Chromatron GCHF-18.3 gas chromatograph (Chromatron, Berlin, G.D.R.) equiped with thermal conductivity detector. Hydrogen at a flow-rate of 50 ml/min was used as carrier gas; the current of the bridge was 200 mA and the temperature of the injector was 250°C.

The studies were carried out on columns of stainless steel (100 cm × 4 mm I.D.). The measurements of retention indices for McReynolds substances were carried out at 140°C, whereas those of specific retention volume, V_m, were made at 180°C. The samples were injected by means of a 1-μl syringe (SGE, North Melbourne, Australia). For individual substances such as benzene, *n*-butanol, pentanone-2, 1-nitropropane and pyridine, trace amounts were injected, by dipping the syringe needle (at the zero position of the piston) in the sample colution for 1 sec[6]. Samples (0.2 μl) of the following mixtures were also injected: (1) aliphatic *n*-hydrocarbons (C_5–C_{12}); (2) ketones: dimethyl ketone, methyl ethyl ketone, methyl *n*-propyl ketone and methyl *n*-butyl ketone; (3) *n*-aliphatic primary alcohols (C_1–C_{10}); (4) *n*-aliphatic carboxylic acids: formic, acetic, propionic, butyric; (5) amines: ethylamine, diethylamine and triethylamine; (6) alcohol isomers: methanol, ethanol, propanol-1, propanol-2, butanol-2, 2-methylpropanol-1, butanol-1, pentanol-2, pentanol-3 and pentanol-1.

From the experimental data the number of theoretical plates, *n*, was calculated according to[7]

$$n = 5.54\,(l_R'/W_{n/2})^2$$

Where l_R = retention distance on the chromatogram and $W_{n/2}$ = peak width at half-height of the peak. The resolution, $R_{i,j}$, for a selected pair of compounds was calculated from[8]

$$R_{i,j} = 2\Delta t_R\,(w_{p,i} + w_{p,j})$$

where $\Delta t_R = t_{Ri,j} - t_{R,i}$, the distance between the maxima of the two peaks, and $w_{p,i}$ and $w_{p,j}$ are the widths of the peaks determined by extrapolating points of inlection of the peaks towards the baseline.

The retention index, I_x, of substance *x* was calculated from[9]

$$I_x = 100 \log (t'_{R,x}/t'_{R,z}) \,/\, (\log t'_{Rz+1} \,/\, t'_{R,z}) + 100\,z$$

in which $t'_{R,x}$ denotes the reduced retention time of the substance, $t'_{R,z}$ the reduced retention time of the homologous alkane with the nearest shortest retention time, $t'_{R,z+1}$ the reduced retention time of the next higher homologue eluted after homologue *z* and *z* denotes the number of carbon atoms in the *n*-alkane molecule. The specific retention volume was calculated according to [10]

$$V_m = \frac{t_R\,\mathrm{W}_{pom} P_{pom}\,T}{m\,T_{pom} P_o} \cdot f$$

where t_R denotes the corrected retention time, W_{pom} the volume velocity of carrier gas in the flow-meter, P_{pom}, T_{pom} the pressure and temperature in the flow-meter, T the temperature of the column and f the James–Martin coefficient.

DISCUSSION

Comparing the retention indices of McReynold substances given Table I, it can be seen that Porapak Q yields the lowest values, and ST-DMN the highest values, which accounts for the polar character of the latter. The high polarity of the ST-DMN packing is confirmed by the data given in Table II, which represent modified McReynolds constants relative those on graphitized carbon black[6,11,12]. The differences are connected with the presence of ester groups in the polymer skeleton.

TABLE I

KOVÁT'S INDICES FOR McREYNOLDS SUBSTANCES ON THE STUDIED POLYMERS AT 140°C

Polymer	*Benzene*	*Butanol-1*	*Pentanone-2*	*1-Nitropropane*	*Pyridine*
Porapak Q	617	607	651	654	660
Chromosorb 101	706	708	747	797	835
ST-DMN	760	782	793	887	886

TABLE II

PROPERTIES OF THE POROUS POLYMERS

S = Specific surface area; t = initial decomposition temperature; n = number of theoretical plates for butanol-1; $R_{i,j}$ = resolution for the pair butanol-2–2-methylpropanol; x, y, z = modified McReynolds constants for benzene, butanol-1 and pentanone-2; I = total selectivity relative to graphitized carbon black. Measurement temperatures: n, $R_{i,j}$, 150°C; x, y, z, 140°C.

Polymer	S (m^2/g)	t	n	$R_{i,j}$	x	y	z	I
Porapak Q	660	200	790	0.48	43	118	86	247
Chromosorb 101	26	220	1053	0.83	132	219	182	533
ST-DMN	90	280	1086	1.08	186	293	228	707

From the data given in Table II it appears that the ST-DMN packing occupies an intermediate position as regards the specific surface area, but is characterized by the highest decomposition temperature as estimated from the TG curve. This feature permits application of the polymer to chromatographic separations at higher working temperatures. This is of particular value in the analysis of compounds with high boiling points.

Comparing the number of theoretical plates, n (Table II), of the substance under the chosen conditions, it appears that the most efficient column is that containing ST-DMN, although the Chromosorb 101 column is only slightly interior. Much lower n values are obtained for the Porapak Q column. A more complete evaluation of the separability of the columns in relation to the given mixture was made on the basis of $R^i{}_j$ for the closest pair of in the given mixture of isomers of aliphatic alcohols. The $R_{i,j}$ values were highest for the ST-DMN packing, which accounts for its highest selectivity in relation to alcohol isomers. The short analysis times for the discussed

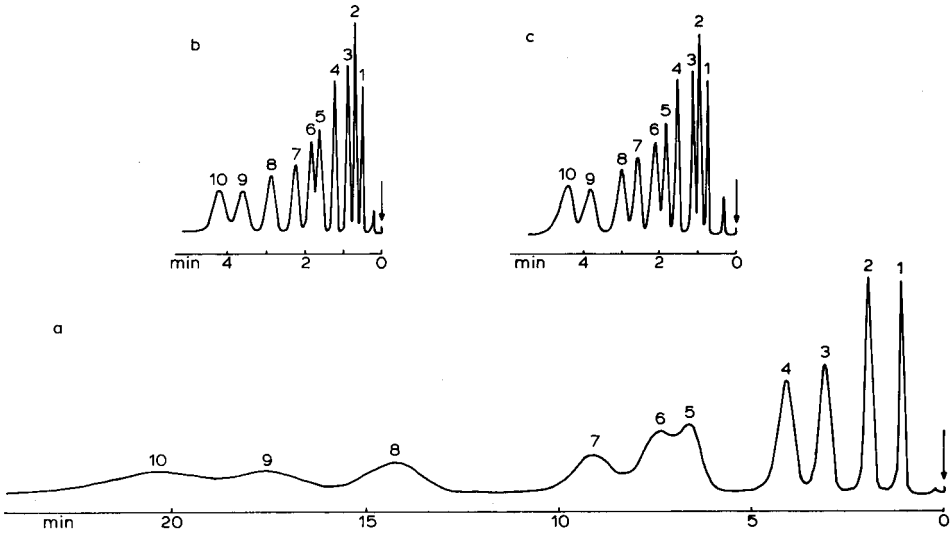


Fig. 1. Comparison of chromatograms of alcohols on Chromosorb 101 (a), Porapak Q (b) and ST-DMN (c). Column: 100 cm × 4 mm I.D., stainless steel; temperature 150°C. Hydrogen flow-rate: 50 ml/min. Injector temperature: 250°C. Catharometer, current: 200 mA. Peaks: 1 = methanol; 2 = ethanol; 3 = propanol-1; 4 = propanol-2; 5 = butanol-2; 6 = 2-methylpropanol-1; 7 = butanol-1; 8 = pentanol-2; 9 = pentanol-3; 10 = pentanol-1.

mixture on Chromosorb 101 and on ST-DMN packings (about 5 min) in contrast to Porapak Q (about 25 min) are of interest (Fig. 1). ST-DMN packings were also found useful for separation of hydrocarbons, ketones, aliphatic acids and amines. In the case of amines, it appeared that on Porapak Q and Chromosorb 101 the heavier amines were adsorbed so strongly that the analysis of the mixtures could not be performed, while on ST-DMN it is possible to obtain a separation of selected amines in a short time (Fig. 2).

In order to evaluate the character of the retention of various classes of compounds on the polymers discussed, the dependences of log V_m on the number of carbon atoms, n_c in molecules of homologous series are used (Figs. 3–9). It appears from Figs. 3–5 that aliphatic *n*-hydrocarbons, ketones and alcohols have the lowest retentions (under the same measurement conditions) on ST-DMN, while the highest values are found on Porapak Q. Carboxylic acids (Fig. 6) however, are more strongly retained on ST-DMN than on Chromosorb 101. A lower retention at the highest

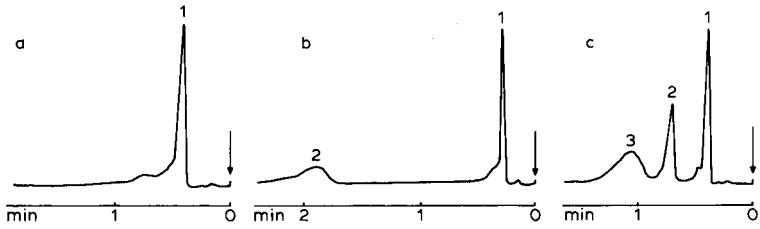


Fig. 2. Comparison of chromatograms of amines on: Chromosorb 101 (a), Porapak Q (b) and ST-DMN (c). Column temperature: 180°C. Other conditions as in Fig. 1. Peaks: 1 = ethylamine; 2 = diethylamine; 3 = triethylamine.

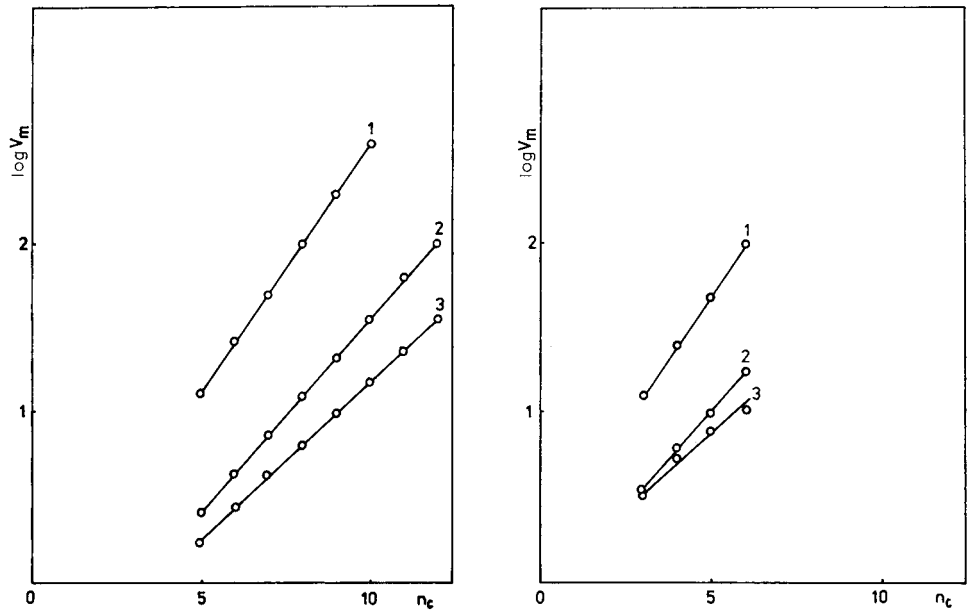


Fig. 3. Dependence of log V_m on number of carbon atoms, n_c, for a mixture of the aliphatic *n*-hydrocarbons on Porapak Q (1), Chromosorb 101 (2) and ST-DMN (3). Chromatographic conditions as in Fig. 2.

Fig. 4. Dependence of log V_m on n_c for a mixture of ketones. Details as in Fig. 3.

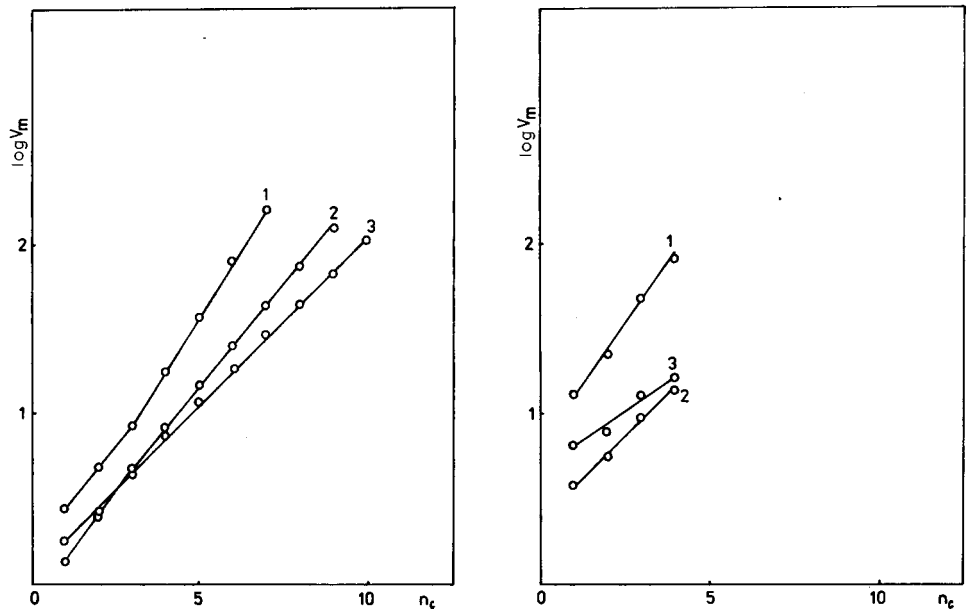
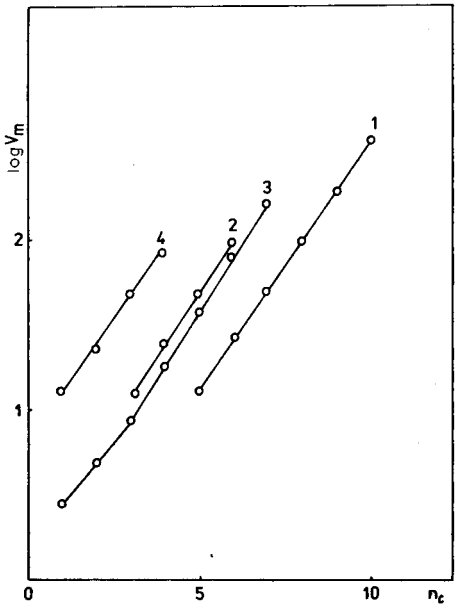


Fig. 5. Dependence of log V_m on n_c for a mixture of alcohols. Details as in Fig. 3.

Fig. 6. Dependence of log V_m on n_c for a mixture of *n*-aliphatic carboxylic acids. Details as in Fig. 3.



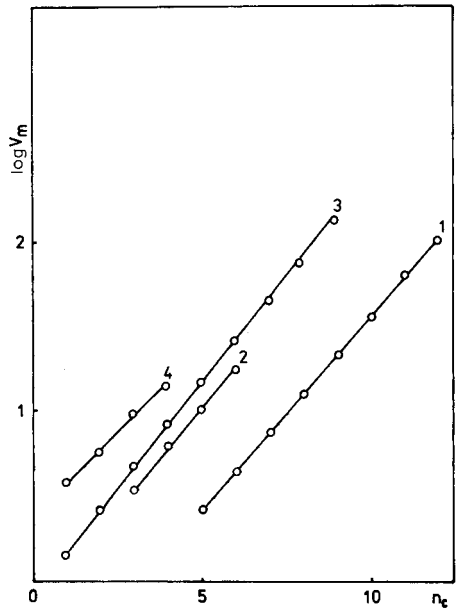
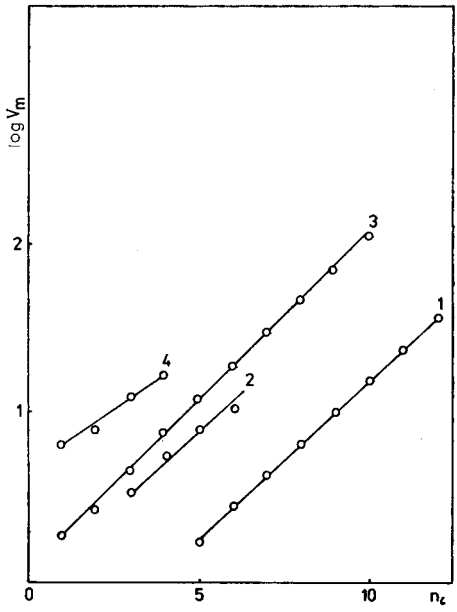


Fig. 7. Dependence of log V_m on n_c for various classes of compounds on Porapak Q: 1 = alipathic *n*-hydrocarbons; 2 = *n*-alcohols; 3 = *n*-ketones; 4 = aliphatic *n*-carboxylic acids. Chromatographic conditions as in Fig. 2.

Fig. 8. Dependence of log V_m on n_c for various classes of compounds on Chromosorb 101. Details as in Fig. 7.



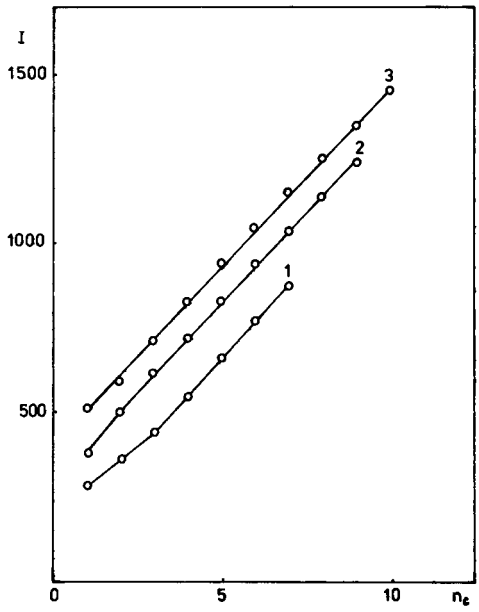


Fig. 9. Dependence of log V_m on n_c for various classes of compounds on ST-DMN. Details as in Fig. 7.

Fig. 10. Dependence of retention index I on n_c for *n*-aliphatic primary alcohols on Porapak Q (1), Chromosorb 101 (2) and ST-DMN (3). Chromatographic conditions as in Fig. 2.

efficiency and resolution results in the best separation on ST-DMN in the shortest time.

The retention sequence on Porapak Q (Fig. 7; *n*-hydrocarbon, alchols, ketones, carboxylic acids) was found to be different from that on Chromosorb 101 and ST-DMN (Figs. 8 and 9; *n*-hydrocarbons, ketones, alcohols, carboxylic acids). This is connected with the non-polar character of Porapak Q, on which strongly polar alcohols are eluted prior to less polar ketones. Further evidence of the high polarity of ST-DMN is the dependence of the retention index on the number of carbon atoms (Fig. 10) for *n*-aliphatic primary alcohols. The curve for ST-DMN contains the highest values and is also the most linear, creating the most favourable conditions for application of the retention index to identification of substances.

From the increasing distances between the log V_m *vs.* n_c lines in Figs. 7–9, the selectivity of the packings for various compound classes increases in the order Porapak Q, Chromosorb 101 and ST-DMN.

CONCLUSIONS

The porous polymer ST-DMN shows favourable properties as a packing in gas chromatography. In comparison to Porapak Q and Chromosorb 101, it has the highest initial decomposition temperature and columns prepared from it have the highest efficiency and separability.

It is suitable for the analysis of various compound classes, hydrocarbons, ketones, alcohols and carboxylic acids, and in contrast to Porapak Q and Chromosorb 101, for amines too. Among the porous polymers studied it has the highest polarity, which is associated with the presence of ester groups in the polymer skeleton. Despite this high polarity, it is characterized by the lowest retention for the particular compound classes, which results in shorter analysis times.

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ETHYLATION OF INORGANIC ANIONS, PHENOLS AND CARBOXYLIC ACIDS FOR GAS CHROMATOGRAPHIC DETERMINATION

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SUMMARY

Some inorganic anions, phenols and carboxylic acids were converted into their ethyl derivatives by reaction with diethyl sulphate, and the ethyl derivatives then determined by gas chromatography with flame ionization detection. For phenols and carboxylic acids, 18-crown-6 was employed as a catalyst for the derivatization reaction. It was observed that 18-crown-6 significantly increases the derivatization yields of phenols and carboxylic acids. From a comparison of this ethylation procedure with the corresponding methylation using dimethyl sulphate, it is concluded that ethylation is more suitable for the determination of sulphide, iodide, phenols and carboxylic acids.

INTRODUCTION

Many gas chromatographic (GC) methods involve the use of derivatization prior to analysis[1–4]. Although this procedure is frequently used for the determination of compounds which are not accessible to direct GC determination, its application to the determination of inorganic anions is as yet relatively unexplored[4–6].

We have investigated the determination of inorganic anions by GC with derivatization[7–10]. In our recent study[9], some inorganic anions were converted into their methyl derivatives by reaction with dimethyl sulphate, etc., and the derivatives were determined by GC with a flame ionization detector (FID). We have also reported a method for determining cyanide (CN^-) or thiocyanate (SCN^-) at trace levels by methylation with dimethyl sulphate and flame thermionic GC[10].

It is interesting to study the ethylation of inorganic anions with diethyl sulphate for GC determination. In this paper, we describe a comparison of methylation and ethylation of some inorganic anions in order to obtain the optimum derivatization system for the GC determination.

Many methods have been published for the GC determination of phenols and carboxylic acids[1–4]: methylation with diazomethane, silylation and acylation have frequently been used. Crown ethers have been used as phase transfer catalysts in the derivatization of organic substances[11–13]. We have also investigated the GC determi-

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nation of phenols and carboxylic acids by using dimethyl or diethyl sulphate and 18-crown-6. The effect of 18-crown-6 on the methylation or ethylation is also reported in the present paper.

EXPERIMENTAL

Apparatus

A Yanagimoto G-180 gas chromatograph equipped with a dual FID (Yanagimoto, Kyoto, Japan) was used. The column and column temperature employed were dependent on the ethyl derivative of the anion; details are given in Table I. The column packing materials, Porapak Q (80–100 mesh) and T (80–100 mesh), were purchased from Waters Assoc. (Milford, MA, U.S.A.), and 5% PEG-HT on Uniport HP (60–80 mesh) and 10% dinonyl phthalate on Chromosorb W (60–80 mesh) were from Gasukuro Kogyo (Tokyo, Japan). Nitrogen was used as the carrier gas at a constant flow-rate of 30 ml/min: the hydrogen and air pressures were 0.5 and 1.0 kg/cm^2, respectively. The injection port temperature was maintained at 250°C. Peak areas were measured by a Chromatopac E1A digital integrator (Shimadzu, Kyoto, Japan).

TABLE I

ETHYLATION AND GAS CHROMATOGRAPHIC CONDITIONS

Stainless-steel column (3 mm I.D.). Column packing: Q = Porapak Q (1 m); T = Porapak T (1 m); PEG = PEG-HT (2 m); DNP = dinonyl phthalate (2 m).

Anion	*Ethyl derivative*	*Extraction (or reaction) solvent*	*Column*	*Column temp. (°C)*
CN^-	C_2H_5CN	Dichloromethane	PEG	60
SCN^-	C_2H_5SCN	Dichloromethane	PEG	100
I^-	C_2H_5I	1,2-Dichloroethane	T	175
Br^-	C_2H_5Br	1,2-Dichloroethane	Q	150
S^{2-}	$(C_2H_5)_2S$	Chloroform	Q	200
C_6H_5OH	$C_6H_5OC_2H_5$	(1,2-Dichloroethane)	PEG*	110*
C_4H_9COOH	$C_4H_9COOC_2H_5$	(Acetonitrile)	DNP*	110*

* In the determination of other phenols or carboxylic acids, the column and column temperature used are shown in Figs. 2 and 3.

Materials

All reagents were of analytical reagent grade and were used without further purification unless otherwise stated. Diethyl sulphate and dimethyl sulphate were commercial grade reagents (Tokyo Kasei Kogyo, Tokyo, Japan). Deionized water, dichloromethane, chloroform, 1,2-dichloroethane and acetonitrile were distilled before use for analysis. All phenols and carboxylic acids were purified by distillation or recrystallization. Solutions of inorganic anions were prepared by dissolving their potassium or sodium salts in water.

Procedure

Determination of inorganic anions. The procedure is analogous to that using

dimethyl sulphate[9], unless otherwise stated. The concentrations of KOH aqueous solutions (0.5 ml) which were added before the diethyl sulphate were 0.5 *M* in the determination of CN^- and 4.0 *M* in that of sulphide (S^{2-}). The extraction solvents employed in each case are given in Table I.

Determination of phenols. To 1.0 ml of an aqueous solution of phenols in a reaction vessel were added 0.5 ml KOH (0.5 *M*) and 1.0 ml 1,2-dichloroethane solution containing diethyl sulphate (0.1 *M*) and 18-crown-6 (0.2 *M*). The vessel was shaken for 30 min in an incubator at 70°C. At the end of the reaction period, after cooling, the organic layer was separated from the aqueous layer. An aliquot (1.0 μl) of the organic layer was injected into the gas chromatograph and the resulting ethyl derivatives were determined with a FID.

Determination of carboxylic acids. A 0.1-ml volume of K_2CO_3 (0.5 *M*) aqueous solution was added to 1.0 ml of an aqueous solution of carboxylic acids. After mixing, the solution was carefully evaporated to dryness. To the residue were added 0.05 ml diethyl sulphate and 2.0 ml acetonitrile solution containing 0.05 *M* 18-crown-6. The mixture was then stirred at room temperature for 30 min. After the reaction, an aliquot (1.0 μl) of the mixture was subjected to GC analysis.

RESULTS AND DISCUSSION

Ethylation of inorganic anions

In order to obtain the optimum ethylation conditions, the effects of the concentration of KOH used, reaction temperature and reaction time on the ethylation yield were investigated for each inorganic anion. From the results, the reaction temperature and reaction time were fixed at 70°C and 30 min, respectively, for ethylation of each anion. The optimum concentration of KOH used in the ethylation of each anion was the same as that used in methylation, Table II, except for the case of S^{2-}. For the latter the optimum KOH concentration was 4.0 *M* in ethylation whereas it was 6.0 *M* (0.5 ml) in methylation. The yield of ethylation of each anion was determined as

TABLE II

DERIVATIZATION YIELDS FOR INORGANIC ANIONS

Sample: 0.10 *M* aqueous solution of inorganic anion. Yields are mean ± S.D. of five replicate analyses.

Anion	*Added KOH**	*Yield (%)*	
		Methylation	*Ethylation*
CN^-	0.5 *M*, 0.5 ml	53.5 ± 1.5	17.4 ± 0.3
SCN^-	None	104.3 ± 2.1	99.8 ± 2.6
I^-	None	73.2 ± 1.8	85.4 ± 1.8
Br^-	None	*ca.* 65**	47.2 ± 1.6
S^{2-}	6.0 *M*, 0.5 ml	42.3 ± 0.8	–
S^{2-}	4.0 *M*, 0.5 ml	–	79.8 ± 1.6

* The optimum derivatization condition is given.

** This value could not be obtained exactly because of the high volatility of the product, methyl bromide.

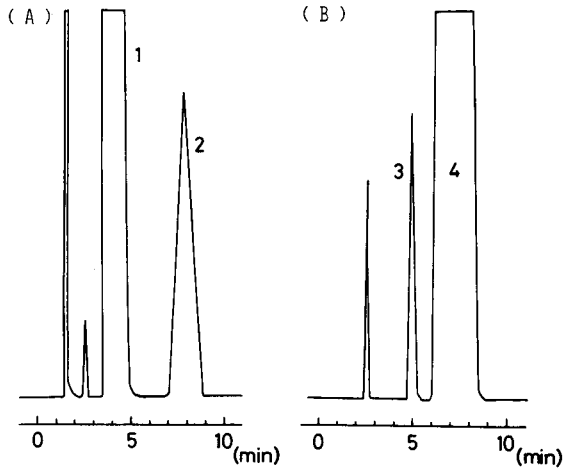

Fig. 1. Gas chromatograms of ethylation products of S^{2-} (A) and I^- (B). GC conditions as in the Experimental section and Table I. Peaks: 1 = chloroform (solvent); 2 = diethyl sulphide; 3 = ethyl iodide; 4 = 1,2-dichloroethane (solvent).

previously reported[9]. The ethylation yields and the yields of methylation using dimethyl sulphate are also given in Table II.

Some anions other than those shown in Table II are also ethylated with diethyl sulphate, for example, nitrite and chloride. However, the yields are fairly low under a variety of derivatization conditions. Ethylation reactions of these anions were not further investigasted because the low yields will not be of practical use for GC determination of the anions.

From Table II, it is seen that both methylation and ethylation of SCN^- proceed quantitatively and that the methylation yields of CN^- and bromide (Br^-) are higher than the ethylation yields of the corresponding anions. On the other hand, iodide (I^-) and S^{2-} have higher ethylation than methylation yields. Therefore, methylation is more suitable for the determination of CN^-, SCN^- and Br^-, whereas ethylation is preferred for S^{2-} and I^-.

Fig. 1 shows typical gas chromatograms of the products of ethylation of S^{2-} and I^- using diethyl sulphate under the optimum conditions described in the Experi-

TABLE III

DERIVATIZATION YIELDS OF PHENOL

Sample: phenol (0.01 *M*). Yields are mean ± S.D. of five replicate analyses.

Reaction temp. (°C)	*18-Crown-6*	*Yield (%)*	
		Methylation	*Ethylation*
70	Added	90.8 ± 1.0	92.3 ± 2.3
	–	81.7 ± 0.5	54.2 ± 0.5
Room temp.	Added	53.8 ± 1.9	2.9 ± 0.1
	–	17.6 ± 1.1	0.9 ± 0.1

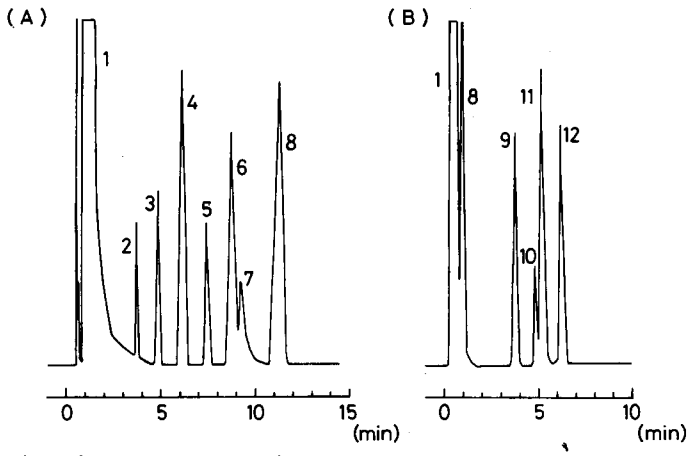

Fig. 2. Gas chromatograms of ethylation products of phenols. Column: 5% PEG-HT (2 m × 3 mm I.D.). Column temperatures: 110°C (A); 210°C (B). Peaks: 1 = 1,2-dichloroethane; 2 = phenol; 3 = *o*-cresol; 4 = *m*-, *p*-cresol and *o*-ethylphenol; 5 = 2,4-xylenol; 6 = *m*- and *p*-ethylphenol; 7 = 3,5-xylenol; 8 = diethyl sulphate; 9 = *m*-nitrophenol; 10 = *o*-nitrophenol; 11 = α- and β-naphthol; 12 = *p*-nitrophenol.

mental section. In each case, a calibration curve was constructed by plotting the peak area of the ethyl derivative *vs.* the concentration of the anion in aqueous solution. The calibration curves were straight lines in the concentration range of 0.1–1.0 mg/ml, passing through the origin.

Derivatization of phenols

In the derivatization of phenols, 18-crown-6 was used as a catalyst. Methylation and ethylation of phenol were performed in the presence and absence of 18-crown-6, both at room temperature and 70°C. The derivatization yields obtained are given in Table III. It is seen that the use of 18-crown-6 increases the derivatization yield in each case. Also ethylation using 18-crown-6 at 70°C gives the highest and most nearly quantitative yield. Therefore, the procedure described in the Experimental section was selected. Fig. 2 shows typical gas chromatograms obtained in the analysis of an aqueous mixture of several phenols. The calibration plots for phenol were linear in the range of 0.1–1.0 mg/ml.

TABLE IV

DERIVATIZATION YIELDS OF CARBOXYLIC ACID

Sample: *n*-valeric acid (0.01 *M*). Yields are mean ± S.D. of five replicate analyses.

Reaction temp. (°C)	*18-Crown-6*	*Yield (%)*	
		Methylation	*Ethylation*
60	Added	99.9 ± 3.3	99.7 ± 0.7
	–	90.3 ± 1.9	83.5 ± 18.1
Room temp.	Added	98.1 ± 2.5	99.6 ± 4.9
	–	53.9 ± 6.6	25.1 ± 2.7

Derivatization of carboxylic acids

Methylation or ethylation of carboxylic acids in aqueous solution gives fairly low yields. Therefore, the derivatization of carboxylic acids was performed in acetonitrile solution after evaporation of an aqueous alkaline solution of the acids, as described in the Experimental section. 18-Crown-6 was also used as the catalyst in these derivatizations. Derivatization yields of *n*-valeric acid were determined under various conditions. From the results in Table IV, it is apparent that 18-crown-6 improves the derivatization yields of *n*-valeric acid, and that this acid is quantitatively methylated or ethylated in the presence of 18-crown-6 even at room temperature. Fig. 3 shows typical gas chromatograms obtained by treating an aqueous mixture of carboxylic acids (C_1–C_7) according to the procedure described in the Experimental section. Higher carboxylic acids can also be detected by this GC method. The calibration plots for *n*-valeric acid were linear in the range of 0.1–1.0 mg/ml.

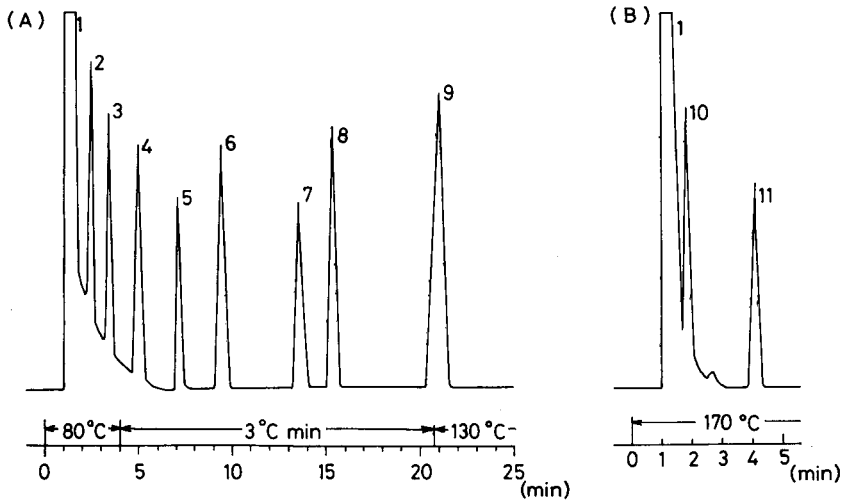

Fig. 3. Gas chromatograms of ethylation products of carboxylic acids. Columns: 10% dinonyl phthalate (2 m × 3 mm I.D.) (A); Porapak Q (1 m × 3 mm I.D.) (B). Peaks: 1 = acetonitrile; 2 = propionic acid; 3 = isobutyric acid; 4 = *n*-butyric acid; 5 = isovaleric acid; 6 = *n*-valeric acid; 7 = isocaproic acid; 8 = *n*-caproic acid; 9 = *n*-heptanoic acid; 10 = formic acid; 11 = acetic acid.

CONCLUSIONS

By ethylation and GC–FID, S^{2-}, I^-, phenol and *n*-valeric acid can be determined at concentrations of 0.1–1.0 mg/ml. It is not expected that this method can be directly applied to these species at much lower levels such as in environmental samples so long as a FID is used. The use of a more highly sensitive detector would enable the determination of S^{2-} or I^- at trace levels.

We have investigated the GC determination of S^{2-} at trace levels by use of ethylation with diethyl sulphate and a flame photometric detector (FPD), and the results will be reported in the near future[14]. Iodide at trace levels can also be determined by this derivatization and electron capture GC. Work is currently being undertaken to apply this method to the simultaneous determination of I^- and Br^- at trace levels by using an electron-capture detector (ECD).

Phenol and *n*-valeric acid are methylated or ethylated almost quantitatively in the presence of 18-crown-6. In general, methyl or ethyl derivatives of phenols and carboxylic acids have no substituents which respond to highly sensitive detectors (*e.g.*, ECD, FPD, flame thermionic detector, etc.). This method, therefore, needs a pre-concentration step in order to determine phenols or carboxylic acids at trace levels. Because certain kinds of phenols, such as nitrophenols and chlorinated phenols frequently found in the environment, have substituents yielding high ECD responses, their ethyl derivatives should be detectable at trace levels by GC–ECD.

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GAS CHROMATOGRAPHY OF CYCLIC AMINO ACID DERIVATIVES

A USEFUL ALTERNATIVE TO ESTERIFICATION PROCEDURES

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SUMMARY

The use of 1,3-dichlorotetrafluoroacetone in combination with reactive anhydrides such as heptafluorobutyric anhydride provides a useful, quick and sensitive method of analysing the twenty protein amino acids by gas chromatography. The chemical treatment proceeds under very mild conditions without the necessity of heat so that both amides, glutamine and asparagine, are preserved and amenable to chromatographic estimation. An ordinary column (2 m) packed with OV-17 stationary phase gave excellent separations of the derivatized compounds in the temperature range of 75–230°C within 20 min. A second short column is required for complete elution of histidine, tryptophan and cystine.

INTRODUCTION

A considerable number of papers dealing with gas chromatographic (GC) or high-performance liquid chromatographic (HPLC) separation of amino acids have appeared over the last few years. While the popularity of GC analysis apparently reached its peak in the last decade, when the numerous previous investigations[1] led to several successful alternative procedures[2–4], in the 1980's the emphasis seems to be turning toward the HPLC technique, with which it is possible to analyse amino acids in their free forms[5–7]. However, with the exception of classical ion-exchange chromatography[8,9], which enables only a moderate acceleration of the separation process because of the limited compressibility of the column packing, and, perhaps, ligand-exchange chromatography[7], the most frequently used alkylated silica supports (reversed-phase, RP) are not so suitable for the separation of highly polar compounds. Thus, in HPLC, as in GC, complex mixtures of amino acids are often separated after conversion of the substances into one of several derivatives.

Precolumn derivatization reduces the chromatographic analysis time to about one-third of that required for ion-exchange amino acid analyzers and provides a greater sensitivity than postcolumn ninhydrin detection, especially if fluorescent compounds are formed. Reversed-phase HPLC is presently the most versatile method of protein sequencing, as the strongly UV-absorbing phenylthiohydantoin (PTH) deriv-

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atives of all protein amino acids can be resolved in a single chromatographic run[10–15] and without additional chemical treatment as it is necessary in the case of GC estimation[1]. However, the lengthy procedure required for the preparation of the PTH derivatives limits their usefulness for routine amino acid analysis.

Two other derivatization procedures may be employed for this purpose, *i.e.*, dansylation[16–18] (or dabsylation[19]) and treatment with *o*-phthaldialdehyde[20–22], a reaction which proceeds very rapidly (2–3 min) in aqueous solution. Strongly fluorescent derivatives are formed in each case and the reversed-phase HPLC separation can be achieved within 30 min. The short analysis time is comparable with present GC methods, but the quality of separation seems to be sacrificed for the high rate of analysis. Moreover, interferences occur due to fluorescent side-products[23], and the imino acids, proline and hydroxyproline, cannot be estimated with the second procedure.

As regards the GC estimation, the higher requirements of the derivatization process often prove to be the Achilles heel of this approach[1]. Whereas in the HPLC analysis not all the reactive groups in the amino acid molecule need to be masked, a complete derivatization is required for satisfactory GC analysis. Nearly 100 chemical treatments have been proposed for this purpose since 1956, when GC was first introduced for amino acid analysis[1]. The N(O,S)-perfluoroacyl amino acid alkyl esters have proved to be the most convenient derivatives enabling the quantitation of all twenty protein amino acids. This is essentially the result of the remarkable work of Gehrke and co-workers[2,24–26], who succeeded with the N(O,S)-trifluoroacetyl *n*-butyl esters first introduced by Zomzely *et al.*[27]. However, certain difficulties were encountered in the determination of histidine[26,28,29], so other procedures were developed with more stable derivatives[3,30,31]. One of the most popular volatile amino acid derivatives is the N(O,S)-heptafluorobutyryl isobutyl ester introduced by MacKenzie and Tenaschuk in 1974[4], which has extensively been studied[32–36] and has recently been used in biomedical applications[37,38].

Progress in capillary technology and especially in the synthesis of new optically active (chiral) phases[39,40] has resulted in an attractive procedure called enantiomer-labelling: the optical antipode to each L-amino acid serves as an internal standard after conversion of the antipodes into the N(O,S)-pentafluoropropionyl isopropyl esters[41–43]. Another novel approach involves determination of D-amino acids by means of deuterium labelling and selected ion monitoring[44]. Because of the special instrumentation required, these procedures are more expensive alternatives to the earlier ones.

Even when the GC analysis of acylated amino acid alkyl esters became routine some negative aspects of the esterification procedures persisted: (1) two incompatible reaction media with an intermediate evaporation step; (2) degradation of the amides, glutamine and asparagine, due to the HCl-catalyzer; (3) dissolution problems with amino acids in the higher alcohols; (4) employment of high reaction temperatures for both reaction steps; (5) stringent requirements of purity of the particular alcohol[45]. In order to avoid these difficulties, some years ago[46–49] the use of 1,3-dichlorotetrafluoroacetone (DCTFA) as a condensation agent was investigated. The results were promising as even the most problematic (with respect to GC estimation) amino acids, *i.e.*, histidine, tryptophan and cystine, could be analysed by this approach[50], and except of the dicarboxylic amino acids[51] even their amides could be preserved[52]. However,

problems were encountered in generalizing the reaction conditions as the various side-chain reactive groups require different chemical treatments[53]. It is now possible to so modify the conditions that all twenty amino acids can be successfully derivatized and quantitatively analysed within 1 h.

EXPERIMENTAL

Reagents and materials

DCTFA was purchased from Fluka (Buchs, Switzerland), HFBA (heptafluorobutyric anhydride) from Pierce Eurochemie (Rotterdam, The Netherlands) and methyl chloroformate from E. Merck (Darmstadt, G.F.R.). Before and after some months of use, the DCTFA was treated with phosphorus pentoxide and distilled (b.p. 45°C). The stock solutions of the reagents were kept in a refrigerator; when required, small amounts (up to 1 ml) were placed in glass vials capped with Mininert valves (Pierce) and kept at room temperature.

The organic solvents, *i.e.*, acetonitrile, pyridine, benzene, dichloromethane, methanol, heptane and petroleum ether (b.p. 60–80°C and 40–60°C), were obtained in the best available quality from Lachema (Brno, Czechoslovakia) and E. Merck. The petroleum ether was redistilled before use, and the pyridine treated with KOH and distilled after some months of use. The mixed solvents, *i.e.*, acetonitrile–pyridine (5:1), benzene–methanol (24:1) and petroleum ether (b.p. 60–80°C)–dichloromethane–methyl chloroformate (200:100:1), were prepared daily in the required amounts (1–10 ml) and kept in Reactivials with the Mininert-valve closures. For application of reagents and the organic solvents, syringes of 50–1000 μl in volume were employed.

Amino acids of grade A quality were supplied by Calbiochem (Lucerne, Switzerland) and E. Merck. The two internal standards, α-aminocaprylic acid and diaminopimelic acid, were purchased from Sigma (St. Louis, MO, U.S.A.). Equimolar mixtures of all protein amino acids, including hydroxyproline, ornithine and S-methylcysteine, were prepared by dissolving the amino acids in 0.1 *M* HCl or water (asparagine, glutamine and tryptophan) to give 10–100 nmoles of each amino acid in 10 μl of the aqueous medium. Approximately 1 *M* aqueous solutions of sodium carbonate (5 g per 45 ml) and hydrochloric acid (dilution 1:11) were prepared monthly from distilled water and chemicals of p.a. quality.

The round-bottomed reaction vials, (height *ca.* 50 mm, capacity *ca.* 2 ml) employed for derivatizations and subsequent extractions were made from 12 mm O.D. glass tubes with ground glass joints 12.5/15 mm. Solid glass or bottom-closed stoppers were used. The reaction tubes were silanized by a treatment with dichlorodimethylsilane in toluene (1:10) for 30 min, and washed subsequently with methanol and acetone.

GC

Commercial low-bleed silicone septa were employed. The bleeding was suppressed further by refluxing in acetone for 6 h and subsequent conditioning at 140°C in a chromatographic oven for 60 h (over the weekend).

The following chromatographic materials were used for the analyses: silanized diatomaceous earth supports, Chromosorb W and G of HP or AW DMCS grade (80–100 mesh); polyorganosiloxane phases, *i.e.*, the methylphenylsilicone fluid OV-17

or SP-2250 and the methylsilicone gum SE-30 or OV-1. These materials were obtained from Applied Science Europe (Oud-Beijerland, The Netherlands) and Supelco (Crans, Switzerland), and coating of a particular support with a phase was performed by the usual rotary evaporator method. The coated supports were then placed in the corresponding analytical columns, the column inlets were filled with silanized glass wool (5–6 cm) and the packings were conditioned under nitrogen (15 ml/min) at 300°C (2°/min linear increase from 60°C) overnight while maintaining the injector port temperature at 250°C.

A dual-column dual-FID Hewlett-Packard 5730 A gas chromatograph connected to a computing integrator 3380 A was employed for the linear temperature-programmed analysis (8°/min increase, injector and detector temperatures 200 and 250°C) of the cyclic amino acid derivatives. Two glass analytical columns (2 mm I.D.) of lengths 2 m (A, filled with 3% OV-17 or SP-2250 on Chromosorb W) and 0.5–0.6 m (B, filled with 1.5% SE-30 or OV-1 on Chromosorb G), operating in the temperature ranges 75–230°C (A) and 150–200°C (B) under nitrogen flow-rates of 20 ml/min (measured at 200°C with column A) and 30 ml/min, were required for a successful determination. As an alternative packing for column A, 3% OV-17–OV-22 (3:1) on Supelcoport (80–100 mesh) can be used. When used as a combustion suporter for the hydrogen flame (30 ml/min), oxygen (60 ml/min) was found to afford a doubled response of the halogen-rich molecules as compared with air (240 ml/min).

Procedure

Condensation. To the dry residue of amino acids (less than 0.3–0.4 mg in total), 70 μl of solvent (acetonitrile–pyridine, 5:1) and 30 μl DCTFA were added and the stoppered sample was held at room temperature for 15 min.

Acylation. To the condensation medium, 100 μl of benzene–methanol (24:1) and two 15-μl volumes of HFBA (approximate time interval, 1–2 sec) were added with continuous gentle mixing. The sample was left to stand for at least 30 sec.

Extraction. A 500-μl volume of extraction medium [petroleum ether (b.p. 60–80°C)–dichloromethane–methyl chloroformate, 200:100:1] followed by 400 μl of 1 *M* sodium carbonate solution were added to the sample and the mixture was shaken until the white precipitation in the organic phase had disappeared (usually after 5–10 sec). The lower aqueous solution was then removed with a Pasteur pipette joined to a microscrew on a fixed stand, and the remaining solution was shaken with 400 μl of 1 *M* hydrochloric acid until the opalescent organic phase had become clear (10–15 sec) and, finally, with 400 μl water. After removal of the aqueous layer, the organic extract was transferred into another vial and reduced in volume to a small drop (10–20 μl) at room or slightly elevated (less than 35°C) temperature using a current of nitrogen (slight moving of the surface of the liquid was decisive for setting of the gas flow). The last drop was then evaporated by manually rotating the vial along its longitudinal axis, followed by addition and evaporation of a small drop (20–30 μl) of light petroleum. This process is necessary and critical, because losses of the simplest amino acids occur if the organic extract is evaporated completely under the gas stream.

Additive acylation. To the residue of the amino acid derivatives were added 50–100 μl *n*-heptane and an aliquot was taken for GC analysis on column B. A drop of HFBA (3–5 μl) was then added to the sample and after heating at 80°C for 2–3 min an aliquot (2–3 μl) was injected on column A.

Comments on the procedure

(1) An effective extraction can be achieved by quickly striking (5–7 times per sec) the tube bottom against a pad on a table. The tubes should be closed tightly. The aqueous phases should be completely removed with the Pasteur pipette, even if a drop of the organic phase is also taken.

(2) The organic extract must be poured into another tube as a thin layer of water firmly adheres to the walls of the reaction tube. Before doing this the ground glass joint should be wiped with a piece of cotton-wool.

(3) Evaporation should be followed visually and carefully at the end. The vial was placed on a thin pile of soft papers on the surface of a sand-bath heated to 80°C. Cooling of the vial, caused by the rapid evaporation of the dichloromethane, was then compensated for by a slight temperature elevation (about 30–35°C) at the bottom of the vial.

(4) If a drop of water appears in the tube after evaporation of the organic phase it should be removed, before the heptane addition, by contacting it with a piece of blotting-paper.

(5) The silanized reaction tubes can be used continuously, provided they are washed with acetone after each derivatization, for 2–3 weeks. If during extraction some drops of water adhere to the walls, the silanization should be repeated.

RESULTS AND DISCUSSION

The separation of the protein amino acids, along with hydroxyproline, S-methylcysteine and ornithine and two internal standards, is shown in Fig. 1. As in previous cases[50,53], three protein amino acids cannot be eluted from column A because of their strong adsorption to the column packing. Another type of column material, together with a shorter column resulted in the successful analysis of these compounds[50]. Since that time, Chromosorb G has been found to be a more convenient support material because even the N^{im}-methoxycarbonyloxazolidinone of histidine could be eluted completly from column B. Thus, this column packing is recommended for analysis of histidine, tryptophan and cystine oxazolidinones.

The analysis of all the other protein amino acids was achieved on 2-m column with a milder polar silicone phase. Chromosorb W HP proved to be the best of the three high quality diatomaceous supports tested (plus Supelcoport and Gas-Chrom Q), as mere coating with the OV-17 phase afforded a satisfactory resolution of the pair proline–hydroxyproline. If Supelcoport is used instead of Chromosorb, an enhancement of the phase polarity (addition of OV-22) is required as stated in the Experimental. Broadening of peaks occurred when Gas-Chrom Q was used.

The influence of phase polarity on the retention behaviour of the derivatives is shown in Fig. 2. Even on the least polar methylsilicone phase (OV-101) the pair leucine–isoleucine is separated completely, while threonine–serine (together with leucine) and some other amino acids are co-eluted. A high-resolution capillary column could be helpful in separating these compounds. As the phenyl group content of the phase increases the separation of alanine–glycine and threonine–serine improves and all the hydroxyl-containing amino acids, together with aspartic acid, exhibit an almost linear decrease in retention times. The best separation of all the compounds is achieved on the OV-17 phase; an improved separation of proline–hydroxyproline is possible by mixing with OV-22, as recommended for Supelcoport.

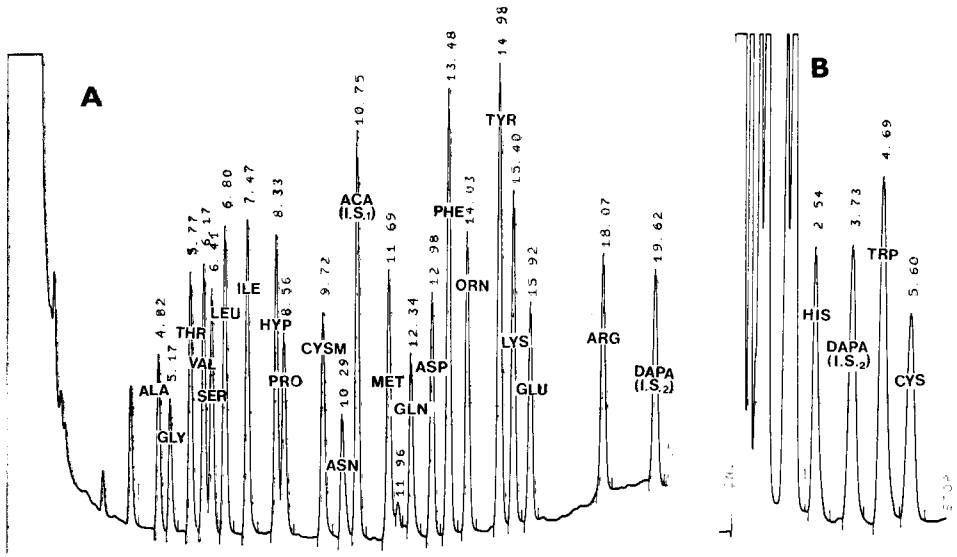


Fig. 1. GC analysis of (N,O)-heptafluorobutyryl amino acid oxazolidinones after derivatization of an equimolar mixture containing 50 nmoles of each amino acid. The brackets around N,O mean that only the reactive side-chain groups are acylated. Temperature ranges: 75–230°C for column A [2 m, 3% OV-17 on Chromosorb W HP (80–100 mesh)] and 150–200°C for column B [0.5 m, 1.5% SE-30 on Chromosorb G HP (80–100 mesh)]. Each peak represents 1 nmole of amino acid. Attenuation: 4×10^{-10} A. ACA = α-aminocaprylic acid; DAPA = diaminopimelic acid.

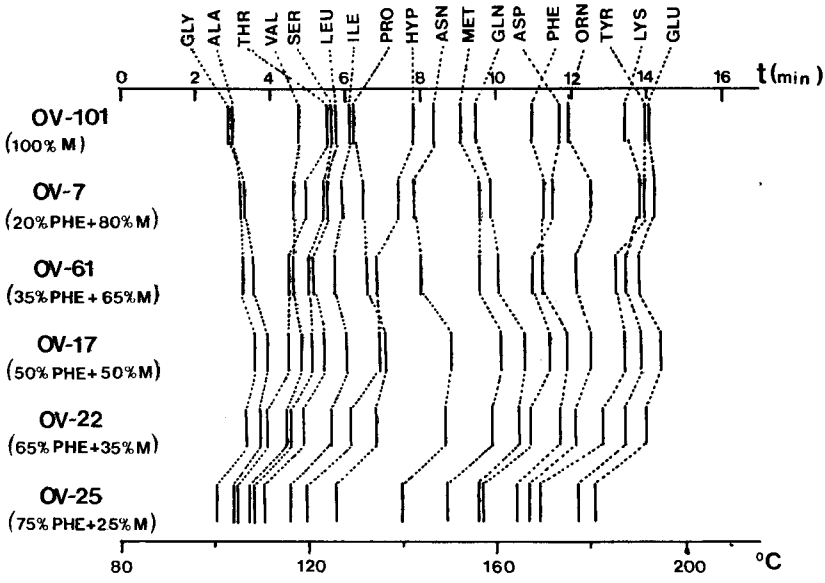
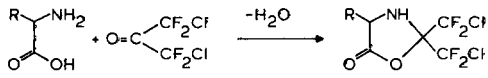


Fig. 2. Retention behaviour of (N,O)-HFB oxazolidinones of eighteen amino acids in a column (2 m × 2 mm) packed with 3% of the particular phase on Chromosorb W HP (80–100 mesh). Temperature range: 80–240°C (8°/min). Carrier gas flow-rate: 25 ml/min. PHE = phenyl; M = methyl.

The retention behaviour of threonine and serine in the analytical column A is influenced by the carrier gas flow-rate and initial temperature: higher flow-rates result in higher retention times for these two compounds; a higher initial temperature leads to the opposite behaviour. However, the separation of the two imino acids is worse under such conditions, as hydroxyproline exhibits higher retention times and tends to merge in proline.

Instead of HFBA, TFAA (trifluoroacetic anhydride) can be used as the acylation agent. The TFAA-treated oxazolidinones have lower retention times only on the phases with lower contents of phenyl groups, while on OV-17 these derivatives are eluted later than the corresponding HFBA-treated forms[53]. Any of the phases studied enabled a complete separation of the TFAA-treated oxazolidinones, so that the employment of HFBA was preferred in general.

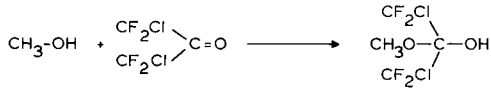
The described procedure is a result of earlier attempts[50–53] to find a chemical treatment that would deal successfully with all of the reactive side-chain groups in a mixture of the protein amino acids. The conversion of free amino acids or their hydrochloride salts into the cyclic oxazolidinones was shown to be dependent on the presence of a convenient condensation medium as the amino acids themselves were not soluble in DCTFA alone. Acetonitrile, in combination with pyridine as a catalyst, proved to be the best medium for this purpose as the presence of the base generally improved the dissolving ability of acetonitrile and the dissolved portion was simultaneously the converted one[46–48]:

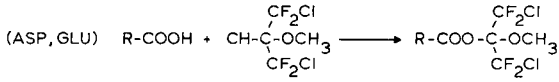


As the rate of dissolution is important, a limited amount of a material should be treated as in the Experimental. The pyridine concentration influences the derivative yields for some amino acids[51,53]. By enhancement of its amount in the solvent, the dissolution of the amino acid residue and cyclization of the two imino acids is more rapid; on the other hand, a progressive yield decline of threonine and serine is accelerated[53]. The selected reaction conditions and condensation time of 15 min represent a compromise in respect of the cyclization of proline: the formation of the cyclic form (retention time 8.56 min) was stimulated even when a small portion remained uncyclized and underwent a second reaction with HFBA to give a side-product (small peak behind methionine with a retention time of 11.96 min, see Fig. 1). Provided the amino acid sample lacks proline, a condensation time of 5 min is sufficient. Oxazolidinones of simple amino acids can be analysed by GC[53] as the polarity of the hydrogen atom in the ring is suppressed by the adjacent halogen atoms to such an extent that this hydrogen cannot be removed even by strong acylating agents[46–48].

A further chemical treatment is required for amino acids with reactive side-chain groups. When only the anhydride was employed, difficulties due to derivatization of the second carboxyl group in the molecules of aspartic and glutamic amino acids were encountered[51]. This problem was solved by addition of a definite amount of methanol to a condensation medium of lower polarity (mixed solvent of benzene in acetonitrile[51]) resulting in conversion of DCTFA into a secondary alcohol, which acts as the specific esterification agent of the additional carboxyl groups. Esterifi-

cation together with acylation of the reactive side-chain groups proceeds readily after addition of the reactive anhdride:

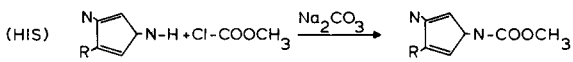




An acylation time of at least 30 sec is required for a full response of glutamine. The two-step HFBA addition improves the results for dicarboxylic amino acids; and the total volume of 30 μl proved to be sufficient for an effective treatment of asparagine. The co-addition of benzene with the alcohol before the HFBA treatment helps to lower the polarity to the extent that the previously recommended combined condensation medium is unnecessary[50–53]. It is a positive factor because acetonitrile alone is the best solvent for the condensation step. Under the chosen acylation conditions the indolyl group of tryptophan is not acylated.

Optimization of the procedure led to a lowering of the amount of methanol added to about 70% of that previously recommended[51,52] in order to get a full response for arginine. Under the specified conditions, the yields were about 10% for the dicarboxylic amino acids, 20% for glutamine and 5–7% for lysine and ornithine lower than the maximal ones[54]. The relative molar responses including the variation coefficients are given in Table I. For comparison with the absolute molar response values see ref. 54. The overall reproducibility averages 4%, the worst cases being glutamine and asparagine (8–9%) and also arginine (6–7%).

Direct evaporation of pyridine with the condensation medium leads to complete loss of the oxazolidinones of most protein amino acids. By means of a convenient extraction medium it was possible to remove the excess of the perhalogenated reagents by shaking with aqueous carbonate solution, and the pyridine salts by shaking with hydrochloric acid, while retaining the derivatives in an organic phase volatile enough to prevent evaporation losses of the simplest amino acids. The use of the higher boiling instead of the lower boiling[50–53] petroleum fraction (b.p. 60–80°C) led to more reproducible extraction yields, to which also the presence of benzene and a higher content of dichloromethane in the phase contribute. The small amount of methyl chloroformate in the extraction medium proved to be an effective means for converting the imidazolyl group in the histidine oxazolidinone to a stable analysable form:



This conversion proceeds instantaneously during shaking of the organic phase with the first alkaline wash, while the free indolyl group of tryptophan as well as the other derivatives are not attacked.

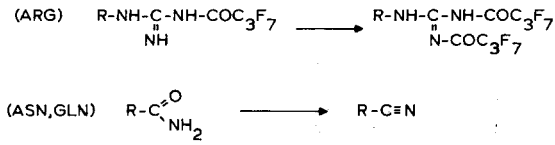
TABLE I

MOLAR RESPONSES OF AMINO ACID OXAZOLIDINONES RELATIVE TO α-AMINOCAPRYLIC ACID OR TO DIAMINOPIMELIC ACID (HIS, TRP AND CYS ONLY).

Values shown are the means for ten individually prepared samples of an amino acid calibration mixture (total amino acid content less than 0.3 mg), together with standard deviations (S.D.) and coefficients of variation (C.V.). The amino acids are ordered according to their elution from the analytical column(s).

Amino acid	*Abbrev.*	$\bar{x}$	*S.D.*	*C.V. (%)*
Alanine	ALA	0.41	0.016	3.93
Glycine	GLY	0.30	0.016	5.25
Threonine	THR	0.65	0.018	2.72
Valine	VAL	0.64	0.029	4.57
Serine	SER	0.60	0.025	4.16
Leucine	LEU	0.76	0.025	3.28
Isoleucine	ILE	0.78	0.008	1.06
Hydroxyproline	HYP	0.73	0.041	5.75
Proline	PRO	0.54	0.020	3.63
S-Methylcysteine	CYSM	0.59	0.012	2.05
Asparagine	ASN	0.36	0.031	8.61
Methionine	MET	0.70	0.030	4.24
Glutamine	GLN	0.43	0.039	8.83
Aspartic acid	ASP	0.65	0.034	5.18
Phenylalanine	PHE	1.12	0.008	0.73
Ornithine	ORN	0.76	0.031	4.08
Tyrosine	TYR	1.17	0.032	2.73
Lysine	LYS	0.84	0.029	3.44
Glutamic acid	GLU	0.59	0.020	3.38
Arginine	ARG	0.72	0.047	6.55
Histidine	HIS	0.80	0.038	4.75
Tryptophan	TRP	1.52	0.031	2.04
Cystine	CYS	0.84	0.029	3.44

Three amino acids, *i.e.*, arginine, glutamine and asparagine, cannot be analysed without additional acylation in heptane at a higher temperature, during which the guanidino group of arginine is acylated fully and the amides are converted into the nitriles (see ref. 55 for mass spectra of amino acid oxazolidinones):



As any change in the asparagine response occurred when the heating was prolonged from 3 to 10 min —unlike an earlier report[52]— short heat treatment is recommended in general. Also tryptophan undergoes a partial acylation (the derivative with the acylated indolyl group being eluted closely after arginine on column A) and the response of histidine declines due to the heat treatment. Analysis in heptane only on column B is therefore preferred. If the above three amino acids are not to be analysed, the other amino acid derivatives can be injected in column A in heptane only (Fig. 3). However,

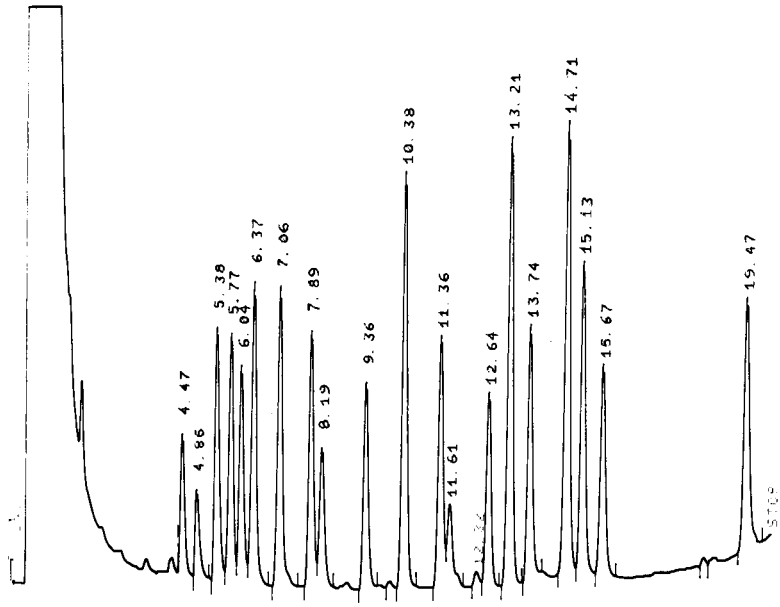


Fig. 3. Amino acid derivatives of the same equimolar mixture as in Fig. 1 analysed on column A [alternative packing: 3% OV-17–OV-22 (3:1) on Supelcoport (80–100 mesh)] in heptane only. Time of condensation shortened to 5 min (see the augmented peak for the second proline derivative, retention time 11,61 min). Nitrogen flow-fate: 25 ml/min. Temperature range: 75–230°C (8°/min).

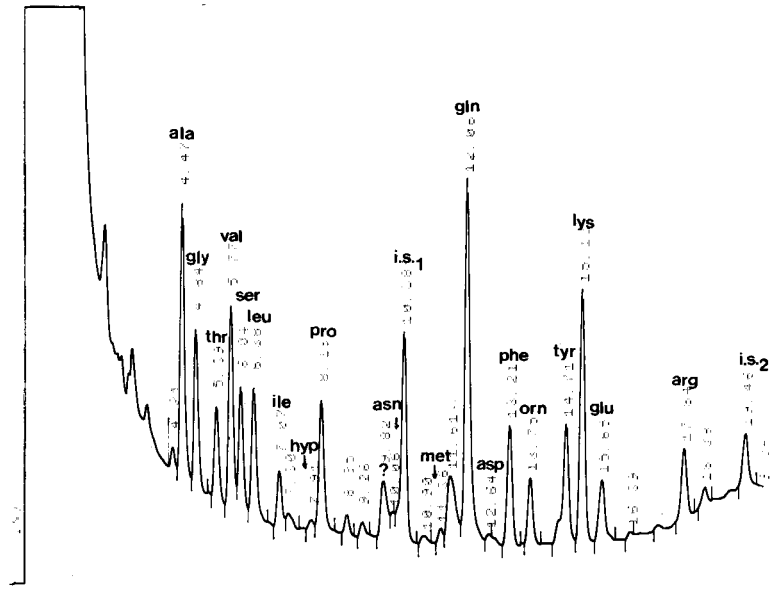


Fig. 4. Analysis of free amino acids in human control serum (100 μl taken off to the clean-up ion-exchange procedure[56]). Internal standards added in amounts of 10 nmoles. GC conditions as in Fig. 3.

as in an earlier study[53], a partial adsorption (10–20%) of the N- and O-HFB acylated forms takes place.

The procedure was especially developed for routine amino acid analysis in minute amounts of biological materials. For this purpose, a pretreatment of the starting material, often involving a clean-up isolation step on ion-exchangers, is carried out prior to derivatization. It was found that the isolation of free amino acids from physiological fluids without precipitation of the protein[3,30] was preferred to the precipitating techniques[2,38] even when the yields for some amino acids were not as expected with the recommended cation-exchange material. By modifying the technique to give a better reproducibility[56], it was possible routinely to analyse free plasma amino acids in 50–100 μl of serum. Moreover, in comparison with the esterification procedures, this derivatization mode proved to be far less sensitive to the presence of interfering material (salts, protein, etc.) because even its one to two order higher excess did not disturb the reaction course. Some examples of biological applications are shown in Figs. 4 and 5.

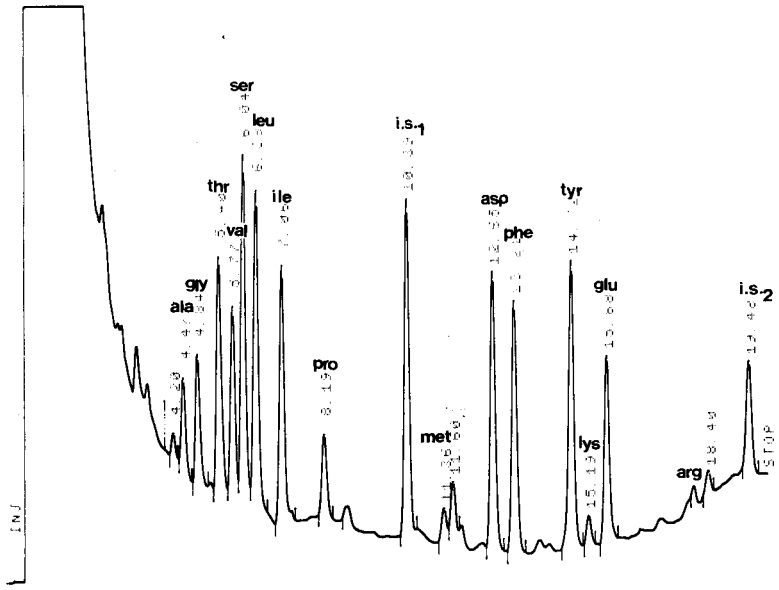


Fig. 5. Analysis of amino acids in acid hydrolysate of pepsin (initial amount hydrolysed in 6 *M* HCl at 110°C for 20 h was 20 μg). Amounts of internal standards added and GC conditions as in Figs. 3 and 4.

The application of this derivatization approach to the analysis of amino acid optical antipodes was also investigated. GC analysis of the enantiomeric amino acid oxazolidinones in a capillary column coated with the recommended chiral phase[39] confirmed a theoretical assumption that because of the planar structure of the oxazolidinone ring it should not be possible to resolve D- and L-antipodes[57].

The electron capture detector (ECD) is highly sensitive to the perhalogenated derivatives. By means of this method picogram amounts could be analysed even when problems were encountered, *e.g.*, adsorption of some perhalogenated forms on the

column packing. The GC-ECD temperature-programmed analysis of cyclic amino acid derivatives will be reported subsequently[58].

CONCLUSIONS

Compared with the esterification procedures[2–4,24–30,32–38], the formation of cyclic derivatives by treatment of amino acids with DCTFA has the following advantages: (1) both characteristic groups, the α-amino and the carboxyl, are blocked by action of a single reagent; (2) the aprotic condensation medium allows one to perform the subsequent acylation step in the same milieu; (3) both reactions proceed very smoothly at room temperature and the acylation is instantaneous; (4) glutamine and asparagine are preserved; (5) histidine, arginine, tryptophan and cystine can be estimated with a good reproducibility; (6) the total time of the chemical treatment including extraction is about half of that previously required; (7) the derivatives show an augmented ECD response and analysis in the picogram range is possible. The only drawback is the requirement for a separate column for the determination of histidine, tryptophan and cystine.

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DISTRIBUTION OF ORGANIC COMPOUNDS ADSORBED ON SIZE-FRACTIONATED MUNICIPAL INCINERATOR FLY-ASH PARTICLES

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SUMMARY

Two different samples of fly-ash have been separated into different size fractions by manual sieving using standard screens. Both samples showed the same trends of relative distribution of organic compounds on fractions. Average particle sizes for the various fractions were: 30 μm, 80 μm, 125 μm, 200 μm, 550 μm and particles > 850 μm. Each fraction was extracted with benzene by ultrasonic agitation, and the concentrated extracts analyzed by gas chromatography and gas chromatography–mass spectrometry for total organic compounds, *n*-alkanes, phthalates, selected polynuclear aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins. The largest concentrations of tetra- and pentachlorodibenzo-*p*-dioxins occurred on the larger (550 μm) particles while the 30-μm particles had greater relative concentrations of octachlorodibenzo-*p*-dioxin. The largest (> 850 μm) sized fraction consisted of two distinctly different types of particles. One of these types was light, black-ash particles such as are obtained from the combustion of newsprint. These light ash particles had high concentrations of total organic compounds and tetrachlorinated dioxins, relative to the other size fractions. Printed words could still be discerned on some of these ash particles, indicating that these particles escaped complete combustion.

INTRODUCTION

The disposal of municipal refuse by incineration is widely practised. Since it is possible to generate large amounts of usable energy from the incineration process, its use and importance will increase. However, it has been shown that the electrostatically precipitated fly-ash residue formed during incineration contains hazardous organic compounds such as the polynuclear aromatic hydrocarbons (PAHs) and polychlorinated dibenzo-*p*-dioxins (PCCDs)[1–4]. The high toxicity of the PCDDs has prompted interest in analysis of the organic compounds found in incinerator fly-ash at trace levels. Of particular interest is the distribution of various compounds on different particle size fractions, since particles of less than 3-μm diameter are respirable by humans and therefore present a special health hazard. In addition it has

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been reported that the fly-ash particles contained in the stack gases contain about ten times the PCDD concentration of the precipitated fly-ash[5,6]. It has been proposed that these particles are the smaller ones with higher surface area to account for this observation. No data are available on their size distrubiton.

Work on the size distribution of pollutants on fly-ash has been for the determination of various trace and ultra-trace elements in fly-ash sampled from coal-burning steam plants[7–10]. Recent work on atmospheric particulate matter has shown that the highest concentrations of PAHs are found on the respirable size fraction of the collected particulate matter[11–14]. One study has reported similar results for PAHs on different size fractions of particulate matter from a coke oven emission source[15].

These previous studies examined particles that were collected and fractionated by cascade impactor devices. None of the studies has investigated bulk samples that contain a broad range of particle sizes, from the respirable size fraction to those in excess of 800 μm diameter. Knowledge of the size distribution of the bulk fly-ash will be helpful in the study of the routes of distribution into the environment of organics sorbed onto these particles. In particular, we wanted to develop data that would indicate the relative concentration of PCDDs on large *versus* small particles to test the hypothesis that small particles contain greater concentrations of organics. In this study a large fly-ash sample is separated into different size fractions by hand-sieving. Organic compounds are extracted by benzene using ultrasonic agitation and the extracts analyzed for organic compounds, including selected PAHs and the various PCDD isomers, by gas chromatography (GC) and combined GC–mass spectrometry (MS).

EXPERIMENTAL

Sample collection and storage

A large (324 g) grab-sample of fly-ash was taken from a southern Ontario municipal incinerator by Ontario Ministry of Environment personnel. The incinerator operating temperature was *ca.* 900°C and electrostatic precipitation was used. The sample was stored in a plastic bag in the dark at room temperature. After extraction, sample extracts were stored in a freezer at *ca.* −15°C. A second sample was obtained by compositing two smaller samples collected from the same incinerator on different days.

Fractionation of fly-ash

Six size fractions of fly-ash were obtained by using five Tyler sieves (W.S. Tyler, St. Catherines, Canada). The brass sieves had metal screens with openings of 850 μm, 250 μm, 150 μm, 106 μm and 63 μm. All sieves, including the top and bottom collector, were cleaned by ultrasnonic agitation with an aqueous solution of Alconox detergent for *ca.* 15 min. This was followed by rinsing with tap water, deionized water and methanol, and then air drying. Hand-sieving was performed and all fractions were stored in polypropylene containers equipped with polypropylene screw-caps that had first been rinsed with small portions of benzene, then air-dried. After sieving of the fly-ash, it was observed that the largest particle fraction (> 850 μm) consisted of two distinctly different types of particles. These two components of the large size fraction were partially separated by hand and were analyzed individually. Hand

separation of these larger particles was difficult and incomplete. However, each portion is estimated to have no more than 10% contamination from the other. One component of this fraction consisted of light, black ash particles. The other component was large agglomerate particles.

Sample extraction and concentration

In a previous study, fly-ash samples were extracted overnight by Soxhlet extraction apparatus[4]. However, fine particle fractions do not allow free solvent flow through the extraction thimble, therefore all extractions were performed using ultrasonic agitation[19]. Samples (10 g) were added to individual flasks with 100 ml of benzene and agitated in an ultrasonic bath for 1 h. Enough water was added to the bath to cover the portion of the flask that contained the fly-ash and benzene. The glass stoppers that were used to cap flasks during the extraction were occasionally loosened to prevent excessive pressure from developing. After 1 h of agitation, the fly ash was allowed to settle and the benzene was decanted into fresh flasks through porous glass frits. This procedure was repeated twice more, for 30 min each time, with 100 ml of fresh benzene added each time. After the third extraction cycle, the fly-ash was transferred to the glass frit and rinsed three times with 10-ml portions of fresh benzene. During each of the three extraction cycles, the water bath temperature increased from *ca.* 30 to 50°C.

The benzene from the three extraction cycles and rinsings was condensed to 2–3 ml by rotary evaporation under aspirator vacuum. This extract was transferred with three benzene rinsings to a 10-ml pear-shaped flask. After rotary evaporation to between 50 and 100 μl, concentrated extracts were transferred with three rinsings to a 1.0-ml reacti-vial equipped with screw-cap and PTFE liner. A final volume of 100 μl was achieved by blowing a stream of helium gas across the top of the vial. All glassware, including reacti-vials and transfer pipets, was cleaned by ultrasonic agitation for 30 min with Alconox detergent. After thorough rinsing with tap water and deionized water, the glassware was then placed in an oven at 300°C for a least 1 h. All equipment was allowed to cool to ambient temperature before use. Solvents were "distilled-in-glass" grade (Caledon Labs., Georgetown, Canada). Benzene solvent (300 ml) was carried through the entire process as a procedure blank.

Gas chromatographic analysis

A Hewlett-Packard 5830 GC with flame ionization detector (FID) was equipped with a 6 ft. × 2 mm I.D. glass column, packed with Aue packing[16]. A temperature program of 90°C initial temperature to 250°C final temperature at 4°C/min was employed for all sample extracts. Injection temperature was 250°C, detector temperature 275°C, and the helium carrier gas flow-rate 37 ml/min, measured at 90°C. A slope sensitivity of 0.1 mV/min was used for peak detection.

For calculation of retention index, a normal hydrocarbon standard mixture was analyzed periodically. Peak areas and retention times were punched onto computer cards, and retention indices were calculated by the Fortran program RICALC[17]. These retention index and peak area values were displayed as bar-graph plots using a Calcomp plotter by the program GCPLOT[17].

GC–MS analysis

Normal hydrocarbons, phthalate esters, selected PAHs and various PCDD isomers were analyzed by a Hewlett-Packard 5992 GC–MS–Calculator using the selected ion monitoring (SIM) technique. In this mode of operation, the quadrupole mass spectrometer was selectively tuned to each of six pre-selected ions for 166 msec dwell time on each ion. The ions monitored for the tetra- through octachlorinated dibenzo-*p*-dioxins were 321.9, 355.9, 389.8, and 459.7, respectively. Chromatographic conditions were as described previously. A 1,2,3,4-tetrachlorodibenzo-*p*-dioxin standard (1,2,3,4-TCDD) was used to quantitate the various tetrachlorinated dioxin isomers, assuming a relative response factor of unity. An octachlorodibenzo-*p*-dioxin (OCDD) standard was used to quantitate this compound. A third standard consisting of selected *n*-hydrocarbons, phthalates and PAHs was also analyzed for the quantitation of these compounds. The PAHs in this standard were biphenyl, fluorene, fluoranthene and benzo[*a*]pyrene.

The GC–MS system was also operated in the scanning mode employing user-developed software[18]. Spectra were scanned from 500 to 40 a.m.u. at 330 a.m.u./sec. A high mass peak threshold was used so that low-level background peaks would be rejected. Spectra taken at the lowest point of the valleys between consecutive peaks were saved for later background subtraction.

RESULTS AND DISCUSSION

Gas chromatographic results

Two samples of fly-ash from the same incinerator but taken on different days were size-fractionated and the organic compounds extracted as described in the Experimental section. The ranges of particle sizes for the different fractions, together with the percentage of total sample weight contained in each fraction, are presented in Table I. Relative concentrations of organic compounds were estimated from GC–FID data. The two fractionated fly-ash samples are referred to as sample A or B. Sample A is the single large sample and sample B is a composite of two smaller

TABLE I

FLY-ASH FRACTIONS OBTAINED BY MANUAL SIEVING

Particle size range (μm)	*Average particle size (μm)*	*Percentage of total sample weight*		*Relative concentration of organic compounds*	
		A	*B*	*A*	*B*
<63	30	37.5	42.7	100	14
63–106	80	26.9	16.8	66	2
106–150	125	9.5	11.9	24	5
150–250	200	8.2	11.5	24	4
250–850	550	14.3	12.1	57	35
Light ash (>850)	–	1.6	2.0	78	100
Agglomerate particles (>850)	–	2.1	3.0	3	17

samples. For both A and B, *ca.* 60 % of the total sample weight consisted of particles smaller than 106 μm. Fig. 1 is a photographic display of the various sized fractions from sample A. For comparison, a sample of fly-ash before size fractionation and a sample of bottom ash from the incinerator furnace grates are also shown. Bottom ash is the residue that remains at the bottom of the furnace grates and is only indirectly related to particles collected by the electrostatic precipitator. The clear lumps of material in the bottom ash are pieces of glass.

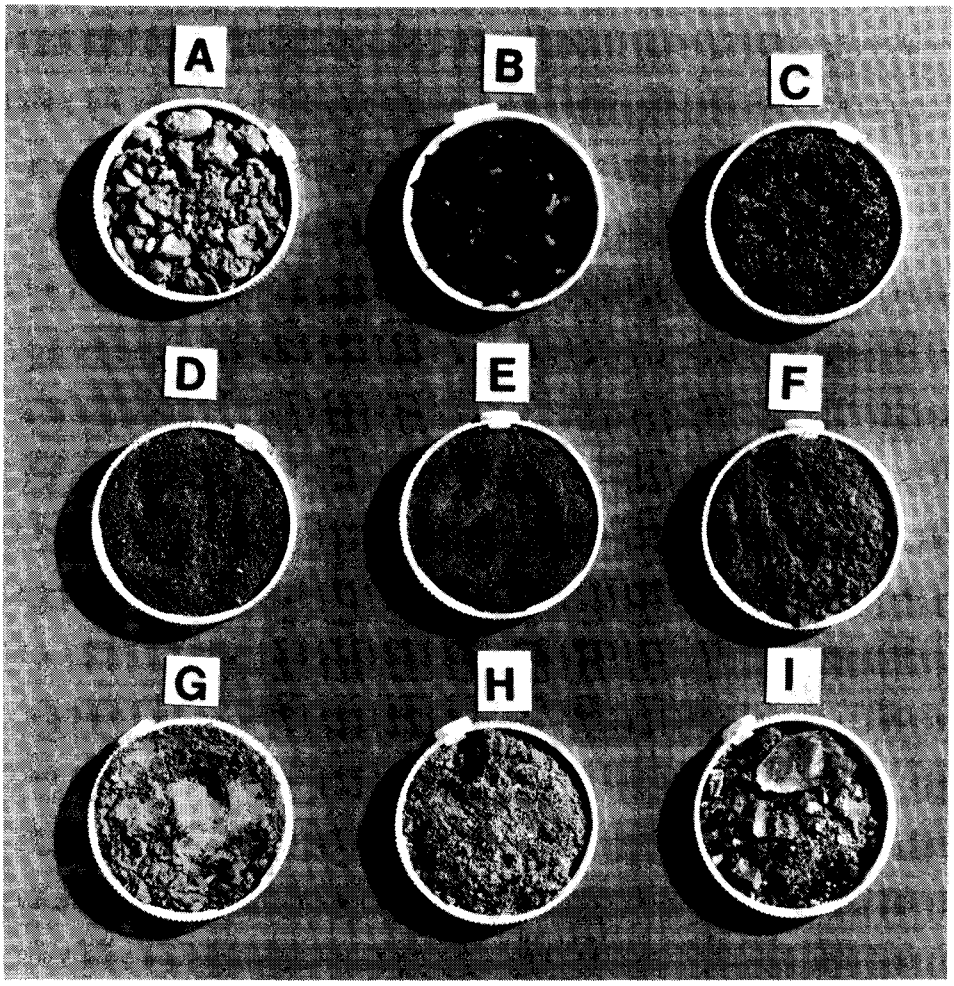

Fig. 1. Size fractions obtained from manual sieving of Ontario fly-ash. A, Agglomerate particles > 850 μm; B, black ash > 850 μm; C, 550 μm particles; D, 200 μm particles; E, 125 μm particles; F, 80 μm particles; G, 30 μm particles; H, original fly-ash; I, residue from bottom of furnace grates (not from electrostatic precipitator).

In the separated fly-ash fractions of sample A, the large, light, black ash particles have the appearance of the ash formed from the combustion of newsprint. Printed words could still be observed on some of these light ash particles, which indicates that they have spent most of their residence time in the cool parts of the

combustion process. The other large particle fraction (agglomerates) consisted of roughly spherical agglomerate particles, which were much heavier than the light ash particles. These could easily be broken into smaller particles, However, this was not done until just prior to extraction.

GC–FID results obtained for the various size fractions of sample A are displayed by the program GCPLOT in Fig. 2. Peak areas were corrected for extraction weights and injection volumes before plotting. Results for the largest size fraction were not included in this plot since particle size boundaries for this fraction are not defined. For convenience, each size fraction other than the largest (particles > 850 μm) is referred to by its average particle size, which is estimated as the approximate

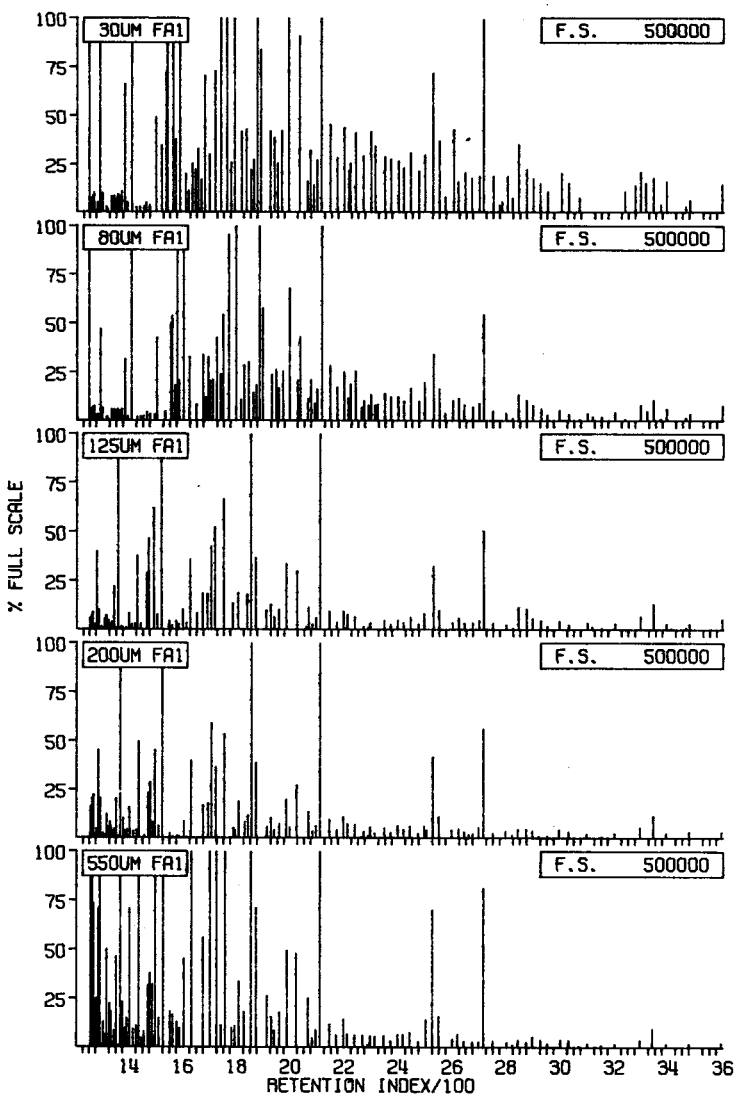

Fig. 2. Comparison of GC–FID data of size fraction extract condensates.

middle value between boundary screen sizes. In the largest size fraction the black ash particles have a high concentration of total organic compounds relative to the other size fractions for both samples. In Fig. 2 it can be seen that many of the major peaks are common to all of the fractions, although large concentration differences are evident GC data primarily show that large differences in the concentrations of individual components and total amount of organic compounds exist between the fractions of different particle size.

GC–MS analysis

The major components of the various extracts of different size fractions identified by GC–MS analysis for both samples are *n*-alkanes, phthalates, and chlorinated benzenes; many other compounds were present, but at relatively low concentrations. However, no differences were observed in the types of compound that were detected in the various size fractions.

Results of SIM analysis of extracts of the various size fractions for phthalates, *n*-hydrocarbons, and selected PAHs are given in Table II for sample A. These analyses were not performed for sample B. Dibutyl phthalate was the largest single component in all the size fractions analyzed. However, its concentration could only be estimated because the abundance of this compound was great anough to cause the mass spectrometer to go into a standby state during SIM analysis. This indicates that the concentration of dibutyl phthalate in each sample must be greater than the highest reported concentration of 3200 ng/g fly-ash for diethyl phthalate in Table II.

TABLE II

CONCENTRATIONS (ng/g) OF PHTHALATES, *n*-HYDROCARBONS AND PAHs EXTRACTED FROM DIFFERENT SIZE FRACTIONS OF SAMPLE A

	Average particle size (μm)					*Light ash (>850)*	*Agglomerate particles (>850)*	*Totals (ng/g)*
	30	*80*	*125*	*200*	*550*			
Diethyl phthalate	240	95	150	650	1200	3200	750	6300
Dioctyl phthalate	690	510	510	680	910	2800	330	6400
Biphenyl	43	35	33	160	240	700	55	1300
Fluorene	24	3.8	3.5	–	69	–	–	100
Anthracene*	16	4.0	2.9	2.9	–	–	–	26
fluoranthene	76	51	68	97	120	150	7.9	570
Pyrene	44	16	13	18	9.1	–	–	100
Normal alkanes	14,000	7900	5200	5400	11,000	5500	900	50,000
Totals	15,000	8600	5900	7000	14,000	12,000	2000	65,000

* Concentrations based on fluoranthene response with relative response factor of unity.

The largest concentrations of the various compounds that were determined were not always associated with the small size fraction as was expected. Comparing results for the 30–550-μm particles, it is observed that the 550-μm particles have the greatest concentration of diethyl phthalate, biphenyl, fluorene, and fluoranthene. The

30-μm particles have the largest concentration of anthracene and pyrene. Dioctyl phthalate and total *n*-hydrocarbons are distributed almost equally among all size fractions. There is some evidence in Table II that the smallest particles contain a greater concentration of higher-molecular-weight PAHs, while the large particles have a greater concentration of the low molecular weight PAHs.

Table III gives for the different size fractions the concentrations of various PCDDs determined by GC–MS–SIM. The first sample (A) presented in Table III is the same one for which earlier reported PAH results apply. Concentrations are reported in terms of nanograms per gram of each particular size fraction. To obtain the amount of PCDDs that would be obtained in a gram of non-size-fractionated fly-ash, each value in Table III must be multiplied by the appropriate weight fraction. Only the 1,2,3,4-TCDD and OCDD standards were available during this study. Values for the penta-, hexa-, and heptachlorinated dioxins were based on the response for 1,2,3,4-TCDD, with a relative response factor of unity. Although the actual response factors of these other dioxins were not measured, the relative amounts in the different size fractions of a particular group of isomers are as represented in Table III. No clear trend is apparent in the concentrations of penta- and hexachlorinated dioxins with respect to particle size, except that the 200-μm and 550-μm particles have about three times more of these dioxins than do the smaller particles (30-μm and 80-μm fractions). Heptachlorinated dioxins concentrations are more nearly uniform throughout all size fractions.

TABLE III

DIOXIN ISOMER DISTRIBUTION IN FLY-ASH SIZE FRACTIONS

Average particle size (μm)	*Weight fraction*	*Concentration of dioxin isomers (ng/g)**					*Total*
		Tetra	*Penta*	*Hexa*	*Hepta*	*Octa*	
Sample A							
30	0.37	2.2	3	3	3	9.5	20
80	0.27	2.0	2	3	3	4.5	10
125	0.09	3.9	4	6	4	6.9	30
200	0.08	7.0	8	10	6	8.2	40
550	0.14	12	10	8	3	3.1	40
>850 (light ash)	0.02	8.4	5	2	0.3	0.2	20
>850	0.02	2.0	2	2	2	8.2	20
Sample B							
30	0.43	2.9	3	3	2	5.0	20
80	0.17	4.3	5	4	1	2.9	20
125	0.12	7.5	9	6	2	4.8	30
200	0.11	8.2	8	5	1	2.0	20
550	0.12	15	10	4	1	0.7	30
>850 (light ash)	0.02	7.3	4	1	0.1	0.2	10
>850 (agglomerate particles)	0.03	4.3	6	5	3	11	30

* Concentrations of penta-, hexa-, and heptachlorinated dibenzo-*p*-dioxins are based on 1,2,3,4-TCDD standard with relative response factor of unity.

Fig. 3 shows the determined concentrations of OCDD and total TCDD with respect to each of the size fractions for both samples. For both samples, the highest concentration of TCDD was found on the larger (550 μm) particles. Conversely, a much lower TCDD concentration was found on the smallest (30 μm) size fraction. There is an almost uniform decrease in TCDD level with respect to particle size, which can be observed in Fig. 3. This trend is observed with both samples. A clear trend is not as evident in the concentration of OCDD, which was detected on each of the size fractions. However, the smallest concentration for OCDD in the well-defined size fractions was detected on the larger (550 μm) particles, while the largest concentration was in the smallest (30 μm) particles. These trends are observed for both replicate sample, though the samples were collected several months apart.

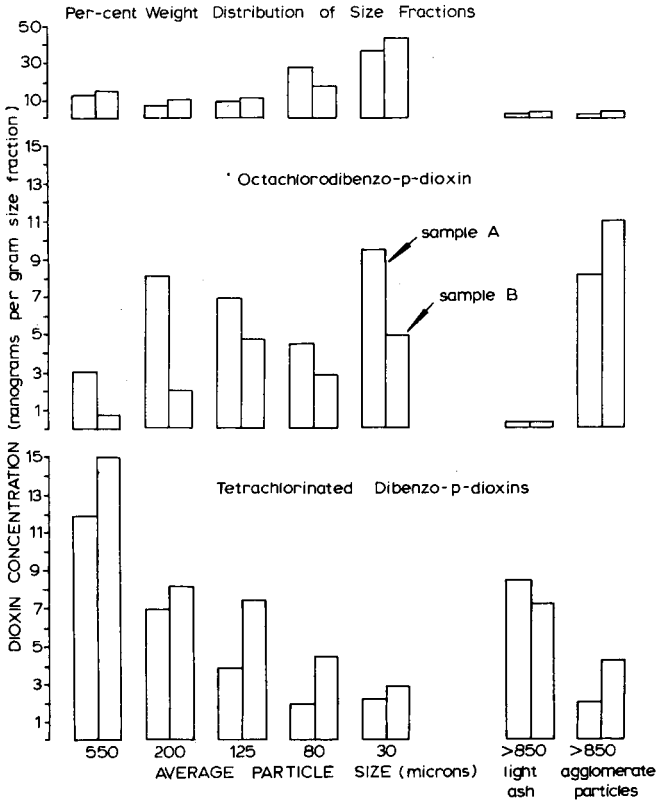

Fig. 3. Comparison of tetra- and octa chlorinated dioxin concentrations in different size fractions.

The largest (> 850 μm) particles, which were sub-divided into black ash particles and large agglomerate particles, also showed some definite trends. Very low levels of OCDD were detected on the black ash particles. However, large concentrations of OCDD were found on the large agglomerate particles, relative to the other size fractions. By contrast, the TCDD concentrations were greater in the ash particles than the agglomerate particles. Both samples studied showed the same trends. The black ash particles were the type of ash residue that remains after combustion of

books and newsprint. Because printed words could still be observed on some of these particles, a definite fraction of the fly-ash consists of particles that have escaped complete combustion. However, as can be seen from Fig. 3, the light ash particles represent a very low proportion by weight of the total fly-ash sample.

It has been reported by Stalling and co-workers[20,21] that recovery of 2,3,7,8-TCDD adsorbed on granular charcoal surfaces is difficult. The carbonaceous surfaces of the light ash particles analyzed in this study are not granular in nature as were the particles in Stalling's work. The ash particles were thin flakes having smooth surfaces; thus extraction of organics from these particles is more efficient than for extraction of organics from irregular granular particles with larger surface areas. Also, an exhaustive ultrasonic extraction was performed using three portions of fresh benzene for a total extraction time of 2 h. Furthermore, the 550-μm particles contained a much larger proportion of the light ash particles than did the 30-μm particles, yet the concentration of total TCDD was much greater for the larger particles than for the smaller particles. Extraction efficiencies for TCDD and OCDD should not vary greatly for different size fractions under the extraction methods employed in this study. Therefore, the very large concentration difference between total TCDD and OCDD on the light ash particles compared with the much smaller differences observed for any of the other fractions indicates that the ash particles were those that experienced very different incinerator conditions from the remainder of the fly-ash particles. This may also explain why the concentration of TCDD is much greater than that of OCDD on the 550-μm particles, since this fraction contained a larger proportion of the light ash than the other size fractions.

Although both samples that were size-fractionated showed the same trends with respect to the relative distributions of organic compounds, the validity of the results was checked further by re-analyzing the extracts of the size fractions of sample

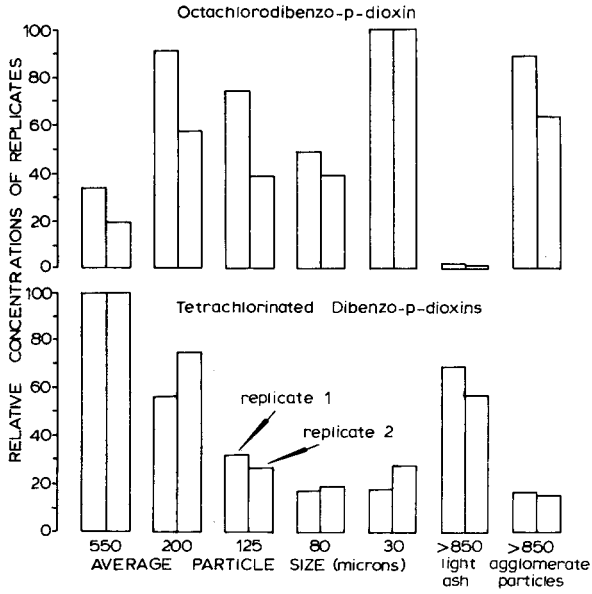

Fig. 4. Results of duplicate determinations of TCDD and OCDD.

A for PCDD by GC–MS, using the same conditions as reported previously. Fig. 4 shows the results of duplicate determinations of each size fraction of sample A for total TCDD and OCDD. Results are normalized to the largest concentration = 100% for TCDD and OCDD. Relative concentrations of TCDD and OCDD for the different size fractions are the same for duplicate determinations, although variations in the results for OCDD are larger than results for TCDD. The possibility of a trend towards enrichment of some compounds on the larger particles does not contradict findings of studies that show a higher concentration of organics on the respirable (< 10 μm) particles, since it is not known in this study what proportion of such particles are present in the 30-μm fraction. Also, other studies have not reported distributions of organics in the very large particles that were examined here. Although the finding of PCDDs in municipal incinerator fly-ash has now been well documented, the results of this study show that there is still much work to be performed in the analysis of this material and in correlating results to incinerator operating conditions.

ACKNOWLEDGEMENT

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETECTION OF MORPHINE BY FLUORESCENCE AFTER POST-COLUMN DERIVATISATION

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the fluorometric determination of morphine in biological samples has been developed. Column effluent is mixed with alkaline potassium ferricyanide to produce the fluorescent dimer pseudomorphine. The method provides higher chromatographic specificity and sensitivity than conventional high-performance liquid chromatography. Morphine can be detected at levels of 10 ng on column.

INTRODUCTION

Fluorescent derivatisation for enhanced detection and selectivity has been in use in chromatography for many years, and the availability of fluorescence detection systems for liquid chromatography systems has allowed the application of fluorescent derivatisation to that field. Derivatives may be formed before the sample is introduced (pre-column) or between the column outlet and the detector (post-column). Pre-column derivatisation is necessary if the reaction is slow, but post-column derivatisation is less likely to form artifacts, allows the simultaneous use of different detection systems, *e.g.* series ultraviolet (UV) detection and fluorescence detection, and does not alter the chromatographic properties of the compounds of interest. Post-column techniques have been extensively used for amino acid analyses[1,2] and several post-column methods have been developed for drug analyses[3–6].

Morphine (I, Table I) can be oxidized to the fluorescent dimer, pseudomorphine, XII, by alkaline potassium ferricyanide. This reaction has been extensively used for the detection of morphine in biological samples[7–9], and the reaction has been applied as a pre-column fluorescence derivatisation method for high-performance liquid chromatography (HPLC)[10]. This paper describes a post-column fluorescence derivatisation method suitable for the detection of morphine in biological samples. The technique is simple, involving the use of a second pump to deliver the derivatising reagent to a post-column mixing volume before passing the eluent through a filter fluorometer. It gives both improved sensitivity and selectivity over UV detection. The method is also compared to the pre-column derivatisation method for morphine described by Jane and Taylor[10].

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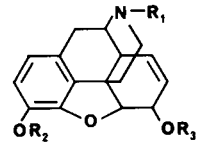
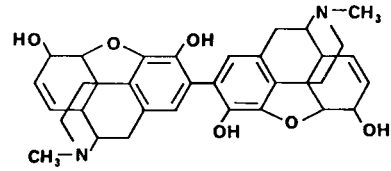
TABLE I

STRUCTURAL FORMULAE OF OPIATES INVESTIGATED FOR OXIDATION BY ALKALINE FERRICYANIDE



Opiate	R_1	R_2	R_3
I Morphine	CH_3	H	H
II Normorphine	H	H	H
III Nalorphine	CH_2–CH = CH_2	H	H
IV Morphine-3-glucuronide	CH_3	$C_6H_9O_6$	H
V Codeine	CH_3	CH_3	H
VI 6-Monoacetylmorphine	CH_3	H	$COCH_3$
VII Diacetylmorphine	CH_3	$COCH_3$	$COCH_3$
VIII Ethylmorphine	CH_3	CH_2CH_3	H
IX Acetylcodeine	CH_3	CH_3	$COCH_3$
X Dihydromorphine*	CH_3	H	H
XI Norcodeine	H	CH_3	H
XII Pseudomorphine			

* Double bond at C-7–C-8 is saturated.

METHODS

Samples and extraction procedures

Ante mortem and post mortem samples arising from forensic inquiries were examined. All extractions of samples were carried out in silanized glassware to avoid loss of drug by adsorption onto the glass surface. The following three extraction procedures were used.

Urine–free morphine. A 100-μl aliquot of a stock solution of nalorphine (0.24 mg/ml) was added to 5 ml of urine. The pH was adjusted to 9 with 2 *M* NaOH, and the urine was buffered with 1.5 ml of *M* K_2HPO_4 and saturated with NaCl (1 g). The urine was then extracted twice with 3 ml of chloroform–2-propanol (9:1) and the organic layer separated by centrifugation. The organic phase was recovered and evaporated to dryness. The residue was then taken up in 100 μl of HPLC eluent, and suitable aliquots were injected onto the HPLC column.

Urine–total morphine. A 100-μl aliquot of nalorphine stock solution was added to 1 ml of urine. To this was added 1 ml of 70% hydrochloric acid, and the solution was digested in a boiling water bath for 30 min. After cooling, the solution was adjusted to pH 9 with ammonia solution, then buffered and extracted as described for free morphine in urine.

Blood. Specimens of blood (10 ml) were digested with acid, filtered and made alkaline as described for total morphine in urine. The solution was then extracted with 50 ml of $CHCl_3$–propan-2-ol (9:1), and the organic phase was separated and back-extracted with 0.1 *M* H_2SO_4. The aqueous phase was separated, made alkaline with ammonia solution, and extracted with chloroform–2-propanol (9:1). The organic phase was separated and evaporated to dryness in preparation for HPLC analysis.

Chromatography

A Waters Model 6000A HPLC pump, Model U6K injector and 440 UV detector (254 nm) were used in normal HPLC configuration. A Metering Pumps Model HM reciprocating piston pump was used as the second pump to introduce the derivatising reagent. The fluorescent response was determined using an Amino Fluorocolorimeter set in the fluorescent mode with an 85-W mercury lamp, a Corning 7-54 excitation filter and a Wratten 2A emission filter and equipped with a 70-μl flow cell. A dual pen recorder was used to monitor the UV and the fluorescent response.

Three columns were used in this study: a 100 × 4.0 mm I.D. Partisil 10 ODS, a 100 × 4 mm I.D. Zorbax ODS (8 μm), and a 200 × 4.0 mm I.D. Kieselgel 60 HPLC (5 μm). The columns were slurry-packed with 2-propanol into glass-lined stainless-steel tubing with 6.35 mm ($\frac{1}{4}$ in.) to 1.56 mm ($\frac{1}{16}$ in.) stainless-steel Swagelok reducing unions as end-fittings, with the 6.35-mm end being drilled out to minimise dead volume. Sintered stainless-steel frits were used to retain the packing. These columns were all operated at ambient temperature. The mobile phase used for the pre-column derivatisation using the Kieselgel 60 column was methanol–2 *M* NH_4OH–1 *M* NH_4NO_3 (30:20:10). The flow-rate was 2.0 ml/min, and the analytical procedure used was as described by Jane and Taylor[10]. The mobile phase for the post-column der-

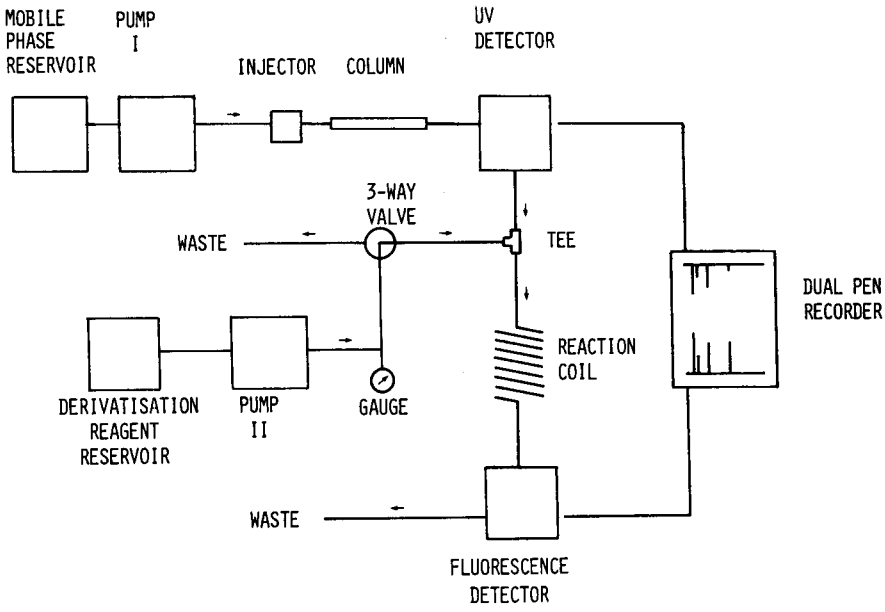


Fig. 1. HPLC system for morphine using post-column derivatisation and fluorescence detection.

ivatisation systems using the reversed-phase columns was methanol–0.1 *M* KBr (12.5:87.5) adjusted to pH 3 with phosphoric acid. The post-column derivatising reagent was 50 mg of $K_3Fe(CN)_6$ in 250 ml of 4 *M* NH_4OH.

The flow diagram for the HPLC post-column derivatisation system is shown in Fig. 1. The outlet from the UV detector was connected to the reagent outlet from the second pump and the reaction coil (5 m of 1.6 mm PTFE tubing of 0.3 mm I.D.) by a 1.6 mm Swagelok tee. The post-column reagent at a flow-rate of 0.4 ml/min, was mixed with mobile phase eluting at a flow-rate of 2.0 ml/min. The reaction coil was kept at room temperature, and its outlet connected to the flow cell of the fluorescence detector.

Quantitative work

The method described by Jane and Taylor[10] was used to determine morphine using pre-column derivatisation and adsorption chromatography, except that nalorphine was used as the internal standard rather than dihydromorphine. For the reversed-phase HPLC and post-column derivatisation, pure drug standards (Table I) were prepared using the mobile phase as solvent, and aliquots were injected onto the column to determine retention times and the fluorescent response after derivatisation.

The post-column reagent flow was optimised by maintaining a constant mobile phase flow-rate and varying the reagent flow-rate while monitoring the fluorescent response of standards. Calibration curves over the range from 10 ng to 2 μg (on column) were obtained for the standards.

The efficiency of the derivatisation was estimated from the calibration curves for morphine and pseudomorphine on the Zorbax ODS column. Quenching of the fluorescent response was estimated by comparing the calibration curves obtained for pseudomorphine with the $K_3Fe(CN)_6$ derivatising reagent, and with this reagent replaced by 4 *M* NH_4OH. The reliability of the whole procedure was determined by extracting morphine-free urine and whole blood samples to which known amounts of morphine and nalorphine had been added, and determining the drug levels. Apart from studies on the derivatisation efficiency and quenching, the Partisil 10 ODS column was used throughout.

RESULTS AND DISCUSSION

The structures of the opiates examined, and their chromatographic properties and fluorescent response determined using the post-column derivatisation technique, are listed in Tables I and II. The oxidation of morphine cogeners by alkaline potassium ferricyanide to form fluorescent pseudomorphine-like dimers is influenced by three structural features: the presence of a free hydroxy group at the O-3 position; the absence of a carbonyl group at the C-6 position; and the presence of the furan oxygen bridge[11]. Thus compounds substituted at the O-3 position (codeine, morphine-3-glucuronide, etc.) or lacking the furan oxygen bridge (pentazocine, levallorphan, etc.) do not form fluorescent derivatives. Optimum conditions for this oxidation in the HPLC system described were selected by varying the derivatising reagent flow-rate while maintaining a constant mobile phase flow-rate through the HPLC column. The results obtained for a number of opiates oxidized by ferricyanide are given in Table III, and the optimal reagent flow-rate was 0.4 ml/min. The five opiates listed in Table

TABLE II

RETENTION, RELATIVE TO MORPHINE, AND FLUORESCENT RESPONSE, RELATIVE TO PSEUDOMORPHINE, OF OPIATES AFTER HPLC SEPARATION ON PARTISIL 10 ODS

Opiate	*Relative retention time*	*Relative fluorescence*
Morphine-3-glucuronide	0.74	nil
Normorphine	0.80	89
Morphine	1.0	100
Dihydromorphine	1.10	81
Nalorphine	1.40	62
Norcodeine	1.48	nil
Codeine	1.83	nil
6-Monoacetylmorphine	2.23	7.5
Ethylmorphine	3.12	nil
Acetylcodeine	7.8	nil
Diacetylmorphine	8.7	nil

III all exhibit an excitation maximum of 323 $\pm$ 1 nm and an emission maximum of 432 $\pm$ 2 nm. The excitation filter, a Corning 7-54, and the emission filter, a Wratten 2A, are both cut-off filters and allow greater light transmission than band-pass filters, and thus gave greater sensitivity than band-pass filters on the fluorescence detector.

The chromatograms obtained for morphine and nalorphine using the Partisil 10 ODS column are shown in Fig. 2. Trace a was obtained after post-column derivatisation using fluorescent detection as described above, and trace b was obtained using UV detection at 254 nm.

The extent of the conversion of morphine into pseudomorphine in the reaction coil was estimated by comparing the calibration curves obtained for morphine and pseudomorphine using the Zorbax ODS column. On this column, pseudomorphine had a retention time relative to morphine of 1.2 compared with 3.9 on the Partisil 10 ODS column. Assuming that a small difference in the retention times of these com-

TABLE III

EFFECT OF POST-COLUMN REAGENT FLOW-RATE ON RELATIVE FLUORESCENT INTENSITY

*Compound**	*Normalised fluorescent intensity*			
	Reagent flow-rate (ml/min)			
	0.2	*0.4*	*0.6*	*0.8*
Normorphine	52	100	85	74
Morphine	74	100	80	64
Dihydromorphine	73	100	82	73
Nalorphine	76	100	83	62
6-Monoacetylmorphine	74	100	59	56
Pseudomorphine**	86	65	42	35

* All results determined on the Partisil 10 ODS column with an eluent flow-rate of 2 ml/min.

** Maximum response was observed when $K_3Fe(CN)_6$ was omitted from the reagent.

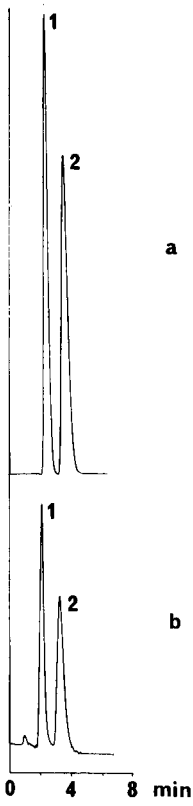


Fig. 2. HPLC separation of morphine (1) and nalorphine (2) on Partisil 10 ODS using (a) fluorescence detection and (b) UV detection under conditions described in the text.

pounds on the Zorbax ODS column should not significantly affect their fluorescent response, the comparison of calibration curves showed morphine to have 53% of the response of pseudomorphine and thus the derivatisation can be estimated as 50% complete. Quenching of the fluorescent signal by the derivatising reagent was also examined using the Zorbax ODS column by preparing calibration curves for pseudomorphine with ferricyanide present and omitted from the derivatising reagent. These curves showed a reduction of 40% in the fluorescent response when the ferricyanide was present (see also Table III).

The combined effect of incomplete derivatisation and quenching of the fluorescent response by the ferricyanide appeared to reduce the optimum response of the system by *ca.* 80%. A similar reduction in response was shown by comparing the on-column detection limit for morphine (10 ng) with that for pseudomorphine when $K_3Fe(CN)_6$ was omitted from the derivatising reagent (2 ng).

The results obtained from biological samples using the post-column method, described here, and the pre-column method, described by Jane and Taylor[10], are listed in Table IV. Agreement between the two methods is good, and using the post-column method replicate determinations of morphine in extracts obtained from a urine sample (3, Table IV) gave a standard deviation of 11% ($n = 9$). Replicate

TABLE IV

MORPHINE LEVELS DETECTED IN BIOLOGICAL SAMPLES BY DERIVATISATION HPLC

Sample	*Method**	*Morphine (μg/ml)*	
		Free	*Total*
1** Urine	A	30	148
	B	34	120
Blood	B	n.d.***	0.03
2 Urine	A	2.4	18
	B	2.0	12
3 Urine	A	14	37
	B	10	35
4 Blood	A	n.d.	0.09
	B	n.d.	0.04
5 Gastric lavage	A	n.d.	26

* A, post-column derivatisation; B, pre-column derivatisation.
** Samples from 1 were post mortem.
*** n.d. = Not determined.

determinations of the peak height ratio of morphine to nalorphine in extracts from a blood sample to which morphine and nalorphine had been added gave a standard deviation of 2.9% (n = 17). The recoveries of both morphine and nalorphine from urine were 80%.

Typical chromatograms obtained from the same urine extract are shown in Fig. 3. Trace a was obtained using UV detection; trace b using fluorescence detection without derivatisation and trace c using fluorescence detection with derivatisation. Peaks 1 and 2 in trace c correspond to morphine and nalorphine.

The compounds eluting later than morphine and nalorphine were not affected by stopping the flow of derivatising agents, indicating that they had a native fluorescence and were not related to morphine. No attempt was made to identify these peaks, and similar chromatograms were obtained from all the urine samples examined. Blood extracts did not contain any significant fluorescent coextractives. Morphine and nalorphine could not be detected in the urine extracts using UV detection because of interference caused by coextractives (trace a). The non-appearance of morphine peaks when $K_3Fe(CN)_6$ was omitted from the derivatising reagent was used as a confirmation of morphine in biological extracts.

The use of a solvent system incorporating halide ions is not generally recommended by HPLC pump manufacturers. However, such use is being increasingly reported[12–16] and corrosion problems can be avoided if adequate precautions are taken. Our procedures involved adequate flushing of the entire chromatographic system with distilled water after a run had been concluded. Solvents containing salts are not allowed to remain in the system unless there is a solvent flow. It is also recommended that the HPLC system, without the column, is passified regularly by pumping 200 ml of 50% HNO solution through it[17].

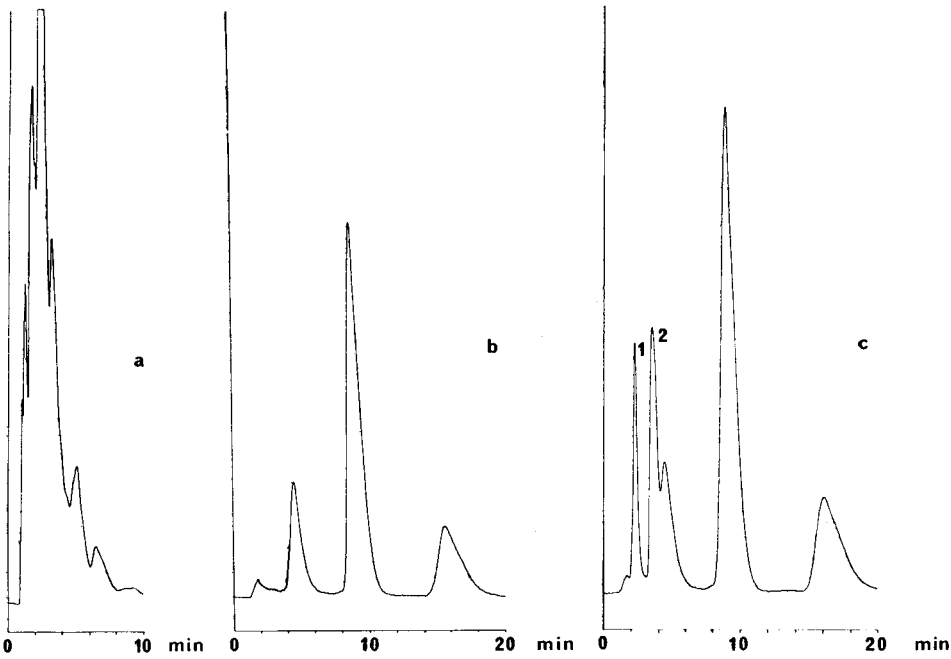


Fig. 3. HPLC of urine extract containing morphine and nalorphine (internal standard) on Partisil 10 ODS under conditions described in the text. (a) UV detection at 254 nm; (b) fluorescence detection, no derivatisation; (c) fluorescence detection with derivatisation. Peaks: 1 = morphine; 2 = nalorphine.

CONCLUSIONS

A reversed-phase HPLC system involving post-column derivatisation has been developed for the detection of morphine. The derivatisation to pseudomorphine followed by fluorescent detection gives the method both added sensitivity and specificity over UV detection, and it is suitable for the detection of morphine in post mortem fluids.

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TRACE ANALYSIS OF EXPLOSIVES IN HANDSWAB EXTRACTS USING AMBERLITE XAD-7 POROUS POLYMER BEADS, SILICA CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A general method for detecting traces of explosives at the low nanogram level in handswab extracts is described. The method involves a preliminary clean-up using Amberlite XAD-7 porous polymer beads to remove interfering lipid material, followed by detection of explosives in the concentrated extracts by capillary column gas chromatography with electron-capture detection. A method for confirming the presence of explosives in the extracts using thin-layer chromatography is also described.

INTRODUCTION

A previous publication from this laboratory[1] described a sensitive method for the trace analysis of explosives using silica capillary column gas chromatography (GC) with electron-capture detection (ECD). Although pure explosives can be analysed reproducibly at the low picogram level by this method their detection in contaminated samples of forensic interest, such as handswab extracts[2], rapidly leads to deterioration of the column and the detector unless the sample is cleaned up prior to analysis[3–5].

No general clean-up procedure suitable for explosives analysis has yet been described in the literature[6]. This paper describes a clean-up technique suitable for the trace analysis of the important commercial and military explosives at the low nanogram level in heavily contaminated samples, such as handswab extracts. The method uses selective charge-transfer extraction of the explosives from solutions of handswab extracts in pentane on to Amberlite XAD-7 porous polymer beads. The explosives are then removed from the surface of the beads with small volumes of ethyl acetate to give extracts suitable for repeated direct analysis by capillary column GC with ECD.

A method capable of detecting explosives at the low nanogram level in the cleaned up extracts using thin-layer chromatography (TLC) with specific reagent sprays is also described.

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EXPERIMENTAL

Reagents

The explosives studied were ethyleneglycol dinitrate (EGDN), nitrobenzene (NB), nitroglycerine (NG), 2,4-dinitrotoluene (2,4-DNT), 2,4,6-trinitrotoluene (TNT), hexogen (RDX), pentaerythritol tetranitrate (PETN), tetryl, octogen (HMX), and nitrocellullose (NC)[1].

All solvents used were pesticide grade (Fisons, Loughborough, Great Britain).

Amberlite XAD-7 (BDH, Poole, Great Britain) was 20–50 mesh grade, and 9 ml (wet volume) of the beads were cleaned by washing in a column successively with 100 ml portions of distilled water, methanol, ethyl acetate, ether, and pentane. The beads were stored under ether until required for use. Quantitative handling of the beads was achieved in the dry state by carrying out all manipulations in narrow-necked screw-topped glass vials. However, the beads were stored under solvent at all other times.

Cotton wool (Vestric, London, Great Britain) was soxhlet extracted with ether for 4 h.

All materials used in the procedure were checked for interferences by running a control experiment.

Gas chromatography

The following conditions were used: column, flexible-fused silica capillary externally coated with polyimide, 21 m × 0.25 mm I.D. (Phase Separations, Queensferry, Great Britain); stationary phase, OV-101; injection port temperature, 165°C; detector oven temperature, 200°C; temperature programme, 25°C held for 30 sec then programmed at 40°/min to 240°C, cooldown time, 4 min; carrier gas, helium; carrier gas flow-rate 30 ml/min (25°C); make-up gas, methane–argon (1:99); make-up gas flow-rate, 13 ml/min; injection solvent, ethyl acetate. The Varian tritium ECD was operated here in the constant current mode, at a potential of 50 V, and a pulse width of 1 μsec, using a Carlo Erba Model 251 control module. Better baseline stability was achieved using the detector in this mode, and a typical analysis of a mixture of explosives is shown in Fig. 1. The injection port liner was cleaned with swabs soaked in ethyl acetate, and the liner was then rinsed with ether and dried at room temperature. By this technique it was possible to avoid the use of high-temperature baking which, under certain conditions, gave rise to undesirable activity.

Thin-layer chromatography

TLC plates were DC-Alufolien Kieselgel 60F 254 (5 cm × 7.5 cm × 0.2 mm) (Merck, Darmstadt, G.F.R.).

The eluting solvent for EGDN, NG, TNT, PETN, and tetryl (eluent A) was a mixture of toluene and cyclohexane (7:3 by volume). The eluting solvent for RDX and HMX (eluent B) was a mixture of chloroform and acetone (2:1 by volume). The eluting solvent for NC (eluent C) was a mixture of acetone and methanol (3:2) by volume.

The specific reagent spray for EGDN, NG, PETN, RDX, tetryl, HMX, and NC was Griess reagent spray[2]. The plates were eluted, and the solvent was evaporated using a stream of warm air. The plates were sprayed with 1*N* sodium hydrox-

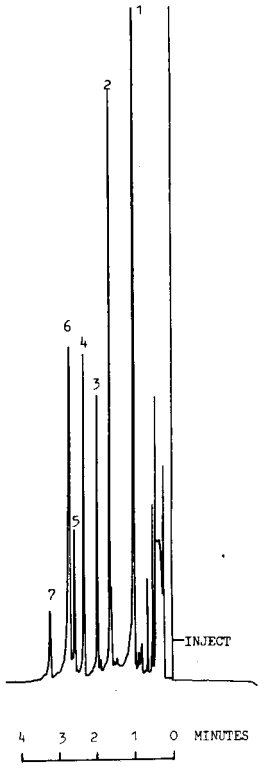

Fig. 1. Mixture of explosives containing 400 pg each of EGDN (1), NG (2), 2,4-DNT (3), TNT (4), PETN (5), RDX (6) and tetryl (7) analysed using the conditions described in the Experimental section.

ide solution and heated to 150°C for 5 min. They were then sprayed with a solution of sulphanilamide (8 g) and N-1-naphthylethylenediamine dihydrochloride (0.4 g) (Sigma, Poole, Great Britain) in 8% orthophosphoric acid (100 ml). The explosives developed a red colouration at room temperature.

The specific reagent spray for TNT and tetryl was a 30% solution of 3,3′-iminobispropylamine[7] (Aldrich, Gillingham, Great Britain) in pyridine. TNT developed a purple colour and tetryl a brown colour at room temperature.

Preparation of handswab extracts

Handswabs were obtained by repeatedly scrubbing the appropriate surface of one hand using a cotton wool swab (40 mg) moistened with ether. The lower surface of the palm and fingers of the hand were swabbed to determine if a subject had handled explosives. The upper surface of the back of the hand was swabbed to determine if a subject had fired a handgun. Spiked extracts of all of the explosives. except NC were prepared by distributing standard solutions of explosives throughout the swab using a syringe. Preparation of spiked extracts containing NC was achieved by spiking the ether-insoluble residue of the handswab extract with a standard solution of NC in acetone and allowing the residue to dry.

Extraction of handswabs

The swab was extracted by successive washing with small portions of ether (total volume 12 ml) in a beaker using a glass rod. The combined extracts were centrifuged to remove traces of skin debris, and the clear supernatant was decanted into a silanized conical tube. The ether was evaporated down to near dryness (5–10 μl) using a current of nitrogen, and the last traces of ether were allowed to evaporate at room temperature. Pentane (3 ml) was added to the residue and the resulting solution was thoroughly mixed and transferred to a screw-capped vial. The insoluble residue removed from the solution of the handswab extract in ether by centrifuging was washed with ether, dried, and extracted with acetone in an ultrasound bath. The acetone extract was then concentrated and analysed by TLC using Griess reagent spray to detect NC.

Clean-up method

First clean-up. Amberlite XAD-7 (10 mg dry weight) was added to the solution of the handswab extract in pentane, and the mixture was gently shaken for 15 min so that the beads circulated throughout the solution. The supernatant was then decanted using a Pasteur pipette, and retained for further processing if required. The beads were thoroughly rinsed with pentane. The residual traces of pentane were evaporated using a current of nitrogen, and the beads transferred to a clean vial. Ethyl acetate (80 μl) was added and after 2 min equilibration the solvent was decanted, and the beads washed with a further two portions of 40 μl of solvent. The extracts were combined and stored in a sealed sample vial.

Second clean-up. The combined ethyl acetate extract was evaporated to near dryness using a current of nitrogen, the residue dissolved in pentane (1 ml) and the Amberlite XAD-7 extraction repeated using 3 mg of fresh beads. (The supernatant pentane solution from this extraction was retained for confirmation of the presence of explosives in the extract by TLC). The beads were then extracted with 30 μl of ethyl acetate, and 1 μl of this extract was analysed by GC with ECD.

Stability of solutions of explosives

Standard solutions of explosives in organic solvents can be unstable under certain conditions and should therefore be regularly monitored for evidence of decomposition. Fresh solutions should always be prepared at regular intervals. The explosives tetryl, TNT and HMX were found to be the most prone to decomposition in solution. For this reason, handswab extracts containing traces of explosives should be processed and analysed without delay.

RESULTS AND DISCUSSION

Attempts to detect traces of explosives in handswabs by analysis of uncleaned-up extracts using capillary column GC with ECD were unsuccessful. Interfering compounds present in the extracts obscured the response for the explosives and heavily contaminated the GC system[3–6]. This contamination prevented the analysis of explosives at low levels without the use of priming[1], and caused rapid deterioration of

the column and the detector. A clean-up method was therefore developed which would allow the routine trace analysis of explosives at the low nanogram level in heavily contaminated samples such as handswab extracts.

Initial experiments using silica gel column chromatography as a clean-up method failed because the wide range of polarity of the explosives prevented their selective elution and gave extracts still heavily contaminated with involatile materials. Experiments using selective charge-transfer extraction[8] of explosives from pentane solutions of handswab extracts with polar non-ionic Amberlite XAD porous polymer beads[9] gave clean samples suitable for GC analysis, and this method of clean-up was further investigated.

Handswab extracts were prepared by the method described in the Experimental section using ether[2], because this solvent could be readily removed by evaporation without the loss of the important volatile explosive EGDN. After evaporation of the ether the extracts were dissolved in pentane, since in these experiments explosives could only be efficiently recovered from saturated hydrocarbon solvents by the porous polymer beads. Three types of bead were investigated (Amberlite XAD-2, -7, and -12)[9] but only Amberlite XAD-7 and XAD-12 showed any potential for the recovery of explosives. Initially, Amberlite XAD-12 (amine oxide polystyrene) was used in these experiments, and useful recoveries for the nitrate ester explosives and RDX were demonstrated. However, these beads were unstable on storage and gave only very poor recoveries of the nitroaromatic explosives at low levels and their use was discontinued. Amberlite XAD-7 [poly(methylmethacrylate)] was found to give practical recoveries of all of the explosives shown in Table I, and optimum conditions were therefore determined using this bead by varying the mass of the beads, the volume of pentane and time required for the extraction. After the extraction, the beads were thoroughly rinsed with pentane to remove unadsorbed handswab material and the beads dried using a current of nitrogen. Ethyl acetate was used to extract the explosives from the surface of the beads since this solvent gave good recoveries of the

TABLE I

CHARACTERISTICS OF THE AMBERLITE XAD-7 EXTRACTION METHOD AND THE ANALYSIS OF EXPLOSIVES BY GAS CHROMATOGRAPHY

Explosive	*Recovery of 200 ng of explosives from a handswab (mean of 3 determinations)*		*Minimum detectable level of explosive in a handswab (ng/swab)*
	First clean-up (%)	*Second clean-up (%)*	
EGDN	19	5	10
NG	77	47	10
2,4-DNT	27	6	50 (25*)
TNT	50	33	20
PETN	80	45	50
RDX	73	47	50
Tetryl	38	25	50

* Using the primary clean-up only.

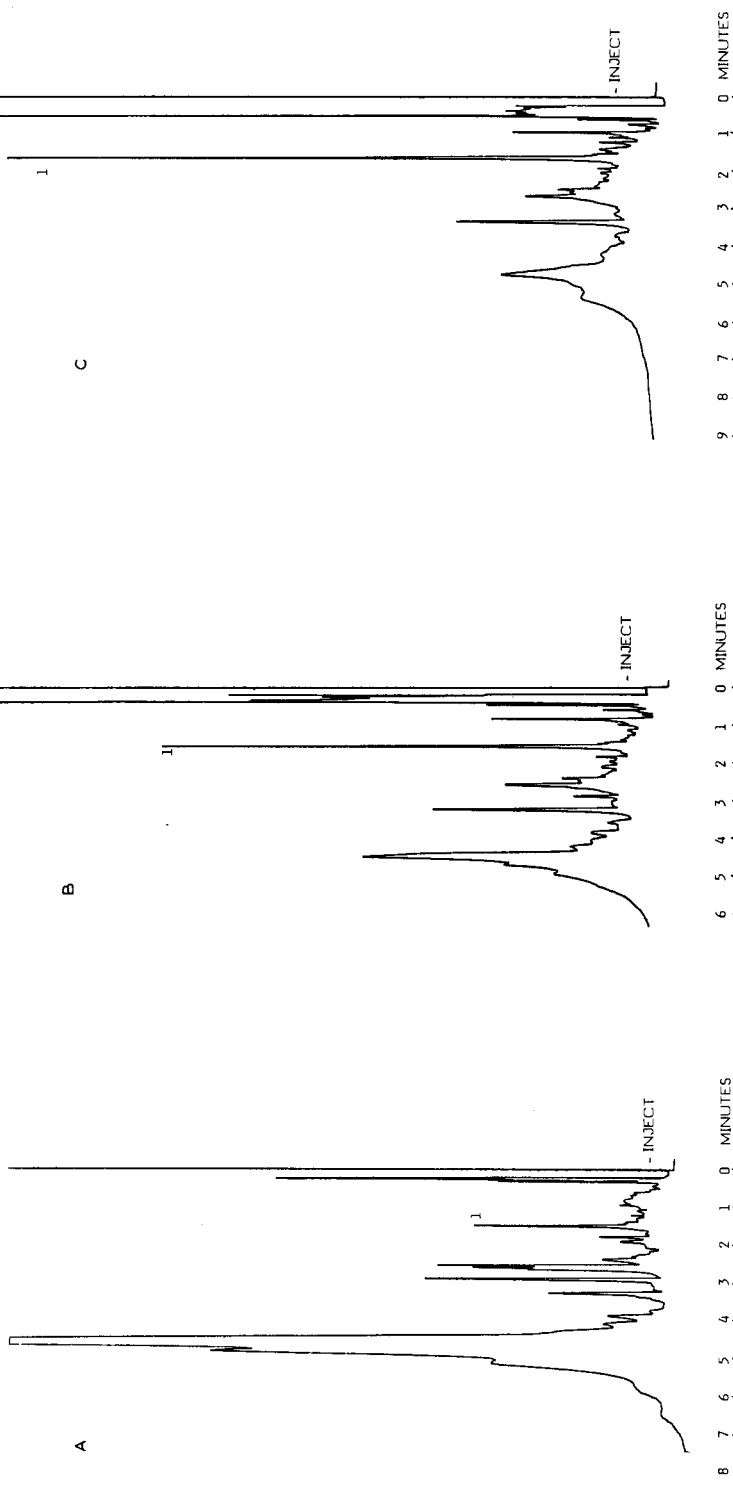

Fig. 2. (A) Unpurified handswab extract taken from a laboratory worker and spiked with 50 ng of NG (1). 0.1% of the sample was analysed, using the standard conditions as described in the Experimental section. (B) The same handswab extract spiked with 50 ng of NG (1) cleaned up once by the Amberlite XAD-7 clean-up method. 0.6% of the sample was analysed, using the standard conditions described in the Experimental section. (C) The same handswab extract spiked with 50 ng of NG (1) cleaned up twice by the Amberlite XAD-7 clean-up method. 2% of the sample was analysed, using the standard conditions described in the Experimental section.

explosives and was also a suitable injection solvent. Only small volumes (15–80 μl) of ethyl acetate were required for the extraction, thus avoiding the need to concentrate the extract prior to analysis by GC. The primary clean-up of the extract by this method gave a sample almost free from lipid material that was clean enough to be analysed by GC.

Because of the sensitivity of the ECD only a few per cent of the sample at most was required for analysis, but because of the relatively poor selectivity of this detector the minimum detectable levels of the explosives in handswabs were limited by the background. Thus it was not worthwhile analysing a larger fraction of the sample since no improvement in the signal-to-background ratio was obtained. However, the level of background could be successfully reduced and the response for explosives present at the low nanogram level enhanced by repeating the clean-up, as demonstrated in Fig. 2, and by this method the minimum detectable levels (MDL) shown in Table I were obtained. Thus using the dual clean-up method it was possible in the case of NG and EGDN routinely to detect levels of explosives in the range 15–20 ng/swab. It should be noted that NB cannot be recovered from handswabs using either Amberlite XAD-2, -7, or -12; however, this explosive can still be detected at high levels in handswabs by analysis of very dilute solutions of uncleaned-up extracts. Although HMX can be analysed by GC under carefully optimised conditions[1], it is an explosive of only relatively minor forensic importance and was not studied in this work.

The general effectiveness of the analytical method was evaluated by analysing a range of handswabs each representing different degrees of contamination. The method was shown to be succesful both in the case of extracts from clean hands (Fig. 3) and heavily contaminated hands. A garage mechanic's hands, covered in dirt and grease, were chosen to approximate the worst possible case of contamination likely to be encountered, and analysis of the cleaned-up extracts showed profiles similar to those of clean hands (Fig. 3) but with an increased level of background. It should be noted that some hands give extracts showing coextractive peaks eluting in the region of the chromatogram after tetryl (Fig. 2) but these do not interfere with the analysis of the explosives described in this paper.

In the Metropolitan Police laboratory the analytical method is being experimentally applied to the detection of firearms residues on hands, and the results of an experiment using a handgun and a double base propellant ammunition demonstrating the presence of NG on the upper surface of the back of the firer's hand are shown in Fig. 4.

The reproducibility of recovery of 100 ng of NG and TNT from ten different handswabs using the primary clean-up were measured and found to be satisfactory: 73%, R.S.D. 10% (n = 10), and 49%, R.S.D. 21%, (n = 10), respectively. The level of recovery of the most important explosive, NG, was shown to be constant within the experimental error over the range 50–300 ng/swab. Recoveries for the explosives obtained using 10 mg of beads at the 200 ng/swab level are shown in Table I.

The extracts obtained by the Amberlite XAD-7 clean-up method are clean enough to allow the routine detection of the explosives shown in Table I at the low nanogram per swab level without degrading the performance of the column or the detector. Regular cleaning of the injection port liner is required to remove septum cores and any traces of involatile residue which can accumulate and give rise to

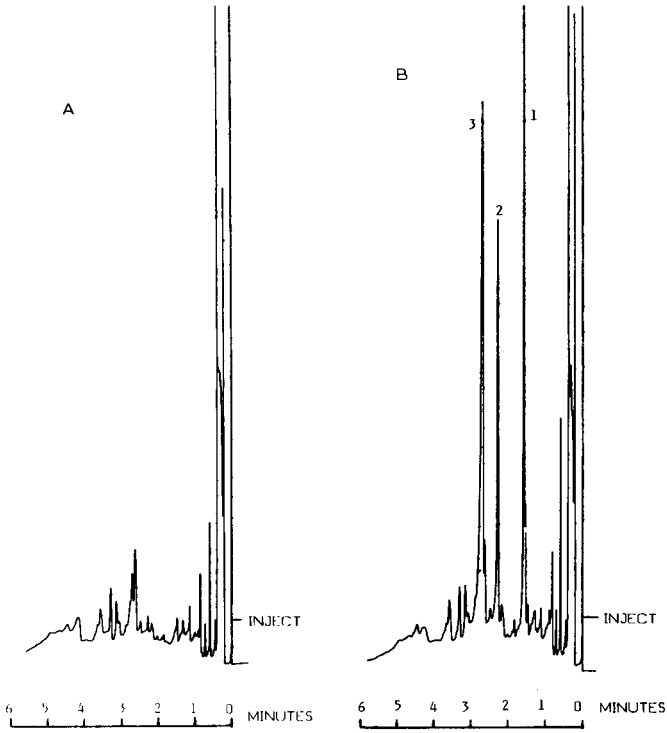

Fig. 3. (A) Unspiked extract of a laboratory worker's left hand, cleaned up twice using the Amberlite XAD-7 clean-up method. Standard conditions as described in the Experimental section. (B) Spiked extract of a laboratory worker's right hand containing 50 ng of NG (1), and 100 ng each of TNT (2) and RDX (3), cleaned up twice by the Amberlite XAD-7 clean-up method. Standard conditions as described in the Experimental section.

undesirable activity. This is especially important when analysing the very readily adsorbed explosive PETN, and it is advisable to test the system regularly for the response of this explosive and to clean the injection port liner regularly to obtain maximum response for this compound. However, for the other explosives described in this paper, the injection port liner need be cleaned only at the end of each working day. Removal of the short length of column protruding into the injector was very effective in restoring the peak shape of the longer-retained explosive RDX should this deteriorate in the long term.

The long-term performance of the system was satisfactory, and in this laboratory one OV-101 silica capillary column has been used over a period of 9 months to analyse over 400 handswab extracts. Regular cleaning of the injection port liner effectively prevents contamination of the detector, and one tritium foil has been in use for over 6 months without deterioration. The analytical traces shown in this paper were obtained at the end of this time using this system. It should be noted that the long useful working life of the fused silica capillary columns in this situation effectively offsets the disadvantage of their high cost.

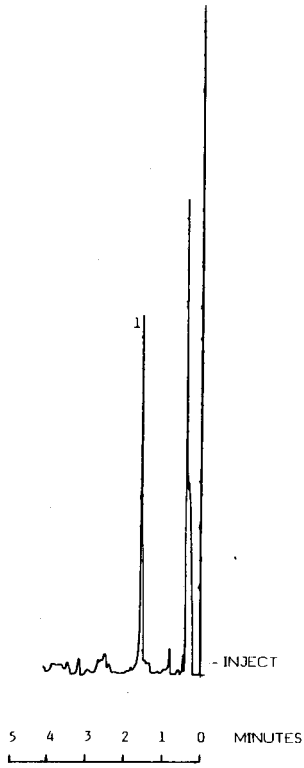

Fig. 4. Extract of the upper surface of the right hand of a laboratory worker who had just fired three rounds of Smith and Wesson Super X double base propellant ammunition, using a Smith and Wesson Model 27, 357 Magnum, showing NG (1). The extract was cleaned up once using the Amberlite XAD-7 method, and analysed using the standard conditions described in the Experimental section.

Analysis of extracts by TLC

Standard mixtures of many of the important commercial and military explosives may be analysed by TLC with sensitivities at the low nanogram level[2,10] (Table II). However, attempts to analyse uncleaned-up extracts by this method were unsuccessful because the lipid material present prevented elution of the extract from the origin because of overloading of the adsorbent.

Analysis of the extracts cleaned-up once by the Amberlite XAD-7 method was successful, with minimum detectable levels in the low nanogram range (Table II). It should be noted that these levels were achieved only by analysing most of the sample in one analytical run. However, this method of analysis could be successfully used to confirm the presence of explosives detected in the extracts by the GC method.

Nitrocellullose (NC) was successfully analysed by TLC using an eluting solvent containing acetone, and Greiss reagent spray. This method of analysis was successfully used to detect NC on the upper surface of the hand of a subject who had fired a handgun (Smith and Wesson Model 27 357 Magnum) using double base propellant ammunition. The NC was readily detected by TLC analysis of the acetone-soluble fraction of the residue obtained by centrifuging the ether extract of the handswab. The feasibility of routinely detecting traces of firearms residue (NG and NC) on

TABLE II

CHARACTERISTICS OF THE TLC ANALYSIS OF EXPLOSIVES

Where an R_F value is not quoted in this table, the explosive concerned eluted close to the solvent front.

Explosive	R_F *eluent A*	R_F *eluent B*	R_F *eluent C*	*MDL of explosives (ng)*		*MDL of explosives in a handswab (ng/swab). Cleaned up once using Amberlite XAD-7*
				Griess spray	*3,3'Iminobis propylamine spray*	
EGDN	0.53	–	–	25	–	300
NG	0.42	–	–	5	–	20
TNT	0.57	–	–	–	10	50
PETN	0.45	–	–	5	–	30
RDX	0.03	0.72	–	5	–	20
Tetryl	0.25	–	–	20	30	60
HMX	0	0.35	–	10	–	500
NC	0	0	0.64	50	–	300*

* This value represents the amount of NC present in the ether-insoluble residue of the handswab.

hands is currently under investigation, and the results will be reported in a future publication.

It should be noted that the majority of the above results (both GC and TLC) were obtained using handswabs spiked with explosives, whereas in reality further losses can be expected to occur in the recovery of explosives from the surface of hands. This will result in higher achievable minimum detectable levels and it will probably be necessary, for some applications, to detect explosives at levels lower than those described in this paper. Two possible approaches to improving the sensitivity of the method are to develop a more selective clean-up method, or to use a more specific detector such as the thermal energy analyser[1] or the mass spectrometer in the chemical ionisation negative specific ion monitoring mode[1]. However, a preliminary clean-up of the samples will still be required to protect the GC system from contamination when using these detectors.

CONCLUSION

This paper describes a general method for analysing traces of important commercial and military explosives at the low nanogram level in handswabs. The clean nature of the extracts obtained by the Amberlite XAD-7 clean-up technique is believed to be responsible for the success of the method, enabling routine reproducible analysis of subnanogram amounts of explosives in the presence of a complex organic background.

Other possible applications of the technique are the detection of explosives in body fluids, environmental samples and post explosion residues, and the detection of firearms residues on hands.

ACKNOWLEDGEMENT

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THE MULTI-RESIDUE DETERMINATION OF COUMARIN-BASED ANTICOAGULANT RODENTICIDES IN ANIMAL MATERIALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The rodenticides brodifacoum, difenacoum, coumatetralyl and warfarin are determined in animal relicta by high-performance exclusion chromatography on porous silica. The first three compounds are not separated, but are subsequently differentiated by adsorption or reversed-phase high-performance liquid chromatography of the appropriate eluate fraction collected from the exclusion column. The method is rapid, and clean-up (on Sep-Pak silica cartridges) is simple. Mean recoveries from spiked substrates were generally above 80% at levels of 0.1–1.0 mg/kg. Routine limits of determination are about 0.05–0.1 mg/kg for warfarin and about 0.02 mg/kg for the other compounds. If analysis for warfarin is not required, the latter limit can be lowered to about 1 μg/kg by a slight modification to the clean-up step.

INTRODUCTION

Methods for the diagnostic determination in animal relicta of the anticoagulant rodenticides warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin] and difenacoum [3-(3-biphenyl-4-yl-1,2,3,4-tetrahydro-1-naphthyl)-4-hydroxycoumarin] by high-performance liquid chromatography (HPLC) have been described[1,2]. A method for determining brodifacoum {3-[3-(4′-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl]-4-hydroxycoumarin} was needed.

Preliminary experiments showed that brodifacoum could be determined at levels of 0.01 mg/kg and above in animal materials by capillary gas chromatography, the brodifacoum being thermally degraded to a mixture of 3-(4′-bromobiphenyl-4-yl)naphthalene and the corresponding tetrahydronaphthalene, both of which were detected[3]. Subsequent results were insufficiently consistent to encourage the develop-

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ment of the method for routine use however, and methods based on HPLC were considered.

Brodifacoum has been determined in baits by reversed-phase HPLC[4] and in rat tissues by adsorption HPLC[5]. Its determination in animal tissues and blood[6] by an adaptation of the reversed-phase method[4] was described during the preparation of the present publication. Exclusion HPLC appeared to offer two advantages: simpler clean-up, because brodifacoum would be eluted before most of the co-extracted material, and more sensitive fluorimetric detection because the use of acetic acid (which quenches fluorescence) in the mobile phase would be avoided. Moreover, determination by exclusion chromatography combined with identification by adsorption or reversed-phase HPLC seemed likely to provide a convenient multi-residue method for the four coumarin anticoagulants used as rodenticides in this country: warfarin, difenacoum, brodifacoum and coumatetralyl [4-hydroxy-3-(1,2,3,4-tetrahydro-1-naphthyl)coumarin].

The present communication describes the determination by HPLC of the four compounds mentioned above in animal tissues and fluids. Extracts are cleaned-up on Sep-Pak silica cartridges and chromatographed on a porous silica exclusion column, from which the rodenticides are eluted before co-extractives. Difenacoum, brodifacoum and coumatetralyl are resolved from warfarin, but not from one another; the three compounds are differentiated by HPLC of collected eluates on an adsorption or reversed-phase column, with UV detection. Since the presence of more than one rodenticide is unlikely, exclusion chromatography normally serves for quantification and reversed-phase or adsorption chromatography for identification. If more than one of the unresolved rodenticides is present, quantification by reversed-phase chromatography is possible, but lower limits of determination can be achieved on the exclusion column.

EXPERIMENTAL

Materials and apparatus

Brodifacoum, mixed isomers of analytical standard grade, was supplied by Sorex (London), Wembley, Great Britain; other rodenticides were from previously specified sources[1]. The *cis*- and *trans*-isomers of brodifacoum and difenacoum were separated for experimental purposes by thin-layer chromatography (TLC) on precoated silica gel plates (Schleicher & Schüll G1500 LS 254, from Anderman & Co., East Molesey, Surrey, Great Britain), with ether–hexane–acetic acid (75:25:1, v/v) as developing solvent.

Methanol was of HPLC grade and chloroform was Distol grade containing about 2% of ethanol as stabilizer (both from Fisons Scientific Apparatus, Loughborough, Great Britain). Other solvents were Analar or of similar quality.

Sep-Pak silica cartridges were from Waters Assoc., Northwich, Great Britain; they were prepared for use by washing successively with methanol–chloroform (15:85, v/v) and chloroform (10 ml of each).

The HPLC columns were of stainless steel, 5 mm I.D., internally polished. Exclusion and adsorption columns were 250 mm long, slurry-packed with Magnusil 5 μm porous silica; reversed-phase columns were 100 mm long, packed with Magnusil 8H C_{22}. All columns and packings were from Magnus Scientific Instrumen-

tation (Sandbach, Great Britain). The liquid chromatograph was as previously described[7], with the addition of a Perkin-Elmer Model 2000 fluorescence spectrophotometer fitted with a 20-μl flow-cell.

Extraction

Liver and stomach contents (10 g) were homogenised with anhydrous sodium sulphate (20 g) and chloroform (30 ml). The extract was filtered through sintered glass, the residue was homogenised with a further 15 ml of chloroform and filtered, and the filtrates were combined. Serum or urine (10 ml) was acidified with hydrochloric acid (5 *N*, 2 ml) and extracted successively with 15 and 10 ml of chloroform. The combined extracts were dried with anydrous sodium sulphate and filtered. Filtrates were concentrated under a stream of nitrogen at 35°C.

Clean-up

The chloroform extract was concentrated to 10.0 ml, a 2-ml aliquot was injected on to a Sep-Pak cartridge, and the rodenticide(s) were eluted with 4 ml of methanol–chloroform (15:85, v/v). The eluate was taken to dryness at 35°C under nitrogen, and the residue was dissolved in methanol (0.1–2.0 ml, according to the expected rodenticide content). If analysis for warfarin was not required, the chloroform extract was concentrated to about 1 ml and transferred to the cartridge, which was eluted with chloroform (4 ml) instead of the mixed eluent; any warfarin present was quantitatively retained by the cartridge.

Determination

Duplicate aliquots (20 μl) of extracts and of standard solutions in methanol were chromatographed on the exclusion column with methanol as eluent at a flow-rate of 1 ml/min. The fluorescence detector was operated at excitation and emission wavelengths of 315 and 410 nm, respectively.

The rodenticides were quantified by reference to standard solutions containing mixtures of warfarin with one of the other three compounds; this procedure was justified because the detector responded equally to equal mass/volume concentrations of brodifacoum, difenacoum and coumatetralyl. If a peak was detected at the retention time of the three excluded rodenticides, the eluate fraction producing it was collected for identification by adsorption or reversed-phase chromatography. It was usually necessary to combine the corresponding fractions from several injections of the same extract.

Identification

The eluate fraction containing brodifacoum, difenacoum or coumatetralyl was taken to dryness under nitrogen, and the residue was dissolved in 50 μl of cyclohexane–dichloromethane–acetic acid (75:25:0.6, v/v) for adsorption chromatography or methanol–water–acetic acid (80:20:0.8, v/v) for reversed-phase chromatography. In either case the solvent was used as the mobile phase for HPLC, at a flow-rate of 1 ml/min. Detection was by UV absorption at 260 nm. If more than one of the three rodenticides was identified, quantification was by reversed-phase chromatography with reference to suitable standards.

RESULTS AND DISCUSSION

Previous work[2] had shown that difenacoum could be separated from both warfarin and most co-extractives in animal materials by exclusion chromatography on porous glass with a mean pore diameter of 200 Å. Coumatetralyl was eluted together with difenacoum and, as was subsequently found, with brodifacoum. Warfarin was eluted later, within the retention volume of the main co-extractive fraction. More recently, the substitution of porous silica (mean pore diameter 60 Å) for porous glass has given better resolution and, because peaks were narrower, higher sensitivity. Difenacoum, brodifacoum and coumatetralyl were still eluted together; warfarin was eluted after these, but well before co-extractives. Exclusion chromatography on porous silica therefore provided a basis for a multi-residue method, provided that brodifacoum, difenacoum and coumatetralyl could be differentiated before or after the determinative step.

Calibration

Chromatography of standard solutions gave a linear calibration curve for warfarin over the range 1.5 ng to 2.5 μg and for the other rodenticides over the range 300 pg to 2.5 μg.

Extraction

Extraction with chloroform was essentially by the procedures previously used for the separate determinations of warfarin[1] and difenacoum[2] in animal fluids and liver. Von Meyer *et al.*[8] have also used chloroform to extract warfarin and coumatetralyl (as well as other anticoagulants) from biological fluids. Koubek *et al.*[5] have shown that incurred brodifacoum is well extracted from rat tissues by methanol–chloroform (1:9, v/v) and it can be assumed that this mixture would be equally effective for difenacoum. In the present work, comparison of chloroform with methanol–chloroform showed that the former yielded cleaner extracts from spiked liver, and was therefore to be preferred if it was no less efficient. Since any advantages of incorporating methanol in the extractant would presumably be at least as marked with the more polar rodenticide coumatetralyl as with brodifacoum, the extraction of this compound was examined. Samples of pheasant liver containing incurred coumatetralyl were analysed after extraction with chloroform and with methanol–chloroform (1:9, v/v). The coumatetralyl levels found were 0.96 and 0.89 mg/kg, respectively; it was therefore concluded that chloroform was a suitable extractant for the substrates analysed in the present work. It should be noted that the Distol chloroform used contained about 2% of ethanol: the effectiveness of pure chloroform was not examined.

Clean-up

Previous work[5,7] had shown the convenience of Sep-Pak cartridges for the rapid clean-up of extracts before HPLC. Such a clean-up step was not strictly necessary for the analyses described here, but it increased the effective life of analytical columns and shortened analysis time by removing co-extractives which were strongly adsorbed by them.

All four rodenticides were eluted with methanol–chloroform (15:85, v/v). When only the three less polar compounds were of interest however, a cleaner extract

was obtained by eluting with chloroform. Fig. 1A and 1B show chromatograms from extracts of unspiked liver and liver spiked with a mixture of all four rodenticides, after elution from the Sep-Pak cartridge with methanol–chloroform; the aliquots injected represent 20 mg of liver. Fig. 1C and 1D are from liver, unspiked and spiked with brodifacoum, after elution from the cartridge with chloroform; the aliquots injected represent 200 mg of liver. The absence of the large peak for co-extractives (numbered 4 in Fig. 1A and 1B) from chromatograms C and D, despite the higher concentrations of the extracts producing them, is notable and there is clearly scope for further concentration without interference. The sharpness of the rodenticide peaks in Fig. 1B and 1D is also striking, particularly in view of the rather large flow-cell volume of 20 μl.

In routine application of the method, clean-up was found to be satisfactory for serum, liver, kidney, brain, muscle and stomach or rumen contents from several species.

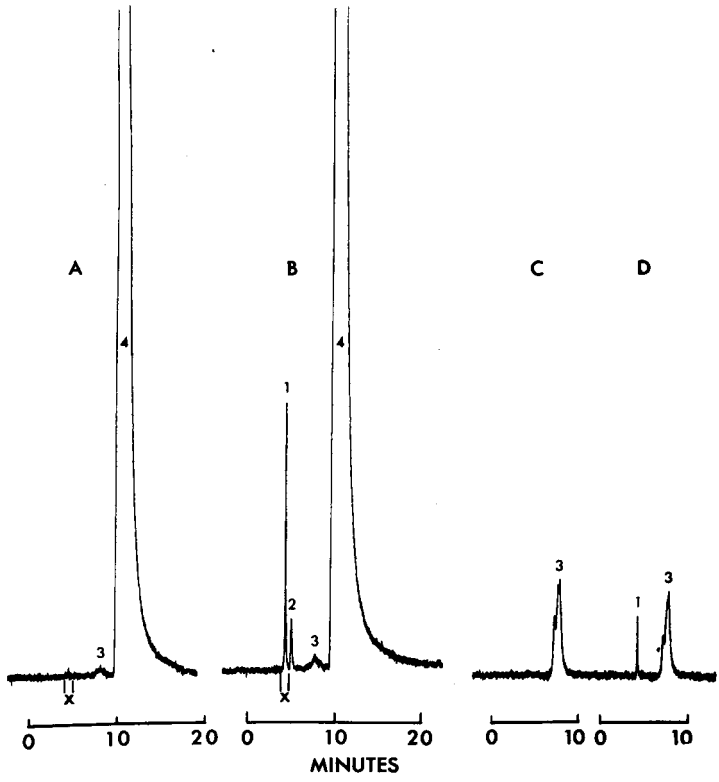

Fig. 1. Exclusion chromatography of liver extracts on porous silica. A, extract equivalent to 20 mg unfortified liver, after clean-up on Sep-Pak silica cartridge and elution therefrom with methanol–chloroform (15:85, v/v); B, as A, but liver spiked with brodifacoum, difenacoum and coumatetralyl (0.1 mg/kg of each) and warfarin (0.5 mg/kg); C, extract equivalent to 200 mg of unfortified liver, after elution from Sep-Pak cartridge with chloroform; D, as C, but liver spiked with brodifacoum (5 μg/kg). Peaks: 1 = brodifacoum, difenacoum and coumatetralyl in B, brodifacoum in D; 2 = warfarin; 3 and 4 = co-extractives. X is the fraction collected for identification by reversed-phase HPLC (see Fig. 3).

Recoveries

Recoveries from spiked liver, serum and stomach contents were examined, as these are normally the most useful materials for diagnostic analysis. Attention was concentrated on liver because it is the more difficult of the two well-defined substrates. In the recovery experiments, five separate samples of each substrate were spiked at each fortification level, either individually with brodifacoum, difenacoum or coumatetralyl or with a mixture of brodifacoum and warfarin.

Recoveries of the four rodenticides added to pig liver at levels of 0.05–1.0 mg/kg, and of brodifacoum added at 5 μg/kg, are given in Table I. Recoveries of brodifacoum and warfarin from serum and stomach contents spiked at 0.05–1.0 and 0.1–1.0 mg/kg, respectively, are shown in Table II. In both Tables, results are presented as the mean percentage recovery at each level of each compound in each

TABLE I

DETERMINATION OF RODENTICIDES BY EXCLUSION HPLC: RECOVERIES FROM SPIKED LIVER

Column, 250 mm × 5 mm I.D., of Magnusil porous silica (5 μm); mobile phase, methanol; flow-rate 1 ml/min. Extraction and clean-up as described in text. Five separate samples at each level analysed. Spiked before homogenization

Fortification level, mg/kg	*Percentage recovery (mean ± 95% confidence interaval)*			
	Brodifacoum	*Difenacoum*	*Coumatetralyl*	*Warfarin*
1.0	86	96	90	80
0.5	81	100	91	84
0.1	87 ± 4.5	100 ± 4.2	87 ± 7.6	76 ± 5.9
0.05	88	87	93	65
0.005	78			

TABLE II

DETERMINATION OF RODENTICIDES BY EXCLUSION HPLC: RECOVERIES FROM SPIKED SERUM AND STOMACH CONTENTS

Conditions as Table I.

Substrate and fortification level, mg/kg	*Percentage recovery (mean ± 95% confidence interval)*	
	Brodifacoum	*Warfarin*
Serum		
1.0	100	98
0.5	91	96
0.1	98 ± 6.5	96 ± 9.0
0.05	86	90
Stomach contents		
1.0	92 ± 10.4	80 ± 5.7
0.1	87	74

substrate, with the 95% confidence interval for each combination of compound and substrate. (Statistical analysis justified the application of a single 95% confidence interval to all fortification levels of each compound–substrate combination.)

Recoveries of brodifacoum, difenacoum and coumatetralyl at levels of 0.05–1.0 mg/kg were all satisfactory, with mean values of 81–100% and only 6 of 120 individual results below 80%. The recovery of brodifacoum from liver at 0.005 mg/kg (range 71–85%, mean 78%) was also adequate, although lower than recoveries at the higher levels. Recoveries of difenacoum from liver were higher than these obtained previously by adsorption HPLC[2], perhaps because the putative causes of the lower recoveries (transfer of the initial chloroform extractives to methanol and delay between spiking and extraction) were avoided.

Warfarin was well recovered from serum at all levels: only one of 20 individual results was below 80%. Recoveries from liver and stomach contents were lower (and lower than those obtained from liver by the method previously used at this laboratory[1]), but sufficient for diagnostic purposes. Mean recoveries at fortification levels of 0.1–1.0 mg/kg ranged from 74 to 84%, and all individual values were 70% or higher. The mean recovery from liver spiked at 0.05 mg/kg was 65%, with a range of 62–72%.

As mentioned above, brodifacoum, difenacoum and coumatetralyl were detected with almost identical sensitivity (response relative to mass/volume concentration) under the fluorescence conditions used. In routine work, therefore, standard solutions of any one of the three can be used for the quantitation of any compound(s) eluted at their common retention time, and the compound(s) can subsequently be identified in the collected eluate fraction. The lower limit of determination for these rodenticides by the normal procedure is apparently about 10–20 μg/kg. If analysis for warfarin is not required, however, lower levels are easily determined by applying a more concentrated extract to the Sep-Pak cartridge and eluting with chloroform (see Fig. 1D), when the limit of determination appears to be below 1 μg/kg. Warfarin is much less sensitively detected by fluorescence: its limits of determination in routine use are about 0.05 mg/kg in serum and 0.1 mg/kg in liver.

Substantially lower levels of all four rodenticides can be determined by collecting eluate fractions from successive injections and combining, concentrating and reinjecting them as described elsewhere[1,7]. An internal standard might be needed: warfarin could be used as internal standard for brodifacoum, difenacoum or coumatetralyl, and any of these three would be suitable as an internal standard for warfarin. Warfarin can also be determined at very low levels by specific-ion mass spectrometry[1] in eluates from exclusion columns.

Identification

Brodifacoum, difenacoum and coumatetralyl in collected eluate fractions can be differentiated by adsorption or reversed-phase chromatography, with mobile phases similar to those used by Yuen[4] and Koubek *et al.*[5], respectively: representative chromatograms are shown in Fig. 2. The *cis*- and *trans*-isomers of both brodifacoum and difenacoum are separated on the adsorption column (Fig. 2A). Adsorption is convenient because it is carried out on the exclusion column, and is perhaps the method of choice for certainty of identification. Reversed-phase chromatography (Fig. 2B) is quicker, however, and the chromatogram is simpler. It is to be preferred

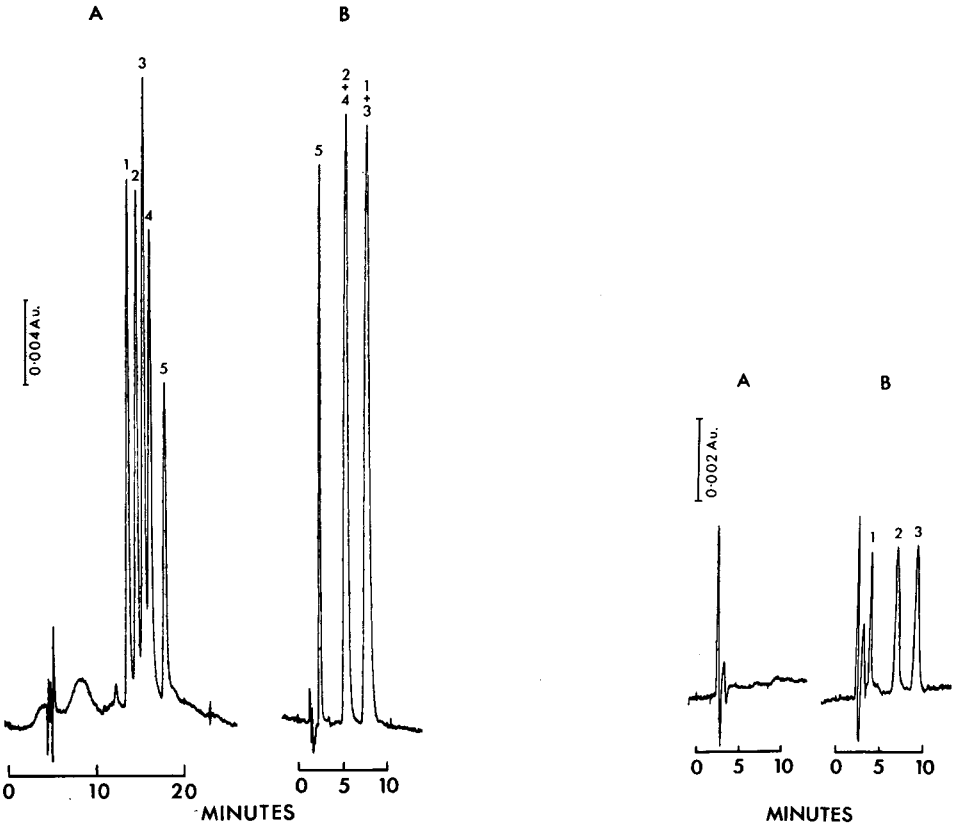

Fig. 2. Separation of brodifacoum, difenacoum and coumatetralyl (40 ng of each) by (A) adsorption chromatography on porous silica and (B) reversed-phase chromatography on a docosyl bonded phase. Peaks: 1 and 3 = isomers of brodifacoum; 2 and 4 = isomers of difenacoum; 5 = coumatetralyl.

Fig. 3. Identification of brodifacoum, difenacoum and coumatetralyl by reversed-phase chromatography. Eluate fractions X (see Fig. 1, A and B) from exclusion column chromatographed on reversed-phase column. A, unfortified liver, as Fig. 1A; B, fortified liver, as Fig. 1B. Peaks: 1 = coumatetralyl; 2 = mixed isomers of difenacoum; 3 = mixed isomers of brodifacoum.

for quantitative determinations if more than one of the three rodenticides is present. Fig. 3A and 3B show reversed-phase chromatograms from the extracts of unspiked and spiked liver whose exclusion chromatograms are shown in Figs. 1A and 1B. Ten successive aliquots of the cleaned-up liver extracts were injected on to the exclusion column, and the eluate fractions marked "X" in Fig. 1A and 1B were collected and taken to dryness, the residues being dissolved in 50 μl of the reversed-phase eluent. Fig. 3B shows the separation of coumatetralyl, difenacoum and brodifacoum in that order.

The sensitivity of detection by UV absorption is much lower than by fluorescence, but is again closely similar, when measured by peak height, for the three compounds in eluates from the reversed-phase column. The brodifacoum and difenacoum peaks are notably broader than those from coumatetralyl (Figs. 2B and 3B), however, possible owing to the incipient resolution of *cis*- and *trans*-isomers.

CONCLUSIONS

Brodifacoum, difenacoum, coumatetralyl and warfarin can be rapidly and sensitively determined in animal materials by exclusion HPLC on porous silica. The method appears to be applicable to a wide range of substrates.

If analysis is for all four rodenticides, the lower limits of determination are about 0.05–0.1 mg/kg for warfarin and about 0.02 mg/kg for brodifacoum, difenacoum and coumatetralyl. If warfarin is not included in the analysis, a more selective clean-up lowers the limit for the other three compounds to about 1 μg/kg or below. The method can be extended to determine sub-μg/kg levels of all four compounds.

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CHROM. 14,166

ZUR BESTIMMUNG VON CARDENOLIDEN AUS GEWEBEKULTUREN VON DIGITALIS-ARTEN

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(Eingegangen am 7. Juli 1981)

SUMMARY

Quantification of cardenolides in cell cultures of Digitalis

As cell cultures of Digitalis seem to form only a few cardenolides, a simple method for the identification and quantification of cardenolides from cell culture material was prepared. The ethanolic extract of the cell material is purified by column chromatography using the reversed-phase sorption material LiChroprep RP-18. The eluted cardenolides are separated by thin-layer chromatography on silica gel using different solvent systems. Quantification of cardenolides is carried out by measuring UV absorption *in situ*. The total analysis requires about 14 h.

EINLEITUNG

Für die Analyse der Cardenolidmuster von Gewebekulturen aus Digitalis-Arten verwendeten wir bislang das zwar sehr genaue und verlässliche, dafür aber zeitraubende Verfahren nach Kaiser[1] mit der Quantifizierung der Hauptglykoside nach Fuchs *et al.*[2]. Als zusätzliche Verfahren benützten wir nach der Fraktionierung über Silicagel[1] die dünnschichtchromatographische (DC) Auftrennung nach Kartnig und Nöhammer[3] und die Auftrennung mittels Hochleistung-Dünnschichtchromatographie (HPTLC) nach Kartnig und Kobosil[4] mit der Quantifizierung durch Remissionsmessung *in situ*.

Da die Cardenolidgehalte der Gewebekulturen aus Digitalis-Arten im allgemeinen wesentlich geringer sind als die der Digitalis-Pflanzen und -Drogen, werden für eine Analyse nach Kaiser 150–250 g Gewebe benötigt. Die Anzucht solcher Gewebemengen nimmt, insbesondere in Oberflächengewebekulturen, relativ viel Zeit in Anspruch.

Ausgehend von der Beobachtung, dass in den im Laufe von acht Jahren von uns angelegten und analysierten, zahlreichen Gewebekulturstämmen aus Digitalis *purpurea* und *lanata* maximal fünfzehn Cardenolide aufgefunden werden konnten, wurde die nachstehende Analysenmethode ausgearbeitet, die nur *ca.* 10 g Untersuchungsmaterial und *ca.* 13–14 h Arbeitszeit benötigt.

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EXPERIMENTELLES

Extraktion des Gewebes

Es werden 10 g Gewebe mit 29 ml Äthanol (96 %) versetzt, mit einem Ultraturrax durch *ca.* 5 min zerkleinert und sodann über 15 min unter Rückflusskühlung am Wasserbad extrahiert. Das blanke Filtrat wird unter vermindertem Druck (Rotavapor) zur Trockene gebracht und in 10 ml Äthanol (70 %, v/v) aufgenommen.

Reinigung des äthanolischen Extraktes

10 g LiChroprep RP-18 (Merck, Darmstadt, B.R.D., Nr. 13900) werden in insgesamt 50 ml Äthanol (70 %) suspendiert und annähernd quantitativ in eine Glassäule (*ca.* 400 × 8 mm I.D.) gefüllt. Nach Abtropfen des überstehenden Äthanols werden die 10 ml Extrakt (s. *Extraktion des Gewebes*) in einem aufgebracht. Elution der Cardenolide mit Äthanol (70 %). Vorlauf 10 ml. Auffangen von 10 Fraktionen zu 2 ml.

DC Ermittlung des Cardenolidmusters

Abdunsten des Äthanols der einzelnen Fraktionen und Aufnehmen der Rückstände in jeweils 0.5 ml Chloroform–Methanol (1:1).

Sorptionsschicht: DC-Alufolien KG 60 (Merck, Nr. 5553), selbst geschnitten in Streifen von 10 × 5 cm ("Mikro-DC").

Auftragemenge: 3.0 µl (Auftragen mit 0.75-µl Mikrokapillare).

Fliessmittelgemisch I (FG I): Cyclohexan–Aceton–Eisessig (4.9:4.9:0.2) (Unter bestimmten Umständen erweisen sich die Volumsrelationen (3.1:6.7:0.2), (3.7:6.1:0.2) oder (4.3:5.5:0.2) als brauchbarer).

Fliessmittelgemisch II (FG II): Chloroform–Methanol (8.0:2.0) (Unter bestimmten Unständen erweisen sich die Volumsrelationen (7.0:3.0), (8.5:1.5) oder (9.0:1.0) als brauchbarer).

Laufstrecke: 7 cm, Entwicklungszeit mit FG I und FG II *ca.* 15 Min.

Detektion: Besprühen mit Trichloressigsäurereagens nach Aldrich *et al.*[5] [3.3 g Trichloressigsäure in 10 ml Chloroform und 2 Tropfen H_2O_2 (30 %)]. Betrachten im UV-Licht (254 nm). Besprühen mit den Reagentien nach Kedde und/oder Baljet.

Quantifizierung der Cardenolide

Durch Messung der Remission *in situ* von einer undetektierten Bahn quer zur Laufrichtung. Spektralphotometer Zeiss PMQ 3 mit Zusatz zur Direktauswertung. Schreiber Servogor Sb, 200 mV, Messung bei 225 nm. Untere Messgrenze für Aglykone *ca.* 150 ng, für Tetraoside *ca.* 300 ng. Relative Standardabweichung für Aglykone ± 2.80 %, für Bioside ± 2.81 % und für Tetraoside ± 2.90 %. Ablesung an den entsprechenden Eichkurven (Fig. 1).

Dauer der Analyse

Die Analysendauer beträgt 13–14 h.

Regeneration der LiChroprep RP-18 Säule

Elution der Verunreinigungen mit Chloroform. Nachwaschen mit 70 % Äthanol. LiChroprep RP-18 ist oftmalig verwendbar.

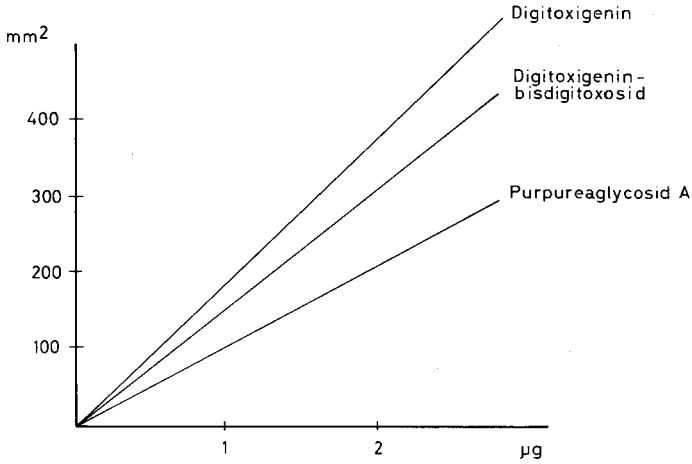

Fig. 1. Eichkurven bei Remissionsmessung in *situ* (225 nm; undetektiert).

ERGEBNISSE UND DISKUSSION

Die vorgestellte Analysenmethode zeichnet sich durch geringen Untersuchungsmaterial- und Zeitaufwand aus. Diese Verbesserungen konnten durch vier Änderungen gegenüber herkömmlichen Verfahren erreicht werden:

(1) Ersatz der üblichen Reinigung des Drogenextraktes mittels Ausschüttelung durch säulenchromatographische Reinigung über dem Reversed-phase-Säulenmaterial LiChroprep RP-18. (Extrakte aus Digitalis-Gewebekulturen enthalten wesentlich weniger Extraktivstoffe als Extrakte aus Digitalis-Drogen).

TABELLE I

R_F-WERTE EINIGER CARDENOLIDE (AGLYKONE, DIGITOXIGENINGLYKOSIDE, TETRAOSIDE)

Dtg = Digitoxigenin, Dgx = Digitoxose.

Cardenolide	*FG I (4.9:4.9:0.2)*	*FG II (8.0:2.0)*	*Fluoreszenzfarbe* (254 nm)*
Digitoxigenin	0.74	0.84	gelb
Gitoxigenin	0.61	0.76	blau
Digoxigenin	0.50	0.71	blau
Diginatigenin	0.41	0.61	blau
Gitaloxigenin	0.68	0.81	blau
Dtg–Dgx	0.54	0.80	gelb
Dtg–Dgx–Dgx	0.50	0.77	gelb
Dtg–Dgx–Dgx–Dgx	0.41	0.76	gelb
Purpureaglykosid A	0.01	0.46	gelb
Purpureaglykosid B	0.00	0.37	blau
Lanatosid A	0.07	0.58	gelb
Lanatosid B	0.04	0.54	blau
Lanatosid C	0.01	0.50	blau

* Nach Besprühen mit Trichloressigsäure-Reagens (Aldrich *et al.*[5]).

(2) Weglassen der säulenchromatographischen Fraktionierung der extrahierten Cardenolide über Silicagel, da 15 bis 20 Cardenolide durch DC- und HPTLC-Systeme ausreichend getrennt werden können.

(3) Ersatz der Papierchromatographie (PC) durch "Mikro-DC" und HPTLC.

(4) Ersatz der kolorimetrischen Quantifizierung durch Remissionsmessung *in situ*.

Reinigungseffekt am äthanolischen, cardenolidhältigen Auszug

Für den Reinigungseffekt der LiChroprep RP-18 Säule am äthanolischen, cardenolidhältigen Auszug aus den Gewebekulturen sind folgende Kriterien ausschlaggebend:

Äthanolkonzentration des zu reinigenden Extraktes. Der beste Reinigungseffekt wird bei einer Äthanolkonzentration von 70% erzielt. Da der Wassergehalt der Gewebe etwas schwankt, wird der primäre Äthanolisch–wässrige Extrakt im Vakuumrotationsverdampfer zur Trockene gebracht und sodann mit 70% Äthanol aufgenommen (*Auftragevolumen*).

Mengenrelation Gewebe–LiChroprep RP-18. Unter Einhaltung einer entsprechenden Säulendimension (s.u. "Säulendimension") reicht der Reinigungseffekt von LiChroprep RP-18 bei einer Äthanolkonzentration von 70% annähernd für die gleiche Gewebemenge frisches Gewebekulturmaterial (Trockenrückstand *ca.* 5%). Zur Reinigung eines Extraktes aus 10 g Gewebekulturmaterial sind demnach 10 g LiChroprep RP-18 nötig. (Bei Digitalis-Drogen reichen 10 g LiChroprep RP-18 nur für die Reinigung eines Extraktes aus 1 g Droge!).

Säulendimension. Für eine ausreichende Reinigung des Drogenextraktes muss die Säulenfüllung eine Mindesthöhe aufweisen. Für 10 g LiChroprep RP-18 (ausreichend für die Reinigung von maximal 10 g Gewebe) darf der Säulendurchmesser nicht mehr als 8 mm betragen.

Auftragevolumen. Der Reinigungs- bzw. Elutionseffekt ist mit vom Auftragevolumen abhängig. Zu grosse Auftragevolumina führen zu einer schlechten Abtrennung der Verunreinigungen. Das kleinstmögliche Auftragevolumen wird von der Löslichkeit des Abdampfrückstandes des Primärextraktes im 70% Äthanol bestimmt. Es beträgt im allgemeinen pro 1 g Gewebekulturmaterial 1 ml Äthanol (70%), d.h. für 10 g Gewebe 10 ml Äthanol (70%), wobei der Rückstand unter leichtem Erwärmen aufgenommen wird. Unmittelbar nach dem Einziehen der aufgebrachten äthanolischen Lösung wird mit der Elution mit 70% Äthanol begonnen.

Bei der Elution mit 70% Äthanol aus der LiChroprep Säule erscheinen die Cardenolide nach ihrer chemischen Struktur beim Austritt aus der LiChroprep RP-18 ml. Die Verunreinigungen verbleiben zum grössten Teil als schmaler, dunkelgrüner Ring am Start. Die Cardenolid-enthaltenden Fraktionen (z.B. zu je 2 ml) sind unter Umständen leicht gelblich–bräunlich gefärbt, jedoch stören diese miteluierten Verunreinigungen die folgende DC-Auftrennung der Cardenolide nicht. Eine Reihung der Cardenolide nach ihrer chemischen Struktur beim Austritt aus der LiChroprep RP-18 Säule konnte nicht beobachtet werden. (Nach Abschluss der Elution werden die Verunreinigungen mittels Chloroform und darnach mittels 70% Äthanol aus der Säule gewaschen und die Säule damit regeneriert. Das LiChroprepmaterial ist oftmalig verwendbar).

Auftrennung und Identifizierung der Cardenolide

Das üblicherweise in Fraktionen zu 2 ml aufgefangene Eluat der LiChroprep RP-18 Säule kann für den Fall, dass nur wenige Cardenolide vorliegen, zusammengelegt und der Untersuchung mittels DC und HPTLC zugeführt werden, die Fraktionen können aber auch —für den Fall, dass mehrere Cardenolide vorliegen— für sich getrennt chromatographiert werden. In jedem Fall wird die Elutionsflüssigkeit (70% Äthanol) abgedunstet und der Rückstand in 0.5 ml Chloroform–Methanol (1:1) aufgenommen.

Die chromatographische Auftrennung der Cardenolide erfolgt auf DC-Alufolien KG 60 in der Grösse von 10 × 5 cm (selbst geschnitten), die bei kürzerer Entwicklungszeit einen besseren Trenneffekt als solche der Grösse 20 × 20 cm erbringen. Als Fliessmittelgemische werden die Systeme Cyclohexan–Aceton–Eisessig und Chloroform–Methanol verwendet, wobei die quantitative Zusammensetzung je nach vorhandenem Cardenolidmuster variiert werden kann (s. Experimentelles). FG I eignet sich besonders zur Auftrennung der Aglykone, FG II besonders zur Auftrennung der Tetraoside (s. Tabelle I).

Zur eventuellen Erhärtung der DC-Befunde kann zusätzlich das HPTLC-System nach Kartnig und Kobosil[4] herangezogen werden.

Die Identifizierung der Cardenolide erfolgt an Hand von authentischen Vergleichssubstanzen, in vereinzelten Fällen an Hand des R_F-Wertes oder durch Interpolieren des R_F-Wertes. Die Fluoreszenzfarben der Flecke (Trichloressigsäure; 254 nm; gelb = Digitoxigeninderivate, stahlblau = Digoxigeninderivate, leuchtend blau = die Derivate der drei übrigen Aglika) sind eine weitere Hilfe. Für die Absicherung fraglicher Ergebnisse kann die beschriebene "Mikro-DC" auch in zweidimensionaler Form unter Verwendung der beiden Fliessmittelgemische I und II oder des HPTLC-Trennsystems nach Kartnig und Kobosil[4] herangezogen werden.

Die Quantifizierung der Cardenolide erfolgt durch Remissionsmessung *in situ* bei 225 nm[6]. Die Messung wird am besten auf einer nicht-detektierten Bahn durchgeführt. Sie kann allerdings auch auf einer mit Trichloressigsäure-Reagens nach Aldrich *et al.*[5] detektierten Bahn erfolgen, jedoch ist dabei die Empfindlichkeit entsprechend geringer. Die Remissionsmessung nach Detektion mit dem Reagens nach Baljet ist bei 225 nm wohl durchführbar, aber ebenfalls wenig empfindlich, während die Messung nach Besprühen mit Keddes Reagens auf Grund der zu unruhigen Nullinie nicht möglich ist. Die Fluoreszenzmessung (Detektion mit Trichloressigsäure-Reagens) brachte keine befriedigenden Ergebnisse, da die Flecken zum Teil inhomogene Fluoreszenzfarben (z.B. im Zentrum blau, am Rande gelb) aufweisen.

Die Remissionsmessung wird quer zur Laufrichtung durchgeführt. Die Spreizung am Schreiber ist je nach vorhandener Cardenolidmenge zu wählen. Durch Verwendung der HPTLC (Auftragen mittels Platin–Iridium Mikrokapillare) kann die Erfassungsgrenze noch weiter (auf *ca.* 50 ng für Aglyka) gesenkt werden (s. Kartnig und Kobosil[4]).

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SEPARATION OF CROCETIN GLYCOSYL ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Different methods for the separation of crocetin glycosyl esters from the ethanolic extract of saffron by high-performance liquid chromatography are discussed. After a clean-up by gel filtration on Sephadex G-50, best results were obtained with LiChrosorb SI 60 as stationary phase and ethyl acetate–isopropanol–water (56:34:10) as mobile phase.

INTRODUCTION

Earlier we reported our investigations of the carotenoid composition in the ethanol extract of saffron (stigma of *Crocus sativus* L.)[2,3]. Beside crocin, which has long been known as the digentiobiosyl ester of the polyene dicarboxylic acid crocetin (1), we isolated four additional pigments. As shown in Fig. 1, the new compounds are mono- and diglycosyl esters of crocetin (2)–(5).

For the isolation of these carotenoids, the ethanol extract was first cleaned up on a countercurrent distribution apparatus according to Signer[4], in the system *n*-butanol–water, and each fraction was purified twice by thin-layer chromatography (TLC). This procedure is very tedious, especially if large amounts of the pigments are needed for spectroscopic investigations. With the same method, we also examined the carotenoid composition of garden crocuses (*Crocus albiflorus*, *Crocus neapolitanus*) with regard to the elucidation of the biosynthesis of C_{20} carotenoids[5]. The investigations showed that these flowers also contain different glycosyl esters of crocetin as main pigments. Compared to saffron, new, more polar compounds were found, of which the main components seemed to be trisaccharide esters of crocetin. In this case, the separation method originally used turned out to be inadequate because of its poor performance. In the present paper, we report about a new method to separate the crocetin glycosyl esters using high-performance liquid chromatography (HPLC). Saffron was chosen for this study, because of its easy availability and the known composition of its pigments.

* Second report on separation of carotenoids by HPLC; for first report see ref. 1.
** Part of the planned Ph D. thesis of Martin Rychener.

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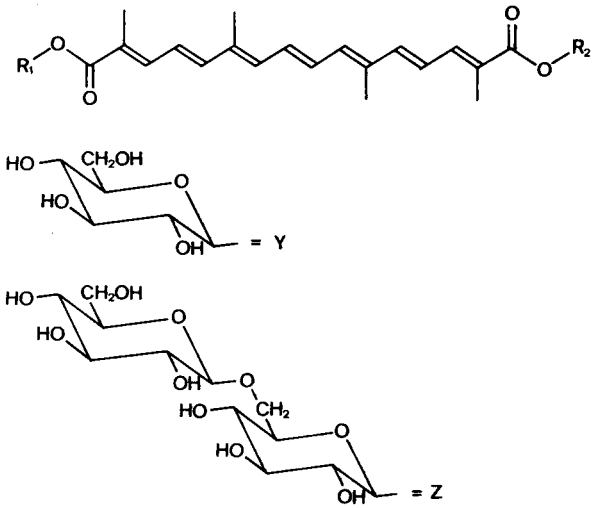

Fig. 1. Structures of crocetin glycosyl esters from saffron (*Crocus sativus* L.). 1, $R_1 = R_2 =$ Z (crocin); 2, $R_1 =$ Z, $R_2 =$ Y; 3, $R_1 =$ Z, $R_2 =$ H; 4, $R_1 = R_2 =$ Y; 5, $R_1 =$ Y, $R_2 =$ H; 6, $R_1 = R_2 =$ H (crocetin).

METHODS AND MATERIALS

General

Extraction and separation were carried out under shielding from light. Solvents were purified by standard methods[6].

Apparatus

An Altex Model 110A pump, a Rheodyne Type 7125 sample inlet system, and a Uvikon 725 detector were used. The columns were SS 316 (250 × 4.6 mm I.D.).

Clean-up

The extraction was carried out according to the details in ref. 2, followed by evaporation of ethanol. The aqueous solution was lyophilised for 24–36 h. A deep red, granular powder was obtained, in a yield of 52 g from 100 g of saffron (*Crocus naturalis pulvis*, Siegfried AG).

Gel filtration

A 5-g amount of extract dissolved in 15 ml of H_2O, was placed on a Quickfit column (80 cm × 38 mm I.D.) charged with Sephadex G-50 (Pharmacia, Uppsala, Sweden) and eluted with H_2O saturated with $CHCl_3$, at an upward flow-rate of 0.2–0.4 ml/min by hydrostatic pressure. The coloured eluate was evaporated and dried under high vacuum. The yield of the deep red powder was 800 mg.

Conditions for HPLC

Method A. The stationary phase was LiChrosorb SI 60, 7 μm (Merck, Darmstadt, G.F.R.). The mobile phase was ethyl acetate–isopropanol–water (56:34:10); UV detection was at 440 nm, and the flow-rate was 0.6 ml/min. The sample consisted of 20 μl of a solution in the mobile phase (crocetin chromophore

titre of 140 nmol/ml). The column was slurry-packed at 400 bar, giving a number of theoretical plates of 6000–8000 with crocetin (Hoffmann-La Roche, Basel, Switzerland) as test substance.

Method B. The stationary phase was LiChrosorb RP-18, 5 μm (Merck). The mobile phase was methanol–water (60:40). UV detection was at 440 nm, and the flow-rate was 1 ml/min. The sample consisted of 20 μl of a solution in ethanol–H_2O (titre: 220 nmol/ml). The column was packed according to the method in ref. 7, giving a number of theoretical plates of 3000–4000 with phenol as test substance.

Method C. The stationary phase was LiChrosorb SI 60, 7 μm (Merck), coated with 0.1 *M* $NaHSO_4$. The mobile phase was ethyl acetate–*n*-hexane (70:30). UV detection was at 440 nm, and the flow-rate was 0.4 ml/min. The sample consisted of 20 μl of solution in the mobile phase (titre: 220 nmol/ml). The column was packed according to the method in ref. 7, giving a number of theoretical plates of 5500–7500 with acetophenone as test substance.

Identification

The compounds were identified by mass spectrometry and UV/visible spectra, and TLC of the carbohydrates. Synthetic crocetin and crocetin-di(β-D-glucosyl) ester were used as reference compounds.

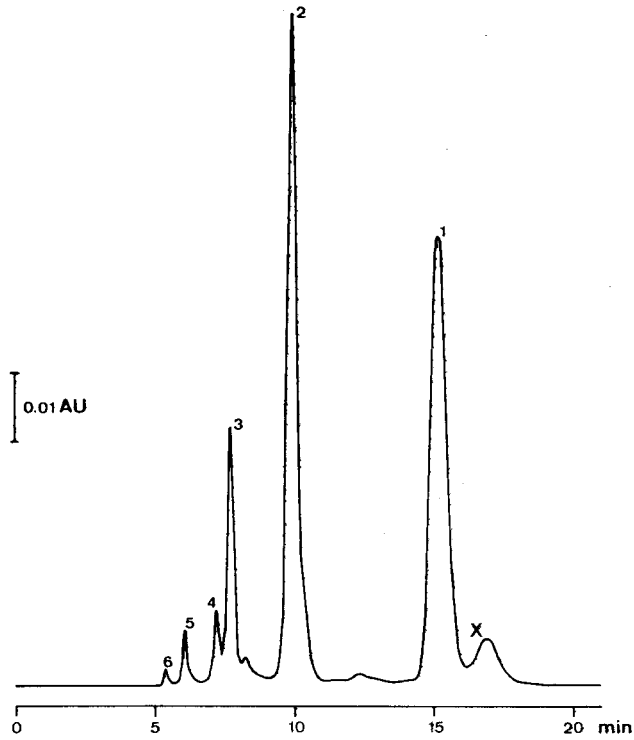

Fig. 2. Separation of precleaned saffron extract (method A).

RESULTS AND DISCUSSION

Experiments to separate the pigments of the ethanol extract of saffron without a clean up were unsuccessful. The best results were obtained after lyophilisation of the extract followed by gel filtration on Sephadex G-60. The lyophilisation of the aqueous extract yielded a deep red, granular powder and the gel filtration removed the free carbohydrates, which disturb the separation of the pigments.

HPLC on LiChrosorb SI 60 with ethyl acetate–isopropanol–water (56:34:10) (method A) proved to be successful for the separation of the carotenoid pigments. The compounds 1–6 could be separated within 20 min, as shown in Fig. 2.

As expected, the separation of the diglucosyl ester from the monogentiobiosyl ester turned out to be very difficult. The separation time could possibly be shortened using a gradient, but because of the unusual addition of water to the mobile phase, which is necessary owing to the high polarity of the glycosylesters, the reconditioning of the column would be time consuming.

Besides the pigments 1–6, which were already known, a new compound X appeared in the chromatogram. This pigment, which is more polar than crocin, is identical with the trisaccharide ester that occurs as main component in garden crocuses. Because of its low concentration in saffron, X was not detected using TLC. The chromatogram also showed the quantitative composition of the pigments in saffron. As expected, the main pigment is crocin (*ca.* 40–45%), followed by the mixed ester 2 (*ca.* 35%), the diglucosyl ester 3 (*ca.* 10%) and the two monoesters 4 and 5 (each *ca.* 2%).

The reversed-phase systems, often used to separate polar compounds, proved to be useful only for the separation of diglycosyl esters. Fig. 3 shows a perfect separa-

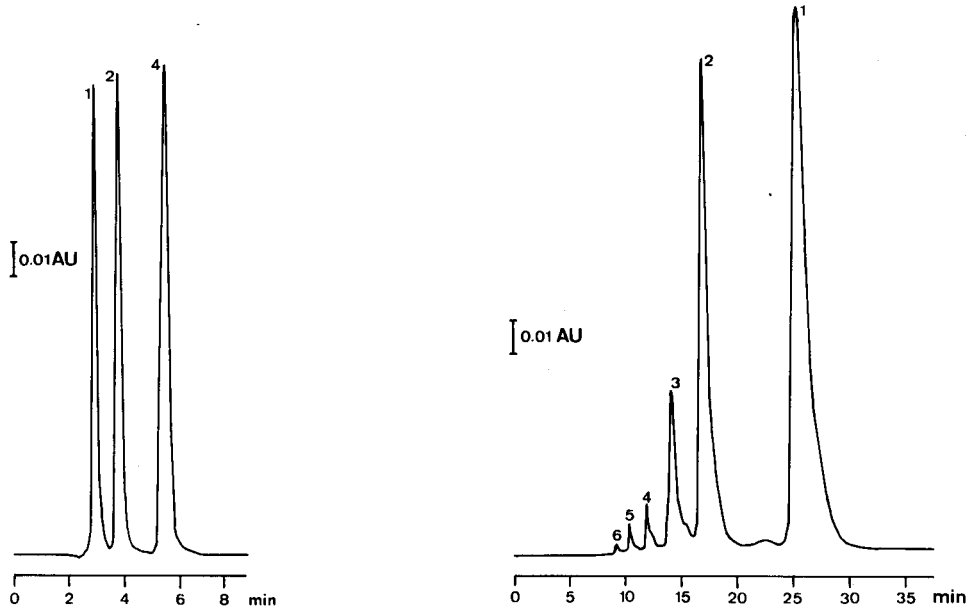

Fig. 3. Separation of diglycosyl esters from saffron (method B).

Fig. 4. Separation of precleaned and acetylated saffron extract (method C).

tion of the digentiobiosyl (1), the diglucosyl (4) and the mixed ester (2) within 6 min, using a LiChrosorb RP-18 column (method B).

For the separation of compounds with a free carboxyl group, *i.e.* the monoglycosyl esters and crocetin, method B was unsuitable because of a partial deprotonation of the carboxyl groups under these conditions. This resulted in strong tailing and extremely long retention times. The addition of an acid to the mobile phase is not appropriate owing to the lability of the glycosyl esters towards hydrolysis.

Because peracetates of the glycosyl esters are generally better separable than the non-acetylated ones, the cleaned up extract was peracetylated with acetic anhydride in pyridine. To avoid chemical tailing, a LiChrosorb SI 60 column was coated with 0.1 *M* $NaHSO_4$ (ref. 8) and ethyl acetate–*n*-hexane (70:30) was used as mobile phase (method C) (Fig. 4). A relatively slow flow-rate (0.4 ml/min) was needed to obtain a satisfactory separation. The separation time, however, was longer compared with that of the non-acetylated pigments, and the polar pigment corresponding to X was not separated from the crocin under these conditions. Moreover, the quality of the coated column decreased quickly.

Our investigations have shown that the best results in the separation of glycosyl esters from saffron can be obtained by using HPLC on silica gel after cleaning up the extract by lyophilisation and gel filtration, because of the higher number of theoretical plates of silica gel columns compared with reversed-phase systems. In comparison with TLC, HPLC gives shorter separation times under very mild conditions. The performance of the system is shown by the fact that for the first time more polar crocetin derivatives than crocin were detected in saffron.

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This work was supported by the Swiss National Science Foundation (Project No. 2.684-0.80). We are also grateful to F. Hoffmann-La Roche & Co., Ltd. (Basle) for their support, to Mrs. V. R. Meyer for helpful discussions, and to Dr. M. Läderach for assistance with the manuscript.

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Note

Isotope effects on solution properties

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The gas–liquid chromatographic (GLC) method of obtaining ΔG^0, ΔH^0 and ΔS^0 for the transfer of vapors into infinitely dilute solution has been shown to be quite reliable when dealing with alkane systems for which interfacial effects are negligible[1]. Thus an extension of the studies of Jakli *et al.*[2] on the vaporization properties of pure deuterium-substituted molecules becomes possible using this method, and we provide herein the necessary data for comparison of the solution properties of three pairs of "light" and "heavy" hydrocarbons in *n*-hexadecane (C_{16}) and in *n*-tetracosane (C_{24}).

EXPERIMENTAL

The apparatus and data handling have been described[3]. C_{16} and C_{24} were used as received from the Chemical Samples Co. The "heavy" hydrocarbons were obtained from Merck, Sharp and Dohme of Canada. The C_5 and C_6 compounds had a stated deuterium content of 98; the C_7, 99 atom %, corresponding to ^1H contents of 0.24, 0.28 and 0.16 atom per "heavy" molecule, respectively. A run involved measurement of the retention times of both members individually at each temperature. In no case was solvent bleeding as great as 0.1 mass %, and no corrections were required. Lack of certainty in the exact mass of solvent in the column may affect the accuracy of the equilibrium pressures, ΔG^0 and ΔS^0 values, but the ratio of pressures for each pair, as well as ΔH^0 values, are independent of this variable.Comparison of the present data for *n*-hexane in the C_{16} solvent with earlier results from this laboratory[1] indicate, in fact, that 3.2 % of the original mass of solvent had bled from the column before it was used for this work. A similar comparison for the C_{24} solvent indicates that 1.5 % had been lost. The values reported in Table I have been adjusted to take this into account. The ΔH^0 values for *n*-hexane in C_{16} and C_{24} in the present work are 0.13 % lower, and 0.23 % higher, respectively, than those observed with the original columns[1]. The thermodynamic properties of solution were derived from quadratic correlations of the logarithm of the specific retention volume *versus* inverse temperature, except in the case of the *n*-pentane pair in C_{16}, for which a linear correlation was used.

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'ABLE I

'APOR PRESSURES OF HYPOTHETICAL PURE LIQUID SOLUTES IN n-$C_{16}H_{34}$ AND n-$C_{24}H_{50}$

' = Temperature; P = pressure.

'olvent	T (°K)	$P_{C_5H_{12}}$ (kPa)	$P_{C_5{}^2H_{12}}$ (kPa)	T (°K)	$P_{C_6H_{14}}$ (kPa)	$P_{C_6{}^2H_{14}}$ (kPa)	T (°K)	$P_{C_7H_{16}}$ (kPa)	$P_{C_7{}^2H_{16}}$ (kPa)
-$C_{16}H_{34}$	291.85	47.56	52.93	292.44	14.17	15.81	293.63	4.441	5.036
	292.47	48.86	54.00	293.66	14.94	16.69	298.52	5.674	6.398
	297.30	58.56	64.56	297.28	17.50	19.49	303.35	7.201	8.111
	302.16	69.47	76.68	303.35	22.54	25.06	308.26	9.023	10.14
				308.23	27.46	30.40	313.10	11.23	12.59
-$C_{24}H_{50}$	329.01	133.2	145.4	325.87	44.99	49.66	329.64	19.24	21.40
	333.91	151.8	165.9	335.07	60.76	66.87	339.35	27.43	30.51
	338.78	173.3	188.6	344.80	81.69	89.64	349.10	38.43	42.57
	343.69	196.1	213.0	349.68	94.22	103.0	358.79	52.52	57.85
	348.52	220.7	239.9	354.60	107.8	117.6	368.50	69.92	76.98
				364.23	138.9	150.8			

RESULTS

The relationship between the specific retention volume, V_g^0, and the equilibrium vapor pressure of the hypothetical pure liquid solute (*i.e.*, the Henry's law constant, k) is

$$k = \frac{273.15\ R}{M_l V_g^0}$$

where R is the gas constant and M_l is the relative molar mass of the solvent in the column. If V_g^0 has units of $cm^3\ g^{-1}$, this equation may be written

$$k\ (\text{kPa}) = \frac{2.271 \cdot 10^6}{M_l V_g^0}$$

Table I presents these pressures for both pairs of solutes at each temperature measured for both solvents. Table II presents the standard thermodynamic property changes for transfer of the ideal vapor at 101.325 kPa to the hypothetical pure solute liquid with intermolecular interactions characteristic of infinite dilution in solvent.

TABLE II

STANDARD THERMODYNAMIC PROPERTY CHANGES FROM VAPOR TO INFINITELY DILUTE SOLUTION IN n-$C_{16}H_{34}$ AND IN n-$C_{24}H_{50}$

Solute	*In n-$C_{16}H_{34}$ at 298.15°K*		*In n-$C_{24}H_{50}$ at 333.15°K*	
	$-\Delta H^\circ$ (kJ mol^{-1})	$-\Delta S^\circ$ (J mol^{-1} °K^{-1})	$-\Delta H^\circ$ (kJ mol^{-1})	$-\Delta S^\circ$ (J mol^{-1} °K^{-1})
n-C_5H_{12}	26.90	85.63	24.94	78.03
n-$C_5{}^2H_{12}$	26.44	85.00	24.64	77.90
n-C_6H_{14}	31.42	90.86	29.57	84.01
n-$C_6{}^2H_{14}$	31.09	90.65	29.27	83.89
n-C_7H_{16}	36.74	98.77	34.40	90.52
n-$C_7{}^2H_{16}$	36.23	98.10	34.14	90.65

DISCUSSION

The deuterium content of the solutes studied is such that if the ^1H were present as one atom per solute molecule, the purity of the "heavy" compounds is quite low: 76, 72 and 84 mole%, in fact, for C_5, C_6 and C_7, respectively. The vapor pressure isotope effect (VPIE) is approximately 0.7% per ^2H atom[2]; for n-$C_6{}^2H_{14}$ at 300°K, a true vapor pressure of 20 kPa would be lowered to 19.96 kPa by the presence of 30 mole% of n-$C_6{}^2H_{13}H$. The effect of isotopic impurity on measured enthalpy of solution would be still smaller. Consequently, no corrections to take into account the presence of protium in the "heavy" compounds were made.

As is true for the VPIE of the pure n-heptanes[2], equilibrium pressures of all solutes herein above both C_{16} and C_{24} are greater for the "heavy" member of each pair. Likewise, the enthalpy of vaporization from these solvents is smaller in every case for the "heavy" solute, in accord with analogous values for the pure n-heptanes. Entropies of vaporization appear to be less positive for the "heavy" than for the "light" alkanes, but the magnitude of the differences is close to experimental error (*ca.* 0.15 J mol^{-1} °K^{-1}). We can state that the isotope effect on entropy of vaporization is considerably smaller than that on enthalpy of vaporization for these compounds, and that the VPIE is the result of energetic rather than entropic factors. This is in spite of the expected role of greater mass and moment of inertia in providing an entropic advantage for the "heavy" alkane in the vapor phase.

In principle, we should be able to produce values for the change in heat capacity upon solution, ΔC_p^∞, from these data, but reliable values of this quantity require more data than were collected here[1]. Qualitatively, it appears that ΔC_p^∞ for the solution process is more positive by roughly 10 J mol^{-1} °K^{-1} for the "heavy" member of each pair, for the C_{24} solvent at 333°K.

The purpose of this note is not the interpretation of the isotope effects observed; others are better qualified. It is to give further evidence for the value of the GLC approach to the study of thermodynamics. In particular, we should note two significant advantages offered by the present approach as applied to the study of VPIE: (1) The environment of the vaporizing molecule can be varied (by using different solvents) allowing an attempt at separating out external from internal contributions to the VPIE. (2) Since the vapor phase solute is at a very low partial pressure in helium, it is behaving essentially ideally, and second virial coefficient data for the "heavy" molecule are not required in order to carry out statistical thermodynamic analysis of the results. This is particularly important because such data are generally not available, and the calculation of reduced partition function ratios has been a fruitful approach to the study of VPIE[4,5].

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Note

Prediction of responses of aromatic hydrocarbons in an electron-capture detector

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In a recent paper[1] we showed that many aromatic hydrocarbons can sensitively be analysed with an electron-capture detector (ECD) and, at the same time, this method can be used for the determination of some physico-chemical parameters of electron + aromatic hydrocarbon reactions[1–4].

The capture coefficients, K, of aromatic hydrocarbons, which determine the molar responses in the ECD, are usually interpreted in terms of electron affinities, EA, on the basis of the equation derived by Wentworth *et al.*[2]

$$K = (k_L/k_D)\, AT^{-3/2} \cdot \exp(\mathrm{EA}/kT) \qquad (1)$$

where T is the detector cell temperature, A is a fundamental constant, k is Boltzmann's constant and k_L/k_D is a constant characteristic of the detector. With this relation it is also possible to predict the detectabilities of aromatics. However, accurate EA values are known only for a rather limited number of compounds, partly because the methods commonly utilized for EA measurements are complicated and tedious, and the results are often erroneous as indicated by the large scatter of values obtained by different methods, especially with low or negative EA. It is worth noting that one of the best methods for measuring the absolute, adiabatic EA value of aromatic hydrocarbons (between EA = 0.1 and 0.9 eV) is supplied by the detector itself, utilizing the temperature dependence of the electron equilibrium[2–4]:

$$AB + e^- \rightleftharpoons AB^-$$

Based on theoretical and experimental investigations[3,5–7], it was found that the EA is closely correlated, among other molecular parameters, with the ionization potential, IP: this relationship can be used to predict EAs because IP values determined by different techniques for a large number (more than 100) of aromatics are available (see, *e.g.*, refs. 6, 8–12). There is, however, a problem that some of the methods (*e.g.*, electron impact, photoelectron spectroscopy) provide principally "vertical" IPs where during the ionization the nuclear coordinates do not change; in contrast, other methods (*e.g.*, photoionization) give an "adiabatic" value, *i.e.*, the energy difference between the equilibrated ground and ionized states.

In Fig. 1 the IPs were selected from the literature as "adiabatic" or "near

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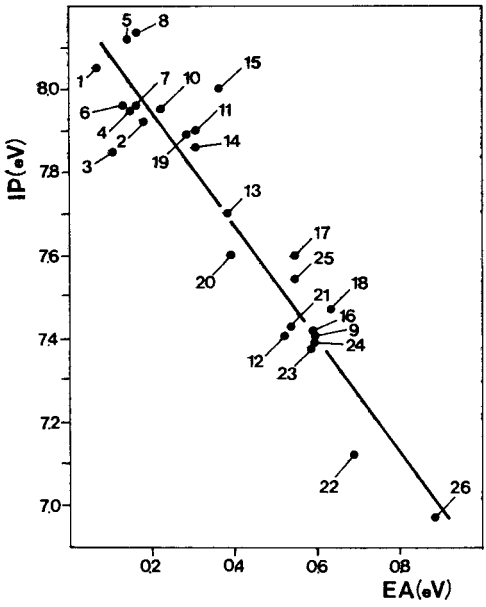

Fig. 1. IP *vs.* EA plot for aromatic hydrocarbons. The ionization potential was taken from the literature cited. Hydrocarbons: 1 = durene (1,2,4,5-tetramethylbenzene)[8]; 2 = pentamethylbenzene[8]; 3 = hexamethylbenzene[8]; 4 = biphenyl[9]; 5 = naphthalene[6]; 6 = 1-methylnaphthalene[6]; 7 = 2-methylnaphthalene[6]; 8 = indene[10]; 9 = azulene[6]; 10 = fluorene[9]; 11 = phenanthrene[11]; 12 = anthracene[12]; 13 = *trans*-stilbene[9]; 14 = diphenylacetylene[8]; 15 = 1,1-diphenylethylene[9]; 16 = pyrene[11]; 17 = benzo[*c*]phenanthrene[12]; 18 = benz[*a*]anthracene[11]; 19 = triphenylene[12]; 20 = chrysene[11]; 21 = benzo[*c*]pyrene[11]; 22 = benzo[*a*]pyrene[11]; 23 = dibenz[*a,h*]anthracene[11]; 24 = dibenz[*a,j*]anthracene[11]; 25 = picene[11]; 26 = naphthacene[12].

adiabatic" values, whereas all EAs were determined by the ECD method. The values for hydrocarbons 1–15 are from our work[1], 16–25 are from Becker and Chen[3] and 26 is from Lyons *et al.*[4].

In the case of highly conjugated polycondensed aromatics possessing large EA values, since the equilibrium internuclear positions in the ground state of the neutral molecule and the positive ion are approximately the same, the difference between the IP values determined by different techniques is not large: "vertical" IP values practically agree with the "adiabatic" values[6,11]. On the other hand, for less conjugated molecules (lower EA values), where large nuclear distance distortions may occur during ionization, the IPs determined by "vertical" and "adiabatic" techniques are considerably different[6]. From the literature [3,6] and also from our studies, it appears that EAs determined by the ECD method give a better correlation with the "adiabatic" IPs than with the "vertical" ones.

The relationship between IP and EA values (in eV) in Fig. 1 is described by:

$$\mathrm{IP} = (8.22 \pm 0.18) - (1.36 \pm 0.09)\ \mathrm{EA} \qquad (2)$$

By combining eqns. 1 and 2, the relation

$$K = (k_L/k_D)AT^{-3/2} \cdot \exp\,[1000(70.17 - 8.539\ \mathrm{IP})/T] \qquad (3)$$

allows the estimation of the approximate order of magnitude of K on the basis of known ionization potentials.

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Note

Isotachophoretic investigation of the binding of 8-anilino-1-naphthalenesulphonic acid to human serum albumin*

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Serum albumin has the ability to bind adsorptively a vast range of ligands, and the transport function of this protein is based on its interaction with small hydrophobic compounds. A listing of the substances whose binding to serum albumin has been investigated is presented by Chignell[1]. Visible and fluorescent dyes in particular have been employed for the experimental characterization of ligand binding to albumin. One such fluorescent dye is 8-anilino-1-naphthalenesulphonic acid (ANS), and a great deal of effort has been invested in the elucidation of the number and types of binding site for this ligand on the albumin molecule.

Typical analytical methods for this problem are fluorescence spectroscopy[2,3] and circular dichroism[4], from which it has been determined that bovine serum albumin possesses at least five binding sites for ANS, with differing affinities[4], and possibly also cooperativities[5,6]. An analysis of equilibrium constants and binding capacities using a modified Scatchard method from fluorescence measurements[3] yielded linear relationships both for human and bovine serum albumins, with $n = 3$ for ANS.

In conventional analytical techniques for binding studies, either the free or bound ligand is determined. As an alternative, analytical capillary isotachophoresis offers the opportunity to analyse simultaneously the protein–ligand complex and the free ligand. Thus, this technique has been applied to the analysis of free and bound indomethacin (1-*p*-chlorobenzoyl-5-methoxy-2-methylindole-3-acetic acid) and sodium dodecyl sulphate[7,8]. The appearance of free ligand at particular stoichiometric ratios was taken to indicate the limit of binding. In the present work, we show that this is not necessarily the case, and that further information on the nature of the binding can be obtained from the isotachopherograms.

MATERIALS AND METHODS

8-Anilino-1-naphthalenesulphonic acid was obtained from E. Merck (Darmstadt, G.F.R.) and human serum albumin (HSA) from Behring (Marburg, G.F.R.). The reagents for the leading and terminating electrolytes, hydrochloric acid, 6-aminohexanoic acid and 3-amino-1,2-propanediol were purchased from E. Merck, and

* This work constitutes a part of the doctoral thesis of G. Bulge.

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hydroxypropylmethyl-cellulose (HPMC) from Ega-Chemie (Steinheim, G.F.R.). All reagents were of the highest available purity, and were used as supplied.

The incubations were carried out in unbuffered neutral solution, since high concentrations of buffer ions lead to longer analysis times in the isotachophoretic analyser. A series of mixtures containing 0.1 mmol/l HSA and ANS in the range 0.1–0.8 mmol/l were employed. A volume of 5 μl of the incubation mixtures was sufficient for qualitative and quantitative analysis by capillary isotachophoresis.

Isotachophoretic analyses were carried out on the LKB 2127 Tachophor (LKB, Bromma, Sweden), fitted with a PTFE capillary (23 cm × 0.5 mm I.D.). The temperature of the capillary block was maintained at 20°C during the analyses. Detection of the zones was both by thermal and UV-signals at 280 nm.

The zones were evaluated as follows. The UV signal (percent transmisson) of the LKB-Tachophor was converted into extinction units using a desk computer. The lengths of the UV-absorbing zones were measured in seconds at half maximal height, with a constant current of 50 μA for the zones passing the detectors. The zones were integrated in terms of (extinction units × seconds). The thermo-signals were used for qualitative analysis of the protein zones. Reciprocal reference unit (RRU) values were calculated from the step heights of the leading electrolyte, h_L, terminating electrolyte, h_T, and sample zone, h_X, by the following expression:

$$\mathrm{RRU} = \frac{(h_T - h_L)}{(h_X - h_L)} \cdot 100$$

The RRU value increases with increasing mobility. A stable plateau of at least 30 sec duration was taken as the minimal criterion for evaluation of this parameter, owing to the slow response and relatively poor resolving power of the thermodetector.

RESULTS

Fig. 1 shows isotachopherograms derived from various stoichiometric ratios of ANS and HSA. From the UV-signals at 280 nm, it is apparent that higher extinctions are obtained with increasing amounts of bound ANS, due to the inherent UV-absorption of the dye itself. Up to a ratio of 3:1, very little free dye, which migrates behind the protein zone, is seen. Thus, it appears that at least three molecules of ANS are bound very tightly to the protein. At ratios greater than 3:1, a further increase in UV-absorption occurs, but very much smaller than the initial increase up to a ratio of 3:1. However, this does indicate that further molecules of dye are bound. With increasing degree of ANS binding, the protein zone loses its characteristic rectangular form, indicating non-homogeneity of the zone. This behaviour, again, is particularly marked at ANS:HSA ratios greater than 3:1. Use of a longer capillary did not influence this pattern, so that the effect is not purely an artefact of the short capillary.

A plot of integrated area of the protein zone against ANS:HSA ratio is depicted in Fig. 2. A linear increase is observed up to a stoichiometry of 3:1, followed by a less steep increase at higher ratios. This is indicative of two types of binding, the first three molecules of ANS being bound more tightly than those at higher ratios. Fig. 3 represents a control plot for that in Fig. 2. Here, the total surface areas of the protein

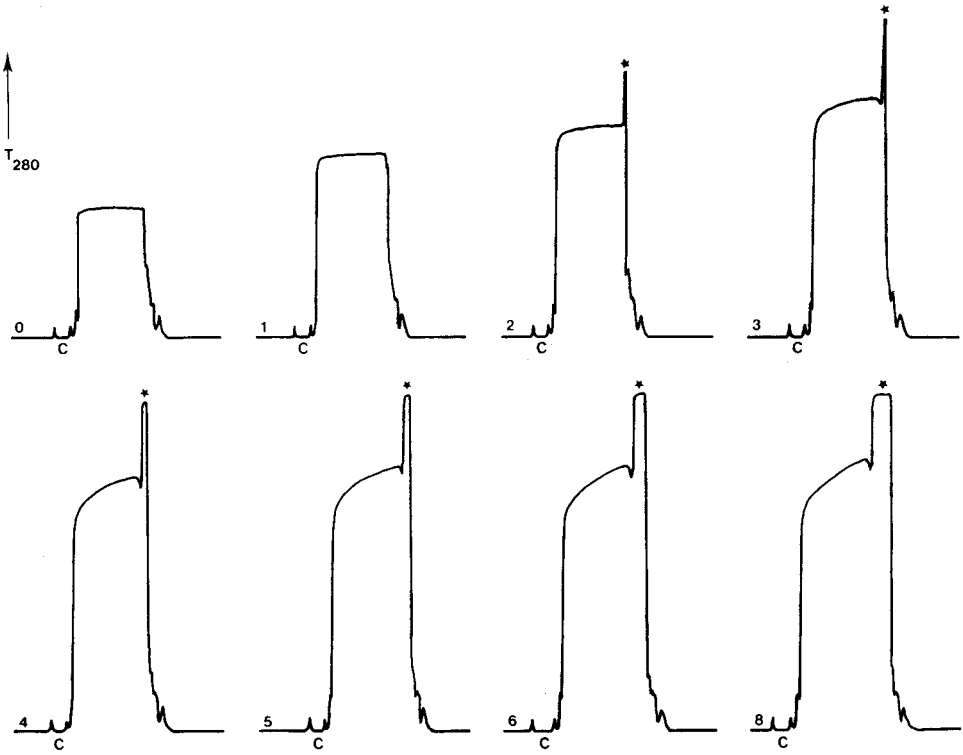

Fig. 1. Isotachopherograms derived from incubations of ANS with HSA at various stoichiometric ratios in the range 0 – 8, as indicated at the left-hand side of each trace. The UV signals at 280 nm are shown. The carbonate zone, always present to a small extent at high pH, is indicated by C, and the free dye zone by ⋆.

and free dye zones at the various ANS:HSA ratios have been added, and, as expected, the plot is linear over the whole range.

Not only the UV signals, but also the thermo signals altered following dye binding. Fig. 4 shows this effect: with increasing degree of dye binding, the thermal step height of the protein zone is lowered, representing a shift to higher mobility.

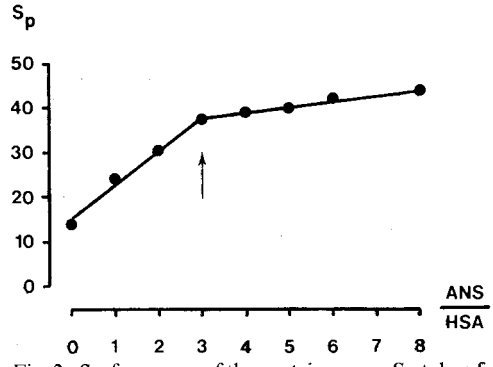

Fig. 2. Surface areas of the protein zones, S_p, taken from the isotachopherograms in Fig. 1, plotted against the respective ANS:HSA ratios.

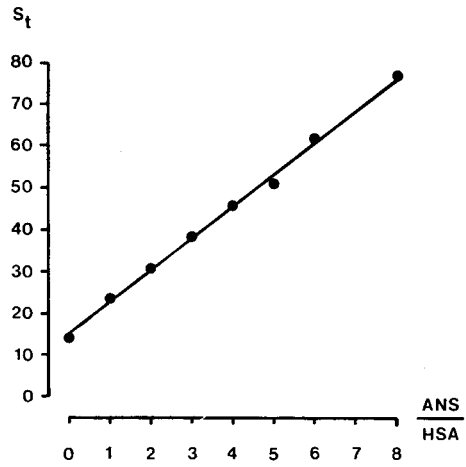

Fig. 3. Sum (S_t) of the surface areas of the protein and free dye zones in the isotachopherograms in Fig. 1, plotted against the respective ANS:HSA ratios.

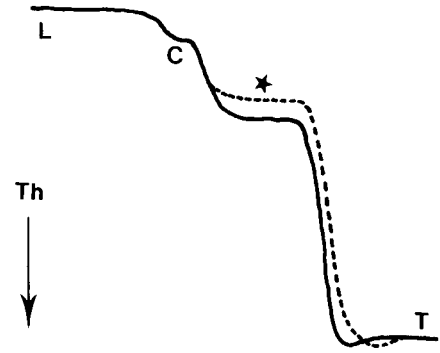

Fig. 4. Thermo signals obtained for HSA without (continuous line) and with (discontinuous line) bound ANS. The protein zone is indicated by *, the zone preceding it, represented by C, is carbonate, L and T are the signals from the leading and terminating ions, respectively.

Thus, under the analytical conditions employed, *i.e.*, separations as anions at high pH, the albumin acquires additional negative charges following binding of the dye, resulting in an increase of the effective anionic mobility of the protein. This is possibly the first direct evidence that the dye binds as an anion rather than the free acid, neutralizing positive charges on the protein molecule. The RRU values, calculated from the expression given in the Materials and methods section, yield the plot shown in Fig. 5 with respect to ANS:HSA ratio. There is an excellent correlation with the form of the plot of surface area in Fig. 2, two straight lines intersecting at a ratio of 3:1. Thus, the mobility shifts are larger following binding of the first three molecules of dye.

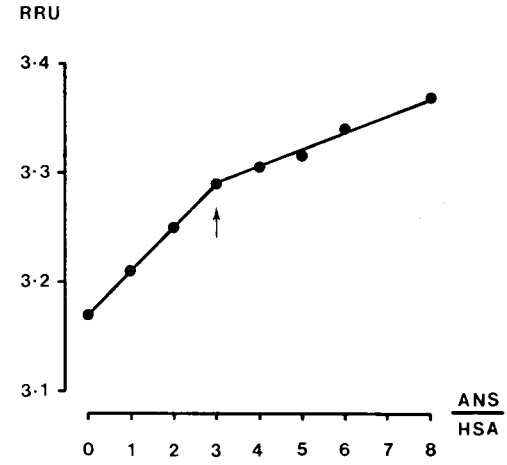

Fig. 5. Plot of RRU values, derived from the step heights of the thermo signals, against ANS:HSA ratios.

In the present work, we have demonstrated that analytical capillary isotachophoresis can be applied to the investigation of ligand–protein interactions. The fluorescent dye ANS has been chosen as a model ligand, since its binding to serum albumin has already been examined in some detail by other methods. Binding of the dye to the albumin leads to an increase in the extinction of the protein zone, due to the intrinsic UV absorption of the dye. Integration of the UV signal gives a measure of the dye binding, whence the binding character can be elucidated from the slope of the relevant plot, and the appearance of free dye. The surface area rather than the extinction itself must be taken as the parameter, since there is a slight alteration of the zone width after dye binding, resulting either from an alteration in the protein conformation (molecular volume) or a mobility shift. The dye binding can also be monitored by use of a detector which responds to differing mobilities, in this case, the thermodetector. Dye binding leads to a shift to higher mobilities, presumably due to an increase in the net negative charge of the protein molecule. Again, a two-phase process is noted, indicating at least two types of binding site.

From work with other techniques[3], the tight binding of three molecules of ANS to a molecule of HSA had been reported. Furthermore, it was shown that ANS:HSA ratios greater than 3:1 can be obtained, since there are lower affinity sites in addition to the three high affinity sites[5,6]. Thus, the results of our isotachophoretic study seem to correlate quite well with these data. The obvious advantage of isotachophoresis over conventional methods is that both the protein–dye complex and the free dye can be analysed separately in a single run.

This technique could find application, for example, in the study of enzyme–substrate interactions, or the identification of a particular binding protein in a complex mixture.

ACKNOWLEDGEMENTS

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Note

Extension of the alkaline end of a pH gradient in thin-layer polyacrylamide electrofocusing gels by addition of N,N,N′,N′-tetramethylethylenediamine

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The use of electrofocusing for the analysis and separation of proteins in complex mixtures and the determination of protein isoelectric points is now very widespread. Polyacrylamide gels in glass tubes ("rods") are often used when a few samples are to be analysed; horizontal thin-layer polyacrylamide gels are, however, preferable when many samples are to be studied together, because of the ease of comparison of band patterns and of handling of both gel and samples.

Because the upper surface of the horizontal gel is open to the atmosphere, carbon dioxide can be absorbed, and it is difficult to produce a pH gradient extending above about pH 9.5 unless special precautions are taken[1]. Thus, electrofocusing cannot readily be used to analyse important basic proteins such as the cytochromes and histones.

In this Note, a simple and economical method for extending the alkaline end of the pH gradient in a horizontal thin-layer polyacrylamide gel is described: namely, the addition of N,N,N′,N′-tetramethylethylenediamine (TEMED), an accelerator of the polymerization of acrylamide in gels used for electrophoresis. TEMED is not required for polymerization of gels used for electrofocusing, but its addition allows the alkaline end of a nominal pH 3.5–9.5 gradient to be extended to above pH 11.

MATERIALS AND METHODS

Acrylamide and N,N′-methylenebisacrylamide (bis), both "specially prepared for electrophoresis", and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from BDH (Poole, Great Britain). Ammonium persulphate, analytical grade, was purchased from E. Merck (Darmstadt, G.F.R.). Ampholine® carrier ampholytes, pH 3.5–9.5 (LKB Cat. No. 1818-101) were employed. Cytochrome *c*, type III from horse heart, and trypsinogen, from bovine pancreas, were obtained from Sigma (St. Louis, MO, U.S.A.). For haemoglobin, a haemolysate from washed human red blood cells was used.

The gel solutions were prepared and the gels cast according to the LKB 1818-P instructions for 0.5-mm thin-layer electrofocusing polyacrylamide gels[2]. The gel solu-

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tion used to fill the mould was prepared by mixing 3.5 ml of 29.1 % (w/v) acrylamide, 3.5 ml of 0.9 % (w/v) bis, 12.0 ml doubly distilled water and 1.5 ml of LKB 1818-101 Ampholine carrier ampholytes, pH 3.5–9.5. After deaeration, 0.5 ml 1 % fresh ammonium persulphate solution (and TEMED, where used) were carefully added, mixed and the solution injected into the prepared mould.

When gels containing TEMED were run under the conditions recommended for gels prepared without TEMED, a burn line was often produced in the gel near the cathode after about 20 min. To avoid this, the LKB 2197 power supply was run at constant power with the following limiting conditions: 25 W, 1600 V and 30 mA. The running time in all cases was 80 min. Gels were run in the LKB Multiphor apparatus, cooled by water circulated at 10°C from an LKB MultiTemp thermostatic circulator. 1 *M* Phosphoric acid was used as anode solution, and 1 *M* NaOH as cathode solution.

The pH gradient across the gel was determined immediately after the power supply was switched off on completion of electrofocusing, using an LKB Multiphor surface pH electrode. The electrode was calibrated with standard buffer solution (Dr. W. Ingold AG, Urdorf ZH, Switzerland; ordering No. 9805) pH 6.841 at 10°C, which was temperature-equilibrated in a cooling jacket connected in series with the Multiphor. The pH gradient was measured at 1-cm intervals (0.5 cm near the cathode) across the 10 cm of gel between the electrode strips; the twelve points were usually measured within a total of 3–5 min. After the pH gradient had been determined, the protein bands were sharpened again by refocusing for another 10 min at the same settings.

The proteins were stained after refocusing, using Serva Blau R (equivalent to Coomassie Brilliant Blue R-250)[2].

RESULTS AND DISCUSSION

The tertiary amine, TEMED, is widely used as an accelerator in the production of polyacrylamide gels by chemical or photochemical polymerization (with ammonium persulphate or riboflavin as initiator, respectively)[3]. As early as 1973, however, it was found[4] that TEMED could be omitted from the gel solution when polyacrylamide gels containing LKB Ampholine carrier ampholytes for electrofocusing were prepared: the Ampholine itself contains enough tertiary amino groups to act as accelerator, although perhaps less efficiently than TEMED.

In our work, polymerization of a 0.5-mm thin-layer gel containing Ampholine carrier ampholytes, pH 3.5–9.5, normally takes 30–40 min in the absence of TEMED, but only 10–15 min when TEMED is present (50 μl in 20 ml of gel solution). TEMED also accelerates polymerization of the gel with Ampholines of other pH ranges, except below pH 4.5 where the addition of silver ions is necessary.

TEMED was also found to increase the pH in the gel at above pH 4.5. Fig. 1 shows the effect of TEMED in the most often used pH range of 3.5–9.5. The increase in pH is greatest at the cathodic end of the gel, and depends on the amount of TEMED used: in 20 ml of gel solution, 50 μl TEMED raises the pH at 0.3 mm from the cathode by 0.7 pH units, and 100 μl TEMED by 1.3 pH units. The highest pH obtainable in the horizontal thin-layer gel system with the nominal pH range 3.5–9.5 is thus raised from pH 9.5 to above pH 11. Basic proteins such as the histones,

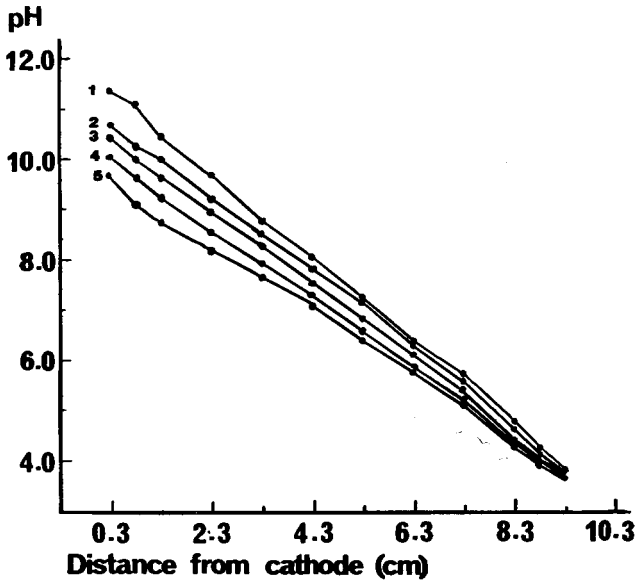

Fig. 1. Extension of a nominal pH 3.5–9.5 gradient upon inclusion of different amounts of TEMED in the gel solution. To 20 ml gel solution were added 100 μl (1), 75 μl (2), 50 μl (3), 25 μl (4) and 0 μl (5) TEMED.

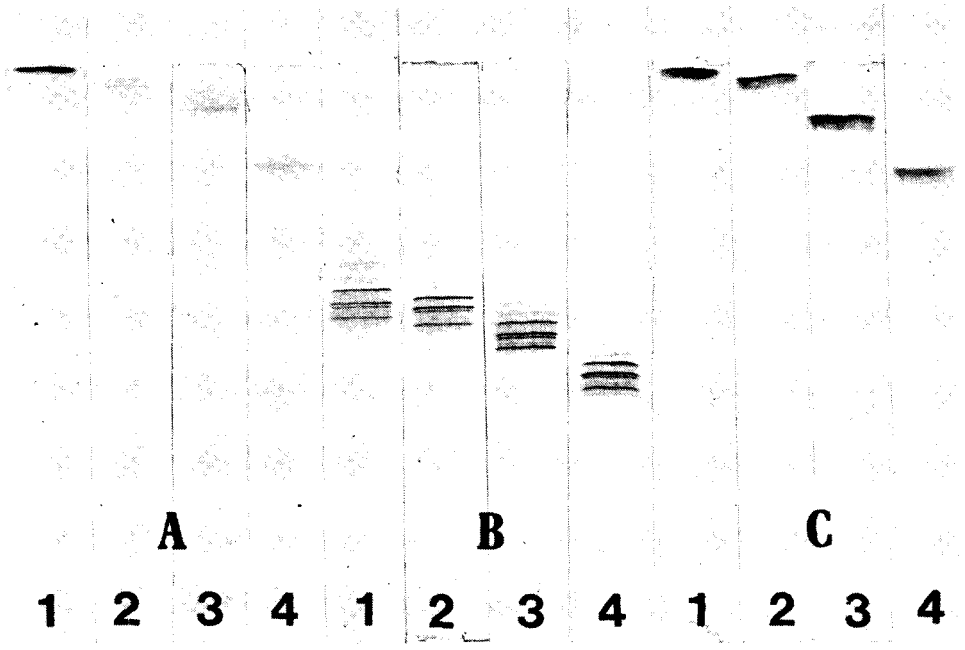

Fig. 2. Shift in the position of focusing of cytochrome *c* (A), haemoglobin (B) and trypsinogen (C) in nominal pH 3.5–9.5 gels supplemented with no TEMED (1), 20 μl (2), 50 μl (3) and 100 μl (4) TEMED. Cathode at the top.

cytochromes and lysozyme should now be easily studied by the high resolution of electrofocusing.

The results shown in Fig. 2 confirm the elongation of the pH gradient upon inclusion of TEMED in the gel solution. As the amount of TEMED is increased, the bands of the three proteins tested focus farther away from the cathode. Cytochrome *c* from horse heart focuses next to the cathode strip in the absence of TEMED, but 17 mm away from it in a gel supplemented with 100 μl TEMED. In gel A2, three bands of cytochrome *c* may be discerned, but in A3 and A4, only one: this is probably due to the fact that resolution is diminished when the pH range is extended[5]. Although elongation of the pH gradient is not necessary when haemoglobin is examined, it has been included here to show that the band pattern is not affected by the concentration of TEMED in the gel, although TEMED does slightly affect the separation of the four major bands.

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Note

Isolation of uniformly labelled fatty acids from *Chlorella pyrenoidosa* grown in an atmosphere of $^{14}CO_2$

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Fatty acids that are labelled in specific positions such as the carboxyl group can be obtained by chemical synthesis[1], but the preparation of uniformly labelled fatty acids is obviously much more difficult. Such compounds can easily be obtained biosynthetically. Several reports have appeared on the preparation of uniformly labelled fatty acids from intact higher plants[2,3], algae[4] and plant cell cultures[5] by growing them photoautotrophically in an atmosphere of $^{14}CO_2$. Since the fatty acid patterns of lipids in plant cells can be modified by controlling the factors prevailing during growth, such as illumination[6], temperature[7], growth regulators[8], as well as the cells age[9], plant cell cultures can be programmed for the production of particular fatty acids. Nevertheless, under any combination of conditions, mixtures of fatty acids rather than pure compounds are obtained. Hence, methods for separating individual compounds are required for in the biosynthetic preparation of labelled fatty acids. The processes of separation described so far involve relatively complicated techniques such as distillation and counter-current distribution of mixtures of labelled fatty acids.

The objective of the present communication is to demonstrate that simple and convenient chromatographic procedures can be used for isolating uniformly labelled fatty acids.

EXPERIMENTAL

Mixture of uniformly labelled fatty acids

The methyl esters of uniformly labelled fatty acids were prepared by methanolysis[10] of the total lipids of *Chlorella pyrenoidosa* (Stock culture No. 7516; American Type Culture Collection, Washington, D.C., U.S.A.) that had been grown in the presence of $^{14}CO_2$ under the conditions specified earlier[4].

Argentation chromatography

As far as possible, all processes were carried out in an oxygen-free nitrogen atmosphere.

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The methyl esters of uniformly labelled fatty acids were fractionated on 0.3-mm layers of silica gel G impregnated with 10% (w/w) silver nitrate, using hexane–diethyl ether (80:20, v/v) as a developing solvent. The plates were dried and scanned for radioactivity. Zones of the sorbent carrying the methyl esters of tri-, di, monoenoic and saturated fatty acids were marked on the layer during scanning. For the purpose of identification, "cold" methyl linolenate, methyl linoleate, methyl oleate and methyl stearate were simultaneously chromatographed on a separate plate and the fractions visualized by charring after spraying the layers with 50% sulphuric acid. The zones of sorbent carrying the various fractions of labelled methyl esters were scraped off and eluted with diethyl ether slightly acidified with concentrated hydrochloric acid (two drops of acid per 100 ml solvent) and washed with water. For this purpose, the sorbent zones were transferred into glass tubes fitted with screw caps, and acidified diethyl ether and water were added. The contents of the tubes were shaken vigorously and the diethyl ether phases containing the eluted methyl esters were withdrawn using Pasteur pipettes. Additional aliquots of diethyl ether were added to the aqueous phases containing the sorbent, and this process was repeated five times. The solutions were then combined and the ether removed by a stream of nitrogen. The methyl esters were dissolved in minimal amounts of hexane and subjected to chromatographic fractionation according to the chain lengths of the various fatty acids.

Reversed-phase chromatography

A solution of 15% (v/v) undecane in hexane was allowed to ascend a layer of silica gel G, 0.3 mm, until the solvent front had reached the upper edge of the plate. The plate can be used immediately after the hexane has been evaporated. For fractionating the methyl esters on such plates, mixtures of acetic acid and water in ratios of 85:15 or 90:10 (v/v) are suitable as developing solvents. It is important that the development is done in the same direction as the plate impregnation. After 4–5 h the front had reached a height of about 15 cm, and acetic acid was evaporated from the chromatograms for 40–60 min at room temperature with a stream of nitrogen. The plates were then scanned for radioactivity and the sorbent zones carrying the methyl esters of individual fatty acids were marked on the layer and then scraped off. The methyl esters were eluted with diethyl ether and washed with water as described above. Diethyl ether was removed by evaporation in a stream of nitrogen and the methyl esters of individual fatty acids were dissolved in small volumes of hexane and tested for purity by gas chromatography (GC)[11].

GC

Gas chromatography was done on a Perkin-Elmer F22 instrument (Perkin-Elmer, Norwalk, CT, U.S.A.) using a glass column (6 ft. × 1/4 in.) packed with 5 CP Silar, 10% on Gas-Chrom Q (80–100 mesh), at a temperature of 220°C with nitrogen as carrier gas.

Measurement of radioactivity

The radioactivity of fractions separated on chromatoplates was determined directly by gas flow counting using a Berthold LB 2760 TLC-Scanner (BF-Vertriebsgesellschaft, Wildbad, G.F.R.). The radioactivity of isolated methyl esters was as-

TABLE I

UNIFORMLY LABELLED FATTY ACIDS FROM *CHLORELLA PYRENOIDOSA*

Chain length: number of double bonds	*Amount*		
	*%**	*mg***	*μCi***
16:0	23.1	46	96
16:1	4.7	10	
16:2	5.2	10	7
16:3	2.9	5	4
18:0	1.0	2	
18:1	37.7	76	201
18:2	15.5	30	27
18:3	8.2	15	10

* Determined by GC analysis.

** Fatty acids were isolated by argentation chromatography followed by reversed-phase chromatography; radioactivity determined by liquid scintillation spectrometry.

sayed by suspending aliquots of these compounds in Aquasol-2 scintillation fluid (New England Nuclear, Boston, MA, U.S.A.) and counting in a Packard Model 2425 Tri.Carb liquid scintillation spectrometer.

Quantitation of the peaks obtained by scanning of chromatoplates and in GC was done by triangulation.

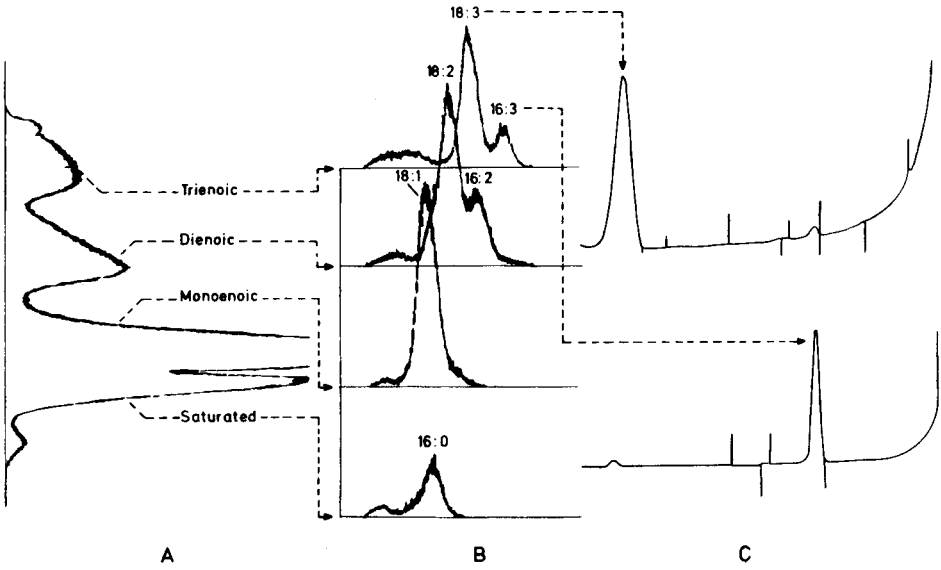

Fig. 1. Separation of uniformly labelled fatty acids by TLC. A, Typical radiochromatogram scan showing the fractionation of the methyl esters into groups with different degrees of unsaturation. Silica gel G containing 10% silver nitrate; hexane–diethyl ether (80:20, v/v). B, Typical radiochromatogram scan showing the fractionation of groups of methyl esters with the same degree of unsaturation into individual compounds. Silica gel G impregnated with undecane; acetic acid–water (90:10, v/v). C, Typical gas chromatograms showing the purity of individual compounds isolated by consecutive TLC in systems A and B.

RESULTS AND DISCUSSION

Table I presents the results of analyses of the total fatty acids in the lipids of *Chlorella pyrenoidosa*. The data are similar to those reported by earlier investigators for the same alga[4,12,13].

Since the basic study by Mangold and Schlenk[4], over two decades ago, on the preparation of uniformly labelled fatty acids, work in this field has been concentrated on improving the methods of analysis for the labelled acids produced[12,13–15]. This has not been paralleled by an improvement in the methods for isolating such fatty acids in pure form. Earlier methods of isolation are tedious and time-consuming. In contrast, conventional thin-layer chromatographic (TLC) methods are simple and fast. Fig. 1 illustrates the successive steps of separation by TLC.

In the first step, A, the mixture of methyl esters was fractioned according to the degree of unsaturation of the components by argentation chromatography. Hexane–diethyl ether (80:20, v/v) gave good resolution of methyl esters with up to three double bonds, although in some cases the separation of methyl esters of saturated and monoenoic fatty acids was not satisfactory. Such compounds were then separated in a consecutive step using hexane–diethyl ether (90:10 or 95:5 v/v) as solvent. Mixtures that contain higher polyunsaturated esters should be resolved by solvents containing higher proportions of diethyl ether, *e.g.*, hexane-diethyl ether (40:60, v/v) or, better, by developing the chromatograms twice with hexane–diethyl ether–acetic acid (94:4:2, v/v).

In the second step, B, methyl esters having the same degree of unsaturation were separated according to the chain lengths of the individual fatty acids by reversed-phase chromatography. Satisfactory separations were obtained with undecane as the stationary phase and acetic acid–water (90:10, v/v) as the developing solvent. An even sharper separation, as shown in Fig. 2, was obtained by slightly increasing the proportion of water in the solvent. However, the quality of separation using the same solvent ratio showed some day to day variation. In general, the most suitable ratio of acetic acid to water lies between 85:15 and 90:10 (v/v).

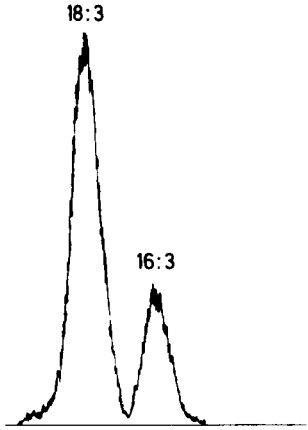

Fig. 2. Typical radiochromatogram scan showing the fractionation of methyl esters of trienoic fatty acids. Silica gel G impregnated with undecane; acetic acid–water (87:13, v/v).

The fractions obtained by reversed-phase chromatography usually contain methyl esters of fatty acids of up to 98% purity as shown by GC analysis, C. Impure fractions are purified either by argentation or reversed-phase chromatography, depending on the identity of the contaminants.

The results of the present study show how convenient conventional TLC techniques are in isolating labelled fatty acids. The entire process of separation is achieved within hours instead of weeks or even months. Thus, not only are time and effort saved but also the labelled fatty acids, especially the unsaturated ones, are less subject to autoxidation due to prolonged processing. Complicated equipment is not needed; for the isolation of the amounts of labelled fatty acids listed in Table I, only 10 silver nitrate-impregnated plates and 30 undecane-impregnated plates of silica gel G were employed.

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Note

Quantitative recovery of polyunsaturated fatty acids on pyrolytic methylation of their trimethylphenylammonium salts

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(Received September 8th, 1981)

We previously demonstrated the superiority of trimethyl(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) over trimethylphenylammonium hydroxide (TMPH) and tetramethylammonium hydroxide (TMH) in the recovery of polyunsaturated fatty acids (PUFAs) as their methyl esters in gas–liquid chromatographic (GLC) analyses[1] and this has been confirmed[2,3]. Ours was the first practical application of the powerful tool of flash alkylation of PUFAs from biological specimens. In order to achieve quantitative recoveries of PUFAs it was necessary to inject the TMTFTH solution of the fatty acids as their TMTFT salts after mixing the solution with methanolic methyl propionate in the injection syringe.

The major function of the short-chain fatty acid methyl ester in the reaction that takes place in the injector of the GLC unit is as a neutralizing reagent. The reaction appears to be an alkaline hydrolysis of the short-chain fatty acid methyl ester to yield methanol and the corresponding short-chain fatty acid. The excess quaternary ammonium hydroxide is neutralized by the released short-chain acid resulting in a sufficiently diminished alkalinity to reduce any alkaline degradation of the PUFAs to a non-detectable level. A minor function of the short-chain fatty acid ester appears to be an alkaline-catalyzed transalkylation, because when ethyl acetate was tested in the process, small amounts of ethyl esters of the long-chain fatty acids were detected.

The methyl propionate, which is 10.4 *M*, was diluted with two volumes of methyl alcohol, making the solution 3.5 *M*, in order to obtain a homogenous solution with the aqueous TMTFTH extract, because of the limited aqueous solubility of methyl propionate. Substitution of neat methyl acetate, which is 12.5 *M* and is soluble in water, for methanolic methyl propionate allows an increase in the concentration of the neutralizing ester by 3.6-fold —a sufficient increase in the neutralizing ability of the system to protect the PUFAs from the alkaline degradation in the hot injector inlet caused by the slightly stronger alkalinity of TMPH over TMTFTH.

Among the advantages of TMPH over TMTFTH are those of ready availability, lower cost, and greater stability. TMPH is easily generated in aqueous solution simply by mixing the appropriate amounts of trimethylphenyl ammonium iodide (TMPI) with silver oxide and water. Hydrolysis of the silver oxide yields silver hydroxide. The silver ion readily precipitates the iodide ion as silver iodide leaving

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trimethylphenylammonium and hydroxide ions in solution. TMPI in high purity is relatively inexpensive and commercially available. Although TMTFTH is generated just as readily from its iodide, TMTFTI is not commercially available. We described a procedure for the preparation of TMTFTI by reaction of α,α,α-trifluoro-*m*-toluidine with methyl iodide, but the yield is low and the carcinogenicity of methyl iodide has probably inhibited the use of TMTFTH with its obvious advantages.

Aqueous solutions of TMTFTH are offered by at least two suppliers, but the solution offered by one supplier, 0.2 *M* in concentration, is too dilute in our opinion. The other supplier offers 0.5 *M* TMTFTH.

Solutions of TMPH are more stable than TMTFTH solutions. This difference is undoubtedly related to the fact that the dimethyltrifluorotoluidine is the better leaving group.

EXPERIMENTAL

Reagents and methods

Trimethyl(α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH), 0.5 *M*, was prepared as described by MacGee and Allen[1] and stored in a stoppered test tube at 4°C in the dark.

Trimethylphenylammonium hydroxide (TMPH), 0.5 *M*, was prepared by mixing 1.3 g of trimethylphenylammonium iodide (Eastman-Kodak, Rochester, NY, U.S.A.) and 1.3 g silver oxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.) with 10 ml of distilled water in a stoppered centrifuge tube. After vigorous mixing by hand and on a vortex mixer to achieve a uniform suspension, the preparation was centrifuged and one drop of the clear supernatant solution was diluted with 10 drops of water and tested for halide with one drop of 0.1 *M* silver nitrate in 6 *M* nitric acid. If a positive halide test resulted, the mixing and centrifugation steps were repeated until a negative halide test was achieved. The TMPH solution was stored in stoppered test tubes at 4°C in the dark.

Methanol–methyl propionate (2:1) was prepared as described previously[1].

Methyl acetate (certified, Fisher Scientific) was shaken in a stoppered test tube with granular anhydrous sodium carbonate (Mallinckrodt, St. Louis, MO, U.S.A.) to remove any water or free acetic acid and was stored at room temperature over the sodium carbonate. Occasional shaking of the mixture is recommended to keep it acid free.

PUFA-rich fatty acids were obtained in hexane by subjecting a human blood clot to a scaled-up modification of the procedure described by MacGee and Allen[1] up to and including the separation of the hexane extract from the neutralized saponified specimen.

GLC analyses were performed on two GLC units, a Bendix Model 2600 (Bendix Corp., Ronceverte, WV, U.S.A.) and a Perkin-Elmer Sigma I (Perkin-Elmer Corp., Norwalk, CT, U.S.A.). Both instruments were used with flame ionization detectors and 6 ft. × $\frac{1}{4}$ in. (4 mm I.D.) coiled glass columns containing pretested 10% Silar 10C on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, PA, U.S.A.).

The injectors were held at 250°C, the detectors at 220°C and nitrogen was the carrier gas at 32 ml/min for the Bendix GLC unit and 50 ml/min for the Perkin-Elmer

GLC unit. The column ovens were temperature programmed from 160°C to 220°C at 2°C/min. Peak areas were measured with an Autolab System I computing integrator (Spectra Physics, Santa Clara, CA, U.S.A.) connected to the Bendix GLC unit and by the Perkin-Elmer Sigma I system.

Procedure

The fatty acids from the total human blood clot from a 7-ml whole blood specimen were obtained in 100 ml of hexane as described above. Aliquots of 5 ml were extracted with 10 μl of either 0.5 *M* TMTFTH or 0.5 *M* TMPH in conical centrifuge tubes by shaking vigorously by hand for 1 min followed by centrifugation at *ca.* 600 *g* for 1 min. The mixing of the TMPH or TMTFTH extract with the neutralizing ester in the injection syringe was as described previously[1], *i.e.* the syringe was prewetted with the short-chain fatty acid methyl ester and an aliquot of the extract was drawn up followed by 0.5 to 1 μl of the neutralizing ester. After drawing the syringe plunger back and forth to mix the contents of the syringe, the sample was injected into the GLC unit. A moderately slow injection, 3–5 sec, was used[1,4].

When more than 1 μl of the quaternary ammonia hydroxide extraction of the fatty acids was required for analysis, it was necessary to employ the following "club sandwich" technique to assure adequate contact between the extract and the neutralizing short-chain fatty acid methyl ester. A 10-μl No. 701SN Hamilton microsyringe with a 3-in. needle (Hamilton, Reno, NV, U.S.A.) was prewetted and filled to the 1-μl mark with methyl acetate (volume of needle plus 1 μl = *ca.* 2.4 μl) followed in turn by 1 μl of the TMPH extract, 1 μl of methyl acetate, 1 μl of the TMPH extract and finally 1 μl of methyl acetate, for a total of 2 μl of the TMPH extract and *ca.* 4.4 μl of methyl acetate. After pumping the syringe plunger back and forth a few times to mix the contents of the syringe, the sample was injected into the GLC unit.

RESULTS AND DISCUSSION

Human blood cells contain large quatities of PUFA, especially arachidonate (20:4) with significant amounts of docosahexaenoate (22:6), making a blood clot a convenient material for this study.

Table I compares the values of the major fatty acids in the blood clot obtained with TMTFTH and TMPH using the procedure described and the Perkin-Elmer Sigma I system. The values are comparable and as can be seen from the table, the amount of the PUFAs recovered by TMPH is the same as the amount recovered by TMTFTH.

In examining the reaction with the two quaternary ammonium hydroxide reagents and methyl acetate on two different GLC units, no differences were observed in the recoveries of the fatty acid methyl esters with the "club sandwich" injection technique. However, small, but significant, losses of PUFAs were noted when the TMPH extract was used with methanolic methyl propionate or when 2 μl of the TMPH extract was sandwiched between two plugs of methyl acetate by the technique originally described[1], but these losses were evident only in the Perkin-Elmer Sigma I instrument. We attribute this small loss to differences in the geometry or composition of the instrument's injector system. The Bendix instrument was configured in such a way that the sample was injected on-column, *i.e.* the sample was introduced into the

TABLE I

COMPARISON OF VALUES OBTAINED WITH TMTFTH AND TMPH AS PERCENT OF TOTAL

	Average value ± S.D. (n = 6)	
	TMTFTH	*TMPH*
16:0	22.43 ± 0.136	22.00 ± 0.493
16:1	2.22 ± 0.177	2.20 ± 0.159
18:0	13.68 ± 0.110	13.44 ± 0.114
18:1	22.29 ± 0.090	22.24 ± 0.101
18:2	14.67 ± 0.101	14.74 ± 0.064
20:3	1.75 ± 0.060	1.79 ± 0.085
20:4	14.48 ± 0.145	14.87 ± 0.275
22:5	1.44 ± 0.019	1.48 ± 0.026
22:6	1.81 ± 0.024	1.90 ± 0.026
Others	5.25 ± 0.175	5.40 ± 0.276

empty inlet end of the column in the heated injector zone. On the other hand, in the Perkin-Elmer instrument the sample was injected into a separate injection zone containing a glass liner and a short stainless steel connector between the injector and the inlet end of the column. There are two simple solutions to this problem, the "club sandwich" technique described in this report or a readily-available reconfiguration to allow on-column injection into the empty heated inlet end of the column.

While the "club sandwich" injection technique is apparently only necessary with TMPH extracts on non-on-column systems, it is simple enough that we recommend it be used with all specimens requiring 2 μl or larger.

CONCLUSION

With the substitution of methyl acetate for methyl propionate, TMPH can be used instead of TMTFTH for the flash alkylation production of fatty acid methyl esters for GLC analysis. The methyl acetate provides a 3.6-fold molar increase in the amount of neutralizing ester which allows the higher alkaline TMPH to be used without destruction to polyunsaturated fatty acids. The advantages of TMPH over TMTFTH include ready availability, lower cost, and greater stability.

ACKNOWLEDGEMENT

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Note

Separation of monoacetyldiglycerides by argentation thin—layer chromatography

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Interest has recently grown in the different molecular species involved in the major phospholipid classes present in cellular membranes, in order to determine the influence of these components on the functional aspects of such membranes[1–3].

One of the essential steps involved in the determination of phospholipids is the production of the corresponding diglycerides and their resolution, preferably as monoacetyl derivatives, according to the number of double bonds in the molecule by argentation thin-layer chromatography ($AgNO_3$-TLC). The composition of the different monoacetyldiglycerides (MADGs) in each fraction is obtained either by direct gas chromatography–mass spectrometry[4] or by computation from gas–liquid chromatographic (GLC) data on the component MADGs and the distribution of fatty acids at positions 1 and 2 of these molecules[5]. Thus, the efficiency of this procedure depends to a large extent on the degree of resolution of MADGs on the TLC plates.

A number of solvents, mostly mixtures of chloroform–methanol[5,6], benzene–chloroform–methanol[7] and benzene–diethyl ether[8], has been used for the TLC separation of MADGs but none of these could completely separate the components. In particular, the compounds containing more than four double bonds tend to segregate in one or two bands.

In the present communication we report the use of a new solvent to separate these highly unsaturated MADGs on $AgNO_3$-TLC plates.

MATERIALS AND METHODS

Reference compounds and reagents

Synthetic L-α-distearoylphosphatidylcholine, methyl pentadecanoate and reference mixtures of fatty acids, all >99% pure, were purchased from Applied Science Labs. (State College, PA, U.S.A.). Phospholipase C (E.C. 3.1.4.3.) from *Clostridium perfringens*, type 1, was a product of Sigma (St. Louis, MO, U.S.A.). Silica gel G for TLC and neutral Al_2O_3 for adsorption column chromatography were obtained from E. Merck (Darmstadt, G.F.R.). Most of the other reagents and solvents used were of A.R. grade. The solvents were in general redistilled. Diethyl ether was freed from peroxides, washed, dried and redistilled before use.

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Preparation of hen egg phosphatidylcholine (PC)

This was extracted[9] from fresh yolk and partially purified by precipitation from cold acetone[10]. Pure PC was isolated chromatographically using a neutral Al_2O_3 column[11], the purity being checked by TLC against reference PC.

Preparation of MADGs from egg PC

sn-1,2-Diglycerides from egg PC were prepared by a method recently developed in this laboratory. The PC was enzymatically (phospholipase C) hydrolysed to the corresponding *sn*-1,2-diglycerides on a preparative TLC plate. The diglycerides were separated from other products by development of the same plate and extracted from the adsorbents using diethyl ether[12]. The diglycerides were acetylated to the corresponding MADGs using acetic anhydride and pyridine, the products being purified by preparative TLC[13].

Subfractionation of MADGs on $AgNO_3$-TLC plates

Glass plates (28 × 14 cm) were coated with 0.5 mm of silica gel G, impregnated with 25% (w/w) $AgNO_3$, dried in air for about 30 min, activated at 120°C for 2 h, cooled and stored in desiccator under silica gel before use. MADGs (10–15 mg) in chloroform were applied on the plate as a narrow band 2 cm from one of the shorter edges. The plate was developed twice up to 22 and 24 cm in light petroleum (b.p. 60–80°C)–diethyl ether–acetic acid (75:35:1, v/v/v) in a saturated chamber at 25°C. The bands were located under UV light after spraying the developed chromatogram with a 1% methanolic solution of 2′,7′-dichlorofluorescein.

The content of each band was isolated from the adsorbents by repeated extraction with diethyl ether. The pooled extract was freed from $AgNO_3$ by washing with water, dried under anhydrous Na_2SO_4 and adjusted to an appropriate concentration.

Determination of the fatty acid compositions of MADG subfractions

Mixed methyl esters corresponding to the MADGs in each band were prepared by alkaline methanolysis[14], and the fatty acid compositions were determined by GLC of the mixed methyl esters, isothermally at 180°C on stainless-steel columns (6 ft. × 1/8 in.) packed with 15% DEGS coated on Gas-Chrom Z (100–120 mesh) (Applied Science Labs.). A dual-column Pye Unicam gas chromatograph, Model GCD, equipped with a flame ionization detector was used. The peaks were identified by comparing their retention parameters with those of the reference methyl esters. Compositions were calculated from peak areas, obtained from the peak height × width at half height and applying suitable correction factors determined from the chromatograms of the reference mixtures of methyl esters. The relative molar percentages of MADGs in the bands were computed from the chromatograms of fatty acid methyl esters with methyl pentadecanoate as internal standard[15].

RESULTS

Fig. 1 shows that MADGs from egg PC can be resolved into ten well separated bands on a 25% $AgNO_3$ treated silica gel G plate developed in the present solvent system. The absence of any fully saturated species in egg PC is evident from the fact that the top band appeared in the chromatogram only when fully saturated MADG

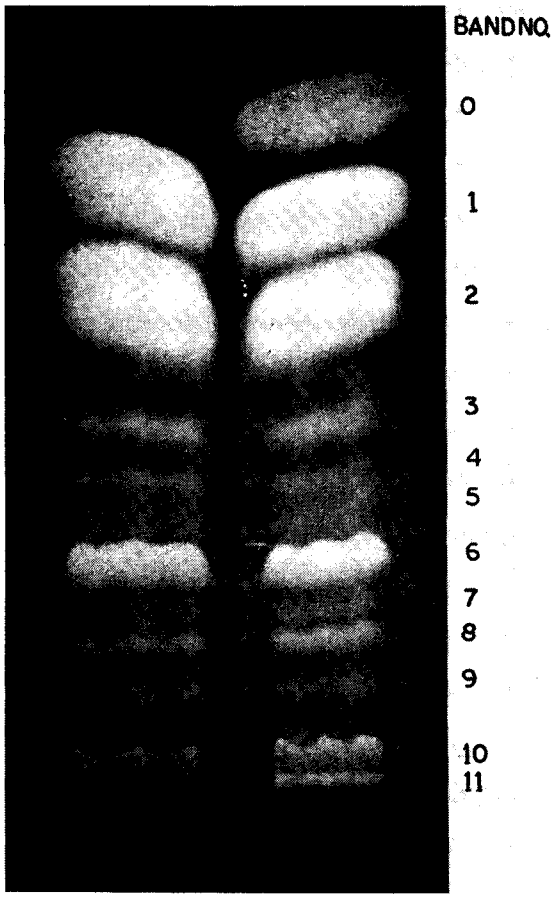

Fig. 1. Separation of monoacetyldiglycerides (MADGs) on an $AgNO_3$-TLC plate. Conditions: 28 × 14 cm TLC glass plate coated with 0.5 mm of silica gel G impregnated with 25% (w/w) $AgNO_3$. MADGs (10 mg) derived from hen egg PC were applied as a small band on the left-hand side of the plate; on the right-hand side, the same mixture together with 1 mg MADGs from authentic 1,2-distearoylphosphatidylcholine was similarly applied. The plate was developed twice up to 22 and 24 cm respectively in light petroleum (b.p. 60 – 80°C) – diethyl ether–acetic acid (75:35:1, v/v/v). Bands were visualized under UV-light after spraying with a 1% methanolic solution of 2′,7′-dichlorofluorescein. Bands: O = 3-acetyl-1,2-distearoylglycerol (OOAC); 1–10 = MAGDs of egg PC; 11 = traces of 2′,7′-dichlorofluorescein not removed during processing.

prepared from reference distearoylphosphatidylcholine was mixed with MADGs of egg PC. From the chromatogram it appears that bands 1, 2, 3, 6 and 10 are major subfractions in the MADGs of egg PC. This was confirmed from the relative proportions of the subfractions estimated by GLC analysis. The very faint band between bands 9 and 10 was taken with band 9 for extraction of the content.

MADGs prepared from rat liver PC, when run side by side with MADGs from egg PC, produced almost identical chromatograms with respect to the number of bands and their R_F values, but the former contained an additional saturated band (the chromatogram is not shown but is available on request).

In Table I the fatty acid compositions (mole%) are expressed in terms of the number of double bonds in the molecule by adding together the percentages of the fatty acids containing identical numbers of double bonds. The average number of double bonds per mole of the MADGs in a band, A, is calculated from:

$$A = \frac{\Sigma \text{ (mole\% of each fatty type} \times \text{number of double bonds in the type} \times 2)}{100}$$

From the acid compositions of the last five bands and the corresponding A values, it is concluded that the present chromatographic method can effectively separate highly unsaturated MADGs containing four or more double bonds. The predominance (over 45%) of each of the two types of fatty acids, one saturated and the other a tetra-, penta- or hexanoic, in bands 6, 8 and 10 respectively indicates that these bands might contain about 90% of the 04AC, 05AC and 06AC types of MADGs. Again the tetra-, penta- and hexaenoic acids are almost entirely distributed in the above three bands respectively, which indicates that the polyunsaturated acids in egg PC remain mainly in combination with saturated fatty acids.

From the fatty acid compositions, applying similar arguments, the 01AC, 02AC and 03AC types of MADGs are considered to be the probable major components of bands 1, 2 and 5 respectively. In the case of bands 3, 4, 7 and 9, where the fatty acid compositions are more complex, additional help was needed from the established elution order of MADGs in $AgNO_3$-adsorption chromatography[16]. For example, the major component of band 4 was considered to be 22AC from the facts that 65% of the fatty acids were of the dienoic type and that the 22AC type of MADG is eluted between 12AC and 03AC.

Considering the fatty acid compositions and the molar proportions of the components of the bands, we conclude that about 50 mole% of egg PC is of the 01 type, the other major types being 02 (32%), 11 (6%), 04 (5.6%), 12 (1.4%) and 06 (1.2%).

DISCUSSION

Resolution of MADGs is a very important step in the determination of the distributions of phosphoglycerides in different tissues; the accuracy of such determinations depends to a large extent on the degree of resolution. The present method is more efficient than the previous ones[4–8]; in the latter, good resolution of highly unsaturated MADGs containing four or more double bonds could not be achieved, whereas these were resolved into four distinct bands by our method.

Ambient humidity is a factor known to have a profound influence on the degree of resolution in $AgNO_3$-TLC. During the development of the present method we found that this effect becomes very prominent when the developing solvent contains hydrophilic components like methanol or ethanol. At high ambient humidity the resolution was poor with these solvent mixtures. With the present solvent system, which does not contain any highly hydrophilic component, well reproducible chromatograms could be obtained under widely varying ambient humidities. High resolution and good reproducibility are the two major advantages of the present method over the others. A third advantage lies in the use of a single solvent for the two

TABLE I

MOLE PERCENTAGES, FATTY ACID COMPOSITIONS, AVERAGE NUMBERS OF DOUBLE BONDS AND THE PROBABLE MAJOR COMPONENTS OF THE DIFFERENT MADG BANDS SEPARATED ON $AgNO_3$-TLC

Band no.	*Mole%*	*Fatty acid composition*							*Average no. double bonds*	*Probable major component**
		Saturated	*Monoenoic*	*Dienoic*	*Trienoic*	*Tetraenoic*	*Pentaenoic*	*Hexaenoic*		
1	49.7	50.1	49.9	–	–	–	–	–	1.0	01AC
2	37.9	42.2	15.9	41.9	–	–	–	–	2.0	02AC
3	1.9	8.2	46.1	41.6	4.1	–	–	–	2.8	12AC
4	0.6	12.2	14.5	65.4	7.9	–	–	–	3.4	22AC
5	0.6	40.8	3.0	6.2	45.4	4.6	–	–	3.4	03AC
6	6.2	46.2	3.5	1.3	1.6	47.4	–	–	4.0	04AC
7	0.4	8.9	31.8	10.4	12.5	36.4	–	–	4.7	14AC
8	0.8	45.1	4.6	–	–	3.4	46.9	–	5.0	05AC
9	0.6	1.8	8.6	2.9	75.1	4.3	7.3	–	5.9	33AC
10	1.3	46.1	1.8	1.0	2.0	–	1.0	48.1	6.1	06AC

* The numerals denote the number of double bonds in the two acyl groups in MADG.

consecutive developments, whereas in most of the previous methods[5,7,8] two different solvents were used.

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Note

Rapid method for the analysis of volatile N-nitrosamines in cigarette smoke by glass capillary chromatography

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It has been well established that many N-nitrosamines are highly carcinogenic[1–3]. Since Druckrey and Preussman[4] first made the suggestion in 1962 that favorable conditions exist in cigarette smoke for the production of N-nitrosamines, there has been considerable interest in the examination of cigarette tar for the presence of N-nitrosamine compounds[5,6]. These studies have required elaborate enrichment procedures for the concentration, detection and quantitative analyses of cigarette smoke nitrosamines. Brunnemann *et al.*[7] reported a fairly simple extraction procedure followed by the use of the highly sensitive and specific thermal energy analyzer (TEA), developed by Fine *et al.*[8]. However, many laboratories, including our own, are not equipped with the expensive TEA. We have, therefore, developed an alternative method for the analysis of volatile N-nitrosamines employing glass capillary gas chromatography (GC) and a nitrogen–phosphorus thermionic detector (NPD). This method has enabled us to monitor dimethylnitrosamine (DMNA), diethylnitrosamine (DENA) and nitrosopyrollidine (NPYR) in the smoke from various types of cigarettes.

EXPERIMENTAL*

Materials

Cigarette smoke condensate (CSC) was collected on a 30-port Borgwaldt smoking machine. Standard smoking conditions were used: 1 puff/min, puff duration of 2 sec, puff volume of 35 ml and butt length of 23 mm. The smoke from 60 cigarettes was collected in 200 ml of a citric acid–sodium phosphate buffer solution (pH 4.5) to which 20 nm of ascorbic acid had been added.

Standard nitrosamines were obtained from Eastman Chemical and used without further purification.

Extraction procedure

The extraction procedure was similar to that reported by Hoffmann *et al.*[9]. The

* Reference to a company or product name does not imply approval or recommendation by the United States Department of Agriculture.

buffer solution was extracted three times with 100 ml of dichloromethane. The combined organic extracts were dried over sodium sulfate, concentrated to 5 ml and chromatographed on 30 g of basic alumina (activity II to III) contained in a 200 × 20 mm glass column. The column was eluted with 250 ml of dichloromethane and 250 ml of dichloromethane–acetone (4:1, v/v). The dichloromethane eluate was concentrated on a rotary evaporator to 1 ml for GC analysis.

Glass capillary GC analysis

The samples were analyzed with a Hewlett-Packard Model 5710A gas chromatograph equipped with a NP detector. The standard 5710A instrument was modified for glass capillary GC analyses as previously described[10]. The nitrosamines were analyzed in the split mode (100:1) with a 1-μl sample on a 10 m × 0.25 mm I.D. wall-coated open tubular (WCOT) column of Superox™-4, a 4,000,000 mol. wt. polyethylene glycol. The column was prepared according to Arrendale and coworkers[11,12]. The NPD was operated under hydrogen and air flow conditions as recommended by the manufacturer. The injection-port temperature was 200°C, the detector temperature was 250°C and the temperature program was 70°C for 8 min, then 2°C/min to 250°C.

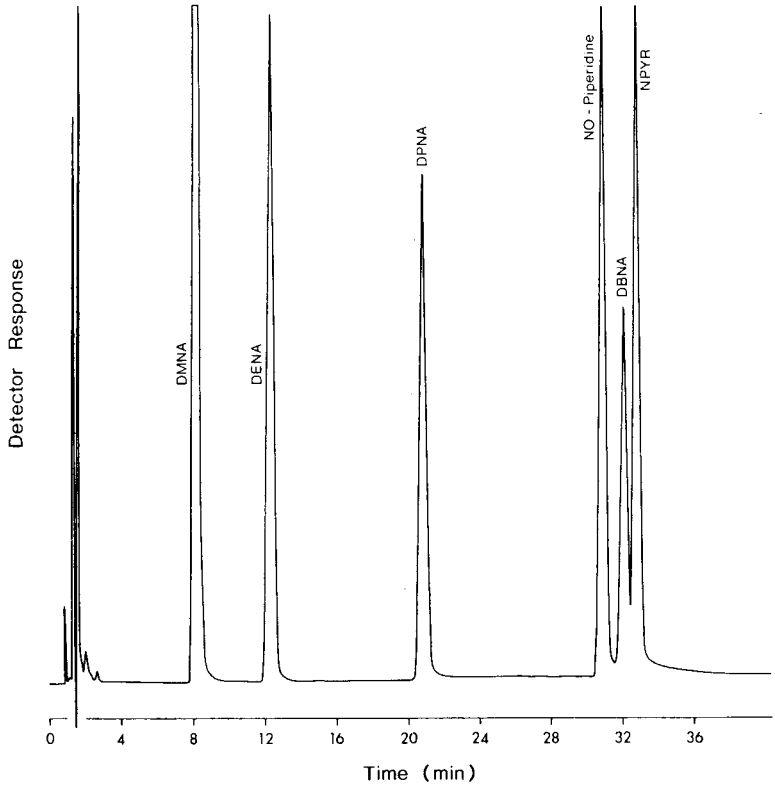

Fig. 1. Glass capillary gas chromatogram of standard nitrosamines on a Superox™-4 WCOT column. DMNA = Dimethylnitrosamine, DENA = diethylnitrosamine, DPNA = dipropylnitrosamine, DBNA = dibutylnitrosamine, NPYR = nitrosopyrollidine.

RESULTS AND DISCUSSION

Brunnemann and Hoffmann[13] used GC on a packed column of 10% Carbowax 20M to separate and quantitate the volatile CSC N-nitrosamines. We felt that because of their separation efficiencies WCOT glass capillary columns could be utilized to resolve better the nitrosamines and to shorten analysis time. The chromatogram of the standard N-nitrosamine mixture on a Superox™-4 WCOT column is shown in Fig. 1.

Since CSC is such a complex mixture almost any separation will still yield an impure and heterogeneous mixture. Therefore, we felt that a NPD would eliminate any interferences of non-nitrogen containing compounds in the quantitation of the volatile N-nitrosamines. Also, the increased sensitivity of the NP detector over the flame-ionization detector would be helpful, as the nitrosamines are present in the ng/cigarette range. As can be seen even with the NPD, the resulting CSC chromatograms were quite complex (Fig. 2). However, the major volatile N-nitrosamines, DMNA, DENA and NPYR, can be detected.

Our primary aim in developing a rapid method for the analysis of volatile nitrosamines was to evaluate the smoke from various tobacco samples for content of these carcinogenic materials. As a comparison to previous methods, quantitative data

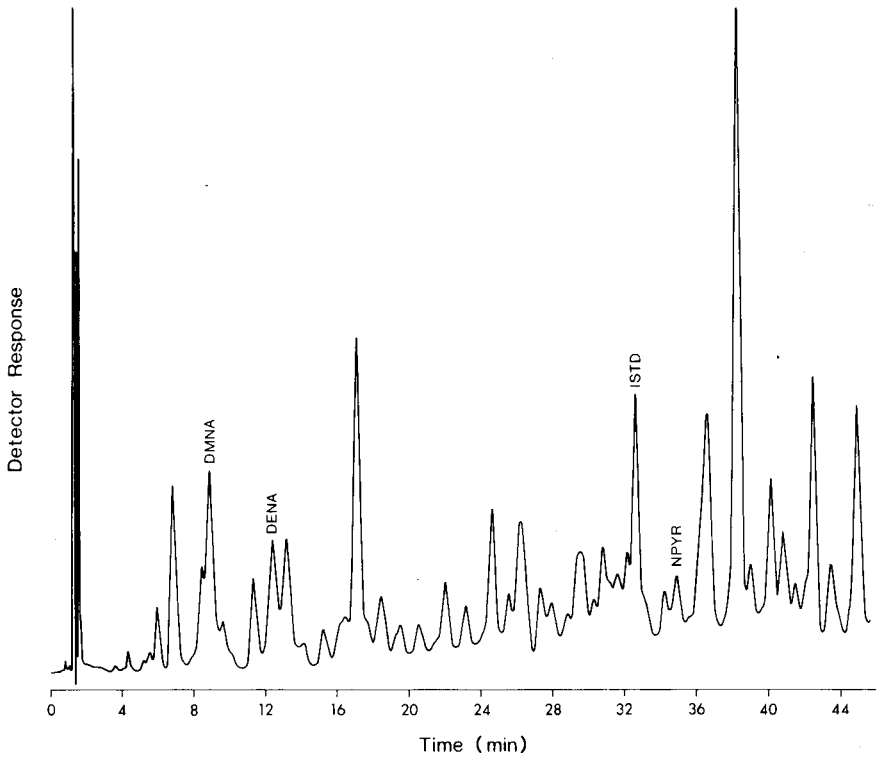

Fig. 2. Glass capillary gas chromatogram of a fraction derived from the CSC of Kentucky 2R1 cigarettes on a Superox™-4 WCOT column. DMNA = Dimethylnitrosamine, DENA = diethylnitrosamine, ISTD = internal standard (nitrosopiperidine), NPYR = nitrosopyrollidine.

from Brunnemann and Hoffmann[13] and our method are compared in Table I for three different types of cigarettes. Except for the filter cigarette our values are higher than those reported by Brunnemann and Hoffmann. These differences may be due to the manner in which the smoke was collected or to some interference from other nitrogen-containing substances in the CSC fraction that were not detected by the TEA. However, we feel this method provides us with an adequate tool for determining differences in volatile nitrosamine contents of various cigarettes. This procedure permits a laboratory not equipped with an expensive TEA system to monitor the contents of the highly carcinogenic volatile nitrosamines in CSC. Also, this method should have equal or better applicability to the analyses of volatile nitrosamines in simpler environmental or food samples.

TABLE I

COMPARISON OF DATA FROM TEA* AND NPD METHODS

	Nitrosamine contents (μg/cigarette)					
	TEA			*NPD*		
	DMNA	*DENA*	*NPYR*	*DMNA*	*DENA*	*NPYR*
Kentucky 1R1	9.0	2.0	6.6	17.0	5.8	1.5
Commercial non-filter	13.0	1.5	11.0	18.2	17.1	9.9
Commercial filter	5.7	1.3	5.1	0.75	9.9	0.08

* Brunnemann and Hoffmann[13].

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Note

Optical resolution of DL-proline by reversed-phase high-performance liquid chromatography using N-(*p*-toluenesulphonyl)-L-phenylalanine–copper(II) as a chiral additive

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High-performance liquid chromatographic (HPLC) resolution of amino acid enantiomers using a chiral chelate additive has been developed for several years[1–3]. However, few systems exhibiting both a highly efficient resolution and a highly sensitive detection for underivatized amino acid enantiomers have been established. We recently reported[4] a reversed-phase liquid chromatographic resolution method using a N-(*p*-toluenesulphonyl)-L-phenylalanine–copper(II) complex [TosPhe-Cu(II)] as chiral additive. Use of the *o*-phthalaldehyde (OPTA) reagent in this method facilitated sensitive detection of common α-amino acids down to pmole levels. However, the reagent could not be applied for the detection of imino acids such as proline. Although Gil-Av *et al.*[5] achieved a reversed-phase liquid chromatographic resolution of enantiomeric amino acids using a proline–copper(II) complex as chiral additive, it may not be possible to combine their system with any detection method effective for proline.

In the present study, DL-proline was resolved on a octadecylsilyl-bonded phase using TosPhe-Cu(II) as chiral additive, and monitored by a fluorimetric post-column derivatization using 7-chloro-4-nitrobenzofrazan (NBD-Cl).

MATERIALS AND METHODS

Proline and other reagents were purchased from Wako (Osaka, Japan). Chemically bonded octadecylsilyl silica gel, Develosil ODS (particle size 5 μm), was obtained from Nomura Chemical (Seto-shi, Japan). Glass-distilled water was used throughout the experiments.

TosPhe was synthesized as described by McChensney and Swann[6]. The mobile phase was prepared by dissolving 319 mg (1 mmol) of TosPhe and 125 mg (0.5 mmol) of $CuSO_4 \cdot 5H_2O$ in 1 l of water and adjusting the pH of the resultant solution with to 6.0 with 5% aqueous sodium carbonate solution. The NBD-Cl reagent was prepared by dissolving 600 mg of 7-chloro-4-nitrobenzofrazan (E. Merck, Darmstadt, G.F.R.) in 300 ml ethanol. Alkalinizing buffer for the post-column derivatization was prepared by mixing ethanol and 0.05 *M* borate buffer, pH 9.5, containing the disodium salt of EDTA (2 g/l) in a volume ratio of 1:1.

Fig. 1 shows the flow diagram of our chromatograph. The mobile phase was

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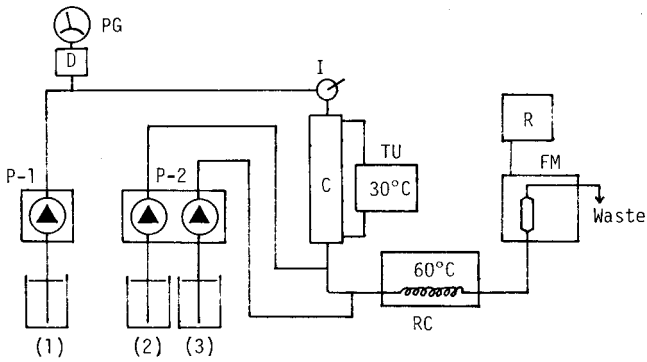

Fig. 1. Flow diagram of the chromatographic system. P-1 = Single plunger pump; P-2 = double plunger pump; D = damper; PG = pressure gauge; I = injector; C = column and column jacket; TU = thermo unit; RC = reaction coil; FM = fluoromonitor; R = recorder; 1 = mobile phase; 2 = alkalinizing buffer; 3 = NBD-Cl reagent.

delivered at a constant flow-rate of 1.0 ml/min using a single plunger pump (Sanuki Industry, Tokyo, Japan). Develosil ODS was packed in the stainless-steel column (10 cm × 4.0 mm I.D.) in our laboratory by the conventional slurry packing technique. The column was operated at 30°C utilizing a Taiyo thermo unit C-600 (Taiyo Scientific Industry, Tokyo, Japan). Both the alkalinizing buffer and NBD-Cl reagent were delivered at a constant flow-rate of 0.6 ml/min using a double plunger pump (Sanuki Industry).

The column eluate was first mixed with the alkalinizing buffer in a T-piece, and then with NBD-Cl reagent in another T-piece. The mixture was then allowed to flow through a PTFE-tubing reaction coil (4 m × 0.5 mm I.D.) immersed in a water-bath at 60°C. The fluorescence intensity of the effluent was measured at 530 nm; excitation of fluorescence was achieved at 470 nm, using an RF-500 LCA spectrofluoromonitor (Shimadzu, Kyoto, Japan) equipped with a xenon discharge lamp.

RESULTS AND DISCUSSION

NBD-Cl was first described by Ghosh and Whitehouse[7] as a fluorigenic reagent for amino acids and amines, and it has been shown[8] to provide a much more intense fluorescence on reaction with imino acids than with α-amino acids. Roth[9] applied NBD-Cl for the specific detection of proline and hydroxyproline in liquid chromatography. In the present study, NBD-Cl was found to be suitable for the micro-detection of D- and L-proline after resolution. TosPhe did not interfere with the fluorimetric detection of proline since NBD-Cl gives only a slow colour reaction with sulphonamides[10]. Precipitation of copper(II) ions in the mobile phase by the action of the alkalinizing buffer was prevented by adding EDTA. With this system, the quantitation of proline down to 1 nmol was achieved.

The conditions for the resolution were essentially those used previously for common amino acids[4]. Fig. 2 shows the resolution of DL-proline when 20 nmol of the racemate were injected into the chromatograph shown in Fig. 1. L-Proline was eluted before the D-isomer, as with the racemates of neutral amino acids described pre-

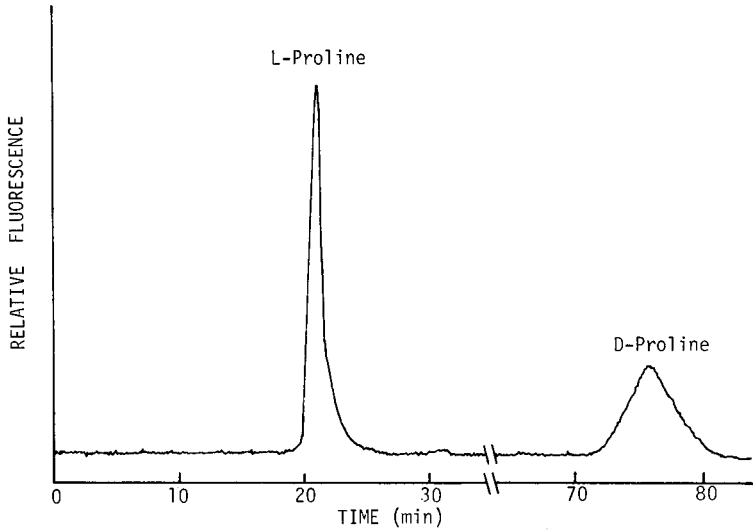

Fig. 2. Separation of D,L-proline with TosPhe-Cu (II) eluent. Mobile phase; aqueous solution containing 1 m*M* TosPhe and 0.5 m*M* $CuSO_4 \cdot 5H_2O$, pH 6.0. Approximately 10 nmol of each isomer were injected.

viously[4]. In addition, DL-proline showed a relatively large separation factor (α = 3.92), corresponding to a difference in the free energies[11] between the solutions of the two enantiomers ($\Delta\Delta G^0 = -RT \ln \alpha$) of 820 cal/mol, whereas other common α-amino acid enantiomers gave α values of less than 2.90[4].

In the mobile phase, TosPhe and copper(II) ion are assumed to form a binary complex, $(TosPhe)_2Cu(II)$ in which TosPhe is in the form of its mono anion. D- and L-proline injected into the column are believed to undergo ligand exchange with the binary complex on the reversed phase, and the corresponding diastereomeric ternary complexes, TosPhe–Cu(II)–D-proline and TosPhe–Cu(II)–L-proline, may be formed. The excellent resolution of DL-proline may be due to the difference in stability between the two diastereomeric species caused by the fixed conformation of the pyrrolidine ring.

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Note

Improved enantiomeric resolution of D,L-Dns-amino acids

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Earlier, we reported[1,2] the resolution of optical isomers of D- and L-Dns-amino acids by mixed complex formation with L-proline in the mobile phase[1,2]. Eight pairs of amino acids were separated. Amino acids with small and/or polar alkyl substituents were not resolved. In this note, the improved resolution of Dns-amino acids with Cu(II)–L-proline mobile phase is described. Several of the small polar amino acids were base line resolved. An efficient column and the proper composition of mobile phase are critical in achieving the separation.

The effect of two different column materials on separation were investigated. The packing materials did not affect the stereoselectivity between the D- and L-isomers, but the retention and selectivity between the amino acids were changed. The Cu(II)–proline system is highly selective. We demonstrated that this system is selective not only for the separation of optical isomers of amino acids but for the individual amino acids as well.

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Model Series 2 liquid chromatographic pump, a Model LC 650-10 fluorescence spectrophotometer and a Model 56 chart recorder (Perkin-Elmer, Norwalk, CT, U.S.A.). The analytical columns (15.0 × 0.42 cm) were packed with either LiChrosorb® RP-8 or Spherisorb® C_{18} by the downward slurry technique. Sample was introduced via a Rheodyne 7105 injection valve. The fluorescence at 480 nm was monitored with excitation at 340 nm.

Reagents

Acetonitrile distilled in glass was bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). D- and L-Dns-amino acids were bought from Sigma (St. Louis, MO, U.S.A.) and Pierce (Rockford, IL, U.S.A.). Some of the Dns-amino acids were prepared as previously described[1]. The mobile phases were made up of 5 m*M* L-proline, 2.5 m*M* $CuSO^4 \cdot 5H_2O$ and 0.5 g ammonium acetate per liter of deionized water with appropriate percentage of acetonitrile as shown in Figs. 1–3.

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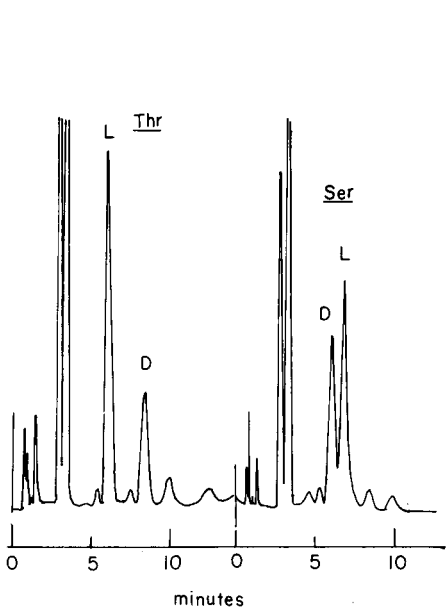

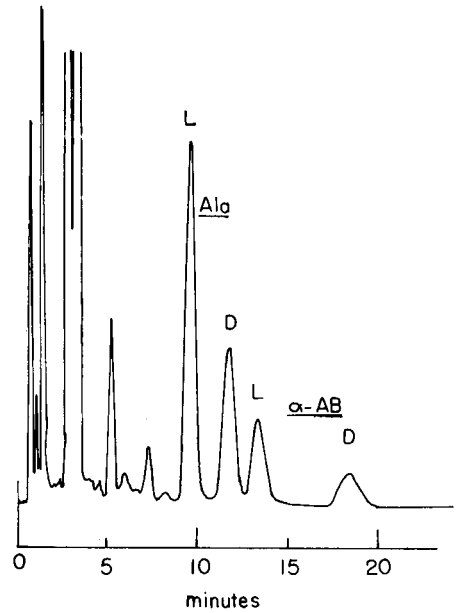

Fig. 1. Separation of D,L-Dns threonine and D,L-Dns serine. Mobile phase: 15% acetonitrile in an aqueous solution containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot H_2O$ and 0.5 g ammonium acetate/l of deionized water. pH 7.0. Column: 15 × 0.42 cm LiChrosorb RP 8. Flow-rate: 2.0 ml/min.

Fig. 2. Separation of D,L-Dns alanine and D,L-Dns α-amino butyric acid. Conditions as in Fig. 1.

RESULTS AND DISCUSSION

Many D- and L-Dns-amino acids can be separated by a copper (II)–L-proline complex mobile phase. Previously, we achieved the complete resolution of 8 pairs of Dns-amino acids which possess non-polar side chains. The selectivity between the D- and L-pairs is dependent on the alkyl substituent on the α-carbon of the amino acid. The greater the carbon content and the bulkier the alkyl group, the larger is the stereoselectivity because of the interaction of the alkyl groups of the bis(amino acid)–Cu(II) complex. By choosing a more efficient column and the proper mobile phase, we have further improved the separation of Dns-amino acids. The present system is more efficient and faster. We achieved the baseline resolution of a group of amino acids that possess small alkyl substitutents with and without polar groups (Figs. 1 and 2), in addition to the amino acids we had previously resolved. The retention and selectivity of these smaller amino acids are very sensitive to the acetonitrile concentration, because the organic modifier influences the distribution of the metal complexes on the hydrocarbonaces stationary phase.

It is of interest that D-serine eluted before the L-isomer. This elution order represents the reverse of what was generally observed for the other amino acids. The free hydroxyl group in serine may participate in coordination and alters the stability of the Dns-serine–Cu(II)–L-proline ternary complex.

The effect of the stationary phase on the selectivity of the amino acids was examined. Table I gives the capacity ratio (k') and the selectivity factor (α) of the Dns-

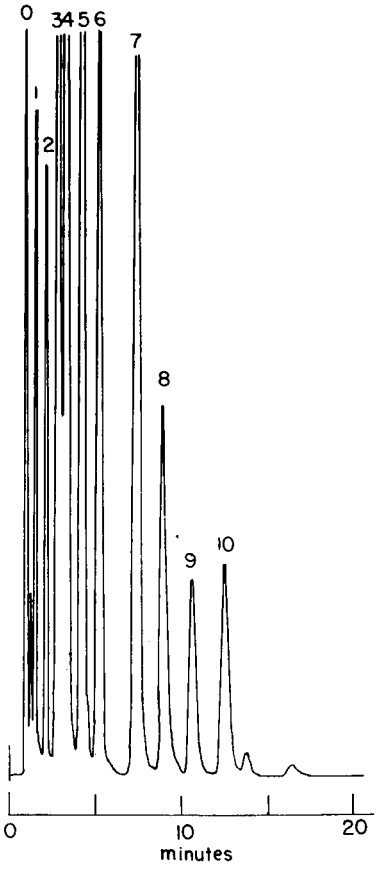

Fig. 3. Separation of L-Dns amino acids. Mobile phase: 20% acetonitrile in an aqueous solution containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 0.5 g ammonium acetate/l of deionized water. pH 7.0. Column: 15.0 × 0.42 cm Spherisorb C_{18}. Flow-rate: 2.0 ml/min. 0 = Reagent; 1 = hydroxyl proline; 2 = serine and threonine; 3 = alanine and α-amino butyric acid; 4 = proline; 5 = valine; 6 = norvaline and methionine; 7 = leucine; 8 = norleucine and phenylalaine (L); 9 = tryptophan; 10 = phenylalanine (D).

amino acids on two reversed-phase packings, LiChrosorb RP-8 and Spherisorb C_{18}. Because the carbon content and chain length on these materials are different, the acetonitrile concentration in the mobile phases was different. It is evident from the k' values that the selectivity between the amino acids is affected by the choice of stationary phases. However, on examination of the stereoselectivity between the D- and L-amino acids, the α values for the same amino acid are in reasonable agreement between the two systems suggesting that the stereoselectivity is independent of the packing used. Thus, the separation of D- and L-amino acids in the two systems occurs by the same mechanism; namely, the formation of Cu(II) ternary complexes with L-proline and the Dns-amino acids, with separation of the ternary complexes on the stationary phase. The selectivity factor which represents the difference in thermodynamic stability of the isomeric metal complexes is constant.

The metal complex system is highly selective. An example of the separation of

TABLE I

CAPACITY RATIO (k') AND SELECTIVITY (α) OF D- AND L-Dns-AMINO ACIDS ON TWO DIFFERENT COLUMN PACKINGS

Column: 15.0 × 0.42 cm. Flow-rate: 2.0 ml/min. LiChrosorb RP-8: Mobile phase: 20% acetonitrile (unless otherwise indicated) in an aqueous solution containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 0.5 g ammonium acetate/l of deionized water. pH 7.0. Spherisorb C_{18}: Mobile phase: 15% acetonitrile in an aqueous solution containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 0.5 g ammonium acetate/l of deionized water. pH 7.0.

Amino acid	*Abbreviation*	*LiChrosorb RP-8*			*Spherisorb C_{18}*		
		k'_L	k'_D	α	k'_L	l'_D	α
Serine	Ser	23.0*	20.0*	0.87	3.7	3.2	0.87
Threonine	Thr	19.0*	31.2*	1.6	3.7	4.6	1.23
Alanine	Ala	4.1	4.3	1.1	5.7	6.6	1.2
α-Aminobutyrate	α-AB	5.4	6.6	1.2	7.3	9.2	1.2
Valine	Val	7.7	10.1	1.3	11.4	15.0	1.3
Methionine	Met	9.7	12.3	1.3	11.4	14.8	1.3
Norvaline	N-Val	10.1	13.7	1.4	14.6	19.0	1.3
Isoleucine	I-Leu	14.7	19.7	1.3	–	–	–
Leucine	Leu	17.0	25.4	1.5	23.9	32.6	1.4
Norleucine	N-Leu	22.1	31.2	1.4	32.6	45.7	1.4
Phenylalanine	Phe	17.4	29.2	1.7	32.6	52.8	1.6
Tryptophan	Trp	23.0	43.4	1.9	41.2	71.2	1.7

* 10% acetonitrile.

many L-Dns-amino acids is shown in Fig. 3. Both D- and L-phenylalanine are included in this chromatogram to demonstrate the efficiency and selectivity of the present separation. The system can be optimized for the separation of natural amino acids.

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Note

High-performance liquid chromatography and thin-layer chromatography of penitrems A–F, tremorgenic mycotoxins from *Penicillium crustosum*

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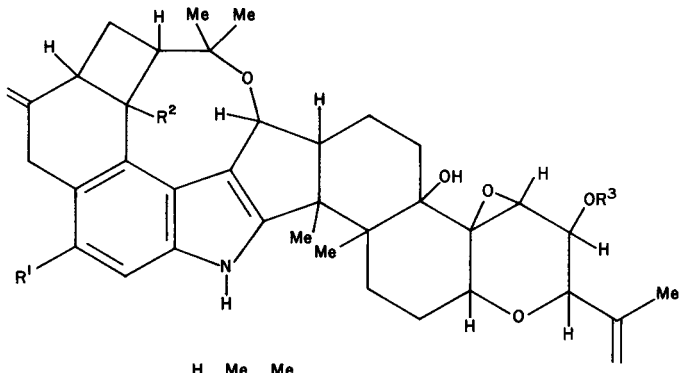
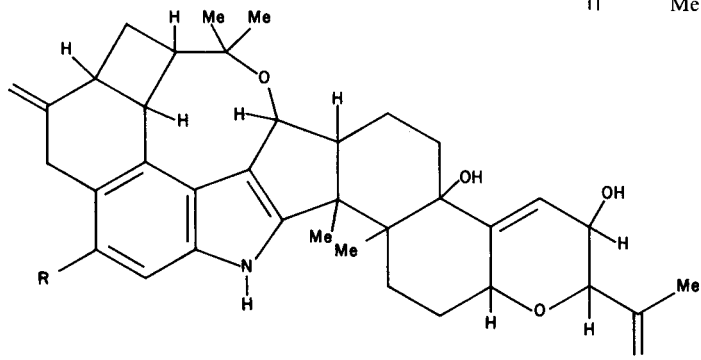
(Received August 6th, 1981)

The structures of penitrems A–F (1–6), metabolites from *Penicillium crustosum**, were recently reported[1]. The penitrems, as well as some other indolic mycotoxins, have received increased attention over the past decade on account of their unique ability to cause sustained tremors in vertebrate animals. Penitrem A, which was also isolated from moulds, previously classified as *P. cyclopium*[2] and *P. palitans*[3],



(1) R^1 = Cl, R^2 = OH, R^3 = H, Penitrem A
(2) R^1 = H, R^2 = H, R^3 = H, Penitrem B
(5) R^1 = H, R^2 = OH, R^3 = H, Penitrem E
(6) R^1 = Cl, R^2 = H, R^3 = H, Penitrem F
(8) R^1 = Cl, R^2 = OH, R^3 = Ac, 25-O-Acetyl penitrem A

Me = Methyl



(3) R = Cl, Penitrem C
(4) R = H, Penitrem D

* Dr. J. I. Pitt, C.S.I.R.O., Australia recently concluded that all of the isolates involved in the production of penitrem A belong to *P. crustosum*.

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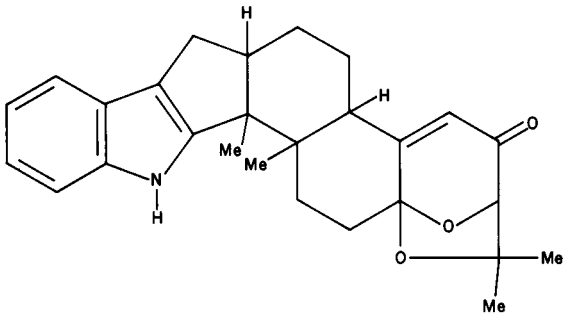

(7) Paspalicine

causes tremors, limb weakness, ataxia and convulsions in mice[4]. Neurochemical studies showed that penitrem A acts by interfering with amino-acid neurotransmitter release mechanisms[5]. The penitrems are structurally closely related to the paspalins [*e.g.*, paspalicine (7)], tremorgenic compounds isolated from *Claviceps paspali*[6].

The production of the penitrems by *P. crustosum* was investigated as a continuation of production and biosynthetic studies, and because of their interesting physiological activity. This necessitated the availability of an efficient method for the identification and quantitation of the different penitrems in culture extracts. Although several solvent systems have been reported for the analysis of penitrem A[7,8], no chromatographic data are available for the other penitrems.

The penitrems are rapidly decomposed in acid media to form blue solutions[3]. This property was utilized by Hou *et al.*[9] for the determination of penitrem A by a colorimetric method. The method has the disadvantages that one penitrem cannot be selectively determined in a mixture of penitrems, and the material is destroyed during the determination.

The present investigation was directed towards development of one or more simple high-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) systems for the separation, identification and quantitation of the various penitrems in a culture extract.

EXPERIMENTAL

All chemicals were of analytical grade and were used without further purification.

Extraction and purification of penitrems

A penitrem-producing isolate of *P. crustosum*, Sol-7, was grown for 8 days at 25°C in a stationary culture in erlenmeyer flasks (500 ml), each containing 100 ml of Czapek medium enriched with 2% yeast extract. The mycelial mats were recovered and homogenized in a Waring blender in acetone. The homogenates were filtered and the acetone removed. The aqueous solution was extracted with dichloromethane. After evaporation of the solvent, the residue was subjected to partition in hexane–90% aqueous ethanol. The methanol was removed under vacuum and the aqueous residue extracted with dichloromethane. The extract was evaporated to dryness. For TLC, the extract was made up to 5 ml with acetone. For HPLC the extract was made up to 10 ml with methanol. To purify the individual penitrems, the extract was subjected to

preparative column chromatography on silica gel in benzene–acetone (9:1). The internal standard used, penitrem A monoacetate (8), was prepared by treatment of penitrem A with acetic anhydride–pyridine (1:1) for 4 h at room temperature and worked up by standard procedures.

TLC

Merck pre-coated silica gel F_{254} plates (Cat. No. 5715, thickness 0.25 mm) were used. Standard solutions of the penitrems were spotted on a baseline 2 cm from the bottom of the plate with a graduated 5-μl pipette, and the plate was then developed 16 cm in the appropriate solvent system in a tank lined with filter-paper. The developed plates were examined under UV light at a wavelength of 254 nm using a Minuvis lamp, and subsequently sprayed with a 1 % cerium(IV) sulphate solution in 3 *M* sulphuric acid and heated at 110°C for 10 min. In this case the final estimations of the toxin concentration relied on a visual comparison with standards of the penitrems.

HPLC

Reversed-phase chromatography was performed using a Hewlett-Packard 1084B HPLC system including a HP 79850 B LC terminal for solvent programming and recording. Separation was achieved on a HP 79918A RP-8 reversed-phase column, particle size 10 μm. The penitrems were detected at 296 nm [penitrem A: λ_{max} (MeOH) 233 and 296 nm (ε 37,000 and 11,600 l mol^{-1} cm^{-1})] with a UV absorbance detector. Water–methanol (22:78), at a flow-rate of 1.5 ml/min, was used as the solvent system. The column pressure was 107 bar, and column temperature 40°C. Solutions of the penitrems in methanol, containing a known quantity of internal standard, were chromatographed directly in 10-μl portions. Comparison of the integrated peak areas with that of penitrem A monoacetate, the internal standard, enabled the quantitation of the amount of penitrems produced by *P. crustosum*.

RESULTS AND DISCUSSION

The penitrems are unstable in chloroform when exposed directly to light, presumably as a result of acid formation in the solvent[3]. Any contact of the penitrems with chloroform was avoided throughout this investigation. Although several solvent systems had been reported for the TLC of penitrem A[7,8], none of these systems was effective in the separation of penitrems A–F. The most efficient solvent systems for the TLC separation of the penitrems were found to be: (a) hexane–ethyl acetate (70:30); (b) dichloromethane–acetone (85:15) and (c) benzene–acetone (85:15). In solvent system a penitrems B and F as well as C and D still overlapped, whereas penitrems C and E overlapped in system b. The only system which gave complete separation of all the penitrems was c. The best results were obtained by developing the chromatogram twice in this solvent system. The general order of the penitrems (with decreasing R_F value) was: F, B, A, E, C and D.

The penitrems showed a high sensitivity towards the spray reagent consisting of a 1 % cerium(IV) sulphate solution in 3 *M* sulphuric acid. The spots immediately turned blue on spraying and after heating at 120°C the colour changed to a stable dark purple. The R_F values of penitrems A–F in different solvent systems and their detection limits by UV light and the spray reagent are given in Table I.

TABLE I

R_F VALUES AND DETECTION LIMITS OF PENITREMS A–F

Solvents: a = hexane–ethyl acetate (70:30); b = dichloromethane–acetone (85:15); c = benzene–acetone (85:15).

Penitrem	R_F			*Lowest amount detectable (g)*	
	a	*b*	*c*	*UV illumination*	$Ce(SO_4)_2$–H_2SO_4
A	0.16	0.49	0.37	10^{-6}	10^{-7}
B	0.18	0.53	0.39	10^{-6}	10^{-7}
C	0.09	0.39	0.28	10^{-6}	10^{-7}
D	0.09	0.37	0.26	10^{-6}	10^{-7}
E	0.13	0.46	0.33	10^{-6}	10^{-7}
F	0.18	0.55	0.42	10^{-6}	10^{-7}

Although TLC provided an excellent qualitative and semiquantitative method, an HPLC method was developed for the quantitative analysis of penitrem mixtures. The separation by HPLC was achieved by reversed-phase chromatography.

In order to develop an efficient mobile phase for the separation, preliminary investigations were carried out on solutions of analytically pure penitrems. The penitrems exhibit a strong absorption in the 275–300 nm region. Therefore, the UV detector at 296 nm is sensitive to small quantities of penitrems, and at a sensitivity of $6.4 \cdot 10^{-3}$ a.u./cm, 20 ng of a pure penitrem produced a significant peak. An even higher sensitivity can be obtained by analysing the eluent at 230 nm. Ten samples (concentration range 100–250 ng) were analysed at both 230 and 296 nm, an increased sensitivity (3.5) being observed at 230 nm. This increase is directly related to the magnitude of the relevant extinction coefficients.In practice analysis at 296 nm is regarded as preferable because less compounds interfere at this wavelength.

By using water–methanol (22:78) as the mobile phase it was possible to separate penitrems A–F in one run. The chromatogram in Fig. 1 indicates a typical separation of the system used. The retention times of the different penitrems are given in Table II.

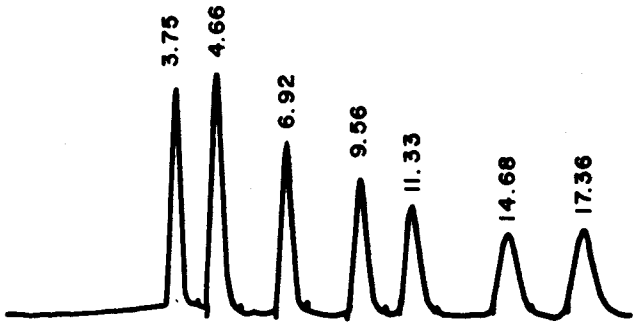

Fig. 1. Reversed-phase HPLC of the penitrems. Column: HP 79918A RP-8 (10 μm). Detector: UV (296 nm). Eluent: water–methanol (22:78); flow-rate 1.5 ml/min. The numbers above the peaks signify the retention times in minutes. The peaks are (with increasing retention times): penitrem E; A; 25-O-acetylpenitrem A; penitrem D; B; C; F.

TABLE II

RETENTION TIMES OF PENITREMS A–F

Metabolite	*Retention time (min)*
Penitrem A	4.66
Penitrem B	11.33
Penitrem C	14.68
Penitrem D	9.56
Penitrem E	3.75
Penitrem F	17.36
Penitrem A monoacetate	6.92

For a quantitative analysis of the penitrems an internal standard (IS) was used. The amount of each component was calculated according to

$$\text{Absolute amount of Y} = \frac{\text{Area Y}}{\text{Area IS}} \cdot \frac{\text{Response Y}}{\text{Response IS}} \cdot \text{Amount IS} \cdot \text{DF}$$

where Response = mass/area and DF = dilution factor. The monoacetate of penitrem A (8) was a suitable internal standard for a penitrem mixture. There was an adequate difference in the retention time of 25-O-acetylpenitrem A from those of penitrems A–F. The factor response Y/response IS for each penitrem was obtained by using analytical samples of the corresponding penitrem and penitrem A acetate.

By the addition of a known quantity of penitrem A acetate to a mycelial extract, it was possible to quantitate the penitrems in the extract. The above approaches should be applicable to the analysis of penitrems produced under natural or laboratory conditions on solid substrates.

ACKNOWLEDGEMENTS

We thank Dr. R. J. Cole, National Peanut Research Laboratory, Dawson, Georgia, for a strain of Sol-7 and Dr. J. I. Pitt, Division of Food Research, C.S.I.R.O., Australia for the identification of this strain as *Penicillium crustosum*.

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Note

High-performance liquid chromatography separation of soybean isoflavones and their glucosides

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Soybeans are known to contain several isoflavones (daidzein, glycitein, genistein) and isoflavone glucosides[1] (daidzin, glycitein-7-β-O-glucoside, genistin) which have been reported to have estrogenic[2], antifungal[3] and antioxidant[4] activity. The quantitative determination of soybean isoflavones has been reported by gas–liquid chromatography (GLC)[1], and more recently high-performance liquid chromatography (HPLC)[5,6,7] has been used.

Carlson and Dolphin[5] separated soybean isoflavone aglycones present in an alcohol extract on a μPorasil column. The isoflavone glucosides were determined after hydrolytic treatment with aqueous acid. In a paper by West *et al.*[6] only two isoflavone aglycones from soybean extracts were separated.

This communication reports the development of a procedure for the separation of the naturally occurring soybean isoflavone glucosides and isoflavone algycones using HPLC with mild solvents and reversed phase packing that may be less likely to catalyze decomposition compared to other packings.

MATERIALS AND METHODS*

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used, comprised of a WISP 710A, M-45 solvent delivery system, Model 660 solvent flow programmer, Model 450 variable-wavelength detector, with a DuPont Zorbax ODS 25 × 0.46 cm I.D. column protected by a 2-cm Corasil C_{18} guard column. The solvent flow-rate was 1 ml/min and the absorption was measured at 262 nm. Solvents were spectral grade, and distilled water was deionized before use. All solvent ratios are on a volume basis.

Genistein and daidzein were obtained from ICN Pharmaceuticals, Life Science Group (Plainview, NY, U.S.A.). *n*-Butyrophenone was purchased from Aldrich (Milwaukee, WS, U.S.A.). Coumesterol was purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.). Genistin and daidzin were synthesized from the aglycones by the pro-

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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cedure of Zemplén and Farkas[8] and were a gift from Dr. L. C. Wang of our laboratory. Purity of the synthesized products determined by HPLC was found to be 99.2 and 94.9%, respectively. Genistin and daidzin were also isolated by preparative HPLC from an alcoholic extract of hexane-defatted soybean meal. Mixtures that contained genistin and daidzin; daidzin and glycitein-7-β-O-glucoside; daidzin, genistin and glycitein-7-β-O-glucoside and the relative proportion of each in the mixtures were generously supplied by Dr. A. Bondi fo the Hebrew University of Jerusalem.

Separations were carried out with an aqueous methanol gradient from 25 to 50% using either curve 6 (linear) or curve 8 (concave) on the 660 gradient programmer. Solvent A contained 15% methanol and solvent B was 65% methanol. By using these combinations of solvents, formation of bubbles in the system was avoided.

RESULTS AND DISCUSSION

Since glycitein, the aglycone, was not available, the mixture of three isoflavone glucosides obtained from Dr. Bondi, which contained naturally occurring concentrations of the glucosides, was hydrolyzed with 2 *N* sulfuric acid in ethanol for 2 h by refluxing. The hydrolyzate was cooled and extracted with diethyl ether, and the ether layer was taken to dryness. By chromatographing both the mixture of natural occurring glucosides and the prepared aglycones, the retention times of the aglycones could be determined and compared with those of their corresponding glucosides.

Fig. 1 shows the separation of soybean isoflavones as observed in the current method. The glycosides eluted first and were followed by the isoflavone aglycones in the same relative order. The eight peaks are daidzin, glycitein-7-β-O-glucoside, genistin, daidzein, glycitein, genistein, coumesterol and *n*-butyrophenone. The methanol

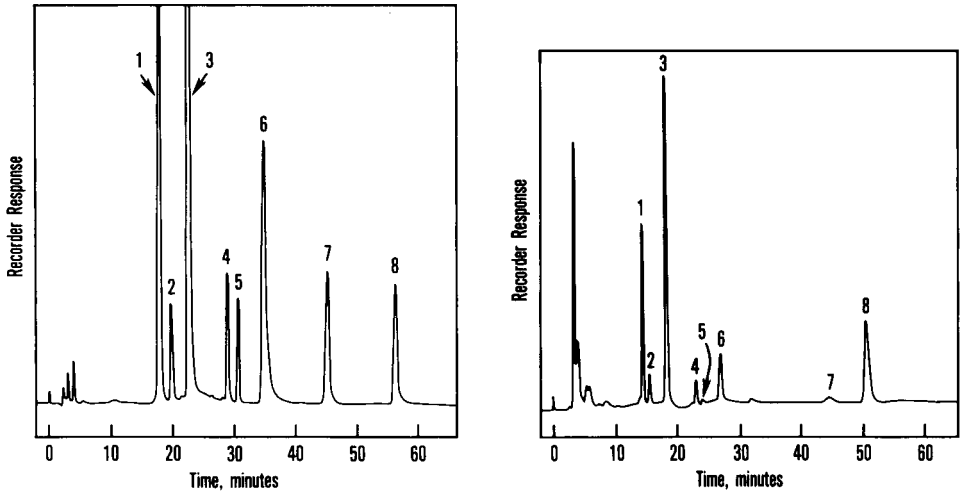

Fig. 1. HPLC elution diagram of daidzin (1), glycitein-7-β-O-glucoside (2), genistin (3), daidzein (4), glycitein (5), genistein (6), coumesterol (7) and *n*-butyrophenone (8), using a linear gradient of methanol from 25 to 50% in 20 min.

Fig. 2. HPLC elution diagram of an aqueous methanolic extract (80%, reflux) of hexane-defatted soybean meal with added *n*-butyrophenone as a standard. Peaks as in Fig. 1.

gradient ran from 25 to 50% in 20 min, and then the solvent remained at 50% for the duration of the separation.

The elution position of coumesterol was of interest, since it is present in soybeans[9] and has physiological activity. However, the concentration is so low in soybean meal that a peak corresponding to coumesterol was not detected in most of my samples.

The elution of *n*-butyrophenone is shown because it was found to be useful as an internal standard in the quantitative determination of soybean isoflavones.

Fig. 2 shows an elution pattern of a hot 80% methanolic extract of hexane-defatted soybean meal. Peaks 1, 2, 3, 4 and 6 were collected from several analysis and their spectra were measured. Each isolated peak had the typical peak at 258–262 nm and shoulder in 320 nm area.

Soybean meal contains at least three isoflavone glucosides (peaks 1, 2 and 3), and the meal also contains at least three isoflavone aglycones, peaks 4, 5 and 6). However, only five of these peaks are present in measurable amounts.

The amounts (mg/100 g) of isoflavones in hexane-defatted soybean meal from Amsoy soybeans, 1979 crop, have been found to be: daidzin, 62; glycitein-7-β-O-glucoside, 18; genistin, 127; daidzein, 48; glycitein, trade; genistein, 40.

This analytical procedure is being applied to the quantitative determination of these biologically active components in soybeans and soybean products, and the results will be reported elsewhere.

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Note

Analysis of isoflavones in Bengalgram by high-performance liquid chromatography

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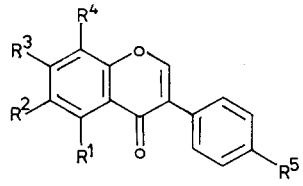
Isoflavones are almost totally confined to the family *Leguminosae* and characteristically to the sub-family *Papilionoidae*[1], which includes a high number of food crops[2]. These compounds are not found in dormant seeds but are present in germinated seeds, presumably synthesised during germination[3]. They are of interest due to their diverse biological properties (anti-fungal, anti-oxidant, anti-haemolytic, oestrogenic[4–6] as well as their importance in chemotaxonomy. Moreover the oestrogenic type isoflavones[6], formononetin (I) and biochanin A (II), common in Bengalgram (*Cicer arientanum*) have been shown to lower significantly serum cholesterol levels in experimental animals[7,8], whereas daidzein (III), and genistein (IV), the principal isoflavone in soya (*Glycine max.*), would appear not to have this property[9,10].



(I) Formononetin : $R^1=R^2=R^4=H; R^3=OH; R^5=OCH_3$
(II) Biochanin A : $R^1=R^3=H; R^2=R^4=OH; R^5=OCH_3$
(III) Daidzein : $R^1=R^2=R^4=H; R^3=R^5=OH$
(IV) Genistein : $R^1=R^3=R^5=OH; R^2=R^4=H$

Previous quantitation of these compounds has been carried out by thin-layer chromatography, gas–liquid chromatography of the trimethylsilyl ethers[11] and gel filtration.

Our work on the beneficial and/or detrimental properties of minor constituents in the human diet has dictated a need for a rapid method of analysis of these compounds in various foods under differing conditions of storage and processing. A facile and rapid method for the determination of these compounds serves as a basis for this report.

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EXPERIMENTAL

A 50-g sample of Bengalgram (commercial variety) was germinated for 72 h. The germ was filtered, dried at 50°C under vacuum and milled (UDY cyclone sample mill, Tecator, CO, U.S.A.). A 10-g sample of the flour was extracted with 85% aqueous methanol (3 × 150 ml) at 60°C. The methanolic extract was evaporated under vacuum and taken up in 70% aqueous ethanol (50 ml) and extracted with hexane (3 × 30 ml). The alcoholic phase was evaporated and the residue taken up in water (10 ml) and extracted with ethyl acetate (3 × 3 ml). The combined ethyl acetate extract was dried (magnesium sulphate), filtered, and made up to constant volume (10 ml). The instrument used was an Applied Chromatography System Model 750 gradient chromatograph. Injection was carried out via a Rheodyne injection valve, Model 7120 (20-μl loop). Detection was made by ultraviolet absorption (Cecil CE 212 detector) at a wavelength of 280 nm. Peak areas were measured using a Hewlett-Packard HP 3390A integrator. Chromatographic columns (10 cm × 5 mm) of Spherisorb-5-ODS were packed in our own laboratory. Methanol was of HPLC grade obtained from Rathburn (Wakeburn, Great Britain). Separations were carried out by using a mobile phase of methanol–water (1:4) initial concentration, rising to (3:2) at 5% min^{-1}. A flow rate of 2 ml min^{-1} was used.

Authentic samples of isoflavones were obtained from Apin Chemicals, Cardiff, Great Britain.

RESULTS AND DISCUSSION

HPLC offers a high degree of separation of the isoflavones as illustrated in Fig. 1. A typical chromatograph for a Bengalgram extract is shown in Fig. 2.

A rapid gradient was employed to obtain the minimal time of analysis (*ca.* 10 min) with adequate separation of the components of interest. The conditions employed were quite sufficient for the method of extraction and food analysed. Although, work with other products may necessitate slightly less severe gradients should interference from other peaks become apparent.

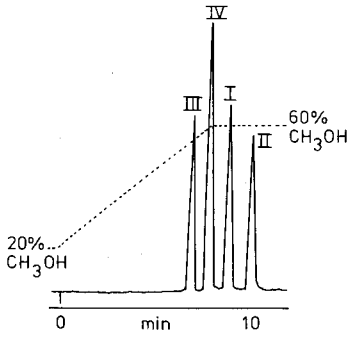

Fig. 1. Chromatogram of isoflavone standards. Solvent gradient, 20 - 60% methanol in water. Detector, × 1. All isoflavones 0.50 mg ml^{-1}.

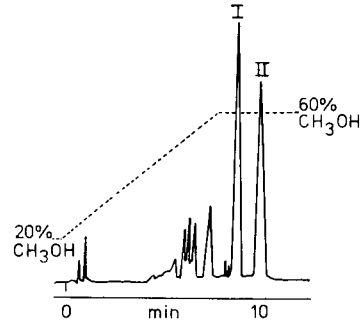

Fig. 2. Chromatogram of Bengalgram extract. Solvent gradient, 20 - 60% methanol in water. Detector, × 1.

Isoflavone concentrations present in the germ as determined by HPLC are shown in Table I. These are in the correct region, although variations are likely to occur with variety and conditions of germination.

TABLE I

ISOFLAVONE CONCENTRATION OF BENGALGRAM

N.D. = Not detectable.

Isoflavone	*Concentration (mg/100 g germ)*	*Literature*[8] *(mg/100 g germ)*
Biochanin A	71	98.6
Formononetin	77	44.1
Genistein	N.D.	N.D.
Daidzein	N.D.	5.1

ACKNOWLEDGEMENT

We are grateful to Dr. J. Ingham, University of Reading, England, and Dr. R. Sharma, National Institute of Nutrition, Hyderabad, India for kindly donating samples of biochanin A and genistein.

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Note

Analysis of olivetol in rabbit serum by high-performance liquid chromatography*

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(Received September 7th, 1981)

Phenolic compounds have been found to be effective inhibitors of prostaglandin cyclooxygenase[1]. Olivetol is a phenolic compound (3,5-dihydroxy-*n*-pentylbenzene) found in substantial quantities in pyrolysis products of cannabidiol and could contribute to harmful effects produced by smoking marijuana[2]. Analysis of cannabinoids by gas chromatography has been shown to produce detectable quantities of olivetol as a result of pyrolytic reactions in the injection port[3]. During the course of a study of the effect of olivetol on uterine blood flow in rabbits it was necessary to develop a method for analysis of olivetol in serum. Gas chromatography did not prove to be useful due to tailing peaks when olivetol was not derivatized and multiple products when it was derivatized (pentafluoropropionate). Therefore, the following straightforward and sensitive high-performance liquid chromatographic (HPLC) method was developed.

EXPERIMENTAL

HPLC was performed on a reversed-phase μBondapak C_{18} column (20 cm × 3.9 mm I.D., 10 μm particle size, Waters Assoc., Milford, MA, U.S.A.) with acetonitrile–water (40:60) as the mobile phase. The flow-rate was 2.0 ml/min at a pressure of 2000 p.s.i. Olivetol was detected at a wavelength of 280 nm (Waters 440 UV detector). Peak areas were calculated by triangulation.

Serum samples (1 ml) were extracted three times with 5 ml of diethyl ether. The combined ether extracts were concentrated to about 1 ml, the sides of the tube were washed down with absolute ethanol, and this was concentrated to near dryness again. Acetonitrile was then used to wash the sides of the tubes several times to replace the ethanol with acetonitrile. This was concentrated to near dryness and 50 μl of acetonitrile were added. Any insoluble material was removed by centrifugation. The total quantity of solution (< 50 μl) was injected into the HPLC column.

* The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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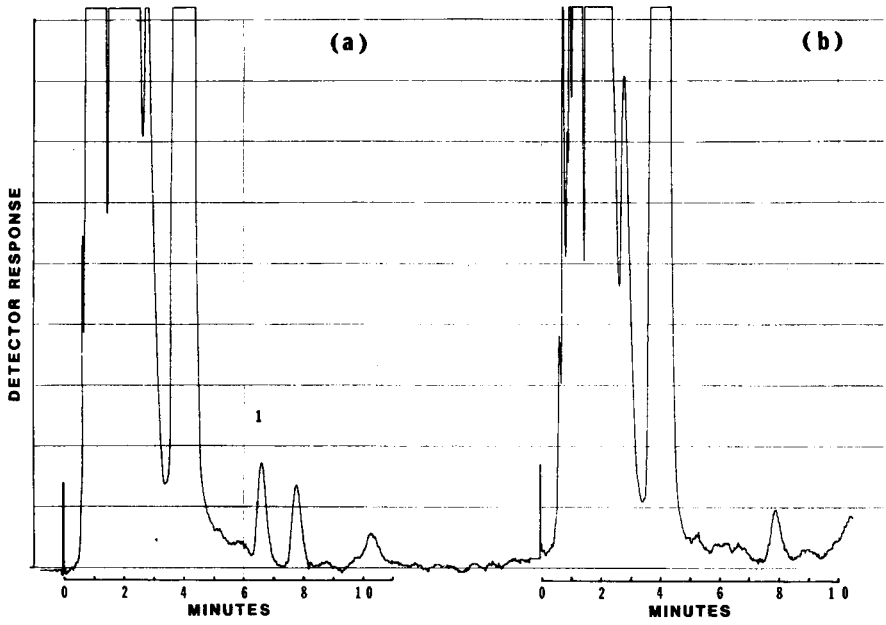

Fig. 1. Chromatogram of rabbit serum extract at maximum sensitivity (0–0.005 a.u.f.s.). See Experimental section for HPLC details. (a), Extract from 1 ml of serum spiked with 100 ng of olivetol (peak 1). (b), Serum blank.

RESULTS AND DISCUSSION

Gas chromatographic analysis of olivetol required derivatization (pentafluoropropionate) for optimum peak shape. However, incomplete derivatization was found to be a problem as evidenced by a broad second peak that appeared when using OV-210 as the stationary phase or a sharp second peak when using SE-30. Also, interferences appeared when serum extracts were derivatized.

Reversed-phase HPLC (μBondapak C_{18}) of olivetol using acetonitrile–water (40:60) as mobile phase gave a symmetrical peak eluting with a retention time of 6.7 min (flow 2.0 ml/min). A 50-mm guard column packed with a reversed-phase packing (Supelco LC-8) was placed in front of the analytical column.

The absorbance maximum of olivetol is 278 nm and the HPLC procedure proved to be very sensitive when monitoring with a 280 nm filter. The standard curve was linear down to 20 ng with a correlation coefficient of 0.998 (each point a mean of 3 determinations). The standards did not deviate from linearity until 1000 ng were injected giving a region of linearity from 20 ng to 800 ng.

No adequate internal standard was readily available. A recovery study was conducted by spiking eight 1-ml serum samples with 500 ng of olivetol standard (Polysciences, Warrington, PA, U.S.A.). This quantity was chosen because the biological sample concentrations were in this range. The mean recovery was 82% with a standard deviation of 6.3% and a range of 72% to 92%.

The greatest source of variability was the dissolution of the extracted olivetol in acetonitrile for injection on the HPLC column. Not all of the components of the

extract were readily soluble in acetonitrile so it was necessary to maintain the solutes in solution by a gradual transfer of the solvent from ether to ethanol to acetonitrile.

The serum blank was exceptionally free of interfering peaks even at maximum sensitivity (Fig. 1). A gradual buildup of highly lipophilic materials necessitated a daily cleaning of the column with acetonitrile as well as periodic cleaning of the guard column.

This procedure proved to be highly sensitive and free of interferences when applied to biological samples. It has been used to satisfactorily quantitate olivetol in a large number of rabbit serum samples.

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CHROM. 14,274

Note

Hochleistungs-Flüssigkeits-Chromatographische Analyse von Digitalis-Blattextrakten

I. Qualitative Analyse

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(Eingegangen am 3. August 1981)

In den letzten Jahren ist für die Analyse einiger Digitalisglykoside, vor allem in Arzneifertigpräparaten, die Hochleistungs-Flüssigkeits-Chromatographie (HPLC) sehr erfolgreich angewendet worden, wobei zunächst Säulen mit Ionenaustauschern[1], dann mit Silicagel[2–4] und schliesslich mit reversed-phase (RP)[5–8] zum Einsatz kamen. Dabei sind auch die Zusammenhänge zwischen Polarität des Cardenolides und seiner Retentionszeit, bzw. der Einfluss der Fliessmittelzusammensetzung auf das Trennergebnis eingehend studiert worden. Für die Detektion zieht man gewöhnlich die UV-Absorption heran; eine beträchtliche Steigerung der Empfindlichkeit wird durch Derivatisierung vor[9] oder nach[10,11] der Säule erreicht.

Allerdings beschränken sich praktisch alle bisherigen Veröffentlichungen auf die Untersuchung der arzneilich bedeutenden Glykoside (vor allem Digitoxin, Digoxin, Acetyldigoxin und Lanatosid C), während die Analyse der übrigen Cardenolide, deren Anteil in Digitalisblättern immerhin 30–70 % des Gesamtglykosidgehaltes betragen kann[12], mittels HPLC bisher nicht beschrieben wurde. Für pflanzenphysiologische und biochemische Untersuchungen ist aber gerade die Analyse der Zusammensetzung des Glykosidgemisches in den Blättern (und nicht nur deren Gehalt an einzelnen, therapeutisch wichtigen Verbindungen) von besonderem Interesse. Bisher sind solche Bestimmungen durch Direktauswertung von Dünnschichtchromatogrammen durchgeführt worden[13]. Für die Untersuchung der Glykosidbiogenese im Verlaufe der Vegetationsperiode haben wir nun ein Verfahren entwickelt, das es erlaubt, mittels HPLC alle mengenmässig bedeutenden Glykoside in den Blättern von *Digitalis lanata* sowie einigen anderen Digitalis-Arten und in Digitalishybriden qualitativ und quantitativ zu erfassen. In der vorliegenden ersten Mitteilung berichten wir zunächst über die Probenaufarbeitung und die qualitative Analyse.

EXPERIMENTELLES

Gerät und Analysendaten

Flüssigkeits-Chromatograph: 1084 B Hewlett-Packard. Säulen: LiChrosorb RP-8 (10 μm) und RP-18 (10 μm), Länge 20 cm, Innendurchmesser 4.6 mm, Stahl. Mobile Phase: Wasser–Acetonitril (75:25; 63:37) isokratisch und Gradientenelution.

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Temperatur: 40°C. Durchfluss: 1.5 ml/min. Variabler Wellenlängendetektor: Hewlett-Packard. Schreiber-Integrator: LC-Terminal 79850 B Hewlett-Packard.

Reagentien

Lösungsmittel: LiChrosolve (Merck, Darmstadt, B.R.D.). Chemikalien z. Analyse (Merck). Die für Vergleichszwecke verwendeten herzwirksamen Glykoside haben wir von den Firmen Boehringer, Mannheim und Sandoz (Basel, Schweiz) erhalten.

Probenvorbereitung

Feingepulverte Digitalis-Blattdroge (1.500 g) werden in einem tarierten Rundkolben mit 15.0 g heissem Ethanol (70%, v/v) übergossen und 15 min unter Rückflusskühlung auf dem Wasserbad erhitzt. Nach Abkühlen auf Raumtemperatur fügt man 25.0 g Wasser und 10.0 g einer Lösung von 15 g $Pb(CH_3COO)_2 \cdot 2H_2O$ in 100 ml Wasser zu, ergänzt mit Wasser auf 60.00 g und mischt gut durch. Hierauf wird der entstandene Niederschlag abzentrifugiert; von der klaren überstehenden Lösung versetzt man 50.0 g mit 12 g einer Lösung von 10 g $Na_2HPO_4 \cdot 2H_2O$ in 100 ml Wasser, mischt gut durch und zentrifugiert. Von der übersteheden klaren Lösung werden 57.0 g (entspr. 1.15 g Digitalis-Blattdroge) mit 1 × 30 und 3 × 20 ml Chloroform–Isopropanol (3:2) ausgeschüttelt. Die vereinigten organischen Phasen bringt man unter vermindertem Druck bei maximal 40°C zur Trockne (Rotationsverdampfer) und löst den Rückstand in 4.00 ml Methanol p.a. Die über Millipore filtrierte Lösung wird für die HPLC-Analyse verwendet.

ERGEBNISSE UND DISKUSSION

Vorbereitung der Proben für die HPLC-Analyse

Obwohl man grundsätzlich mit Methanol oder Methanol–Wassergemischen hergestellte Extrakte aus Digitalisblättern direkt für die HPLC verwenden kann, so scheidet dieses Verfahren in der Praxis doch zumeist aus und zwar aus zwei Gründen: erstens enthält ein ungereinigter Blattextrakt beträchtliche Mengen an Begleitstoffen ("Ballaststoffe"), von denen ein Teil als mit der Front wandernder peak erscheint, in dem aber einige Cardenolidglykoside "verschwinden"; zweitens belegt ein anderer Teil dieser Begleitstoffe die Oberfläche des Säulenfüllmaterials, sodass die Säulen sehr schnell unbrauchbar werden. Auf solche Schwierigkeiten haben übrigens auch Jurenitsch *et al.*[14] bei Convallaria-Analysen und Tittel und Wagner[15] bei anderen Herzglykosiddrogen hingewiesen. Versuche zur Abtrennung der "Ballaststoffe" mittels Vorsäulen, Sep–Pak-Kartuschen oder Extrelut®-Säulen brachten keine brauchbaren Resultate. Beim Eluieren der Sep–Pak-Kartuschen mit Methanol werden UV-absorbierende Substanzen herausgelöst; selbst wenn man mit Methanol gewaschene Kartuschen benützt gelingt es nicht, Chlorophyll quantitativ von den herzwirksamen Glykosiden abzutrennen.

Die besten Ergebnisse erhält man, wenn man die seit langem bewährte Reinigung von Blattauszügen mit Bleiacetat vornimmt. Dabei werden Chlorophyll und verschiedene phenolische Begleitstoffe (Flavonoide, Gerbstoffe) ausgefällt; aus dem Filtrat lassen sich die Cardenolidglykoside durch Ausschütteln mit organischen Lösungsmitteln isolieren (Einzelheiten siehe Experimentelles).

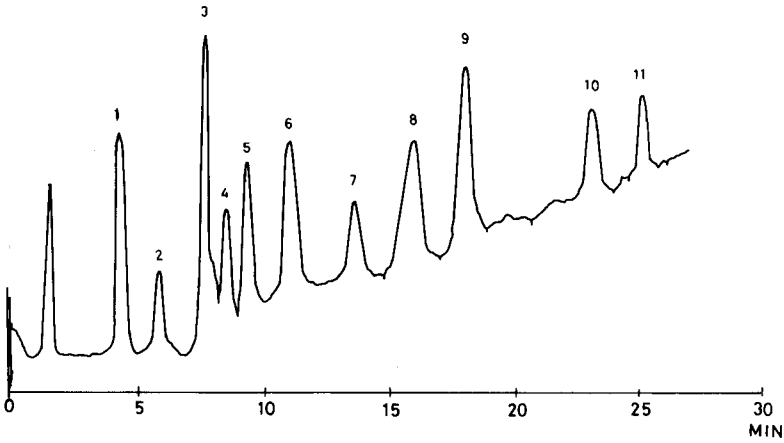

Fig. 1. Trennung eines Gemisches von reinen Digitalisglykosiden mittels HPLC. Bedingungen: LiChrosorb RP-18 Säule (10 μm); 4 min isokratisch Acetonitril–Wasser (25:75), dann Gradientenelution in 30 min bis Acetonitril–Wasser (37:63); Fluss 1.5 ml/min; Detektor 230 nm. 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Glucogitorosid; 4 = Odorobiosid G; 5 = Lanatosid C; 6 = Glucoevatromonosid; 7 = Glucodigitoxigeninbisdigitoxosid; 8 = Lanatosid B; 9 = Acetylgitoxin; 10 = Lanatosid A; 11 = Digitoxin.

Qualitative HPLC-Analyse

Bei der Untersuchung des in den Blättern von Digitalis-Arten vorkommenden Glykosidgemisches ist mit einem weiten Polaritätsbereich zu rechnen, der von stark hydrophilen Glykosiden wie z.B. Glucogitofucosid oder Digitalinum verum bis zu extrem lipophilen wie z.B. Digitoxin reicht. Wir haben deshalb das Verhalten ein-

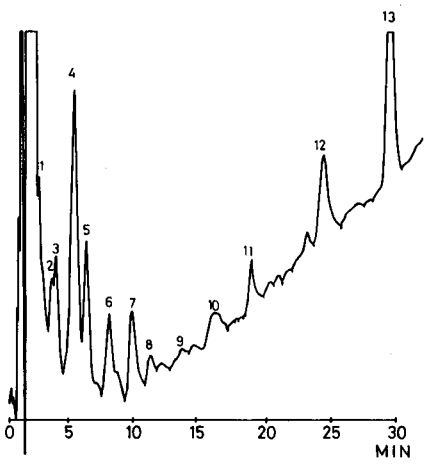

Fig. 2. HPLC-Trennung der Glykoside in einem Blattextrakt von *Digitalis lanata* EHRH. Bedingungen: LiChrosorb RP-Säule (10 μm); Gradientenelution 30 min Acetonitril–Wasser von (27:73) bis (37:63). Fluss 1.5 ml/min; Detektor 230 nm. 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Glucogitorosid; 4 = Glucolanadoxin; 5 = Glucodigifucosid; 6 = Odorobiosid G; 7 = Lanatosid C; 8 = Glucoevatromonosid; 9 = Glucodigitoxigeninbisdigitoxosid; 10 = Lanatosid B; 11 = Lanatosid A; 12 = Digitoxin; 13 = nicht identifiziert.

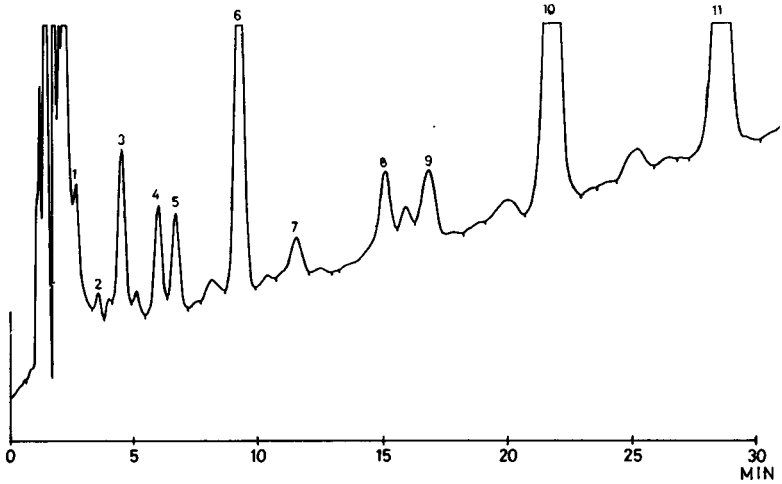

Fig. 3. HPLC-Trennung der Glykoside in einem Blattextrakt von *Digitalis heywoodii* P. et Silva. Bedingungen wie in Fig. 2. 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Glucogitorosid; 4 = Glucolanadoxin; 5 = Glucodigifucosid; 6 = Odorobiosid G; 7 = Glucoevatromonosid; 8 = Glucodigitoxigeninbisdigitoxosid; 9 = Lanatosid B; 10 = Lanatosid A; 11 = Digitoxin.

zelner Glykoside unterschiedlicher Polarität bei verschiedenen Bedingungen geprüft. Hierbei erwiesen sich RP-8- und RP-18-Säulen als besonders geeignet; für die mobile Phase kamen vor allem die Gemische Acetonitril–Wasser und Tetrahydrofuran–Dioxan–Wasser zur Anwendung, mit denen die besten Resultate erzielt wurden. Allerdings gelingt unter isokratischen Bedingungen keine völlige Trennung, da jeweils kritisch Substanzpaare auftreten [Beispiele: Lanatosid C und Digoxigenin oder La-

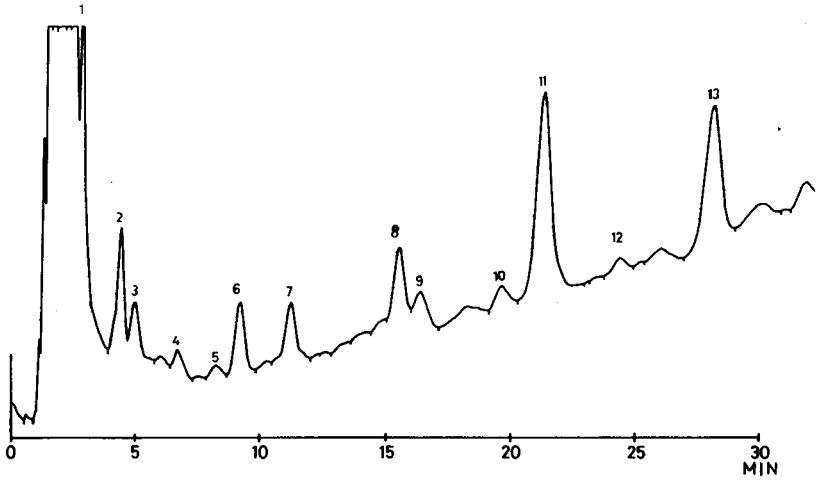

Fig. 4. HPLC-Trennung der Glykoside in einem Blattextrakt der Hybride *Digitalis heywoodii x Digitalis lanata*. Bedingungen wie in Fig. 2. 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Glucogitorosid; 4 = Glucolanadoxin; 5 = Glucodigifucosid; 6 = Odorobiosid G; 7 = Glucoevatromonosid; 8 = Glucodigitoxigeninbisdigitoxosid; 9 = Lanatosid B; 10 = nicht identifiziert; 11 = Lanatosid A; 12 = Acetylgitoxin; 13 = Digitoxin.

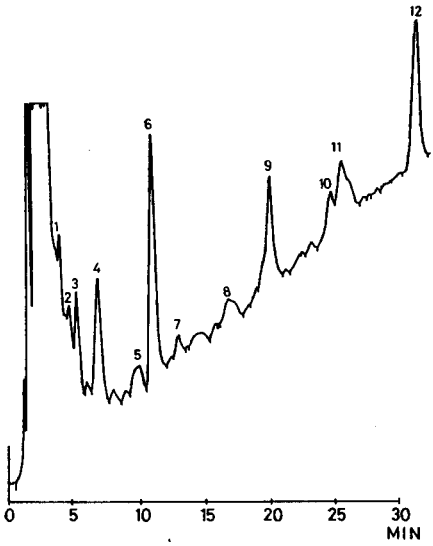

Fig. 5. HPLC-Trennung der Glykoside in einem Blattextrakt von *Digitalis lutea* L. Bedingungen wie in Fig. 2. 1 = Digitalinum verum; 2 = Glucoverodoxin; 3 = Glucogitorosid; 4 = Glucolanadoxin; 5 = Lanatosid C; 6 = Glucoevatromonosid; 7 = Glucodigitoxigeninbisdigitoxosid; 8 = Lanatosid B; 9 = Acetylgitoxin; 10 = Lanatosid A; 11 = Digitoxin; 12 = nicht identifiziert.

natosid B und Lanatosid E an RP-18-Säulen bei Verwendung von Acetonitril–Wasser (37:63)]. Durch Gradientenelution kann man diese Schwierigkeiten ausschalten und zu einer vollständigen Auftrennung des in Blattextrakten vorliegenden Glykosidgemisches gelangen. Von den als Referenz verwendeten Digitalisglykosiden haben wir 2–5 µl einer 0.08%igen methanolischen Lösung eingespritzt, von den gereinigten Blattextrakten wurden 2–10 µl einer methanolischen Lösung verwendet, entsprechend *ca.* 0.5–2.5 mg trockener Blattdroge. Als Messwellenlänge haben wir 230 nm gewählt, obwohl dies nicht dem Absorptionsmaximum der Cardenolide (218–220 nm) entspricht. Während man bei reinen Glykosiden noch relativ gut bei 220 nm arbeiten kann[5–7], empfiehlt es sich bei gereinigten Drogenextrakten und bei Gradientenelution die Detektion bei 230 nm vorzunehmen, da das Signal–Rausch-Verhältnis hier günstiger liegt. Lindner und Frei[3] haben schon früher für die Messung den Bereich von 225 ± 10 nm vorgeschlagen und auch Erni und Frei[6] haben etliche Chromatogramme bei 230 nm aufgenommen. Die Fig. 1–5 zeigen die Trennungen der wichtigsten in *Digitalis lanata* EHRH., *Digitalis heywoodii* P. et Silva und *Digitalis lutea* L. vorkommenden Glykoside. Die Identität jeder Substanz bzw. jedes peaks wurde durch Cochromatographie mit authentischem Material gesichert.

Die ausgezeichneten Resultate, die man durch Derivatisierung der Glykoside vor der Säule erhält —Umwandlung in die 4-Nitrobenzoylester nach Nachtmann *et al.*[9]— lassen sich nach unseren Erfahrungen nicht auf Gesamtextrakte übertragen. Offenar enthalten selbst gereinigte Extrakte noch beträchtliche Mengen an acylierbaren Begleitstoffen, die bei der HPLC-Analyse als nicht identifizierbare peaks erscheinen[16]. Wir ziehen deshalb bei der Untersuchung von Blattextrakten die Analyse der underivatisierten Cardenolide vor, während wir bei der Prüfung von Reinglyko-

sidpräparaten die HPLC-Trennung der 4-Nitrobenzoylderivate als besonders vorteilhaft ansehen.

In der zweiten Mitteilung berichten wir über die quantitative Bestimmung der herzwirksamen Glykoside in Digitalisblättern mittels HPLC.

DANKSAGUNG

Wir danken der Deutschen Forschungsgemeinschaft (Bonn-Bad Godesberg) für die Bereitstellung des HPLC-Gerätes und für finanzielle Unterstützung. Herrn Dr. F. Kaiser, Boehringer-Mannheim danken wir für die Überlassung von Glucoevatromonosid, Glucogitorosid, Glucolanadoxin, Glucoverodoxin und Glucogitofucosid, Herrn Dr. A. Angliker, Sandoz AG, Basel für die Lanatoside A, B, C und für Digitalinum verum.

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Note

Isolation and characterization of the trimethyl ester of 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid, a degradation product of naphthomycin A, semisynthetically obtained from *Penicillium gladioli* cultures

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High-performance liquid chromatography (HPLC) is extensively used in isolating and analyzing natural substances of biological and pharmaceutical interest. In the present work, HPLC allowed the comparison of 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester (Fig. 1, compound I), already known[1], with a product, not yet unequivocally identified, obtained by transformation of naphthomycin A (Fig. 1). Naphthomycin A is an antibacterial antibiotic, an antimetabolite of vitamin K, isolated from cultures of *Streptomyces collinus*[2]: a structure has been proposed, on the basis of spectroscopic results[3,4]. On degradation of naphthomycin A, a group of homologous fragments is obtained[5], all derived from the naphthoquinonic nucleus, according to spectroscopic characterization. Since compound I is presumably identical to one of these fragments, its semisynthesis was done according to Grove[1], starting from the fermentation of *Penicillium gladioli*, and leading to the production of gladiolic acid (Fig. 1, compound II), an antibacterial and fungistatic substance, as an intermediate.

EXPERIMENTAL

Penicillium gladioli (IMI 38567), purchased from the Commonwealth Mycological Institute, was grown in a liquid medium, according to Brian *et al.*[6], but gave only a small amount of a complex mixture of metabolites, as shown by HPLC analysis (Fig. 2), which is described in detail in *Procedures*. Owing to the low yield of the reaction, we did not attempt to isolate the gladiolic acid (compound II).

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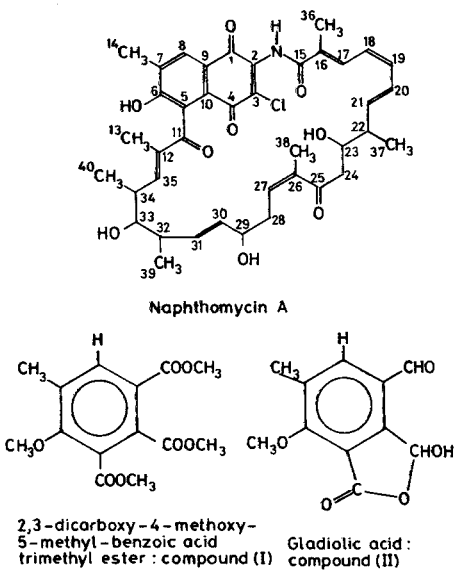


Fig. 1. Structural formulae of naphthomycin A, 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester and gladiolic acid.

The extract of *Penicillium gladioli* growth medium was then directly transformed by oxidation and methylation and the new mixture was analyzed by HPLC after first checking by high-performance thin-layer chromatography (HPTLC). The peak corresponding to compound I was isolated by preparative column chromatography at medium pressure.

All the solvents utilized (unless stated otherwise) were Carlo Erba RS for HPLC; chloroform was stabilized with amylene.

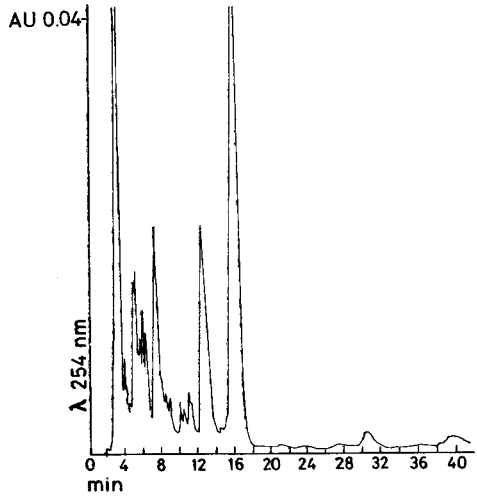


Fig. 2. HPLC of the extract of *Penicillium gladioli* growth medium before oxidation and methylation. Chromatographic conditions: LiChrosorb RP-8 (10 μm) column; eluent, acetonitrile containing 20% of 0.01 *M* H_3PO_4 at pH 3; flow-rate, 1 ml/min; pressure, 3500 p.s.i.; UV detection, λ = 254 nm.

Procedures

HPLC analyses of the extract of *Penicillium gladioli* growth medium before (a) and after (b) oxidation and methylation were performed. All separations were accomplished with an apparatus consisting of a Waters M 6000 solvent pump, a Rheodyne Model 70-10 injector valve and a Perkin-Elmer LC-75 UV–VIS detector. The columns (25 cm × 4.5 mm I.D.; Policonsult Scientifica, Rome, Italy) were packed with LiChrosorb RP-8 or Si 60 (10 μm) (E. Merck, Darmstadt, G.F.R.). The silica gel column was preceded by a pre-column (5 cm × 4.5 mm I.D.) packed with the same material.

Conditions: a, reversed-phase separation on RP-8 column, eluent acetonitrile (Merck LiChrosolv) containing 20% of 0.01 *M* H_3PO_4 at pH 3, UV detection at λ = 254 nm; b, normal-phase separation, after oxidation and methylation, on Si 60 column, eluent chloroform–2% ethyl acetate, UV detection at λ = 254 nm.

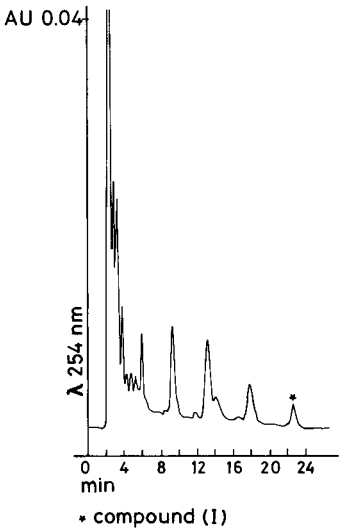
HPTLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation was carried out on Merck pre-coated plates Si 60 F_{254}, with chloroform–2% ethyl acetate as eluent. Chromatographic conditions were optimized by utilizing the degradation product of naphthomycin A as a reference.

The compound 2,3-dicarboxy-4-methoxy-5-methylbenzoic acid trimethyl ester was isolated by medium-pressure preparative column chromatography. The apparatus consisted of a Perkin-Elmer Series 2/1 solvent pump, a Waters UK 6000 injector and a LKB Ultrorack fraction collector. For the separation, we utilized a LiChrosorb Si 60 (40–63 μm) Lobar B column (E. Merck; 310 × 25 mm I.D.) eluted with chloroform at 0.8 ml/min. The amount of mixture injected was 50 mg in 0.5 ml eluent. As a suitable detector for preparative chromatography was not available, the fractions collected were compared by TLC with the degradation product of naphthomycin A, utilizing sheets of Si 60 (Merck F_{254}) with chloroform–4% ethyl acetate as eluent. The R_F calculated for compound I under these conditions was 0.31.

RESULTS AND DISCUSSION

As Figs. 3 and 4 show, HPLC and HPTLC allowed the separation of compound I from a complex mixture of products obtained by transformation of the crude extract of cultures of *Penicillium gladioli*. In the HPTLC chromatogram the spot corresponding to compound I was preceded by one intense spot, running with the solvent front, and by several other spots. The R_F of compound I in the mixture is slightly lowered with respect to the R_F value of the degradation product of naphthomycin A utilized as a reference (R_F = 0.18 instead of 0.21), owing to a spot of lower polarity running very close to it. HPLC under the same conditions gave a better separation and the retention times for the degradation product of naphthomycin A and compound I in the mixture were the same (t_R = 22.4 min).

Preparative medium-pressure chromatography allowed the isolation of the reference product, which was characterized as compound I by elemental analysis, nuclear magnetic resonance (NMR), infrared (IR) and mass spectrometry (MS), $C_{14}H_{16}O_7$: calc. C 56.75, H 5.45; found C 57.07, H 5.47%. MS: M^+ 296, 265, 249, 233, 205, 191, 161, 146. IR (Nujol): 1760, 1450 cm^{-1}. NMR (C^2HCl_3), relative to tetramethylsilane (0 ppm): 2.34 (s, 3H), 3.84 (s, 3H), 3.87 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 7.83 (s, 1H). These data correspond perfectly to those obtained for the degrada-



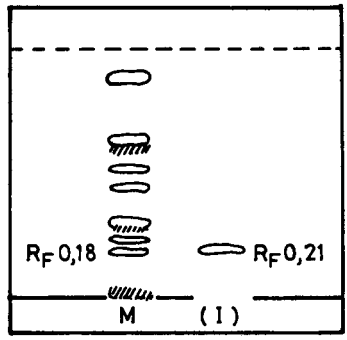


Fig. 3. HPLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation. Chromatographic conditions: LiChrosorb Si 60 (10 μm) column; pre-column packed with the same material: eluent, chloroform–2% ethyl acetate; flow-rate, 2 ml/min; pressure, 1000 p.s.i. The peak marked with an asterisk has the retention time of the degradation product of naphthomycin A and has been identified as compound I; t_R = 22.4 min.

Fig. 4. HPTLC of the extract of *Penicillium gladioli* growth medium after oxidation and methylation on Merck Si 60 F_{254} pre-coated plates. Eluent: chloroform–2% ethyl acetate. M = Extract of *Penicillium gladioli* growth medium after oxidation and methylation; (I) = degradation product of naphthomycin A.

tion product of naphthomycin A[5] whose structure (as that of one of its related compounds, previously isolated[5]) is thus unambiguously demonstrated. From these results it is therefore possible to determine the substituent pattern in the chromophore of naphthomycin A.

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Note

High-performance liquid chromatographic separation of the two estrogen isomers of estradiol with electrochemical detection

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Catechol estrogens, the 2- or 4-hydroxylated metabolites of phenolic estrogens, have been the focus of increasing attention in recent years. They are major metabolites of estrogens present in the circulation and are formed within certain estrogen target organs[1]. Investigation of the labile catechol estrogens is complicated by rapid oxidative degradation that occurs during isolation and purification. This degradation can be decreased, but not eliminated when separations are carried out under reducing conditions, for example, by using ascorbic acid-saturated plates for thin-layer chromatography[2]. In the course of developing an assay for estrogen-2/4-hydroxylase[3], we explored the use of high-performance liquid chromatography (HPLC), coupled with electrochemical detection, for separating the labelled products, 2- and 4-hydroxyestradiol, formed during an *in vitro* incubation of brain tissue with a radioactive estradiol substrate.

The polarographic (electrochemical) detection method, coupled with HPLC is considered to be one of the most sensitive methods for detection of catechol compounds[4–6]. Greater sensitivity and reproducibility and lower interference from impurities make this an attractive system for separation of the catechol metabolites of estrogen. We report here on the HPLC separation of the two isomers of catechol estrogens, 2-OHE_2 from 4-OHE_2, with electrochemical detection. This technique was shown to be applicable to isolation of small amounts of radioactive product formed by estrogen-2/4-hydroxylase from brain *in vitro*[3], and for verifying the purity of radioactive enzymatically synthesized 2-OHE_2 (ref. 7).

MATERIALS AND METHODS

A Hewlett-Packard Model 1081 B liquid chromatograph (Palo Alto, CA, U.S.A.) was used, equipped with an automatic injector, and with a 10-μm HP C_{18} reversed-phase column (250 × 4.6 mm I.D.). A digital multimeter (Hewlett-Packard 3465 A) was used for zeroing the detector and for on-line monitoring of the output. The control of a Rheodyne 5001 (Cotati, CA, U.S.A.) three-way pneumatic valve and a LKB Ultrarack (Rockville, MD, U.S.A.) fraction collector was integrated into the events board of the instrument. These additions, along with the auto-injector, allowed fully automated separation and collection of samples. For detection, a glass-

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TABLE I

RELATIVE RETENTION TIMES OF ESTRADIOL AND METABOLITES

Mobile phase: varied as indicated and made 0.2 *N* with respect to acetic acid.

Solvent system		*Compounds*			
Water (%)	*Methanol (%)*	*4-OHE$_2$*	*2-OHE$_2$*	*E$_2$*	*2-Methoxy-E$_2$*
65	35	0.40	0.50	1.00 (24.25 min)	–
62	38	0.39	0.50	1.00 (21.36)	–
60	40	0.41	0.51	1.00 (19.31)	1.32
55	45	0.44	0.53	1.00 (12.11)	–
35	65	–	0.69	1.00 (8.65)	1.15

carbon electrochemical device (Bio-Analytical Systems, West Lafayette, IN, U.S.A.), was used at an applied potential of +0.86 V *vs.* the reference electrode.

Glass-distilled methanol (Burdick & Jackson, Muskegon, MI, U.S.A.), acetic acid (Baker, Phillipsburg, NJ, U.S.A.) and deionized water were filtered through a Millipore vacuum filtration device (Millipore, Bedford, MA, U.S.A.). The solvent systems consisted of methanol and water in various proportions, made 0.2 *N* with respect to glacial acetic acid (pH 2.9). Solvents were degassed immediately before use. Separations were carried out at ambient temperature at an attenuation level of 10. The flow-rate of the mobile phase was 3 ml/min.

The steroids and their sources were: estradiol [1,3,5(10)-estratriene-3,17β-diol], Steraloids; 2-methoxy-estradiol [1,3,5(10)-estratriene-3,17β-diol-2-methylether], Research Plus; 2-OHE$_2$ [1,3,5(10)-estratriene-2,3,17β-triol] and 4-OHE$_2$ [1,3,5(10)-estratriene-3,4,17β-triol] were supplied by Dr. K. I. H. Williams (Worcester Foundation, Shrewsbury, MA, U.S.A.). All steroids were injected in 10 μl methanol containing ascorbic acid (1 m*M*) as an anti-oxidant.

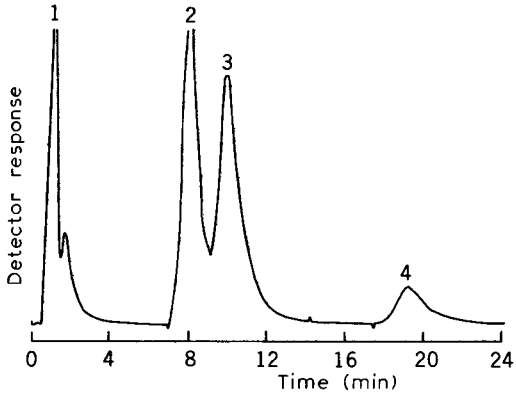

Fig. 1. Separation of isomeric catechol estrogens. Peaks: 1 = ascorbic acid; 2 = 4-OHE$_2$; 3 = 2-OHE$_2$; 4 = E$_2$. Conditions: reversed-phase C$_{18}$ column (250 × 4.6 mm I.D.); mobile phase water–methanol (60:40) 0.2 *N* with respect to acetic acid; electrochemical detection at +0.86 V; flow-rate 3 ml/min; quantity of steroids 1 μg each.

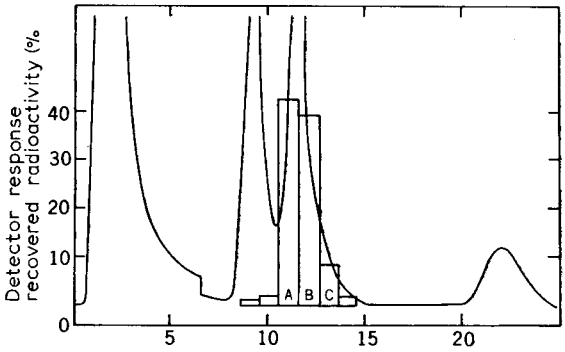

Fig. 2. Correspondence of radioactivity from bio-synthesized 2-OHE_2 with authentic 2-OHE_2. Conditions as in Fig. 1. Bars, radioactivity in percent of total recovered per minute. Bars A, B and C account for approximately 91 % of recovered radioactivity. Fractions not represented by bars contained less than 1 % of the recovered radioactivity.

RESULTS AND DISCUSSION

Different absolute retention times for estradiol and its metabolites were obtained by varying the proportions of methanol and water in the mobile phase. The relative retention times remained constant over the range of concentrations shown in Table I until the ratio of methanol to water exceeded 1. To separate 2- and 4-OHE_2 from each other and from estradiol, we chose water–methanol (60:40). This solvent system offered the shortest absolute retention times while maintaining adequate separation (>1.5 min) of the catechol isomers of E_2. The ascorbic acid used as an antioxidant was eluted from the column near the solvent peak and did not interfere with the detection of the estrogens (Fig. 1).

The separations afforded by this system allowed us to examine the chemical purity of our enzymatically synthesized radiolabelled 2-OHE_2. It was injected with non-labelled carrier, and fractions were collected directly in scintillation vials. Purity was established by correspondence of the radioactivity eluted with the authentic 2-OHE_2 standard (Fig. 2).

This separatory technique was also applied to a quantitative assay for brain 2/4-hydroxylase activity[3]. The tissue was incubated with 6,7-tritiated estradiol as the substrate. The quantities of radiolabelled catechol estrogens formed during the incubations were in the picogram (fmole) range. Therefore, they were diluted with authentic catechol estrogens to permit visualizing the compounds in the chromatogram and to protect them from spontaneous oxidative degradation. By injecting the radioactive products with microgram amounts of carrier and using a low attenuation level, recordings with minimal noise were obtained. Degradative losses were also minimized and corrected for by using an internal ^{14}C-labelled standard.

Separation of estrogen metabolites by HPLC coupled with electrochemical detection has been recently reported by Shimada *et al.*[8,9]. The present paper is the first report of separation of the hydroxylated metabolites of estradiol from the parent molecule by HPLC with the benefits of electrochemical detection. We have taken advantage of this resolution, and here report on the efficacy of this fully automated separatory system to measure the enzymatic hydroxylation of estradiol.

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Note

High-performance liquid chromatography of inorganic and organic anions using ultraviolet detection and an amino column

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Silica-based high performance liquid chromatography (HPLC) has been used only sparingly for routine analysis of inorganic anions. With the advent of variable-wavelength detectors, those anions which absorb below 254 nm can be analyzed.

Ion-exchange techniques have been used on inorganic systems[1] utilizing a conductivity detector after removing the background conductance of the eluent; however, chromatography obtained with polymer-based ion exchangers is not normally as efficient as that obtained on micro-particulate supports[2]. Richards[3] showed that organic anions could be separated on cation-exchange resins by ion exclusion.

Inorganic anions have been determined on reversed-phase systems after derivatization to organic species[4]. Using ion-pair formation, inorganic anions have been separated on a cyano column[5]. Anions were determined using tetrabutylammonium hydroxide as an ion-pairing reagent on a reversed-phase column[6]. Nitrate and bromide were determined in foodstuffs using an amino column[7]. The purpose of the present work is to expand the use of an amino column to the analysis of various organic and inorganic anions.

EXPERIMENTAL

Chemicals

Inorganic chemicals used were reagent grade available from J. T. Baker (Philipsburg, NJ, U.S.A.) All organic acids were of technical grade or better. Water used to prepare eluents was deionized and filtered through a 0.45 μm membrane filter.

HPLC conditions

The liquid chromatograph used consisted of an Altex Model 100A pump, a Rheodyne Model 7010 injection valve equipped with a 20-μl loop, a LC-55 (Perkin Elmer) variable-wavelength detector and a Sargent Model SRG recorder.

The column used throughout this work was a 250 × 4.6 mm I.D. Zorbax® NH_2 (DuPont Instruments, Wilmington, DE, U.S.A.). The eluent consisted of 0.03 *M* H_3PO_4 (J. T. Baker) adjusted to pH 3.2 with NaOH. Flow-rate was 2.0 ml/min. Detection was by UV at 205 nm for all chromatograms except Fig. 4, which was monitored at 214 nm. Detector sensitivity was 0.1 a.u.f.s. Column pressure was 1500 p.s.i. and operating temperature was 25°C.

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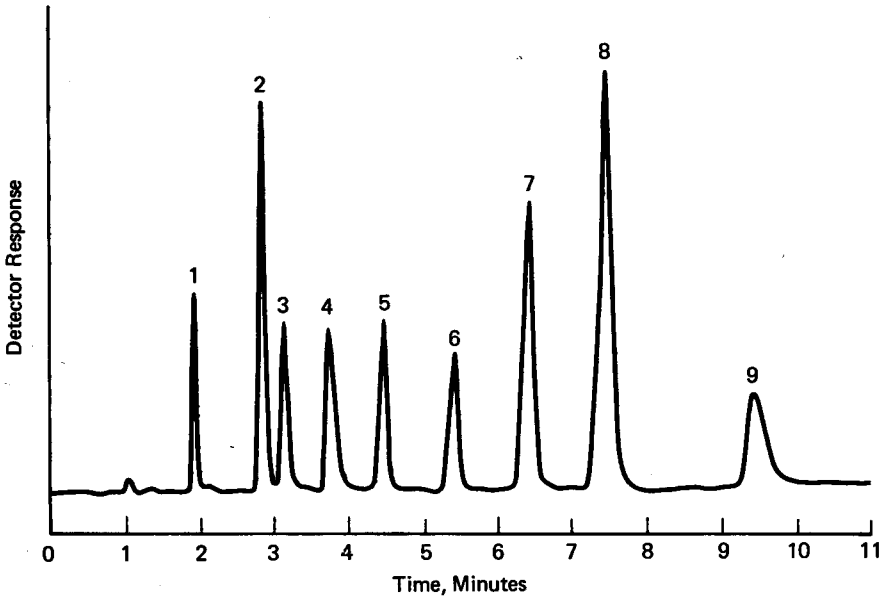

Fig. 1. Separation of acetate (1), acrylate (2), glycolate (3), formate (4), nitrite (5), bromide (6), nitrate (7), iodate (8) and dichloroacetate (9). Columns, Zorbax NH_2 (250 × 4.6 mm I.D.); mobile phases, 0.03 *M* H_3PO_4 adjusted to pH 3.2 with NaOH; flow-rate, 2.0 ml/min; temperatures, 25°C; detection, UV at 205 nm and 0.1 a.u.f.s.; pressure, 1500 p.s.i. Injected sample was prepared in water to give concentrations of 25–100 μg/ml.

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF INORGANIC AND ORGANIC ANIONS

Columns, Zorbax NH_2, eluent, 0.03 *M* H_3PO_4, pH 3.2, at 2.0 ml/min. The calculations were performed using chromatograms obtained at a 5-mV full scale setting.

Anion	*Retention time (min)*	*Calculated detection limit* (μg/ml)*
Acetate	1.9	15
Acrylate	2.8	2
Glycolate	3.1	18
Formate	3.7	18
Nitrite	4.5	0.4
Bromide	5.5	0.5
Nitrate	6.4	0.3
Iodate	7.4	1.0
Dichloroacetate	9.4	16
Iodide	5.5	0.5
Propionate	1.9	16
Bromate	6.2	3
Trichloroacetate	11.6	20
Thiocyanate	3.6	–

* Calculated as three times baseline peak-to-peak random noise level.

RESULTS AND DISCUSSION

An example of the separations obtained is shown in Fig. 1. The estimated detection limits, together with the observed retention times, are listed in Table I.

The mechanism of separation is weak base ion exchange. Thus, changes in pH of the eluent dramatically affect retention times, because of varying degrees of pro-

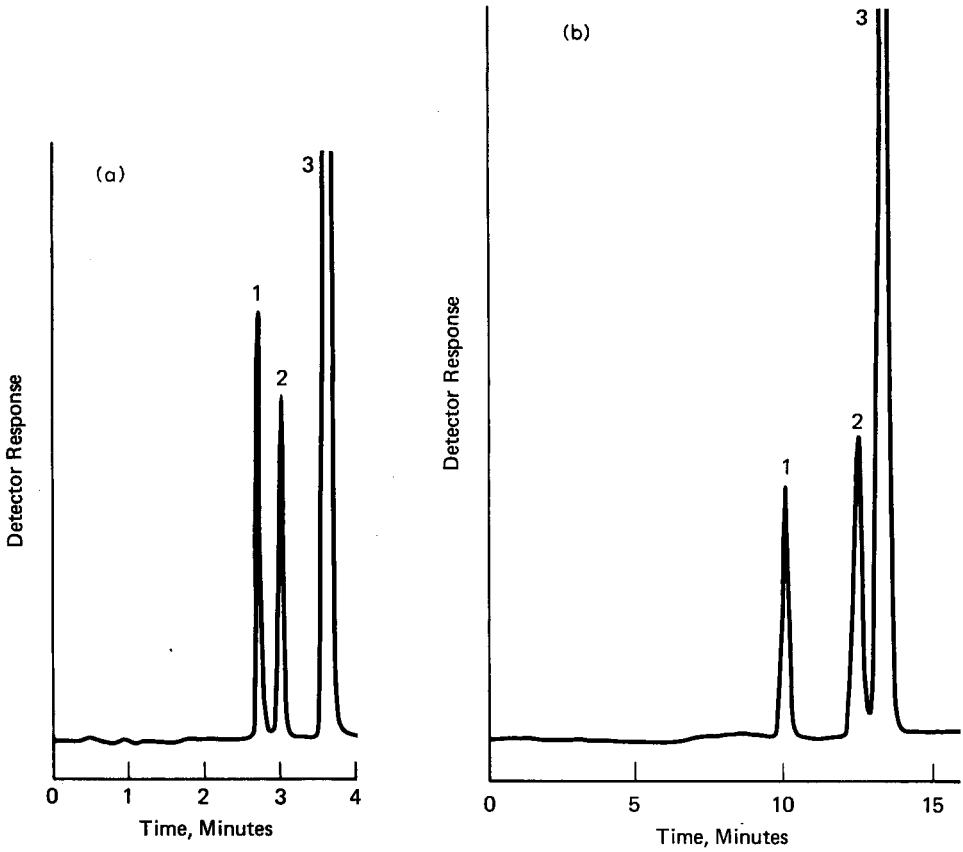

Fig. 2. (a), Separation of nitrite (1), bromide (2) and nitrate (3), showing the effect of pH on retention times. Mobile phase, 0.03 *M* H_3PO_4 adjusted to pH 5.0 with NaOH. Injected sample was prepared in water to give concentrations of 10 μg/ml for nitrite and bromide and 35 μg/ml for nitrate. Other conditions as in Fig. 1. (b), As (a), except mobile phase: 0.03 *M* H_3PO_4 adjusted to pH 2.8 with NaOH.

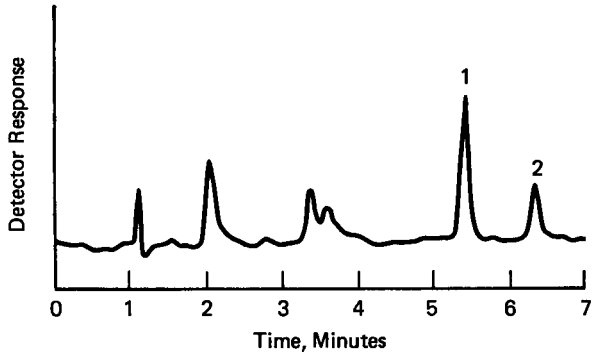

Fig. 3. Separation of bromide (1) and nitrate (2) in 95% sulfuric acid. Injected sample was prepared by diluting 1 ml in 1 ml of water. Other conditions as in Fig. 1.

tonation of the amino functionality (Fig. 2a and b). Column efficiencies obtained were in the range of 4000 theoretical plates, calculated at a capacity factor of 4.5 for bromide ions. The separation technique was applied to the analysis of real samples.

Fig. 3 illustrates the determination of bromide and nitrate in 95% sulfuric acid. The sample was diluted by placing 1.0 ml of acid in 1.0 ml of water prior to injection. Brine solution (20% NaCl) was analyzed for organic and inorganic ions by injecting the solution neat (Fig. 4).

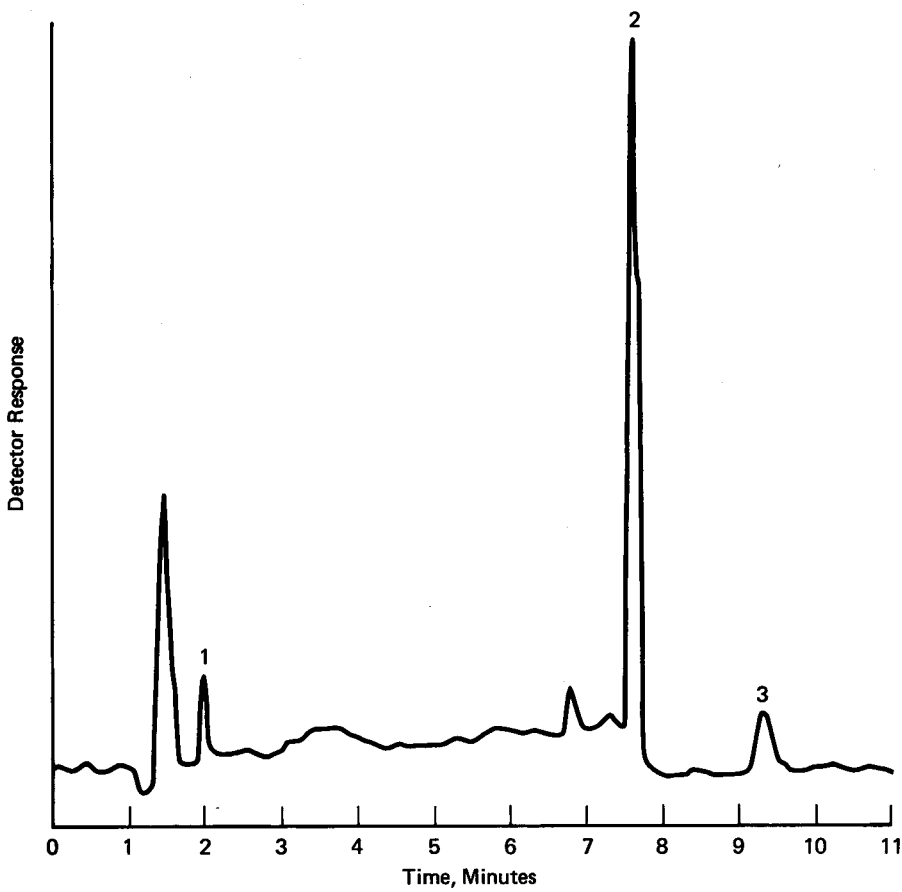

Fig. 4. Separation of anions in 20% NaCl solution. Sample was injected neat. Detection, UV at 214 nm and 0.1 a.u.f.s. Other conditions as in Fig. 1. Peaks: 1 = acetate; 2 = bromate; 3 = dichloroacetate.

CONCLUSIONS

A sensitive method for organic and inorganic ions that respond in the UV region has been expanded. It offers an advantage over previously available procedures in that separations can be obtained on commercially available columns, and samples high in UV-transparent ions, such as chloride and sulfate, can be analyzed with ease. The variety of ions separated is an indication of the broad applicability of this techmque.

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Note

Thin-layer and short-column chromatography of partially reduced Cinchona alkaloids

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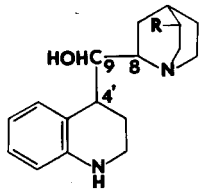
(Received August 13th, 1981)

Partial reduction of Cinchona alkaloids in the 1′,2′,3′,4′ positions of the molecules gives rise to a new chiral centre at C-4′, and consequently from each parent alkaloid two epimeric 1′,2′,3′,4′-tetrahydro derivatives, designated α and β, are

TABLE I

STRUCTURE OF PARTIALLY REDUCED CINCHONA ALKALOIDS



In the 9-deoxy compounds the chiral centre at C (9) is replaced by a methylene group. This structural modification is designated in abbreviation by the prefix 9D. The absolute configuration around the 4′ chiral centre was unambiguously established for α-1′,2′,3′,4′,10,11-hexahydrocinchonine[11]. Other assignments were derived from data for αHHC, comprehensive optical information and chemical interconversions being available. In a pair of epimeric free bases, the prefix α is given to the one having the more positive specific rotation.

Compound	*Abbreviation*	*Absolute configuration*			*R*
		C (8)	*C (9)*	*C (4′)*	
					10 11
Derivatives of cinchonine	C	R	S	–	$CH=CH_2$
and 10,11-dihydrocinchonine	HC	R	S	–	$CH_2–CH_3$
α-1′,2′,3′,4′-Tetrahydrocinchonine	αTHC	R	S	R	$CH=CH_2$
β-1′,2′,3′,4′-Tetrahydrocinchonine	βTHC	R	S	S	$CH=CH_2$
α-1′,2′,3′,4′,10,11-Hexahydrocinchonine	αHHC	R	S	R	$CH_2–CH_3$
β-1′,2′,3′,4′,10,11-Hexahydrocinchonine	βHHC	R	S	S	$CH_2–CH_3$
Derivatives of cinchonidine	Cd	S	R	–	$CH=CH_2$
and 10,11-dihydrocinchonidine	HCd	S	R	–	$CH_2–CH_3$
α-1′,2′,3′,4′-Tetrahydrocinchonidine	αTHCd	S	R	R	$CH=CH_2$
β-1′,2′,3′,4′-Tetrahydrocinchonidine	βTHCd	S	R	S	$CH=CH_2$
α-1′,2′,3′,4′10,11-Hexahydrocinchonidine	αHHCd	S	R	R	$CH_2–CH_3$
β-1′,2′,3′,4′,10,11-Hexahydrocinchonidine	βHHCd	S	R	S	$CH_2–CH_3$

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formed (Table I). These partially reduced alkaloids are of interest for their antiinflammatory activity[1,2] and their synthetic utility for a number of transformations which are not otherwise possible directly[3–7].

Previous methods of separation of the C-4′ epimers employed conversion into crystalline derivatives, fractional crystallization of the latter and, in a few cases, subsequent regeneration of a free base[2,8–10]. These methods are rather tedious and frequently failed to provide both epimers. This was due mainly to the complexity of the mixtures after reduction, the desired compounds having usually accompanied by a pair of 9-deoxy analogues.

In the present paper we report a simple thin-layer chromatographic (TLC) method for complete separation and identification of a series of partially reduced Cinchona alkaloids derived from cinchonine and cinchonidine, together with TLC and short-column preparative procedures for their isolation.

The possibilities of chromatographic separation were studied with mixtures obtained by chemical reduction with sodium and ethanol. This method of reduction gives the most complex mixtures.

EXPERIMENTAL

Compounds and reagents

Parent alkaloids were reduced with sodium and ethanol as described earlier[12]. The resulting crude product was reduced again using 18 g sodium and 250 ml ethanol per 5 g of starting alkaloid. All solvents were purified by standard methods and redistilled.

Solvent systems

Solvent systems suitable for the TLC separation of other Cinchona alkaloids[13], together with several new ones, were tested on the crude mixtures of reduction products. The following systems gave the best separations:

S1 chloroform–methanol–25% ammonia (85:14:1)
S2 dichloromethane–diethyl ether–diethylamine (20:15:5)
S3 acetone–25% ammonia (58:2)
S4 ethyl acetate–isopropanol–25% ammonia (45:35:5)
S5 carbon tetrachloride–*n*-butanol–methanol–10% ammonia (12:9:9:1)
S6 dichloromethane–isopropanol–triethylamine (90:10:15)
S7 chloroform–methanol–triethylamine (85:5:10)
S8 ethyl acetate–isopropanol–triethylamine (5:2:1)
S9 chloroform–methanol–triethylamine (85:14:1)
S10 ethyl acetate–isopropanol–triethylamine (17:2:1)

Analytical TLC

The TLC plates were 20 × 20 cm or 20 × 5 cm silica gel 60 F_{254} pre-coated glass plates, with a layer thickness of 0.25 mm (E. Merck, Darmstadt, G.F.R.). They were not activated before use. The chromatograms were developed at room temperature in a chamber lined with filter-paper and pre-saturated with the solvents for at least 30 min. The alkaloids were spotted 3.0 cm above the bottom of the plates and the plates were developed over a distance of 10 cm. The hR_F values given in Table II were determined using pure compounds isolated by preparative TLC (see below) and calculated from at least four chromatograms.

Preparative TLC

The TLC plates were 20 × 20 cm silica gel 60 F_{254} Merck glass plates with a layer thickness of 2 mm, not activated before use. Samples (0.15 g) of the mixture of alkaloids were dissolved in ethanol and applied as a thin band 3 cm from the bottom of the plate, and developed in a carefully saturated chamber with solvent S7. The solvent was allowed to rise 15 cm from the starting line. The plates were dried at ambient temperature. The bands of resolved compounds were located in UV light, and then scraped off with a spatula and eluted with ethanol. The composition of the resulting eluates was checked by TLC in systems S4, S7 and S8. The separation was not complete. Mixed fractions were rechromatographed in solvent S4 in the case of the reduction products of cinchonine and dihydrocinchonine, and in solvents S6, S7 and S8 for derivatives of cinchonidine and dihydrocinchonidine (Table III). The isolated components of the mixture after reduction of dihydrocinchonine were identified by comparison with reference compounds isolated earlier by chemical procedures[14,15]. Other assignments were derived from mass spectra and optical rotation data.

Short-column chromatography

The columns were prepared as follows. A slurry of silica gel (Macherey, Nagel & Co. HF_{254}, *ca.* 70 g) in solvent S9 (300 ml) was poured into a glass column (3.5 cm I.D.) and allowed to settle. Solvent S9 was then pumped through the system at flow-rate 1.5 ml/min until the absorbent became uniform, the flow was stopped and the column was kept overnight before use. The crude mixture of alkaloids (0.6–0.9 g) was dissolved in solvent S9 (5 ml), applied on the column and eluted with the same solvent. The volumes of the eluted fractions collected and the flow-rates varied for individual groups of reduction products (Table IV).

The completeness of separation was monitored by TLC in solvents S7, S8 and S9. To achieve complete separation, some fractions were rechromatographed —the most mobile fraction from the mixture of cinchonidine derivatives in solvent S10, and the less mobile fraction from the mixture of dihydrocinchonidine derivatives in solvent S7 (Tables III and IV). Chromatographically homogeneous fractions were pooled, evaporated to dryness and coevaporated with isopropanol until the smell of triethylamine was no longer present. The individual components were identified as described under *Preparative TLC*.

RESULTS AND DISCUSSION

Table II summarizes the results with eight solvent systems found to give the best separations of partially reduced derivatives of cinchonine and cinchonidine. Two TLC systems, S6 and S7, are suitable, with all the groups of investigated modified alkaloids, for the analytical separation of four partially hydrogenated products within the particular groups. Two other solvents, S1 and S4, also give good separations with the exception of HCd derivatives.

There are distinct differences in the separations between cinchonine and cinchonidine derivatives. The latter show much higher differences in chromatographic mobility between oxy and deoxy compounds than do the former, *e.g.*, in solvents S1, S6 and S7. Nevertheless, system S7 gives satisfactory results also for cinchonine

TABLE II

hR_F VALUES OF PARENT AND PARTIALLY REDUCED CINCHONA ALKALOIDS

The values were calculated from at least four chromatograms run under the following conditions: silica gel 60 F_{254} pre-coated glass plates, 20 × 20 cm (Merck); temperature 21 ± 3°C; normal chromatography chamber, saturated for 30 min before use; distance travelled 10 cm.

Alkaloid	*Solvent*							
	S1	*S2*	*S3*	*S4*	*S5*	*S6*	*S7*	*S8*
Cinchonine (C)	40	37	41	52	76	53	37	41
αTHC	30	47	44	47	56	51	37	41
βTHC	23	47	36	33	35	46	31	31
α9DTHC	50	58	53	60	73	71	60	50
β9DTHC	34	47	37	39	46	55	49	32
10,11-Dihydrocinchonine (HC)	27	28	34	42	58	38	23	31
αHHC	19	43	34	36	41	40	23	33
βHHC	14	43	24	22	22	32	20	23
α9DHHC	38	57	44	49	57	66	57	45
β9DHHC	24	43	29	30	35	46	36	25
Cinchonidine (Cd)	36	27	38	48	73	42	30	34
αTHCd	29	50	44	45	51	56	41	43
βTHCd	23	42	41	41	50	46	29	38
α9DTHCd	41	54	45	51	62	64	58	44
β9THCd	45	60	52	55	66	70	62	51
10,11-Dihydrocinchonidine (HCd)	25	25	33	44	65	35	22	30
αHHCd	18	46	35	34	35	43	25	32
βHHCd	17	38	33	31	42	37	21	32
α9DHHCd	30	55	33	44	48	62	51	38
β9DHHCd	36	61	44	46	57	69	60	46

derivatives. In contrast, the separation of the epimers within each pair is much easier to achieve in the series of cinchonine derivatives (solvents S4, S5, S6 and S7 for pairs of deoxy compounds, S4 and S5 for pairs of oxy derivatives) than in the cinchonidine series.

The expected order of mobility is that deoxy alkaloids precede compounds with 9-hydroxy substituents. This is found in the cinchonidine series, but only partly for cinchonine derivatives. In some chromatographic systems (S3, S4, S5, S8), αTHC and αHHC are more mobile than their β-deoxy congeners. Because of this effect the differences in hR_F values between epimers become favourable, *e.g.*, Δ21 for α- and β9DTHC in solvent S4 and for α- and βTHC in solvent S5.

Of the epimeric reduced alkaloids, the α compounds, *i.e.*, those whose partial rotatory contribution at C-4′ is positive, usually have higher R_F values than their counterparts in all solvent systems tested. In two deoxy pairs derived from cinchonidine this order is reversed, β9DTHCd and β9DHHCd being more mobile than the respective α compounds. Solvent S5 is exceptional, in that βHHCd is more mobile than αHHCd.

All the newly developed solvent systems S6, S7 and S8 contain triethylamine,

TABLE III

ROUTES OF CHROMATOGRAPHIC SEPARATIONS OF MIXTURES OF CINCHONA ALKALOIDS PARTIALLY REDUCED BY SODIUM AND ETHANOL

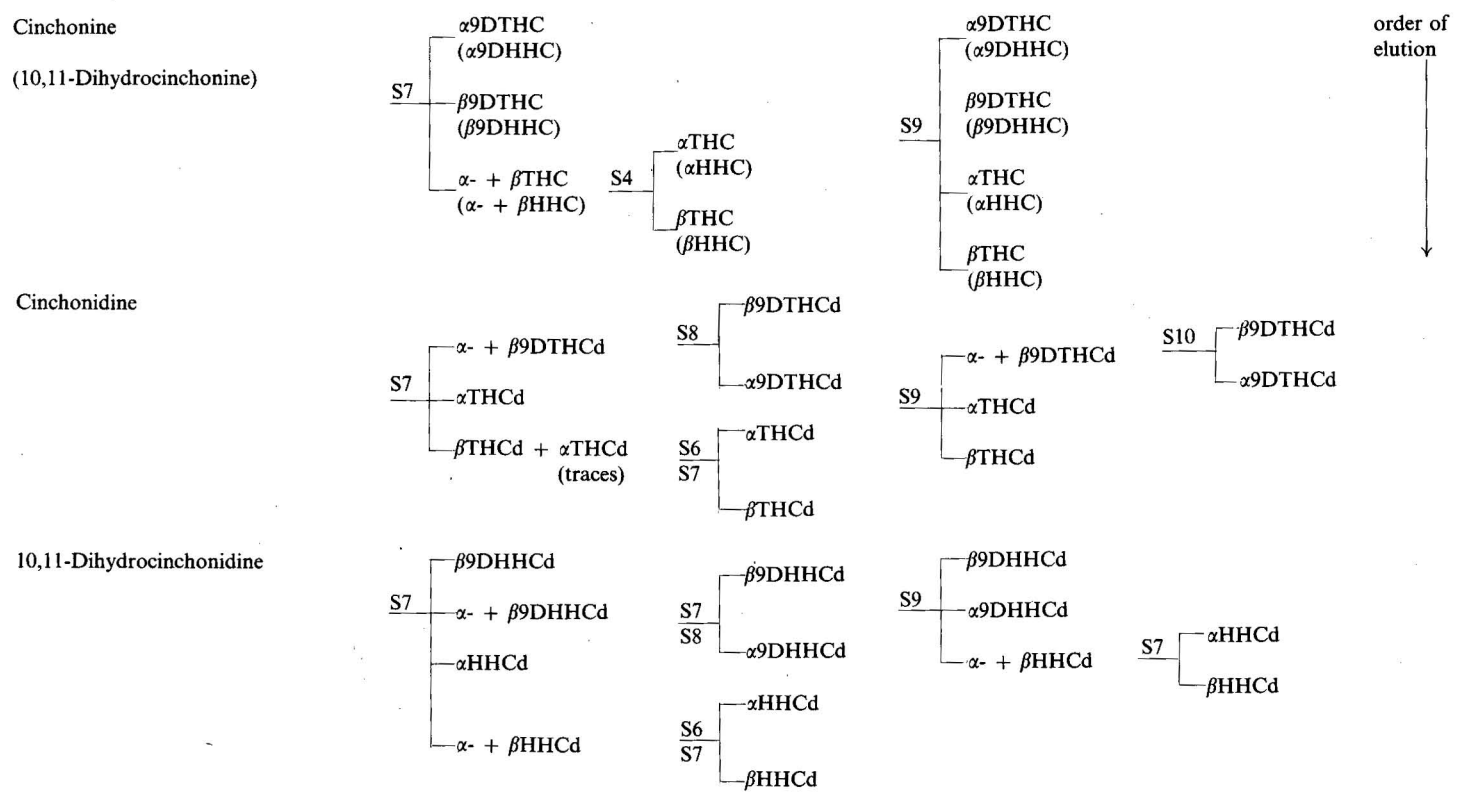

TABLE IV

SHORT-COLUMN CHROMATOGRAPHY OF MIXTURES OF CINCHONA ALKALOIDS PARTIALLY REDUCED BY SODIUM AND ETHANOL

Solvent: S9

Parent alkaloid	*Height of column (cm/0.1 g)*	*Flow-rate*	*Volume of fractions collected (ml)*	*Components isolated* and % of starting mixture*				*Total recovery (%)*
Cinchonine (C)	3.0	1.1	5	α9DTHC	β9DTHC	αTHC	βTHC	70
				19	10	11	30	70
Dihydrocinchonine (HC)	2.2	1.2	15	α9DHHC	β9DHHC	αHHC	βHHC	
				9	16	16	26	67
Cinchonidine (Cd)	2.2	1.4	10	β9DTHCd**	α9DTHCd**	αTHCd	βTHCd	
				11	4	26	13	54
Dihydrocinchonidine (HCd)	3.0	1.1	10	β9DHHCd	α9DHHCd	αHHCd***	βHHCd***	
				17	18	22	6	63

* In order of elution.

** After rechromatography in solvent S10.

*** After rechromatography in solvent S7.

the presence of which is necessary to obtain successful separations of partly reduced alkaloids. Triethylamine was not used as a component of the systems giving the best results in the TLC of Cinchona alkaloids. Solvents S6 and S7 may find broader application as new systems for the separation of the vinyl and dihydro alkaloids. The differences between the R_F values in these systems are equal to or higher than those in the literature[13].

For preparative purposes, thin-layer and short-column approaches were investigated (Table III) TLC may be recommended for small samples or for fast isolation of some compounds e.g. α9DTTC or α9DHHC. Generally, however, the short-column procedure is superior because of the smaller number of chromatographic steps needed to obtain chromatographically homogeneous compounds. This approach was therefore studied in more detail.

The results are given in Table IV. Solvent S9 on a short silica gel column is able to separate in one operation four partly reduced alkaloids derived from cinchonine or 10,11-dihydrocinchonine. In the case of the cinchonidine series, α- and β9DTHCd as well as α- and βHHCd are not resolved and have to be rechromatographed in solvents S10 and S7 respectively.

CONCLUSION

The application of the described chromatographic methods enables the rapid, simple and complete separation of partially reduced Cinchona alkaloids. In addition to known 10,11-dihydrocinchonine derivatives, tetra- and hexahydro bases derived from cinchonine, cinchonidine and 10,11-dihydrocinchonidine were isolated for the first time. Their chemical and spectroscopic characterization will be described elsewhere.

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Errata

J. Chromatogr., 213 (1981) 337–339

Page 338, Table I, "ε_{255}" should read "ε_{225}" and "λ_{ex}^{max}" should read "λ_{em}^{max}".

Page 338, Table II, "acetonitrile–phosphate buffer (pH 5.05) (65:35)" should read "acetonitrile–phosphate buffer (pH 5.05, 10 m*M*) (65:35)" and "Effect of ionic strength" should read "Effect of ionic strength [with "weak" solvent as acetonitrile–water (65:35)]".

J. Chromatogr., 217 (1981) 463–471

Page 469, Table I, "*Detection limit (ng)*" should read "*Detection limit (pg)*".

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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Electron Capture – Theory and Practice in Chromatography

edited by A. ZLATKIS, *Houston, TX, USA and* C.F. POOLE, *Detroit, MI, USA*

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This book provides the first comprehensive coverage of all aspects of the theory, design, operation and applications of the electron capture detector (ECD) from the chromatographer's point of view. In addition, an up-to-date look at the ancillary techniques of selective electron-capture sensitization, atmospheric pressure ionization and plasma chromatography has been included. ECD users will find the solutions to instrumental and technical problems which arise during practice particularly valuable. These have been derived from the experiences of the internationally distinguished team of authors.

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