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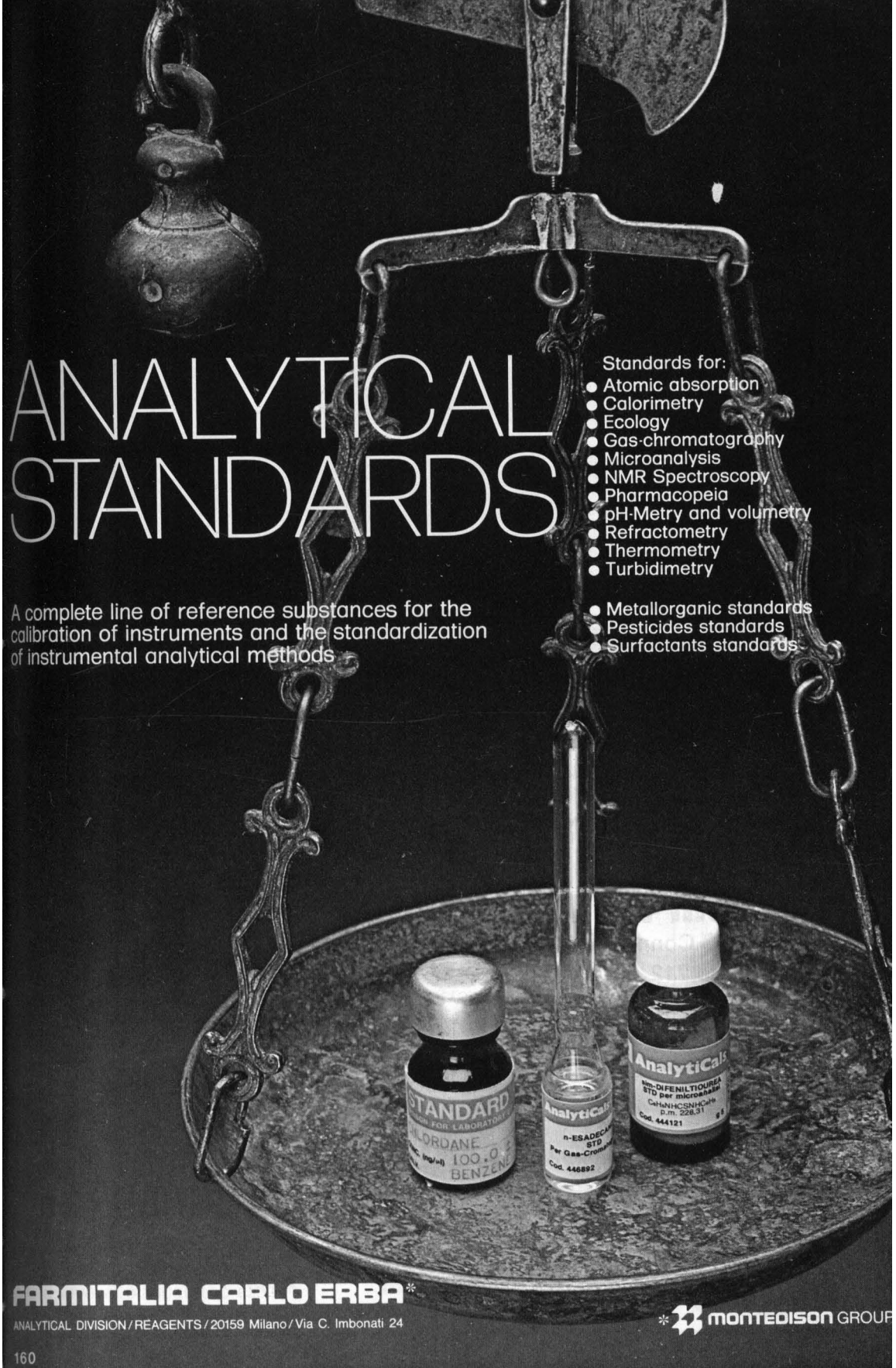
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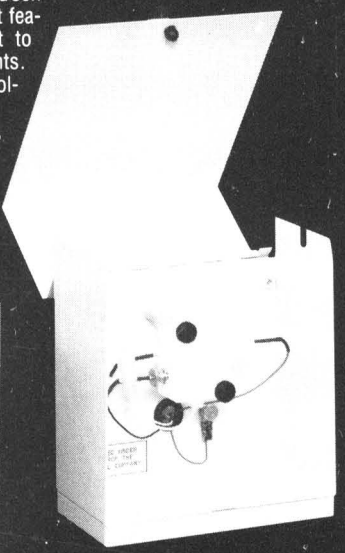

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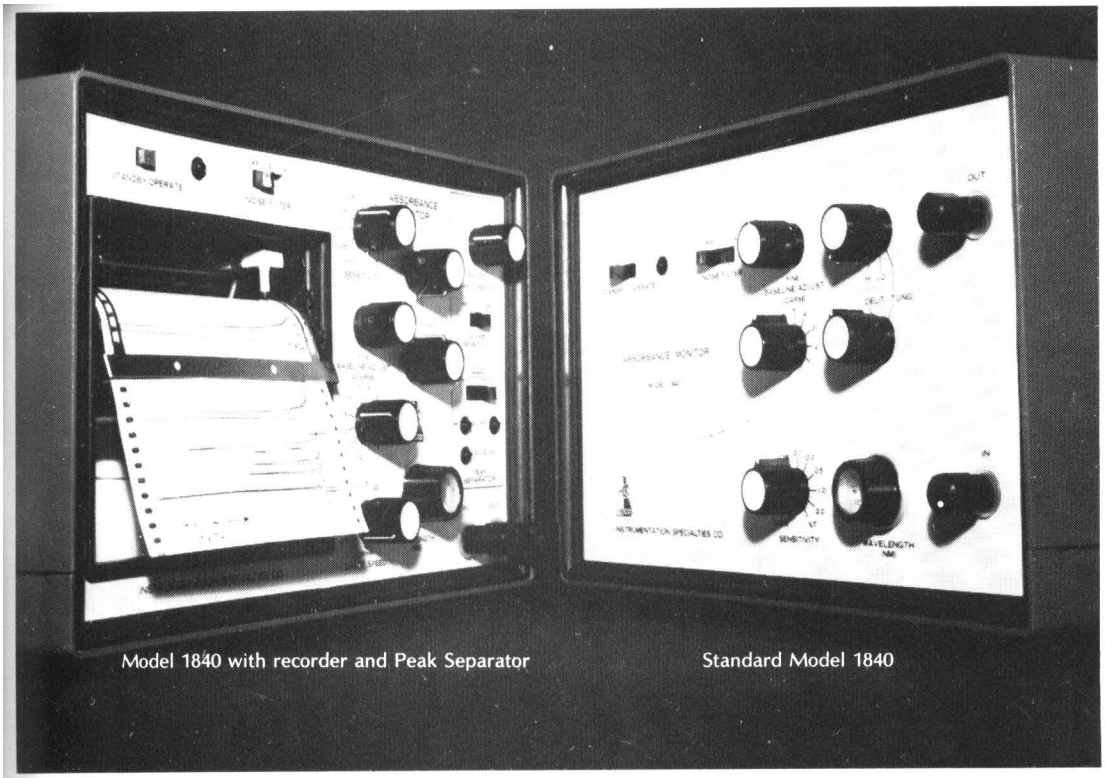
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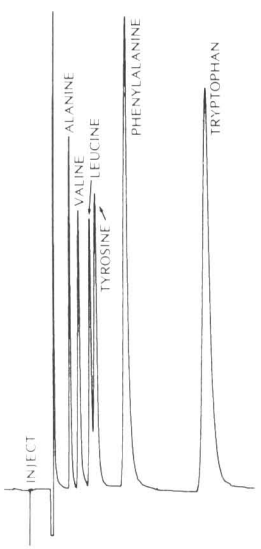
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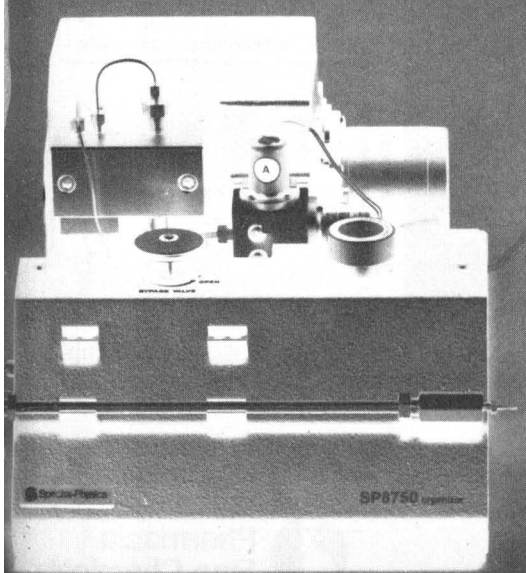
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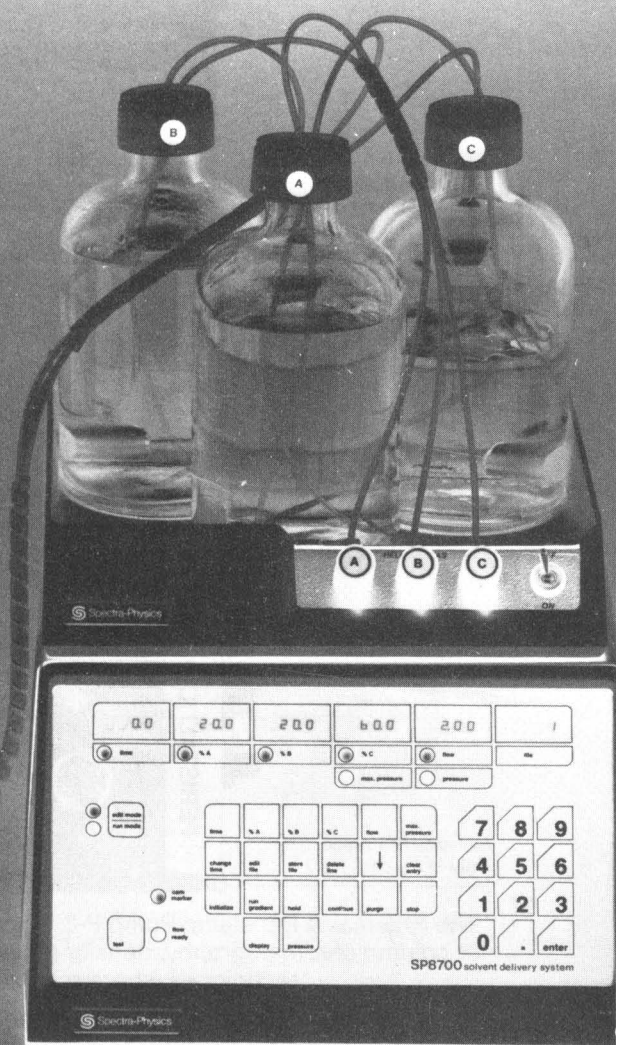
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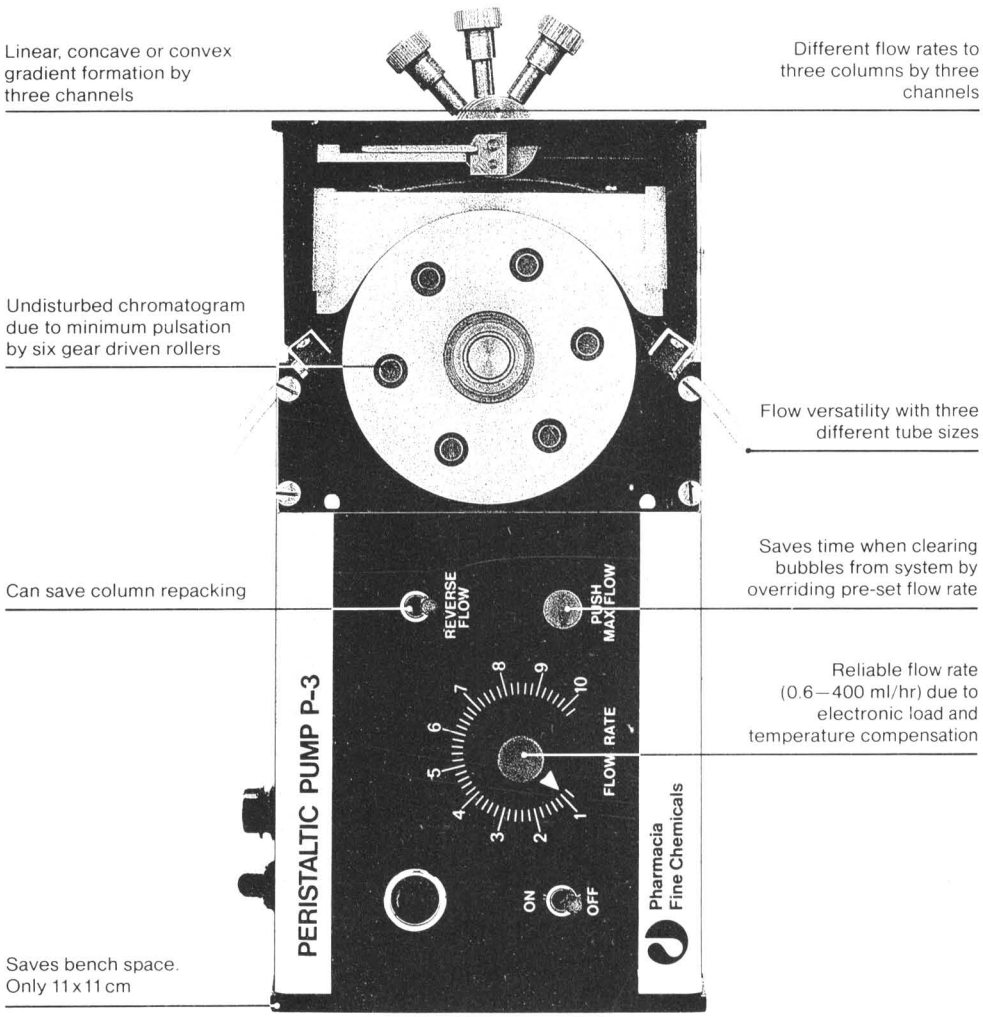




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THEORY AND RETENTION MECHANISMS

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MECHANISM OF ION-PAIR LIQUID CHROMATOGRAPHY OF AMINES, NEUTRALS, ZWITTERIONS AND ACIDS USING ANIONIC HETAERONS

JOHN H. KNOX* and RICHARD A. HARTWICK*

Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ (Great Britain)

SUMMARY

The basis of retention in reversed-phase ion-pair chromatography is examined using C_{18}, C_{10} and C_{12} alkyl sulphates as hetaerons (pairing species) with a range of cations, neutrals, zwitterions and anions as solutes. The basic eluent comprises water–methanol (80:20) containing 20 m*M* phosphate buffer pH 6.00 and 50 m*M* sodium ion. Measurements were made of k' (column capacity ratio) as a function of hetaeron concentration in the mobile (C_m) and stationary phases (C_s) the latter being determined by the break-through method.

While the adsorption isotherms were non-linear, plots of k' *versus* surface concentration of hetaerons were essentially linear for cations up to a level at which the hetaeron formed micelles in the eluent whereupon k' declined. k' for neutrals fell slightly as C_s increased as did k' for small zwitterions; k' for anions fell drastically as C_s increased whereas small peptides behaved like cations. k' values for all types at any given surface concentration (molar) were more or less independent of the chain length of the hetaeron.

It is concluded that the degree of retention is directly related to the surface charge arising from the adsorbed hetaeron and that under most practical conditions ion-pair formation in the eluent is unimportant.

INTRODUCTION

Recently several papers have appeared on the so-called "mechanism" of ion-pair chromatography[1–6]. Two "mechanisms" were clearly distinguished by Horváth *et al.*[1] who wrote: "Retention of eluite in ion-pair chromatography on non-polar bonded phases can occur by dynamic ion-exchange, *i.e.* ion-pair formation takes place between the eluite and hetaeron (the pairing ion) bonded to the stationary phase, or by ion-pair formation in the mobile phase and binding the complex to the non-polar stationary phase". Deelder *et al.*[2] repeated this distinction in very similar words. Bidlingmeyer *et al.*[3] were more specific: "Two hypotheses are presently embraced. The first view stipulates the formation of an ion pair in the mobile phase

* Present address: School of Chemistry, Rutgers University, Piscataway, NJ 08854, U.S.A.

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prior to its adsorption onto the bonded hydrophobic stationary phase. The second view stipulates an ion-exchange mechanism ... (in which) ... the unpaired lipophilic alkyl ions adsorb onto the bonded surface and cause the column to behave as a dynamic ion exchanger". Terweij-Groen *et al.*[5] also accept the existence of two "mechanisms" arguing in favour of the first.

These statements and others similar to them[6–8] seem to imply that there are two alternative kinetic processes whereby partition of solute ions can occur in ion-pair chromatography. Thus in the "ion-exchange" mechanism the solute ions are supposed to approach the bonded surface and there to unite with adsorbed hetaerons to form ion pairs, whereas in the "ion-pair-formation-in-the-mobile-phase" mechanism the ion-pairs are supposed first to be formed in the eluent and subsequently to migrate to the bonded surface where they become adsorbed. *It can be stated categorically that, whether these are the processes occurring or not, they have no relevance to the basis or "mechanism" of retention.* The kinetics of processes occurring in chromatography affect only the peak width not peak retention[9]: peak retention is determined exclusively by thermodynamic equilibria[4,9,10]. How the equilibria come to be established is irrelevant provided that the processes are fast enough not to cause additional peak spreading. Thus it is a fundamental tenet of chromatographic theory that no deductions about the kinetics of chromatography can be made from measurements of solute retention.

The ion-exchange analogue as proposed by Knox and co-workers[11–13] in connection with the use of strongly hydrophobic hetaerons, referred to the idea that, at thermodynamic equilibrium, the hydrophobic surface of the support was nearly saturated with respect to adsorption of pairing agent so that the effect of counter ions added to the eluent was analogous to the effect in ion-exchange chromatography, namely a strong reduction in retention. It was also recognised[13] that under many conditions, especially with short-chain hetaerons, the surface would be far from saturated. While retention still arises from the partition of solute–hetaeron ion pairs into the bonded layer, the effect of added counter ions in the eluent is now relatively minor. Through a confusion of roles of thermodynamic equilibria and rate processes in chromatography these two thermodynamic situations appear to have become associated (wrongly) with two possible kinetic mechanisms for equilibration of solute ions between the eluent and stationary phase.

The main equilibria which are generally recognised to be important in ion-pair chromatography of an ionized species[1,2,4–7,11–13] are the following: (P^- is a hetaeron which can equally well be a cation with appropriate reversal of the charges on other ions, S^+ is a solute ion, C^+ and B^- are counter ions normally also present in the eluent, L represents the hydrocarbon or ligand groups bonded to the internal surface of the support, and the subscripts m and s refer to the mobile and stationary phases, respectively).

Adsorption of solute ion onto L	$S_m^+ + B_m^- + L_s \overset{1}{\rightleftharpoons} SBL_s$
Ion-pair formation in eluent	$S_m^+ + P_m^- \overset{2}{\rightleftharpoons} SP_m$
Adsorption of haeteron onto L	$P_m^- + C_m^+ + L_s \overset{3}{\rightleftharpoons} PCL_s$
Adsorption of solute and hetaeron giving ion pairs in stationary phase	$S_m^+ + P_m^- + L_s \overset{4}{\rightleftharpoons} SPL_s$

If it is assumed that S^+ is present in trace concentrations and that the total available ligand concentration [L] is constant, that is

$$[L] = [L_s] + [PCL_s] = \text{constant} \tag{1}$$

one can readily derive

$$k' = \varphi[L] \frac{K_1[B_m^-] + K_4[P_m^-]}{\{1 + K_2[P_m^-]\}\{1 + K_3[P_m^-][C_m^+]\}} \tag{2}$$

where k' is the column capacity ratio and φ is the ratio of stationary to mobile phase. This equation is essentially the same as that derived by Horváth *et al.*[1] except that their equilibria 1 and 3 did not contain the counter ions B^- and C^+ necessary to ensure the balance of electrical neutrality. Melin *et al.*[4] give a similar equation but do not include equilibrium 2.

In some circumstances it may be useful to consider other equilibria involving the hetaeron

Ion-pair exchange	$PCL_s + S_m^+ \overset{5}{\rightleftharpoons} PSL_s + C_m^+$
Ion-pair adsorption	$PS_m + L_s \overset{6}{\rightleftharpoons} PSL_s$

These equilibria are not, however, independent of 2, 3 and 4 since

$$K_4 = K_3K_5 = K_2K_6 \tag{3}$$

From the five equilibria 2, 3, 4, 5, 6 there are, in fact, eight possible combinations of three which are strictly equivalent. These may be represented as

$$\begin{aligned} 2 + 3 + 4 \equiv 2 + 3 + 5 \equiv 2 + 3 + 6 \equiv 2 + 4 + 5 \equiv 3 + 4 + 6 \\ \equiv 2 + 5 + 6 \equiv 3 + 5 + 6 \equiv 4 + 5 + 6 \end{aligned} \tag{4}$$

where the numbers represent the equilibria. Eqn. 2 is unalterable except for substitutions of K_4 derivable from eqn. 3. It is thus incorrect to maintain as do Terweij-Groen *et al.*[5] that equilibrium 5 is important while equilibrium 4 is unimportant, since 4 is the sum of 3 and 5.

In eqn. 2 the cause of retention on addition of the hetaeron P^- is seen unequivocably to be the operation of equilibrium 4, that is the partition of S^+ into the stationary phase by formation of solute–hetaeron ion pairs. The term $K_1[B_m^-]$ arises from the retention of S^+ in the absence of added hetaeron, and in many situations is negligible. Retention is counteracted by advancement of the equilibria 2 and 3 which correspond to formation of ion pairs in the eluent and to saturation of the ligand L by adsorption of counter ion-hetaeron ion pairs. In no sense can "formation of ion pairs in the mobile phase" be said to contribute to increasing retention: indeed it can only act to reduce retention, as was demonstrated by Terweij-Groen *et al.*[5].

When one, but not the other, of K_2 and K_3 is small so that the appropriate

factor is the denominator is always near unity, then k' will initially rise in proportion to $[P_m^-]$ but will flatten off to a constant value when $[P_m^-]$ is high. This behaviour is widely observed in practice[1–7,11–13].

Under these conditions the limiting value of k' is given by either

$$k'_{max.} = \varphi[L]\, K_5 \qquad (\text{when } K_2 = 0) \tag{5}$$

or

$$k'_{max.} = \varphi[L]\, K_6 \qquad (\text{when } K_3 = 0) \tag{6}$$

It is these two limiting conditions which may have led to Horváth *et al.*[1] to define their two "mechanisms". From circumstantial evidence on the effect (or rather lack of effect) of the chain length of the hetaeron on the concentration of P^- at which k' became constant, they deduced that K_3 was negligible in their system, that equilibrium 2 caused the flattening off, and therefore that eqn. 6 held. Since reaction 6 represents the equilibration of solute–hetaeron ion pairs between the eluent and stationary phase they deduced that ion-pair chromatography "proceeds through the formation of ion pairs in the mobile phase followed by adsorption onto the non-polar stationary phase". While the first deduction (*i.e.* that K_3 is negligible) may well be correct, the second is incorrect if it is meant to refer to the kinetics rather than the thermodynamics of the process. Taking K_3 as zero, Horváth *et al.* could then deduce that K_6 increased by a factor of about 1.7 for each CH_2 group added to the alkyl chain of the hetaeron.

It was unfortunate that in this work[1] the degree of adsorption of P^- by the surface was not directly measured since this would have at once established the shape of the isotherm and given a value for K_3. Terweij-Groen *et al.*[5] and subsequently Deelder *et al.*[2] measured this directly. Using the equilibria already given it is readily shown that k' can equally well be expressed by eqn. 7, which involves the concentration of adsorbed hetaeron, $[PCL_s]$, rather than its concentration in eluent $[P_m^-]$.

$$k' = \varphi \frac{K_5 + (K_1/K_3)\,[B_m^-]/[P_m^-]}{1 + K_2[P_m^-]} \cdot \frac{[PCL_s]}{[C_m^+]} \tag{7}$$

Terweij-Groen *et al.*[5] demonstrated that eqn. 7 held for the chromatography of acids using cetyltrimethylammonium as hetaeron. Deelder *et al.*[2] demonstrated that with aqueous eluents containing hexyl, octyl and dodecyl sulphonates as hetaerons, plots of k' against $[PCL_s]/[C_m^+]$ were linear, particularly when PCL_s was high ($\approx 200\ \mu mol\ g^{-1}$ or $\approx 1\ \mu mol\ m^{-2}$), having chosen conditions so that retention of S^+ was negligible in the absence of hetaeron. From both studies it may be concluded that $K_2[P_m^-] \ll 1$ in these systems. Deelder *et al.*[2] found that K_5 (their K_e) increased about twice going from the C_6 to C_8 sulphonate and by another twice from C_8 to C_{12} sulphonate.

In the present work we were concerned to provide further evidence on the basis of ion-pair chromatography and to test a very simple hypothesis, namely that the predominant factor causing enhanced retention in ion-pair chromatography is the interaction of the charge of adsorbed hetaeron with the opposite charges of the solute ions. If this is so then, to a first approximation, retention should be a linear function of the charge density on the surface of the packing material, which arises from adsorption of the hetaeron, and it should be more or less independent of the chain

length of the hetaeron at any surface concentration. As a further consequence of this hypothesis retention of ions of the same charge as the hetaerons (co-ions) should be reduced while that of neutrals should be almost unaffected. The retention of zwitterionic species would be particularly interesting since they might behave as either counter ions, or co-ions or neutrals showing increased, decreased or constant retention as the hetaeron concentration was charged.

EXPERIMENTAL

The high-performance liquid chromatographic (HPLC) equipment comprised the following components: a Waters Assoc. (Croydon, Great Britain) Model M6000 pump, a Waters Assoc. U6K valve, or a Shandon syringe/septum injector and a 100 or 125 × 5 mm I.D. internally polished stainless-steel column (Shandon Southern Products, Runcorn, Great Britain). For UV absorbing compounds, a Cecil Instruments (Cambridge, Great Britain) Model 212 UV photometer with an 8-μl flow cell was used; for fluorescent compounds, a Schoeffel Model FS 970 fluorometer (Kratos, Manchester, Great Britain) was used; for breakthrough curves, an Optilab Multiref 902 refractive index monitor (Techmation, London, Great Britain) was used.

Columns were slurry packed in the laboratory with 5-μm ODS-Hypersil (Shandon Southern Products) at 6000 p.s.i. using propanol followed by hexane, methanol and finally water–methanol (80:20, v/v). Columns were thermostatted by water jackets connected to a circulating bath at 25 $\pm$ 1°C. In addition the eluent was thermostatted before entry to the column by passage through a heat exchanger consisting of 1 m of 1/16 in. O.D., 0.020 in I.D. stainless-steel tubing immersed in the circulating bath.

For microelectrophoresis a Mark II electrophoresis equipment (Rank Bros, Bottisham, Great Britain) was used employing a rectangular quartz cell 0.80 mm deep, 9.7 mm wide and $\approx$50 mm long. Platinum electrodes were used in the end compartments with a constant potential difference of 50 V. Microscopic observations of the electrophoretic mobilities, U_e, of ODS-Hypersil particles were made at the so-called stationary levels in the cell to eliminate electroendosmotic effects, at least ten measurements being made in each direction. From the electrophoretic mobility the zeta potential, ζ, was obtained from

$$\zeta = \frac{U_e \eta}{\varepsilon_0 \varepsilon_r E} \tag{8}$$

where η = viscosity, ε_r = dielectric constant, ε_0 = permittivity of a vacuum, E = field strength and U_e = true electrophonetic velocity.

Reagents and standards

The alkyl sulfates used as pairing ions (sodium salts), were purchased from Sigma (Poole, Great Britain). HPLC-grade methanol (Rathburn Chemicals, Walkerburn, Great Britain) and AnalaR-grade phosphates (BDH, Poole, Great Britain) were used in the preparation of buffers.

The various amino acids, peptides and amine compounds were purchased from Sigma.

Preparation of eluents

Eluents were prepared in single large batches of sufficient quantity for experiments on a single pairing ion so that intra-study variations were eliminated. The standard eluents consisted of water–methanol (80:20) mixtures containing 0.018 *M* KH_2PO_4 and 0.002 *M* $NaHPO_4$ calculated to provide a pH of 6.0. A pH of exactly 6.00 was finally produced by small additions of either NaOH or HCl to this solution. After addition of the desired concentration of pairing agent as the sodium alkyl sulphate, the total sodium ion concentration was adjusted to exactly 0.050 *M* by the addition of NaCl. In this way, when the pairing salt concentration was changed, only the relative concentrations of the pairing ion and chloride were altered. In a few experiments other sodium chloride concentrations were used as described in the text.

Adsorption isotherms

The amounts of sodium alkyl sulphate adsorbed by the stationary phase from the standard eluents were determined by the break-through method using the Optilab Multiref 902 refractometer as detector. A "T-piece" leading to an on/off valve was fitted to the chromatograph immediately upstream of the injector so that when changing the pairing-ion concentration, the entire system could be flushed up to the point of injection with new eluent. The new eluent was then directed onto the column by closing the valve leading from the "T-piece". The breakthrough volume, V_b, was measured from the moment of closing the valve to the mid point of the eluted front. After subtraction of the void volume, V_m, the amount of pairing ion adsorbed, q_{ads}, is readily obtained[14] from

$$q_{ads} = (V_b - V_m)\, C_m = V_b' C_m = AC_s \tag{9}$$

where C_m is the concentration of pairing ion in the eluent, and V_b' is the net retention volume corresponding to the front, A is the surface area of the packing within the column and C_s the surface concentration of adsorbed hetaeron.

For the longer chain alkyl sulphates it was impossible to wash the pairing ions off the column between each concentration increase and a step-wise increase in concentrations was used with each previous concentration being used as the baseline. The total amount adsorbed onto the stationary phase was the sum of the individual increases obtained by successive applications of eqn. 9.

RESULTS AND DISCUSSION

Adsorption isotherms

Isotherms for the adsorption of the three alkyl sulphates, octyl, decyl and lauryl (dodecyl) from the standard eluent [that is water–methanol (80:20) containing 0.02 *M* phosphate buffer at pH 6.00 and sodium ion at 0.05 *M*] are shown in Fig. 1. To calculate the surface concentration, C_s, the surface area of ODS-Hypersil was required. This was measured by the BET method[16] and found to be 105 $m^2\ g^{-1}$. Taken with a measured weight of ODS-Hypersil in the column of 1.13 g the total surface area within the 4.7 × 125 mm column was 120 m^2. Fig. 2 shows that the adsorption isotherms of Fig. 1 are well fitted by the Freundlich equation as found by Deelder *et al.*[2]:

$$C_s = \alpha C_m^{\beta} \tag{10}$$

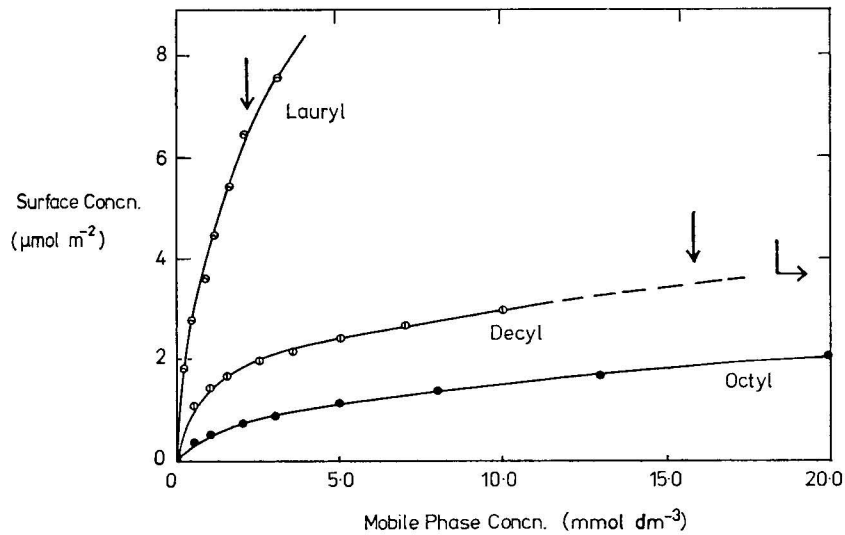


Fig. 1. Isotherms for adsorption of alkyl sulphates by ODS-Hypersil from standard eluent (water–methanol, 80:20 made 0.02 *M* in phosphate buffer pH 6.0 and 0.05 *M* in Na^+). Surface area of ODS-Hypersil 105 $m^2\ g^{-1}$. Temperature, 298°K. Estimated critical micelle concentrations[15] are indicated by arrows.

From Fig. 1 it may be noted that the maximum concentrations of the adsorbed alkyl sulphates were approximately 2, 3 and 8 μmol m^{-2} for the C_8, C_{10} and C_{12} pairing agents, respectively even though the isotherms show no sign of reaching saturation (as seen by Fig. 2). These surface concentrations are relatively high and compare with the maximum concentration of trialkyl silyl groups bonded to silica surfaces of around 2 to 5 μmol m^{-2} (ref. 17) depending upon chain length of the alkyl groups. It

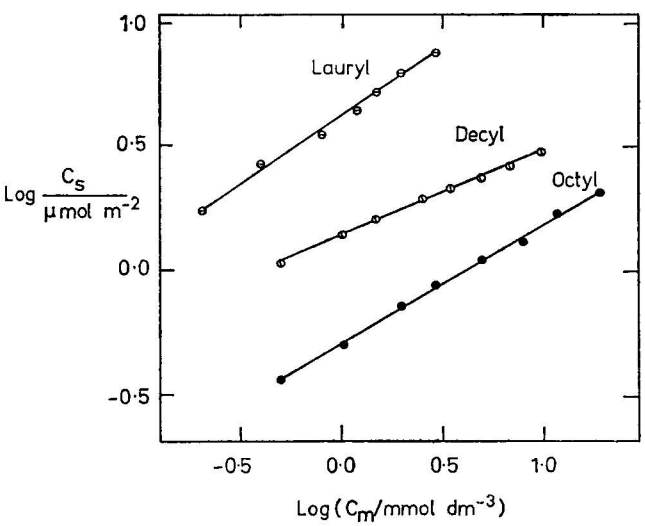


Fig. 2. Logarithmic plot of data of Fig. 1 according to eqn. 10. Values of β are: octyl sulphate, 0.49; decyl sulphate, 0.35; lauryl sulphate, 0.57.

may be noted that with lauryl sulphate the estimated critical micelle concentration (CMC) of sodium lauryl sulphate[15] is around the highest concentration used and therefore that catastrophic adsorption may be occurring. For octyl and decyl sulphates the CMC values are significantly above the highest concentration employed. Here the maximum surface concentrations are comparable to those of bonded groups indicating a near monomolecular layer of adsorbed hetaeron. For comparison it may be noted that the maximum surface concentrations of cetyltrimethylammonium observed by Knox and Laird[11] at an eluent concentration of 2% (w/v) were ≈ 0.25 μmol m^{-2} on SAS silica and by Terweij-Groen *et al.*[5] up to 1.5 μmol m^2 (300 μmol g^{-1}). Deelder *et al.*[2] measured maximum surface concentrations of 50 and 250 μmol g^{-1} for C_8 and C_{12} sulphonates adsorbed by Partisil 10 ODS corresponding to between 0.4 and 1.25 μmol m^{-2} of surface area.

The surface coverages found in this and other comparable studies thus show that surface active agents are strongly adsorbed by bonded phases to give coverages approaching those of the chemically bonded groups themselves.

Dependence of retention upon concentration of the pairing ions in mobile and stationary phases

Fig. 3 shows the effect of the mobile phase concentration of lauryl, decyl and octyl sulfates on the k' values of tyrosine amide. A maximum in retention occurs for

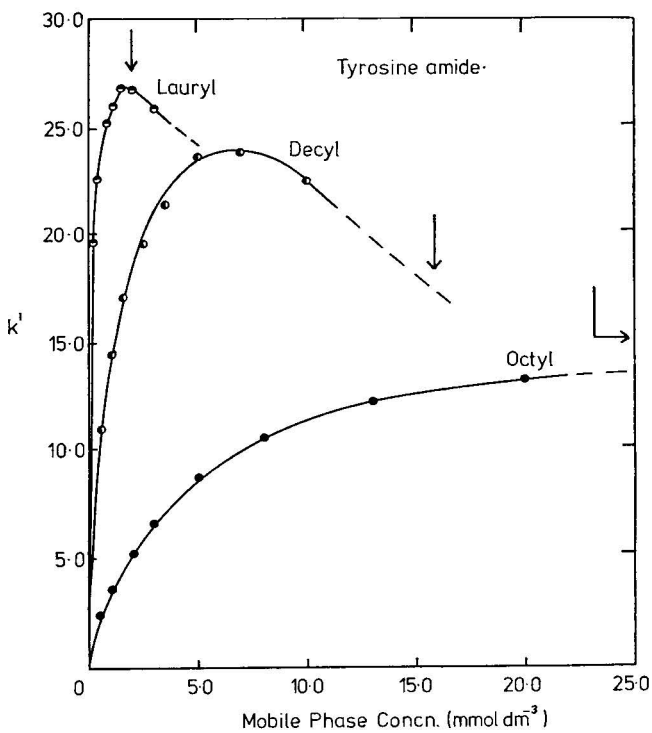


Fig. 3. Dependence of k' for tyrosine amide upon concentrations of alkyl sulphates added to standard eluent. Packing ODS-Hypersil. Estimated critical micelle concentrations[15] indicated by arrows.

the decyl and lauryl sulfates which is similar to that observed by Horváth *et al.*[1] with decyl sulphate as hetaeron. It is interesting to note that these maxima occur at lower mobile phase concentrations the longer the chain length of the pairing ion. These results contrast with those of Horváth *et al.* who found that the concentration of hetaeron at which k' became constant was independent of chain length for C_4 to C_8 alkyl sulphates and about 40 mM. However, for C_{10} sulphate a clear maximum was observed at a significantly lower hetaeron concentration of about 15 mM. In the present work the maxima occurred at $\approx$20 mM for octyl sulphate, $\approx$7 mM for decyl sulphate and $\approx$2 mM for lauryl sulphate. Estimated values of the CMC[15] in the eluent used by Horváth *et al.* and in our standard eluent are 100 mM for octyl, 20 mM for decyl and 2.5 mM for lauryl sulphate. It seems most likely that the eventual fall in k' as hetaeron concentration is increased in both this work and that of Horváth *et al.*, arises from the incipient micelle formation coupled with ion-pair formation in the eluent as proposed earlier to explain similar effects by Knox and Laird[11] and by Terweij-Groen *et al.*[5]. It is, however, notable that the adsorption of the alkyl sulphates is still increasing when k' is past its maximum value and indeed even when C_m exceeds the estimated CMC.

Fig. 4 shows how the k' values of positive, neutral and negatively charged solutes depend upon the concentration of the hetaeron decyl sulphate in both eluent and stationary phase.

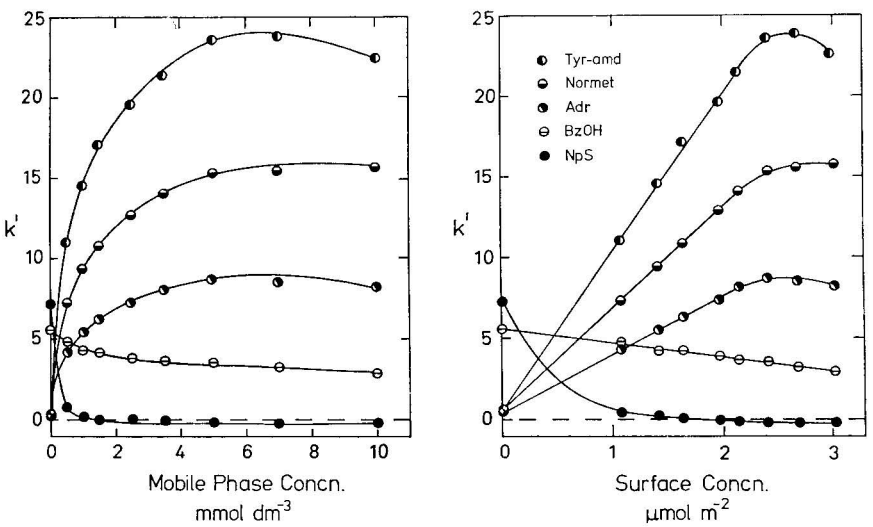


Fig. 4. Dependence of k' for various solutes upon concentrations of octyl sulphate in standard eluent. Left: k' *versus* C_m; right: k' *versus* C_s. Tyr-amd = tyrosine amide, Normet = normetadrenaline, Adr = adrenaline, BzOH = benzyl alcohol, NpS = naphthalene-2-sulphonate. Pairing ion: decyl sulphate.

For positively charged species k' increases with C_m in a concave manner to a maximum value when C_m is around 8 mmol dm^{-3}. When k' is plotted against C_s, however, an excellent linear relationship is observed almost up to the concentration corresponding to the maximum in k'. This result is in close agreement with those of previous workers[2,5] and strongly suggests that the major factor contributing to reten-

tion in this form of ion-pair chromatography is a direct interaction between the adsorbed pairing species and the solute ion of opposite charge, with interactions in the mobile phase being negligible until C_m reaches some critical concentration which is close to the CMC.

For the neutral species, benzyl alcohol, k' decreases slightly with increase in C_s as found by Melin *et al.*[4] indicating a slight lessening of the interaction with the stationary phase as this becomes progressively more polar through adsorption of the hetaeron. For the negatively charged solute naphthalene-2-sulfonate (NpS^-), on the other hand, there is a dramatic loss of retention when decyl sulfate is adsorbed onto the stationary phase, until finally it is partially excluded from the packing material (that is its k' value becomes negative). This occurs at about 2.7 μmol m^{-2} of decyl sulfate. This result is similar to those previously found by Knox and Jurand[13], Bidlingmeyer *et al.*[3] and by Melin *et al.*[4].

The dramatic decrease in k' of negatively charged solutes, the slight loss in k' of neutral species, and the linear increases in k' of the positively charged species with C_s, all point strongly to surface charge as the dominant factor governing retention, the surface becoming increasingly negatively charged with increasing adsorption of the hydrophobic negatively charged hetaeron ions.

The profound effect observed in practice from the addition of pairing ions on the retention of the solutes of various charges is well illustrated by the chromatograms shown in Fig. 5. When no octyl sulfate is present in the mobile phase, the positively charged species adrenaline (Adr^+), normetadrenaline ($Normet^+$) and tyrosine amide

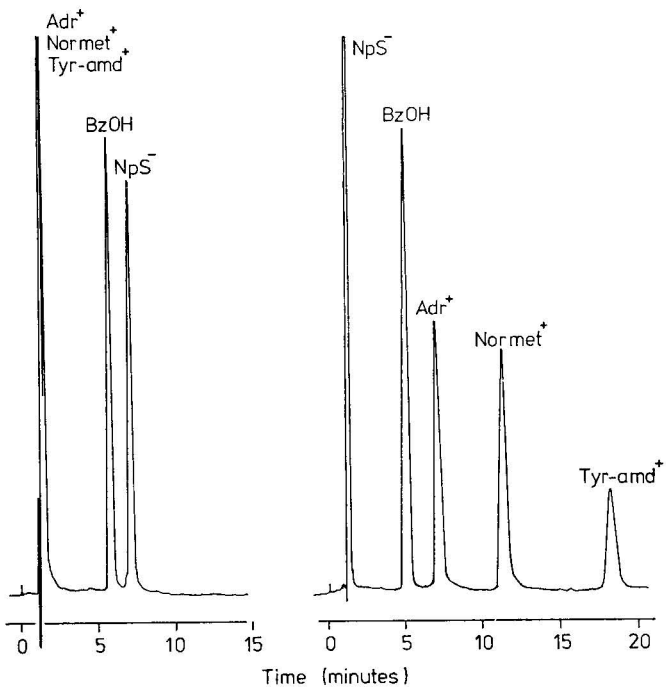


Fig. 5. Effect on k' of various species (for identification see Fig. 4) of the addition of octyl sulphate to standard eluent. Left: no octyl sulphate; right: 14 mmol dm^{-3} in octyl sulphate.

(Tyr-amd$^+$) are almost unretained, whereas the neutral solute benzyl alcohol (BzOH) and the negatively charged napthalene-2-sulfonic acid (NpS$^-$) have k' values of about 6 and 7, respectively. When the mobile phase contains 0.014 mol dm^{-3} octyl sulfate, NpS$^-$ loses almost all retention, while Adr$^+$, Normet$^+$ and Tyr-amd$^+$ become well retained. The retention of BzOH decrease slightly.

The type of effect shown in Fig. 5 is not, of course, limited to negatively charged pairing ions. Fig. 6 shows the effect of the addition of tetrabutyl ammonium iodide (TBAI) upon the retention of NpS$^-$, phenol (PhOH) and the O-methyl ester of tryptophan (Trp-OMe$^+$). Again, reversal of the elution order occurs, this time with the positively charged Trp-OMe$^+$ losing retention, while that of NpS$^-$ increases, and that of the neutral PhOH slightly decreased.

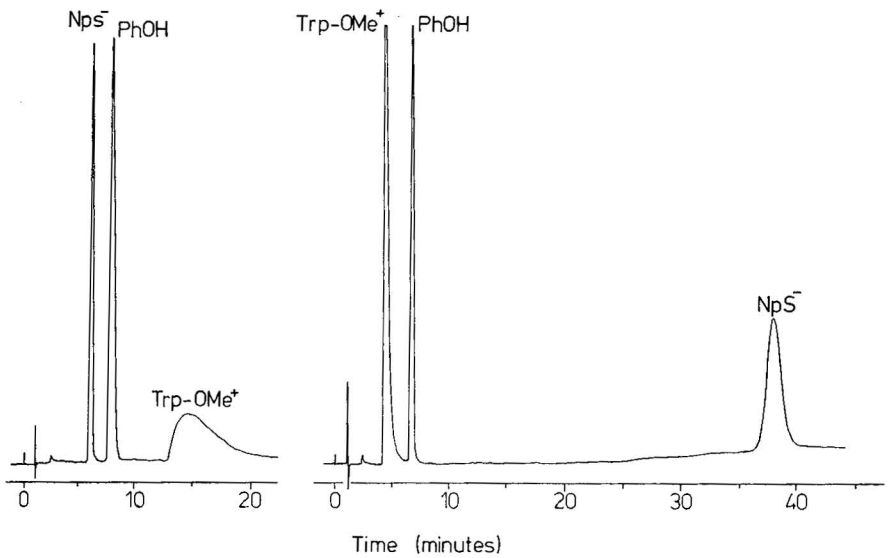


Fig. 6. Effect on k' of various species of the addition of tetrabutyl ammonium (4 mM). Eluent, water–methanol (75: 25, v/v) containing 0.10 M phosphate buffer pH = 6.0. NpS$^-$ = naphthalene-2-sulphonate, PhOH = phenol, Trp-OMe$^+$ = methoxy tryptophan. Left: No TBAI; right: 4 mmol dm^{-3} in TBAI.

Effect of pairing ions on zwitterionic solutes

Fig. 7 shows the retention behaviour of zwitterions of various chain lengths, in the presence of decyl sulfate at various concentrations. The simple amino acids tryptophan (Trp) and phenylalanine (Phe) show ambiguous behaviour like that of neutral species with no significant change in retention when the pairing-ion concentration is increased. However, with peptides, as the chain length is increased, the effect of addition of pairing ion becomes progressively greater so that with PheGlyGly and TrpGly the retention behaviour resembles that of the positively charged species of Fig. 4; that is, there exists a linear relationship between k' and the surface concentration of pairing ion. Apparently with the longer peptides, the positive charge on the amino group is able to function more or less independently of the negatively charged carboxyl group. This suggests that a singly charged pairing ion of either charge could effect the separation of the short chain peptides on reversed-phase materials, and that

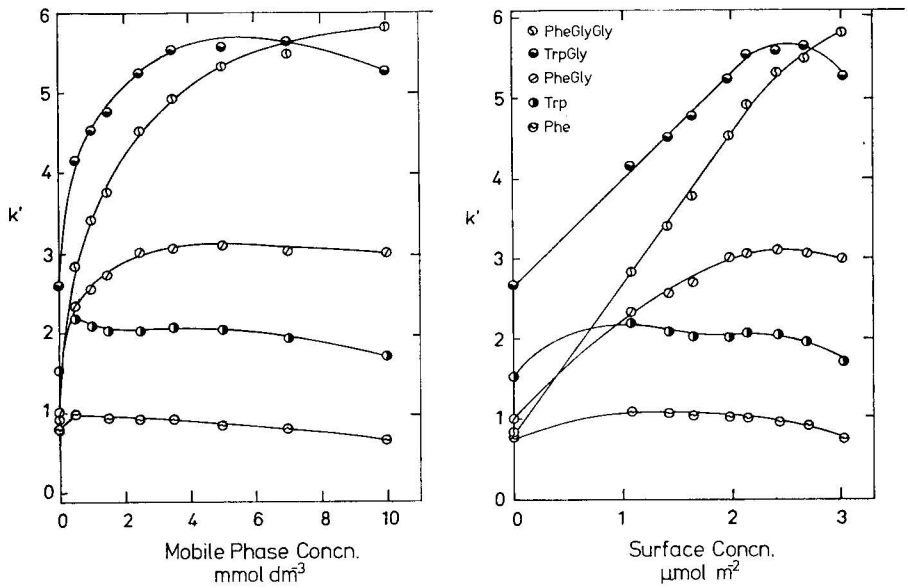


Fig. 7. Dependence of k' for various zwitterionic solutes upon concentration of decyl sulphate in standard eluent. Left: k' *versus* C_m; right k' *versus* C_s. Pairing ion: decyl sulphate.

it is unnecessary to employ extreme pH as previously though necessary[5] in order to suppress the ionization of either the amino or the carboxyl groups.

Dependence of retention upon chain length of pairing ion

If retention in reversed-phase ion-pair chromatography is predominantly dependent upon the interaction of a charged surface produced by adsorption of pairing ions, with solute ions of opposite charge, retention of a given solute ought to be independent of the chain length of the pairing ion *per se*. Figs. 8 and 9 illustrate the degree to which this was found to be true. Fig. 8 shows that when normetadrenaline (Normet$^+$) is chromatographed at various concentrations of lauryl, octyl and decyl sulfates in the presence of constant sodium ion concentration, k' shows the usual concave dependence upon C_m, the curves being steeper and showing maxima occurring at lower values of C_m the greater the length of the alkyl chain. However, when the k' values are plotted against surface concentration, C_s, fairly good linear relationships are observed up to the maximum k', but more important, the curves for the three hetaerons are nearly coincidental. Fig. 9 shows that this behaviour also occurs with neutral, negatively charged and other positively charged species.

Evidently the major factor governing retention is the interaction of charges and not of the non-polar alkyl groups with the solute ions. A secondary influence of chain length can nevertheless be detected in that for a given C_s, the retention is slightly greater with lauryl sulphate (C_{12}) than with octyl or decyl sulphates. This study thus indicates that K_5 is little dependent upon chain length of the hetaeron, when the eluent is water–methanol (80:20) made 0.05 M in NaCl and 0.02 M in phosphate buffer. The results contrast with those of Deelder *et al.*[2] who found a two-fold increase in K_5 in going from C_8 to C_{12} sulphonates.

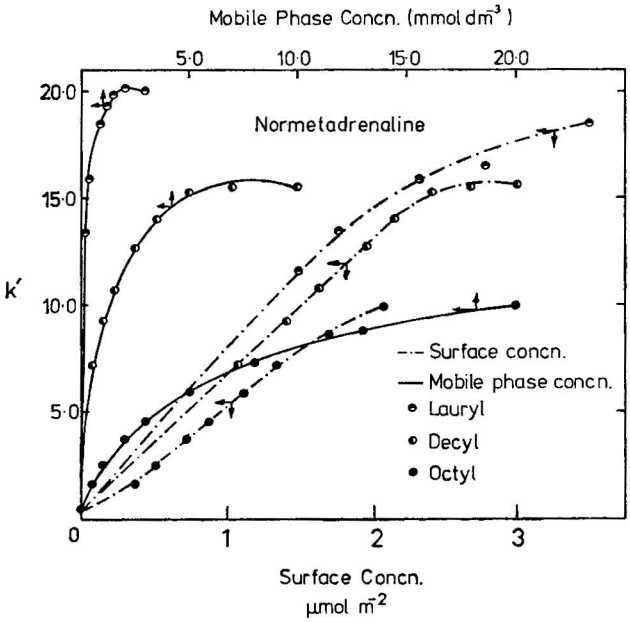


Fig. 8. Dependence of k' for normetadrenaline on concentration of alkyl sulphate for alkyl sulphates of different chain length. Full lines, plots of k' *versus* C_m; broken lines, plots of k' *versus* C_s.

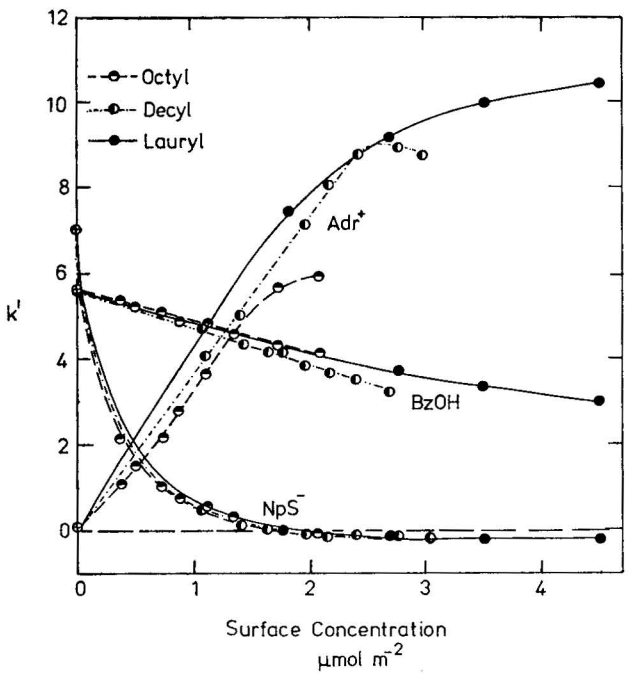


Fig. 9. Dependence of k' for positively charged, neutral and negatively charged solutes (for identification see Fig. 4) upon concentration of alkyl sulphate for alkyl sulphates of different chain length.

Effect of counter-ion concentration

According to eqn. 7, if K_1 and K_2 are taken as effectively zero, plots of k' for an ionized solute S^+ against $C_s/[Na_m^+]$ should be linear. This is demonstrated by Fig. 10 in which $[Na_m^+]$ was varied while keeping the mobile phase concentration of octyl sulphate constant at 5 mmol dm^{-3}. For cationic species excellent linear relationships are observed with small positive intercepts at $C_m = 0$. The results are similar to those found previously[2,5] except that in some cases[2] some curvature was found indicating a dependence of K_5 upon surface coverage by the hetaeron. Neutral benzyl alcohol and simple amino acids show indecisive behaviour as was observed when C_m was varied at constant $[Na_m^+]$. The behaviour of the dipeptide TryGly is, however, interesting in that increase of $C_s/[Na_m^+]$ brought about by changing $[Na_m^+]$ at constant C_m causes a slight decrease in k' whereas increase in C_s brought about by changing C_m at constant $[Na_m^+]$ increases k' (Fig. 7). These two apparently contradictory results are rationalised by observing that in the former case, because of the non-linearity of the adsorption isotherm, C_s increases as $C_s/[Na_m^+]$ decreases. Thus for both cases k' increases with C_s and $[Na_m^+]$ has little influence on k'. This is not unexpected for with a zwitterionic solute increase of ionic strength will have compensating effects on the two oppositely charged parts of the molecule so that charge interaction will appear more important than ion-exchange effects.

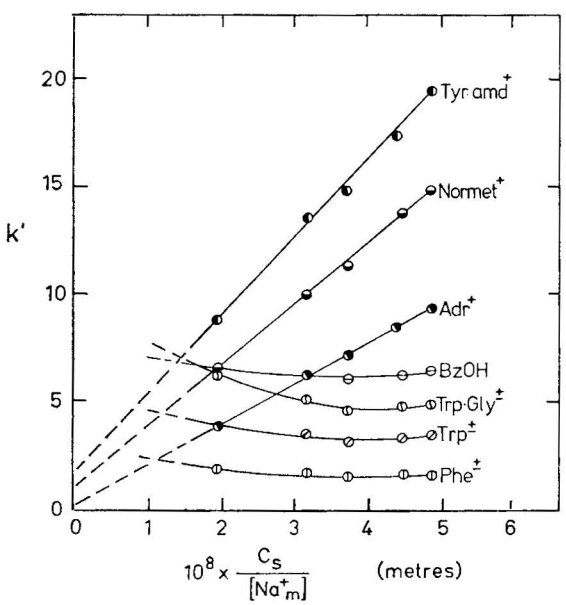


Fig. 10. Effect of counter-ion concentration (10–70 mM Na^+) on retention of various solutes in presence of octyl sulphate (5 mM). C_s determined by breakthrough technique. Plot according to eqn. 7 (simplified).

Buffer capacity

In choosing the optimum eluent for ion-pair chromatography several types of buffering must be considered. Throughout the chromatographic band the concentration of solute will very from zero to some maximum which will normally be in the

range of 10^{-3} to 10^{-6} *M* depending upon the concentration in the injected sample, the distance migrated along the column, the column volume and its plate efficiency. If the solute is wholly or partially ionized its retention or k' values may vary over this concentration range for some or all of the following reasons: (a) inadequate pH buffer capacity which allows the ratio of ionized to unionized form to vary, (b) too small an ionic strength of eluent which allows the ionic strength to vary within the peak, (c) inadequate pairing-ion capacity which causes C_s to vary throughout the peak as a result of additional ion pairs brought into the stationary phase associated with the adsorbed solute ions.

If any of (a) to (c) occur peak tailing or fronting will be observed. The combined importance of ionic strength and pH buffering is demonstrated by the two chromatograms of Fig. 11. Severe tailing of the ionized solutes but not of the neutral benzyl alcohol is observed when the ionic strength of the phosphate buffer is 0.005 *M*. This is largely eliminated when the ionic strength is increased to 0.10 *M*, the concentration of the pairing agent being 0.01 *M* in both cases. The initial sample size was around 10^{-8} mol injected in 10 μl of eluent (*i.e.* initial concentration was about 10^{-3} *M*). This emerged from the column with a peak maximum concentration of about 10^{-5} *M*.

Loading of the pairing ions onto the column

A direct consequence of adsorption of the pairing ions onto the stationary phase is that finite volumes of eluent must be passed before the column surface is

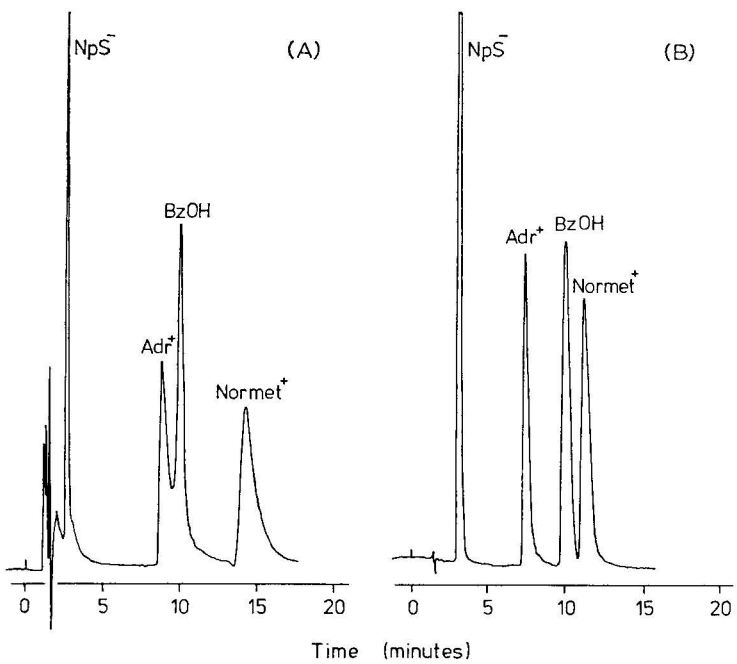


Fig. 11. Effect of inadequate buffer capacity in ion-pair chromatography. Eluents (A) 5 m*M* phosphate buffer, (B) 100 m*M* phosphate buffer in water–methanol (85/15, v/v) containing 10 m*M* octyl sulphate.

fully coated. Obviously, the more packing surface area there is to be covered, the more strongly adsorbed the pairing ion is, and the lower is its concentration in the mobile phase, the more volume will be required to coat the packing surface, as shown by eqn. 9. Since the adsorption isotherms follow Freundlich behaviour, eqns. 9 and 10 give

$$V_b' = A\alpha C_m^{(\beta-1)} \tag{11}$$

Fig. 12 shows the approximate V_b' values calculated from eqn. 11 for a 100-mm column packed with 5-μm particles of ODS-Hypersil containing a total surface area of 100 m^2. As the concentration of pairing ion in the mobile phase is decreased, greater volumes are required for equilibration and, for example, with lauryl sulfate at 0.1 mmol dm^{-3} $\approx$1.5 dm^3 of eluent are required. At a flow-rate of 1.0 cm^3 min^{-1}, 25 h would be needed to reach full equilibration! For the less strongly adsorbed octyl sulfate, equilibration is not such a problem with only about 100 cm^3 being required, even at this low concentration.

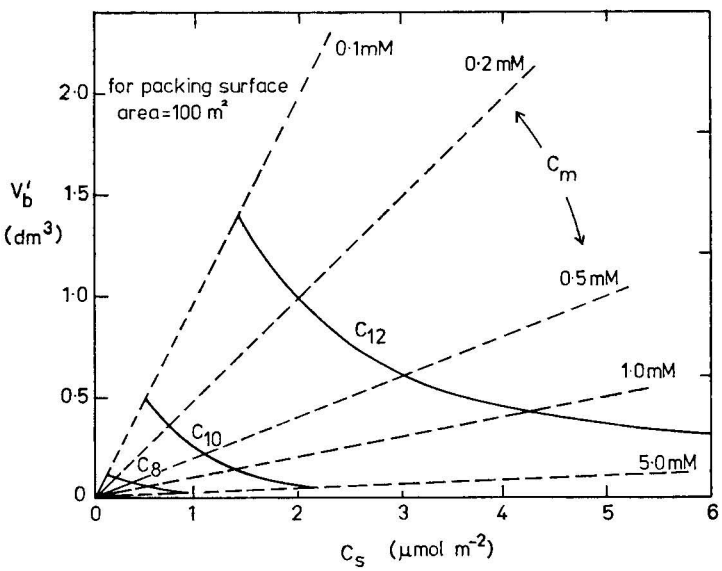


Fig. 12. Breakthrough volumes for alkyl sulphates at various mobile phase concentrations for a references surface area of 100 m^2 using standard eluent.

From Fig. 12 it may also be deduced that, for the longer chain pairing ions, it is preferable to load the column at high concentrations and then decrease the mobile phase concentration to the desired level, again waiting for re-equilibration to occur (see Knox and Jurand[13]).

The different rates of desorption of the three pairing agents are clearly shown in Fig. 13 which establishes that strongly adsorbed hetaerons, such as lauryl sulfate, require almost infinitely large volumes for complete desorption as a consequence of the high curvature and extreme slope of the adsorption isotherm at low concentrations of hetaeron.

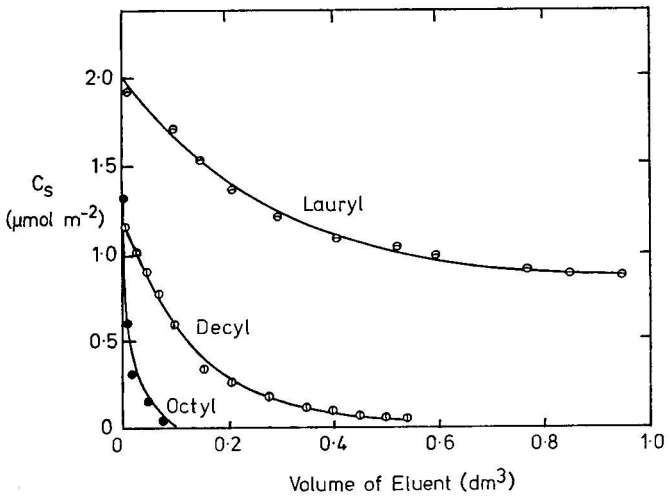


Fig. 13. Desorption of various alkyl sulphates from ODS-Hypersil by standard eluent containing no alkyl sulphate.

The practical consequence of this is that a column is often irreversibly altered if strongly adsorbed pairing ions are used: in this work, for example, we were unable to wash the lauryl ion off the column packing with even pure methanol and isopropanol.

Microelectrophoresis

Microelectrophoresis of ODS-Hypersil was carried out using standard eluents containing various concentrations of octyl sulphate in the range 10 to 20 mM. The results are shown in Fig. 14 from which it may be seen that zeta potential at first rises gradually from about 15 mV and then fairly rapidly when C_s is in the range 1.5 to 2 μmol m^{-2} corresponding to a surface charge of 0.15 to 0.2 C m^{-2}. The zeta potential for ODS-Hypersil in standard eluent containing no octyl sulphate is in agreement with that obtained in a wider ranging study by Knox and Kaliszan[18].

In a recent paper Cantwell and Puon[19] were able to explain the adsorption isotherm of diphenylguanidinium ($DPGH^+$) on Amberlite XAD-2 resin on the basis of the Stern-Gouy-Chapman (SGC) theory of the electrial doublelayer (see for example ref. 20). They showed that the charge density in the inner part of the double layer (the "molecular condenser") was that obtained by assuming that virtually all the adsorbed $DPGH^+$ contributed to the charge. From their data they were able to show that the Outer Helmholtz Plane (OHP) was about 0.4 nm from the surface. Their surface potentials, ψ, calculated from SGC theory and the observed isotherms, ranged from 30 to 120 mV, for activities of $DPGH^+$ from $3 \cdot 10^{-4}$ to $3 \cdot 10^{-2}$ M, and more or less fitted eqn. 12, where α_{DPGH^+} is the activity of $DPGH^+$. However, values

$$\psi = \psi^{\ominus} + (RT/F) \ln \alpha_{DPGH^+} \tag{12}$$

obtained from measurements of zeta potentials were much smaller and showed less variation being in the range 25–40 mV. For this latter calculation it was assumed

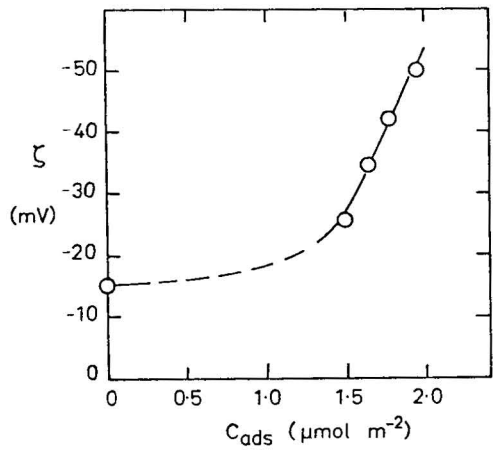


Fig. 14. Zeta potential of ODS-Hypersil as a function of surface concentrations of octyl sulphate for C_m between 10 and 30 mM and $[Na^+] = 50$ mM. All values of ζ are negative.

by the authors that the zeta potential was the same as the potential at the OHP. The discrepancy between these values suggests that this assumption may be fairly seriously in error. This may arise from the porous nature of the particles and the fact that the zeta potential is determined by a surface dividing those ions which on average move with the particle during electrophoresis from those that remain with the surrounding fluid. This particular surface must reside entirely outside the porous particles whereas the OHP will penetrate and suffuse the pores within the particles. The plane defining the zeta potential will therefore separate the inside of the particle from the surrounding fluid by a potential barrier (whose height is given roughly by the zeta potential). The situation is then very similar to that associated with the Donnan membrane equilibrium when specific ions (such as $DPGH^+$) are restricted to one side of a membrane causing a chemical and electrical potential difference for any ions which are not so restricted (*e.g.* Na^+ and Cl^-). To a first approximation it may be appropriate to regard the entry ports to the inside of a porous particle as being protected by a potential barrier which will exclude ions of the same charge as those producing the barrier (negative in the case of alkyl sulphates) and which will welcome ions of opposite charge.

CONCLUSIONS

Eqns. 2 and 7 appear to give a good explanation of the enhancement of retention of ionized solutes arising from addition of hydrophobic ions (hetaerons) to the eluent. It appears that the enhanced retention can be attributed to the adsorbed charge and is to a first approximation independent of the hydrophobic part of the hetaeron. For ions of the same charge as the hetaeron eqns. 2 and 7 clearly do not apply and some form of electrostatic repulsion must be invoked.

At the same time it is clear that the electrical properties of the packing material change considerably as the concentration of adsorbed ions increases. Thus the absolute values of both the surface potential and the zeta potential increase with surface charge.

Since the zeta potential is determined by a surface external to the porous particles its existence should affect the chemical potential of solute ions distributed between the interior and exterior. The degree of retention or exclusion must be related exponentially to the potential rather than linearly. In the case of retention the operation of eqn. 12 produces a linear relationship between retentivity and surface charge and justifies eqns. 2 and 7. However, ions of like charge will become increasingly excluded from the particles so that superimposed on any changes in the partition ratio for similar but uncharged species will be a factor $\exp(-z\zeta F/RT)$, where z is the integral ionic charge and F is Faraday's constant. Typically ζ is of the order of 50 mV which will produce roughly a tenfold reduction in k'. This can, of course, be only a rough approximation (a) because the zeta potential may represent only a proportion of the potential difference between the pore volume and external volume, and (b) because there will be variations of potential within the pores, particularly close to the internal surface arising from the tightly held adsorbed hetaeron ions. Clearly further investigation of ionic exclusion will be necessary before this phenomenon is fully explained.

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GENERATION OF ABSORPTION SITES IN THE β-Ni(NCS)$_2$(4-METHYLPYRIDINE)$_4$ PHASE BY THE MOBILE PHASE COMPONENTS

W. KEMULA*, D. SYBILSKA, J. LIPKOWSKI and K. DUSZCZYK
Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01-224 Warsaw (Poland)

SUMMARY

The chromatographic separation of nitroalkanes, dinitrobenzenes and nitronaphthalenes on a column filled with β-Ni(NCS)$_2$(4-methylpyridine)$_4$ clathrates has been studied. These investigations were followed by an examination of the composition and the structure of the sorbents. It has been found that the influence of organic solvents on the sorption is dependent on the nature and the concentration of the solvent, and on the type of compounds to be separated. An attempt is made to explain these effects in terms of the existence of two types of sorption centres and of a competitive sorption mechanism.

INTRODUCTION

Since the clathrates formed by Werner complexes were first applied in chromatography[1], various isomeric compounds have been separated on these sorbents[2,3]. The chromatographic and physicochemical investigations of the clathrate sorbents have led not only to the understanding of the composition and structure of the sorbents[4,5] but also to the partial understanding of the mechanism of the chromatographic separation processes on the clathrates[6–8].

We have recently found that it is possible to control the chromatographic processes occurring on the clathrates by changing the composition of the mobile phase. However, it remains to be determined whether such changes influence only the dimensions of the cages and of the channels in the structure of the clathrates, or whether there are any effects on the guest molecules.

We present here the results of attempts to solve this problem by examining the influence of an organic solvent (added to the aqueous mobile phase) on the process of elution from clathrate columns of aliphatic and aromatic compounds. The clathrate sorbents were formed from Ni(NCS)$_2$(4-MePy)$_4$, where 4-MePy = 4-methylpyridine.

EXPERIMENTAL

The solvents and all the reagents were p.a. grade and their physicochemical

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constants agreed with those in the literature. The 4-MePy used contained 0.05% of 3-methylpyridine but no 2.5-dimethylpyridine, as detectable by gas chromatography.

Nitroethane, nitropropanes and 1-nitro-*n*-butane were taken as model aliphatic compounds, dinitrobenzenes and 1- and 2-nitronaphthalenes as the aromatic ones.

The basic chromatographic investigations were carried out by means of the chromato-polarographic method and apparatus described by Kemula[9].

The sorbents were prepared from the clathrate $[Ni(NCS)_2(4\text{-}MePy)_4]\cdot(4\text{-}MePy)_{0.7}$ by the method described earlier[6–8]. The columns (30–35 or 60–70 × 5 mm I.D.) contained 300 or 600 mg of the clathrate. The flow-rate was 8 ml/h. The 5–10-μl samples of the solutions to be investigated, about $2\cdot10^{-3}$–$4\cdot10^{-3}$ M, were injected by means of a Hamilton microsyringe. The temperature of the chromatographic column during the experiments was 25 ± 1°C.

Standard formulae[10] were employed for calculation of the values of the distribution coefficient, K, capacity factor, k', and the separation coefficient, α. The mean density of the sorbent was assumed to be 1.25 g/cm^3 in these calculations.

The X-ray measurements were carried out using a Rigaku-Denki (CuK_α) diffractometer. The phase analyses of the sorbents were carried out as described previously[11].

RESULTS

Chromatography

Fig. 1 shows a plot of the logarithm of the distribution coefficient K of nitropropanes and of 1-nitro-*n*-butane *vs.* the logarithm of the concentration of methanol

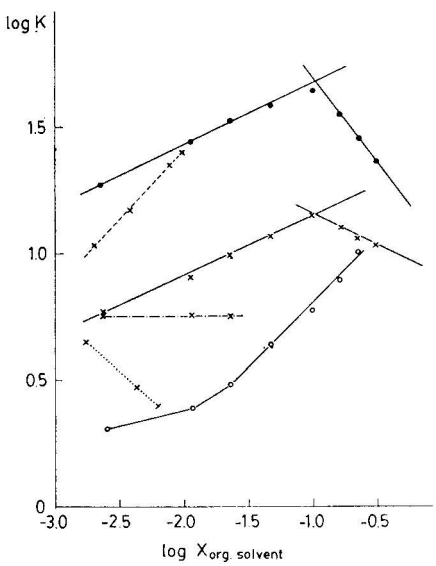


Fig. 1. Plot of log K of 1-nitro-*n*-butane (●), 1-nitropropane (×) and 2-nitropropane (○) *versus* log $X_{org.solvent}$, where $X_{org.\ solvent}$ is the mole fraction of an organic solvent in the aqueous mobile phase solution. Organic solvents: methanol (———); ethyl acetate (– – –); formamide (–·–·–); furfuryl alcohol (· · ·).

in the aqueous mobile phase. The values of concentration are given as mole fractions. The influence of ethyl acetate, furfuryl alcohol and formamide on the distribution coefficient (logarithmic scale), taking 1-nitropropane as an example, is also shown. In each case the mobile phase contained 0.2 mole/l of NH_4SCN, 0.1 mole/l of 4-MePy and various amounts of methanol or of the other investigated solvent.

Table I compares the properties of the clathrate, when contacted with different solvents. The values of the distribution coefficients of nitroethane, nitropropanes and 1-nitro-*n*-butane, determined in the aqueous solutions of the mobile phase, containing 5% (v/v) of different solvents, are shown. The conditions were the same as those corresponding to Fig. 1. The comparatively low solvent concentration [5% (v/v)] was used to avoid too great a variation of the value of the distribution coefficient, caused by the change in solubility.

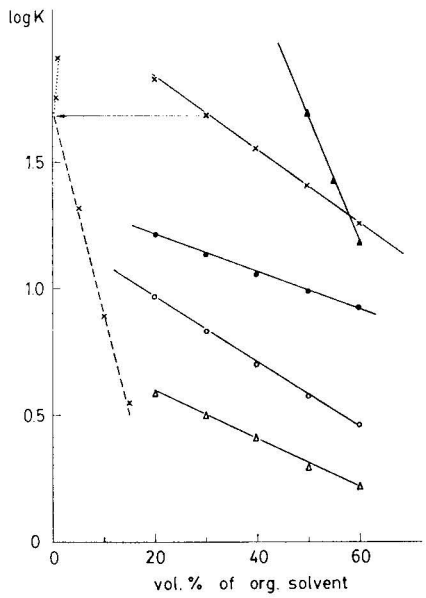


Fig. 2. Plot of log K of *o*-dinitrobenzene (○), *m*-dinitrobenzene (●), *p*-dinitrobenzene (×), α-nitronaphthalene (△) and β-nitronaphthalene (▲) *vs.* % (v/v) methanol in aqueous mobile phase solution. The values of log $K_{p\text{-dinitrobenzene}}$ (×) as a function of % (v/v) ethyl acetate (– – –) and furfuryl alcohol (· · ·) in 30% (v/v) methanol in water are also shown.

Fig. 2 shows a plot of the logarithm of the distribution coefficient K of dinitrobenzenes and nitronaphthalenes *vs.* the concentration of methanol expressed in % (v/v). The influence of ethyl acetate and furfuryl alcohol on the distribution coefficient, taking *p*-dinitrobenzene as an example, is demonstrated. In each case the mobile phase contained 0.1 mole/l of NH_4SCN, 0.2 mole/l of 4-MePy and various concentrations of methanol in water. When other solvents were used, the mobile phases contained 30% of methanol. Methanol was added because of the poor solubility of dinitrobenzenes and nitronaphthalenes in water, which resulted in the very high values of their capacity factor ($k' > 100$) in relation to water.

TABLE I

DISTRIBUTION COEFFICIENTS, K, OF NITROALKANES AND CRYSTAL LATTICE PARAMETERS AND MOLAR VOLUMES OF THE CLATHRATES, β-[Ni(NCS)$_2$ (4-MePy)$_4$]·(n 4-MePy + m org. solv.)

Mobile phase: 0.2 M NH_4SCN + 0.1 M 4-MePy in water containing 5% (v/v) of the given solvents.

Solvent	*K*				*Crystal lattice parameters (Å)*		V_{mol} (cm^3)
	Nitroethane	*2-Nitropropane*	*1-Nitropropane*	*1-Nitro-n-butane*	*a*	*c*	
Formamide	0.5	1.5	5.7	17.0	17.26	23.63	530.0
Dimethyl sulphoxide	0.8	1.9	6.5	20.0	17.29	23.58	530.7
Ethylene glycol	1.1	2.1	7.6	24.0	17.31	23.55	531.3
Methanol	1.2	3.0	9.9	34.0	17.28	23.54	529.2
Ethanol	1.3	3.7	10.9	38.5			
Acetone	1.7	4.2	12.8	39.5			
Diethylene glycol monomethyl ether	2.7	10.2	23.0	68.0	17.34	23.71	536.7
Ethyl acetate	2.1	15.0	25.0	91.0	17.24	23.20	519.1
Pure water	0.5	1.9	5.7	17.5	17.08	23.26	510.9
2% (v/v) Furfuryl alcohol		1.5	3.0	8.0			

Investigations of the solid phases

It has been found that all the column fillings were tetragonal clathrates of the formula β-[Ni(NCS)$_2$(4-MePy)$_4$]·G, where G is a mixture of 4-MePy and the organic solvent. The composition of G depends on the concentration of both components in the mobile phase.

Table I gives values of the lattice parameters and the molar volumes of the sorbents.

DISCUSSION

The investigated clathrates, which comfortably accommodate the molecules of *o*-, *m*- and *p*-disubstituted benzene derivatives, still enable the separation of the isomeric nitroaliphatic compounds, which have very different shapes and volumes.

The results of the present work show that these different selectivities may result from the existence of two types of sorption centres in the crystal lattice of the sorbents. Both centres are generated by the clathrated guest: one by aromatic molecules, such as α-picoline and furfuryl alcohol, and accounts for the sorption of aromatic molecules; the other by molecules of aliphatic solvents such as methanol and ethyl acetate, and is the potential sorption centre for other aliphatic molecules. Thus, when the methanol concentration is first increased there is a corresponding increase of the value of the distribution coefficient of the nitroalkane; also, the plot of log K *vs.* log (mole fraction of methanol) is a straight line, which can be taken as evidence that the new centres are being generated by methanol at the expense of those occupied by 4-MePy, and the capacity towards aliphatic molecules increases. With further increase of the concentration of methanol ($C > 20\%$) the direction of the plot for nitroalkanes reverses. Perhaps in this region the most important processes are the competitive sorption of methanol and the solubilities of nitroalkanes in the mobile phase. The molar volumes of the clathrates can be taken as evidence for this. After the sorbents had been allowed to equilibrate with the mobile phases of different methanol concentrations, the molar volumes were: $V_{10\%} = 519.9$ cm^3; $V_{20\%} = 514.7$ cm^3; $V_{30\%} = 515.2$ cm^3; $V_{40\%} = 512.8$ cm^3; $V_{H_2O} = 510.9$ cm^3. The maxima in the molar volumes of the clathrate sorbents and in the K coefficients for 1-nitro-*n*-alkanes fall approximately in the same range of methanol concentration in the mobile phase.

It should be stressed that only the changes in the composition and structure of the clathrate can be responsible for the increase of the value of K of nitroalkanes in the initial range of concentration of such solvents as methanol, ethanol, acetone and ethyl acetate. Interactions in liquid solution are not responsible, as was confirmed in reversed-phase systems[12].

In the case of furfuryl alcohol, only a rapid decrease of the distribution coefficient is observed for all the investigated nitroalkanes. The molecules of furfuryl alcohol behave in a similar manner to those of methylpyridines, generating centres able to sorb aromatic compounds at the cost of the other centres occupied by an aliphatic solvent or water.

The opposite behaviour is found in the case of aromatic compounds. In methanol the values of the distribution coefficients decrease, *i.e.*, the plot of log K *vs.* % (v/v) methanol is linear. This suggests that the predominant effect is the increase

of the solubility of nitroaromatic compounds in the mobile phase, while the corresponding changes in the structure of the sorbent are not large: there is no competitive sorption, as there are different sorption centres for methanol and for nitroaromatic compounds. Other solvents such as ethanol, acetone, diethylene glycol monomethyl ether, ethylene glycol and ethyl acetate give similar results.

One might expect that in the initial region aromatic compounds should facilitate the sorption of nitroaromatic compounds, generating the appropriate sorption centres. We found this effect in our previous study of the influence of various pyridine bases, which accompany 4-MePy (ref. 13). Also in the case of furfuryl alcohol, we found an initial increase in K for *p*-dinitrobenzene (Fig. 2).

It should be noted here that organic solvents influence not only sorbent capacity but also selectivity. The changes of the retention of 1- and 2-nitropropanes caused by methanol are a good example of this (Fig. 1).

Some support for the above conclusions can be obtained from the results of the phase analysis of the sorbent. From an examination of the structure, two kinds of holes can be distinguished[14]. There is a lack of structural data concerning the occupation of these holes by the differently shaped guest molecules. However, the changes of the lattice parameters (Table I) and the molar volume indicate that the solvents are clathrated: the composition of G changes, but it is difficult to find any simple relation between it and the retention of the investigated compounds.

It appears that the chromatographic investigation of the sorption in clathrates, under appropriate conditions, can be very effective for the detection of minute changes of the structure and the occupation of the holes; this requires low concentrations of a guest, to avoid distortion of the host structure.

ACKNOWLEDGEMENT

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GROUP SEPARATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RAPID DETERMINATION OF REMAINING ALKANES IN ALKANOLS

W. ECKNIG*

Zentralinstitut für physikalische Chemie der Akademie der Wissenschaften der D.D.R., Rudower Chaussee 5, Berlin-Adlershof (D.D.R.)

K. METZNER

VEB Chemische Werke Buna, Schkopau (D.D.R.)

and

I. NEHLS and Z. STANKE

Zentralinstitut für physikalische Chemie der Akademie der Wissenschaften der D.D.R., Rudower Chaussee 5, Berlin-Adlershof (D.D.R.)

SUMMARY

The dependence of the group separation of *n*-alkanol–*n*-alkane mixtures using reversed-phase systems on both the water content of the methanol used and the chain length of the alkyl groups chemically bonded on the silica surface has been investigated. Considering the experimental results in terms of the ratio $\log \alpha_{21}/\Delta \log k'_{CH_2}$, it seems to be possible to choose group separation conditions systematically. Low contents of water together with highly inert silica substituted by short carbon chains seem to favour group separation. The conditions elaborated permit the determination of the sum of remaining alkanes using a refractive index detector.

INTRODUCTION

As a result of increasing efforts to achieve the more intensive utilization of the high-boiling hydrocarbons contained in petroleum or coal products there is increasing interest in group separations by modern chromatographic methods[1–5]. In order to solve such separation problems, in addition to size-exclusion chromatography[6] adsorption liquid chromatography[7] has mainly been applied. However, reversed-phase (RP) systems have been less investigated for the separation of groups of homologous substances from complex mixtures. Nevertheless, the application of reversed-phase systems can be interesting in view of the sensitive refractive index detection of pure and substituted alkanes, using highly polar mobile phases, *e.g.*, methanol–water mixtures.

The investigations described here were carried out in order to obtain a more systematic choice of the reversed-phase chromatographic conditions suitable for group separation.

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THEORETICAL

In order to achieve an efficient separation of two groups of homologous substances a chromatographic system should be characterized by a high alkane–alkanol selectivity, α_{21}, but low methylene group increments, $\Delta k'_{CH_2}$, in order to separate the groups as effectively as possible but to elute each series of homologue within a small retention interval; α_{21} is the separation factor of the two species, alkanol (1) and alkane (2), with equal chain lengths, n, but different functional groups X1 (C–OH) and X2 (C–H), respectively. Therefore, a high value of the ratio

$$\frac{\log \alpha_{21}}{\Delta \log k'_{CH_2}} = \sigma_G \tag{1}$$

is required for a chromatographic system to yield an effective group separation.

Additivity of group contributions to the retention can also be assumed in reversed-phase chromatography[9]. Therefore, the σ_G relationship can be written in terms of group contributions of the free energy:

$$\frac{\Delta G^0_{X2} - \Delta G^0_{X1}}{\Delta G^0_{CH_2}} = \sigma_G \tag{2}$$

According to eqn. 2, both low hydrophobic interactions and small interactions of the CH_2 groups should be favoured group separations of an alkane–alkanol mixture.

EXPERIMENTAL

The measurements were carried out by means of the Hewlett-Packard 1010 A high-pressure liquid chromatograph, fitted with an LDC 1107 differential refractometer detector, at ambient temperature. SGE high-pressure syringes were used for sampling.

Each alcohol was injected pure or mixed with the alkane of the same chain length. The injection of pure alkanes yielded asymmetric peaks, whereas symmetric peaks were obtained if the alkane-alkanol mixtures were injected. The columns (stainless steel, 250 × 4 mm I.D.) were packed using the fast filtration technique.

The polymeric reversed phases RP-1 and RP-4 were prepared according to Hastings *et al.*[10]. RP-6 and RP-16 were synthesized by Dr. Tänzer (Merseburg) and Dr. Eppert (Leuna), respectively. The chemical modification was monitored by IR spectroscopy and elemental analysis. The other products were brush-type reversed phases. RP-2, RP-8 and RP-18 were commercial materials (Merck, Darmstadt, G.F.R.).

The sum of remaining alkanes was determined quantitatively in groups of n-alkanols with similar chain length ($n \pm 2$). Calibration was carried out by constructing a calibration graph for a representative alkane of medium chain length in the sample. On determining the peak area by planimetry, the relative standard deviation of the results was about 5%. Better results should be obtained if electronic integration is used.

RESULTS AND DISCUSSION

In order to examine the dependence of the group separation on the chromatographic conditions, experiments were carried out by changing both the eluent composition and the type of chemically modified silica. The results in Fig. 1 and Table I are given in terms of the σ_G ratio corresponding to eqn. 1.

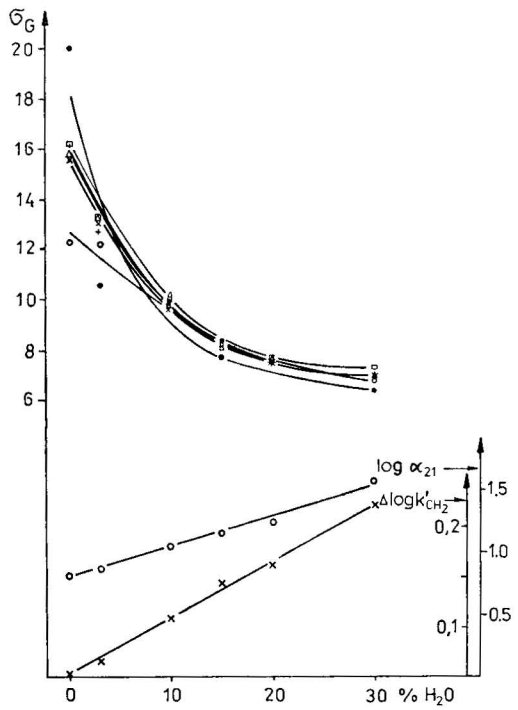


Fig. 1. Influence of the water content in methanol on σ_G ratio. Length of the carbon chain: upper curves: ●, C_6; ○, C_9; ×, C_{11}; +, C_{13}; △, C_{15}; □, C_{17}; lower curves: ○, C_{11}; ×, average C_6 to C_{17}.

Fig. 1 demonstrates the strong influence of the hydrophobic nature of the eluent on the σ_G ratio. The stationary phase used was RP-4. As expected, an increase in the content of water in the methanol gives an increase in both the selectivity ($\log \alpha_{21}$) and the contributions of the alkyl groups ($\Delta \log k'_{CH_2}$) to the retention of the aliphatic species. However, because the $\Delta \log k'_{CH_2}$ values increase twice as fast as $\log \alpha_{21}$, the σ_G ratio decrease rapidly up to a content of 15% (v/v) of water. Higher contents of water give only small alterations in σ_G, because the relative change becomes smaller if the absolute values of α_{21} and $\Delta k'_{CH_2}$ increase.

Accordingly, in order to achieve efficient group separations methanol containing only small contents of water should be applied. The dependence of σ_G values on the type of chemically modified silica from RP-1 to RP-18 is shown in Table I. The relative retentions, α_{21}, increase with increase in both the percentage of carbon bonded to the silica and the carbon chain length, as expected.[11] In comparison, the methylene group increments, $\Delta k'_{CH_2}$, seem to be nearly independent of the carbon content, although dependent on the length of the carbon chains.

TABLE I

SEPARATION OF HOMOLOGOUS *n*-ALKANOLS AND *n*-ALKANES ON CHEMICALLY MODIFIED SILICA

Eluent: methanol–water (97:3). 1 = *n*-Dodecanol; 2 = *n*-dodecane.

Stationary phase	*Carbon content (%, w/w)*	*RP*	*log* α_{21}	$\Delta \log k'_{CH_2}$	σ_G
Si-methyl*	19.0	1	0.696	0.064	10.9
Si-butyl*	16.3	4	0.863	0.066	13.1
Si-ethyl	6.4	2	0.538	0.055	9.8
Si-butyl**	8.1	4	0.33	0.05	6.6
Si-hexyl	10.2	6	0.63	0.09	7
Si-octyl	13.1	8	0.568	0.086	6.6
Si-decyl**	14.8	10	0.49	0.75	6.5
Si-hexadecyl	16.9	16	0.651	0.149	4.4
Si-octadecyl	22.1	18	0.773	0.252	3.1
Si-octadecyl**	22.3	18	0.75	0.135	5.5

* Polymer type.

** Eluent: pure methanol (data from ref. 8).

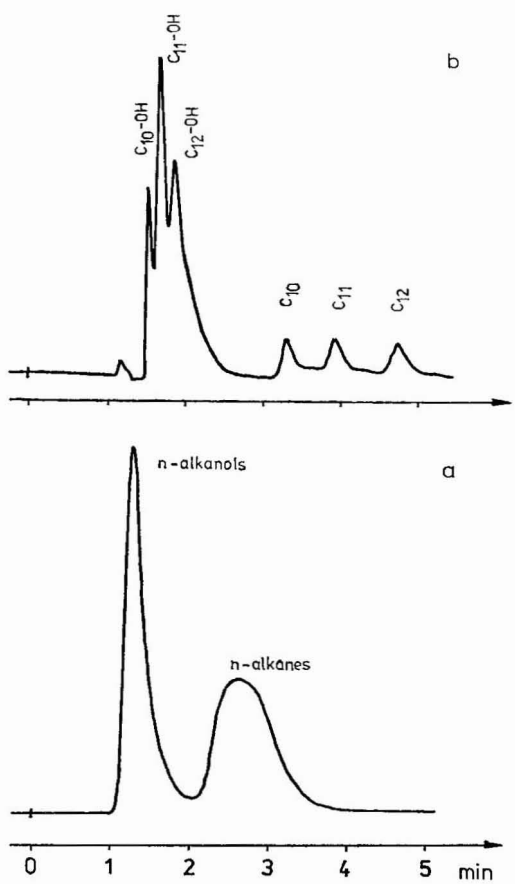


Fig. 2. Influence of the bristle length on the group separation of *n*-alkanols–*n*-alkanes using methanol–water (97:3) as eluent. (a) RP-4 (polymer, flow-rate 0.34 cm/sec, pressure 4.6 MPa; (b) RP-18, flow-rate 0.34 cm/sec, pressure 3.5 MPa.

However the log α_{21} values vary only by a factor of 1.33, whereas the contributions of the CH_2 groups, $\Delta \log k'_{CH_2}$, change systematically by a factor of 5. Therefore ,the σ_G ratio decrease significantly from RP-1 to RP-18, owing primarily to this strong increase in the interaction of the alkyl groups. As confirmed by Fig. 2, it can be concluded that reversed phases characterized by short alkyl chains yielding more efficient group separations than those substituted by long carbon chains.

From Fig. 2, in addition to the higher carbon content, the high α_{21} values may also be caused by a greater inertness of the polymers in comparison with the brushes, yielding lower retentions of the alkanols.

CONCLUSION

In group separation of *n*-alkanol–*n*-alkane mixtures, the possibility of improving the separation factor, α_{21}, by changing the chromatographic conditions seems to be limited, because higher values of α_{21} are connected with higher contributions of the CH_2 groups, which make the group separation worse by spreading the homologous species. Therefore, the contributions of the CH_2 groups to the retention must be kept as small as possible by selecting an eluent involving only weak hydrophobic properties. These eluents must be combined with reversed phases, which offer a low surface area for the dispersion interactions between the CH_2 groups of the sample molecules and the organic layer of the modified silica. In order to separate a group of strongly polar species, for example alkanols, from weakly polar species, polymeric modified silica seems to provide some adventages. Together with further systems investigated in our laboratory, these results offer some general conclusions for the systematic choice of chromatographic systems that yield more efficient group separations of hydrocarbons (for example, by using fluorocarbon-substituted silica). Further basic experimental and theoretical investigations have been carried out.

Using the conditions established here, in analytical practice alkanes can be separated from alkanols in 3–5 min. The reversed-phase system used involves methanol modified by 1–3% of water as eluent, and silica substituted by short alkyl chains. The remaining alkanes can be determined quantitatively in the technically required range down to 0.1% (w/w) using a refractive index detector.

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CHROM. 13,272

EQUILIBRIUM DISPLACEMENT IN A CHROMATOGRAPHIC REACTOR

B. MILE*, L. MORTON and P. A. SEWELL

Department of Chemistry and Biochemistry, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF (Great Britain)

SUMMARY

The catalytic dehydrogenation of cyclohexane to benzene ($C_6H_{12} \rightleftharpoons C_6H_6 + 3H_2$) has been studied in a gas chromatographic reactor. An enhancement of product yield above equilibrium has been observed and direct evidence for equilibrium displacement obtained by the observation of a maximisation of yield at an optimum flow-rate. This optimisation is a result of the effects of flow-rate on peak broadening by longitudinal diffusion, an aspect which has been omitted in theoretical treatments of chromatographic reactors.

Further studies with a longer reactor showed considerably greater yields but no maximisation. In this case three product peaks were obtained. However, it has been shown that all three peaks are in fact benzene and not benzene pre-cursors as previous workers have assumed. A study of the decomposition of such precursors, *e.g.* cyclohexane and cyclohexa-1,3-diene has been made and the mechanistic and chromatographic implications of this have been discussed.

INTRODUCTION

The yields of useful products from conventional flow reactions are ultimately limited either by the value of the equilibrium constant or by the consumption of primary products by secondary reactions. By combining in one reactor both catalytic and chromatographic functions and by pulsing the reactants both of these limitations can be removed. The back reaction can be minimised by chromatographically separating the products from each other, while secondary reactions may be reduced by retaining the primary product on the column relative to the reactant pulse.

Several experimental and theoretical studies of equilibrium displacement on small bore columns have been made[1–8], but the evidence of displacement is equivocal. The comparison between experimental yield and estimated equilibrium yield involves many assumptions, and in addition there are unusual and unexplained features in some of the reported experiments (*e.g.* the effect of the ratio of pulse size to column length on conversion)[2].

The careful study of the dehydrogenation of cyclohexane to benzene reported here provides more direct evidence for displacement, and is in accord with the theoretical predictions of a maximisation by Gore[8] who used digital methods to treat

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the effects of finite equilibrium rates on the extent of displacement. However, we believe the maximisation is the result of a chromatographic aspect which Gore specifically did not include in his model.

EXPERIMENTAL

The catalyst chromatographic bed used was 0.72% platinum on silica (chromatography grade; Woelm, Eschwege, 6.F.R.) and was prepared by the "ammine complex" method of Dorling and Moss[9]. The BET surface area was 525 $m^2\ g^{-1}$ and the platinum crystalite size was *ca.* 1.5 nm.

The apparatus was home-made, based on a Pye Unicam "Research" chromatograph. Detection was by a Taylor-Servomex micro-katharometer and the peak areas were measured with a Hewlett-Packard Model 3373B electronic integrator.

The reactor was constructed from 4 mm diameter glass tubing and consisted of two sections: the catalyst section contained the platinum/silica catalyst and was varied in length from 0.06 to 1.0 m; the analytical section was 0.30 m and was packed with silica (Woelm).

The temperature of the two sections could be varied independently; that of the catalyst section, requiring precise control, was achieved to better than $\pm 0.5°C$ by using an air thermostat linked to a Mini-Therm proportional controller as a temperature trimmer. The temperature of the analytical section was controlled by the Pye oven controller. Helium was used as carrier gas after removed of impurities by passage through a one metre tube of silica. It was found that the degree of conversion was very sensitive to impurities in the carrier gas and to impurities inadvertently admitted to the system. To prevent this, and also to reduce the build up of carbonaceous matter on the catalyst, a stream of helium was maintained through the column at all times. Even with this precaution it was found necessary to reactivate the catalyst at intervals by further reduction in hydrogen.

The reactant was pulsed into the reactor using a microlitre syringe or a gas sampling valve (Pye-Unicam).

In some experiments it was necessary to investigate the integrity of the emergent chromatographic peak. This was done by collecting the emergent peak in a capillary tube packed with glass wool, extracting the sample with toluene and injecting into a Perkin-Elmer Model F.17 analytical chromatograph with a flame-ionization detector.

RESULTS AND DISCUSSION

The degree of conversion, α

The degree of conversion, α, of cyclohexane to benzene was usually determined from the benzene and cyclohexane peak areas after sensitivity corrections had been made,

$$\alpha = \frac{\text{benzene peak area}}{\Sigma\ \text{benzene} + \text{cyclohexane}} \tag{1}$$

Although a hydrogen peak (negative) was usually observed its calibration and measument was more prone to error. A check on carbon balance was made routinely by

adding *n*-pentane as an internal standard (*n*-pentane is inert under the present conditions) to the cyclohexane. This showed that all the cyclohexane lost appeared as benzene (to within 2%) and thus allowed us to use eqn. 1 for α determination and hence dispense with the need for the internal standard for most experiments.

Theoretical equilibrium yields

Both the presence of inert gas (helium) and pressure affect the equilibrium yields. It can be shown for the cyclohexane dehydrogenation reaction that

$$\frac{K_p(1-\alpha)}{27p^3} = \frac{\alpha^4}{\left\{3\alpha + 1 + \frac{n_{He}}{n_0}\right\}^3} \tag{2}$$

where K_p is the equilibrium constant, p is the total pressure and n_{He}/n_0 is the ratio of the number of moles of helium to number of moles of benzene in the pulse.

K_p was calculated from the free energy functions, for benzene, cyclohexane and hydrogen at the relevant temperature; the pressure p was measured at the reactor inlet, (the correction for pressure drop in the reactor was trivial); the ratio n_{He}/n_0 was calculated from the width of the cyclohexane peak at half peak height corrected for band broadening in the analytical column, together with the flow-rate and pressure to determine n_{He}. This gives an average value of n_{He}/n_0 in the reactor. Equilibrium values of α (α_{eq}) were obtained by graphical and numerical solutions of eqn. 2. It is of interest to note that the effect of the inert gas can give quite high values of α even when the equilibrium constant is very low (*e.g.* K_p at 160°C is $3.1 \cdot 10^{-6}$ atm^3 but α is still 0.098).

Effects of temperature and flow-rate on the theoretical and experimental value of α

A typical chromatogram from a 1-μl cyclohexane pulse is shown in Fig. 1a.

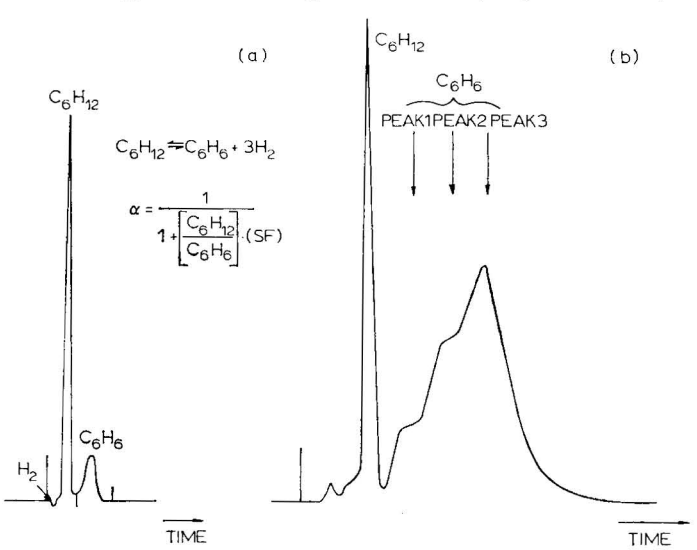


Fig. 1. Typical chromatograms from the chromatographic 0.06-m (a) and 1.0-m (b) reactors.

Values of the degree of conversion (α) at 160, 180, 200 and 240°C calculated from such chromatograms were measured with a standard deviation of less than 2%. The results for the short bed reactor (0.06 m) are shown in Fig. 2 together with the equilibrium values at 160, 180 and 200°C calculated from eqn. 2.

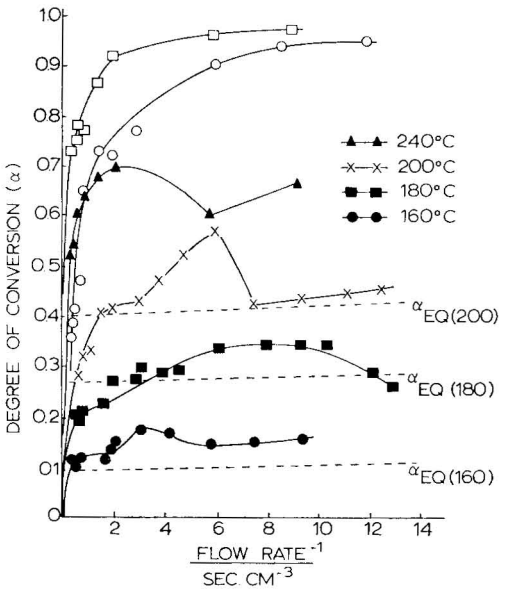


Fig. 2. Degree of conversion (α) and α_{EQ} as a function of flow-rate and temperature. Open symbols: results from the 1.0-m reactor; closed symbols: results from the 0.06-m reactor. *Note*: the 180, 200 and 240°C curves (0.06-m reactor) have been displaced upwards by 0.1 unit of α for clarity.

There are two features of interest, the occurrence of a maximisation of α at certain flow-rates and the fact that this maximum is well above (*ca.* 50%) the equilibrium conversion. Both are indicative of the displacement of the equilibrium by the chromatographic process and it appears to us that the occurrence of the maximum at an optimum flow-rate provides unequivocal proof of such a displacement since no theoretical estimates and experimental results are being compared and we can see no way of explaining the maximum in terms of equilibrium conditions on the column.

We are undertaking a full quantitative analysis of the system by solving the differential equations involving flow and reaction using numerical methods but the effects of flow-rate on α can be discussed semiquantitatively as follows: The displacement of equilibrium by reduction of the back reaction will be favoured by minimising the overlap between the product benzene and hydrogen peaks. Thus the effect of flow-rate on the displacement is essentially a chromatographic effect until the flow-rates become so large that short residence times coupled with finite reaction rates reduce conversions even to below the equilibrium value. The spatial separation between the band centres of the benzene and hydrogen peaks, Δz, is given by eqn. 3 and will be independent of flow-rate to a first approximation.

$$\Delta z = L[1 - k_{H_2}/k_B] \tag{3}$$

where L is the column length, and k_{H2} and k_B are the partition coefficients for hydrogen and benzene, respectively.

The width of the bands, Δx, will increase as a result of axial diffusion and will be given by eqn. 4

$$\Delta x = \sqrt{\sigma^2 + \frac{2DL}{uk_{H_2}}} \tag{4}$$

where σ is the initial band width, u is the linear velocity of the carrier gas and D the diffusion coefficient for the relevant component in the carrier gas. Because the pressure increases with increasing flow-rate the diffusion coefficient will decrease somewhat with increasing flow-rate. The relationship between D and the flow-rate is of the form $D = A(1 + Cu)^{-1}$, where A and C are constants. The value of σ will increase with increasing flow-rate. Adding all these functions together gives eqn. 5 where B is a constant.

$$\Delta x = \sqrt{Bu + \frac{AL}{u(1 + Cu)k_{H_2}}} \tag{5}$$

Experimentally we find that Δx is approximately proportional to $u^{-\frac{1}{2}}$ for the cyclohexane peak indicating that the effect of diffusion and initial band widths are small compared with axial diffusion in the reactor, *i.e.* $\Delta x \propto u^{-\frac{1}{2}}$. Th overlap between the benzene and hydrogen peaks will be a function of the separation of the band centres Δz and the peak widths Δx. Since Δz is independent of flow-rate the overlap will only show a flow-rate dependence because of its effects on peak width Δx. An increase in flow-rate results in a reduction in Δx, less overlap and hence more displacement of the equilibrium.

Of course at very high flow-rates two kinetic features become important. Firstly at high flow-rates the rates of adsorption and desorption will be two low for phase equilibria to occur and secondly the rates of the dehydrogenation steps will become too low for the full benefit of displacement to acrue. The occurrence of these two kinetic limitations may explain the occurrence of a knee on the curves shown in Fig. 2 rather than a maximum dropping without interruption to zero conversion.

It is of interest to note that the maximum observed arises because of axial diffusion. Although a similar maximum has been predicted by Gore[8] who modelled such systems using an analogue computer we are unable to discover the physical basis of the maximum in Gore's work since he specifically ignores the effects of axial diffusion.

Effect of the length of the reactor bed

As shown in Fig. 2 the degrees of conversion are markedly increased in the 1-m bed compared with the 0.06-m bed, values of α of 0.9 to 1 being obtained at flow-rates below 10 ml min^{-1}. Such an increase in displacement is expected because the peak separation Δz increases with L while the peak broadening Δx increases only as $L^{\frac{1}{2}}$. Thus in the longer bed lengths peak overlap is reduced to zero at the reactor outlet even at very slow flow-rates. The effects of peak broadening which result in a

maximum in α in the small reactor are now insignificant compared with the peak separation. The fall in α at high flow-rate is again a result of the two kinetic effects.

The complex nature of the benzene peak in the long reactor

A typical chromatogram from the reactor shown in Fig. 1b is seen to have three poorly resolved peaks following the cyclohexane peak. We, like other workers, thought that these were benzene and its two precursors, cyclohexane and cyclohexa-1,3-diene. However, when the three peaks were collected and reanalysed on the analytical chromatograph we found that only one peak, that of benzene, was observed under conditions where the four C_6 hydrocarbons were readily resolved. Thus the three peaks emerging from the 1-m chromatographic reactor are all due to benzene.

Our tentative explanation of this unusual phenomenon is in terms of three processes leading to benzene at three different rates. These could involve three different types of catalytic site or three different routes although the occurrence of two peaks in addition to benzene does suggest that the two benzene precursors, cyclohexene and cyclohexa-1,3-diene are involved. The retention time of pure benzene injected into the reactor corresponds to the last and major peak. The route to this peak must be fastest so that the benzene is formed early in the bed and then behaves chromatographically like an injected pulse of benzene. The first peak after cyclohexane is due to benzene formed by the slowest process while the mid peak is formed at an intermediate rate. In summary we believe that three distinct kinetic processes lead to the formation of three separate bands of benzene at three different locations in the reactor and that these three bands are eluted as three distinct but poorly resolved peaks.

We have undertaken a careful study of the dehydrogenation of cyclohexene and cyclohexa-1,3-diene in the same reactors and have found these to react muxh more rapildy than cyclohexane (even at temperatures as low as 50°C) and to result in disproportionation. Thus is the case of cyclohexene, cyclohexane and benzene are formed and at sufficiently slow rates the ratio is exactly 2:1 as expected from the stoichiometry of the reaction

$$3C_6H_{10} \rightarrow 2C_6H_{12} + C_6H_6 \qquad \text{(a)}$$

In the case of the diene, cyclohexane, cyclohexene and benzene are formed. At sufficiently long retention times no cyclohexene is observed and the ratio of cyclohexane to benzene is now 1:2 as expected from the reaction

$$3C_6H_8 \rightarrow 2C_6H_6 + C_6H_{12} \qquad \text{(b)}$$

The free energy changes for these reactions are so large ($>$150 kJ/mol) that they are the only reactions that need to be considered. Tentatively we suggest that the three peaks involve three reaction pathways: (1) direct rapid conversion of cyclohexane to benzene and hydrogen (peak 3), (2) conversion to cyclohexene followed by reaction a and then further dehydrogenation of cyclohexane to benzene (peak 1), (3) conversion to cyclohexadiene followed by reaction b, again followed by cyclohexane dehydrogenation to benzene (peak 2).

The results in the long column certainly show that considerable caution needs to be exercised in the interpretation of chromatograms where reactions are occurring and which can involve several precursors.

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REVERSED-PHASE LIQUID–LIQUID COLUMN CHROMATOGRAPHY OF ORGANIC ACIDS WITH A COMPLEXING STATIONARY PHASE CONTAINING TRI-*n*-OCTYLPHOSPHINE OXIDE

H. W. STUURMAN*, K.-G. WAHLUND and G. SCHILL

Department of Analytical Pharmaceutical Chemistry, Biomedical Center, University of Uppsala, Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

A reversed-phase system for the liquid chromatographic separation of hydrophilic organic acids was developed. A solution of a strong hydrogen acceptor, tri-*n*-octylphosphine oxide, in *n*-decane was coated on LiChrosorb RP-8 as support, and aqueous buffer solutions were used as mobile phases. Three methods for loading the stationary phase on to the support were tested. The retention of the solutes can be described by a liquid–liquid distribution model, based on complex formation between the solutes and the hydrogen acceptor in the stationary phase. The retention and the separation selectivity can be regulated by the concentration of the hydrogen acceptor in the stationary phase and by the pH of the mobile phase.

INTRODUCTION

Hydrophilic acids are difficult to retain in reversed-phase liquid–liquid chromatography, both as acids and as ion pairs. A considerable increase in retention can be obtained by addition of a complexing agent, *e.g.* a strong hydrogen acceptor, to the stationary organic phase. The enhancement of the degree of extraction of carboxylic acids in liquid–liquid extractions after the addition of tri-*n*-octylphosphine oxide (TOPO) to the organic solvent has been studied[1]. In the present study, we used TOPO in the stationary phase of liquid–liquid chromatographic systems for the separation of organic acids of physiological importance.

Complexing stationary phases have been extensively used in the reversed-phase liquid–liquid chromatography of metal ions, often termed extraction chromatography[2]. The complexing agents have been neutral compounds, such as TOPO, or charged compounds such as long-chain amine salts and quaternary ammonium salts ("liquid ion exchangers"). The stationary phase has been either the pure complexing agent or a solution of it in an organic diluent, and the mobile phases have been aqueous solutions.

These reversed-phase systems have been applied in column chromatography as well as in paper and thin-layer chromatography, and have been used mainly for

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the separation of ionic compounds. Applications to the separation of organic substances seem to be few. Some compounds of biochemical origin[3], and sulphonic acids[4], were separated in columns with a "liquid ion exchanger" in the stationary phase. Similarly, steroidal glucosiduronic acids were separated by paper chromatography[5]. Aminophenols were separated with a stationary phase of di-(2-ethylhexyl)-phosphoric acid (HDEHP) in chloroform[6]. Rapid separations of sulphonic acids, carboxylic acids and phenols were obtained by high-pressure column chromatography with tri-*n*-octylamine as the stationary phase[7].

Up to now, most of the column chromatographic work has been done with supports of large particle diameter, which give columns of relatively low efficiency. However, Kraak and Huber[7] used a 5–10-μm support, which was dry packed after coating with the stationary phase. Horwitz *et al.*[8,9] made very efficient columns for the separation of radioactive metal ions by coating HDEHP dissolved in dodecane on a 5-μm support, the coated support being slurry packed (suspended in the eluent) by a rather specialized technique. Modern microparticulate liquid chromatographic supports are usually slurry packed[10] suspended in organic liquids that are miscible with an organic stationary phase. This means that the stationary phase has to be coated on the already packed column by *in situ* methods. This has been done with the neutral extractant tributyl phosphate, which was coated as the undiluted liquid on a 5-μm support for the separation of carboxylic acids[11] and catecholamines[12].

In the present work, we have been able to apply, by *in situ* coating, a stationary phase consisting of a solution of the neutral organophosphorus extractant TOPO in a hydrocarbon; the support was a hydrophobic microparticulate silica. We report the retention of aromatic carboxylic acids and phenol. One of the advantages of this separation system is that it can be extended to other types of complexing agents dissolved in the stationary phase, whereby the separation selectivity can be changed and different types of solutes can be retained. Further, the retention can be varied within wide limits by altering the concentration of the complexing agent.

CHROMATOGRAPHIC SYSTEM

Our system has LiChrosorb RP-8 (7 μm) as support, a solution of TOPO in *n*-decane as stationary phase and aqueous buffer solutions with different pH (ionic strength 0.1) as mobile phase. The columns are of length 150 mm and I.D. 4.6 mm.

COATING OF THE SUPPORT WITH STATIONARY PHASE

Three techniques were tested for the coating of the support with the stationary phase: pumping, injection and precipitation.

In the first method (*cf.* ref. 13), the column is equilibrated with the stationary phase by pumping (*ca.* 50 ml); the mobile phase is then pumped through the column until the excess of stationary phase is removed. With decane as stationary phase, it took about 1 week before the system was stable. The amount of stationary phase deposited in the pores of the support was very reproducible, but the method was limited to stationary phases with relatively low viscosity.

Injection of small volumes of stationary phase while the mobile phase was

being pumped (*cf.* ref. 11) was a rapid coating technique. It gave, however, unstable systems with decreasing capacity ratios and a much lower retention than systems prepared by the pumping method.

In the precipitation method (*cf.* ref. 14), the column was first equilibrated with a solution of the stationary phase in acetone (3:1). The mobile phase was then pumped through the column; the acetone dissolved and the stationary phase was precipitated on the support. With this method, only a low degree of coating could be obtained.

A fourth method, based on adsorption of the stationary phase from the mobile phase[15], was not usable, as the solubility of the stationary phase in the eluent was too low.

The results presented below were obtained on columns prepared by the pumping method. So far, the columns have been used only over short periods (not more than 10 days). The systems were stable with respect to the volume of the mobile phase (V_m). Thus, the phase ratio (V_s/V_m) can be considered constant (V_s = volume of stationary phase). The capacity ratios changed by less than 1% per day.

RETENTION

Model

When an acidic solute (HX) is retained by distribution between an aqueous mobile phase and a stationary organic liquid phase containing a complexing agent (TOPO), the capacity ratio can be expressed by:

$$k'_{HX} = \frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n} \cdot [TOPO]_o^n}{(1 + K'_a/a_{H^+})} \quad (1)$$

(The subscript o refers to the organic phase; symbols without subscript relate to the aqueous phase.)

where $K_{D(HX)} = \frac{[HX]_o}{[HX]}$ is the distribution constant, and $K_{HXTOPO_n} =$

$$= \frac{[HXTOPO]_o}{[HX]_o \cdot [TOPO]_o^n}$$

is the formation constant of the complex between the solute and the complexing agent. The number of TOPO molecules in the complex is n. The model is based on the assumption that one single complex is formed, which contains as many TOPO molecules as the number of hydrogen-donating groups in the solute.

According to this model, the retention can be regulated by (1) the nature and concentration of the complexing agent and (2) the pH of the aqueous phase.

Results

The effect of TOPO in the stationary phase is demonstrated in Fig. 1A. A linear relationship is found between log k'_{HX} and log $[TOPO]_o$. This is in accordance with eqn. 1, which can be transformed into:

$$\log k'_{HX} = \log K + n \cdot \log [TOPO]_o \quad (2)$$

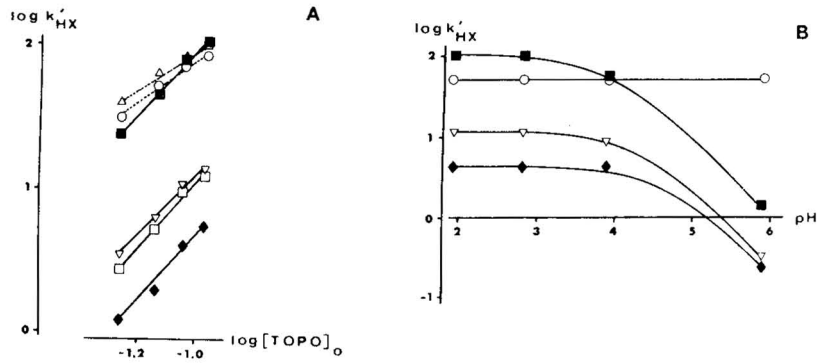


Fig. 1. Influence of TOPO (A) and pH (B) on the retention. Solutes: △, benzoic acid; ○, phenol; ■, 3-hydroxybenzoic acid; ▽, 3-hydroxy-4-methoxybenzoic acid; □, 4-hydroxyphenylacetic acid; ♦, 5-hydroxyindole-3-acetic acid. A: Stationary phase, TOPO in decane; eluent, formate buffer solution of pH 3.9. B: Stationary phase, 0.1 *M* TOPO in decane; eluents, phosphate and formate buffer solutions.

in which

$$K = \frac{V_s}{V_m} \cdot \frac{K_{D(HX)} \cdot K_{HXTOPO_n}}{(1 + K'_a/a_{H+})}$$

The slope of the line represents n, and $K_{D(HX)} \cdot K_{HXTOPO_n}$ can be evaluated from the intercept using the known values of a_{H+} and K'_a.

The results are presented in Table I, which also gives results obtained by batch-extraction experiments with the same liquid phases. There is a fairly good agreement between the values of n and of log $K_{D(HX)} \cdot K_{HXTOPO_n}$ obtained by the chromatographic and by the batch-extraction experiments, indicating that the retention is mainly governed by liquid–liquid distribution. The found and the expected values of n also agree well. For benzoic acid and phenol, each containing one hydrogen-donating group, n is close to unity, whereas it rises to *ca.* 2 when a phenolic and a carboxylic group are present. The indole function in 5-hydroxyindole-3-acetic acid, which might be weakly hydrogen donating, does not affect the value of n.

TABLE I

COMPLEX FORMATION WITH TOPO

Chromatographic conditions: $V_s/V_m = 0.7$; pH 3.9.

Solute	*log* $K_{D(HX)} \cdot K_{HXTOPO_n}$		*Number of TOPO molecules in complex (n)*		
	Chrom.	*Batch*	*Chrom.*	*Batch*	*Expected*
5-Hydroxyindole-3-acetic acid	3.14		2.3		2
4-Hydroxyphenylacetic acid	3.59	3.56	2.2	2.0	2
3-Hydroxy-4-methoxy-benzoic acid	3.41		2.0		2
3-Hydroxybenzoic acid	4.54	4.81	2.2	2.0	2
Benzoic acid	3.64		1.3		1
Phenol	3.46		1.4		1

The retention and the separation selectivity depend on the complex formation constants and on n.

Fig. 1B shows the relationship between the retention of the acids and the pH of the mobile phase. The carboxylic acids are protolyzed on increase of pH, and, in accordance with the model, the capacity ratio then decreases. The retention of the weak acid phenol ($pK'_a = 9.9$) is not affected in the pH region studied. The pK'_a values of the solutes can be estimated from the retention data in Fig. 1B by use of equation 1. The values found (Table II) are in fairly good agreement with the literature values.

Further details on the chromatographic system and on the liquid–liquid distribution of organic acids with TOPO in the organic phase will be given in forthcoming papers.

TABLE II

PROTOLYSIS OF SOLUTES

Temperature: 25°C. Ionic strength: 0.1. The literature values are corrected for the ionic strength.

Solute	pK'_a	
	Chrom.	*Literature*[16]
5-Hydroxyindole-3-acetic acid	4.65	—
4-Hydroxyphenylacetic acid	4.38	—
3-Hydroxy-4-methoxybenzoic acid	4.35	4.38
3-Hydroxybenzoic acid	4.07	3.98
Phenol	—	9.90

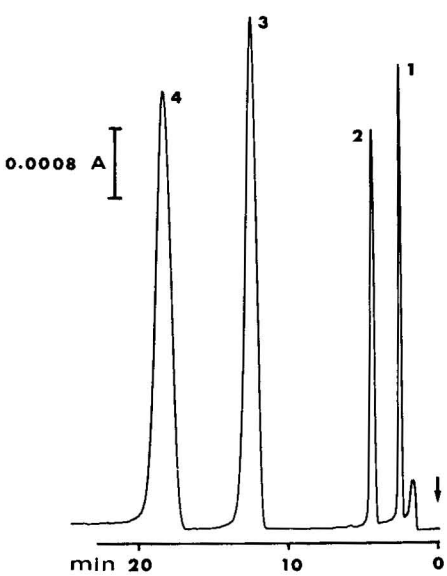


Fig. 2. Separation of hydrophilic aromatic acids. Stationary phase: 0.15 M TOPO in n-decane. Mobile phase: phosphate buffer solution of pH 1.8; 0.8 ml/min. Solutes: 1 = 4-hydroxy-3-methoxymandelic acid; 2 = 3,4-dihydroxymandelic acid; 3 = 3-hydroxymandelic acid; 4 = 5-hydroxyindole-3-acetic acid.

SEPARATIONS

The separation of some hydrophilic aromatic acids is demonstrated in Fig. 2. The mandelic acid derivatives, differing only in the number of aromatic hydroxy groups, are well separated.

CONCLUSIONS

(1) The pumping method is suitable for the coating of LiChrosorb RP-8 with a hydrophobic liquid stationary phase.

(2) The retention of acidic solutes can be described by a liquid–liquid distribution model, based on complex formation between the solutes and TOPO in the stationary phase.

(3) The retention depends on the number of TOPO molecules in the complex and on the magnitude of the complex formation constant. This gives possibilities for controlling retention and selectivity.

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PREPARATION OF COLUMNS, SAMPLING AND DETECTION PROBLEMS

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FIRST AND SECOND DERIVATIVE RECORDING OF THIN-LAYER CHROMATOGRAMS

APPLICATION TO THE ASSAY OF UNRESOLVED COMPOUNDS

J. TRAVESET, V. SUCH and R. GONZALO
Departamento de Análisis y Control, Lacer S.A., Cerdeña 350, Barcelona-25 (Spain)
and
E. GELPI*
Analytical Neurochemistry Unit, Instituto de Química Bio-orgánica (CSIC), Jorge Girona Salgado, s/n., Barcelona-34 (Spain)

SUMMARY

The practical advantages of direct first or second derivative recording of thin-layer chromatographic (TLC) or high-performance TLC separations to expand the qualitative and quantitative scope of TLC are considered. By coupling TLC scanning photodensitometry with a derivative recording technique, trace analysis and detailed studies of unresolved compounds can be significatively simplified without introducing any additional experimental burden.

INTRODUCTION

In parallel with liquid chromatography, thin-layer chromatography (TLC) has recently experienced a significant optimization of instrumental and operational parameters, resulting in what has been considered to be the second generation of TLC generally referred to as high-performance TLC (HPTLC)[1]. Thus, the new technology in coating materials, developing chambers and dosage methods has led to a considerable improvement in separation power and analytical capacity, all with relatively short running times. This, coupled with the performance of modern photometric scanning instruments, has facilitated direct quantitative evaluations of HPTLC plates in a number of applications in various fields[2–5].

Speed and simplicity are of utmost importance in the development of analytical methods, especially for product quality control purposes, and in this regard the advantages of HPTLC are well proven. However, speed and simplicity usually necessitate operating conditions that do not favour selectivity, resolution or sensitivity.

In our recent experience with the application of derivative spectroscopic techniques to different complex mixtures[5,6] we came to the conclusion that the coupling of HPTLC with first or second derivative spectrophotodensitometric detection of the components separated on the plates could enhance the resolving power and detecta-

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bility in standard HPTLC separations. This is demonstrated here with a few examples of interest in the field of pharmaceutical sciences.

To our knowledge, derivative techniques have not been systematicaly applied to chromatographic systems, despite the fact that they could improve detectability and resolution without the need to resort to extensive modifications of the operating parameters. The paucity of reports in this field is perhaps a consequence of the technical difficulties associated with the generation of derivative curves by either electronic or computer methods.

However, the derivative signals generated by a computer from a given TLC spot have been used to define its centre (R_F) and width[1] and recently derivative spectroscopy has been used to enhance the discriminatory capibility of size exclusion chromatography[7]. Second derivative has also been succesfully coupled with an HPLC fluorimetric system for the detection of porphyrins[8]. Although the electronically generated first and second derivative signals were obtained in both instances from circuits designed and built in the authors' laboratories, it is now possible to simplify the whole operation by using standard derivative instrumental units designed for UV spectroscopy.

EXPERIMENTAL

Samples

The active ingredients of different pharmaceutical formulations were assayed by TLC in order to establish (a) the residual content of *p*-chloroacetanilide in commercial batches of the phenacetin used in various pharmaceutical applications; (b) the content of xanthinol nicotinate and hydrochlorothiazide in sugar-coated tablets (Lacer, Barcelona, Spain); and (c) the content of reserpine and rescinamine in commercial samples of *Rauwolfia* alkaloids (Giulini Pharma, Hannover, G.F.R.). The samples were diluted to the appropriate concentration prior to their application to the TLC plates.

Methods

The HPTLC and TLC separations were performed on Merck (Darmstadt, G.F.R.) 60 F254 silica gel plates (10 × 20 cm or 20 × 20 cm, layer thickness 0.25 mm), except for reserpine–rescinamine mixtures, for which the plates used had a concentrating zone (Merck, K 60 F254).

Samples were spotted with an Evachrom sample applicator and the plates were developed by ascending elution in closed glass chambers, using the following solvent compositions (by volume) and running conditions: (a) acetone–benzene–light petroleum (1:1:1), solvent front migration distance 15 cm, time 90 min; (b) methanol–25% ammonia solution (100:0.25), solvent front migration 4 cm, time 10 min; (c) methanol–methyl ethyl ketone–*n*-heptane (84:33.6:58), solvent front migration 4 cm, time 10 min. The separations were carried out in the dark.

Spectrophotodensitometric detection

The plates (air-dried in an oven for 5 min) were read on a Zeiss KM 3 chromatogram spectrophotodensitometer (Carl Zeiss, Oberkochen, G.F.R.) operated in the reflectance mode for cases (a) and (b) and in the fluorescence mode for case (c) with slits of (a) 8 × 0.5 mm, (b) and (c) 3.5 × 0.5 mm. The scanning speeds were (a) and (b) 100 mm/min and (c) 50 mm/min. The plates were read in the direction of solvent flow.

Derivative TLC or HPTLC

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

RESULTS AND DISCUSSION

Trace analysis, which is always a difficult task, becomes impossible in cases such as that illustrated in Fig. 1. In this particular instance it was necessary to determine the residual content of *p*-chloroacetanilide (PCA) in commercial batches of phenacetin so as to verify compliance with the accepted upper limit of 0.03 or 0.05% set by the US and European Pharmacopoeias, respectively[9,10]. These two compounds can be separated by TLC with the R_F values indicated in Fig. 1, except when the relative amount of phenacetin in the samples is such that it completely masks the peak of PCA. Accordingly, the samples would either have to be re-run on another solvent system, not always easy to prepare without resorting to an empirical trial-and-error approach, or alternatively the PCA would have to be isolated by usually tedious

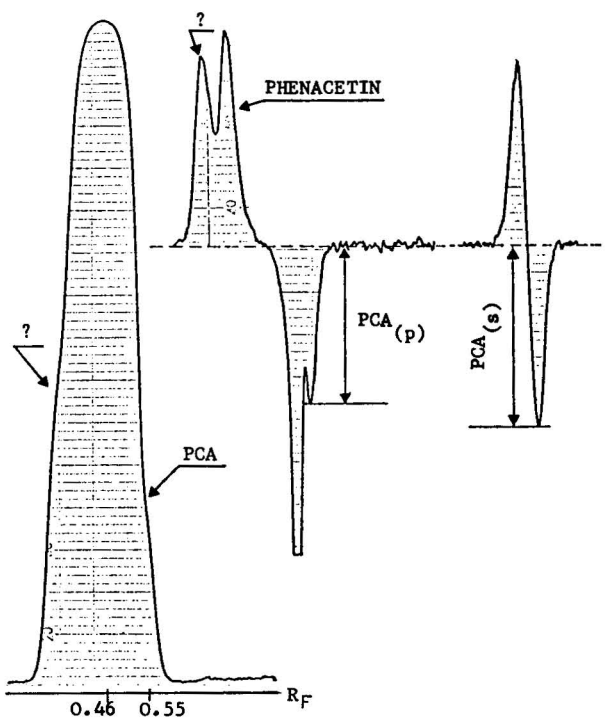


Fig. 1. TLC assay of traces of *p*-chloroacetanilide (PCA) (R_F = 0.55) in a commercial batch of phenacetin (R_F = 0.46). The photodensitometric response shown corresponds to a sample containing microgram amounts of phenacetin applied on the origin of the plate. $PCA_{(s)}$ indicates the measurement taken on the second half of the first derivative curve generated from a standard. $PCA_{(p)}$ indicates the corresponding measurement on the first derivative curve of the HPTLC spot at R_F = 0.44–0.55. The unknown product was not quantitated.

extraction procedures. However, this problem can be solved in a much simpler and rapid way by the application of first derivative techniques to detect the small slope change on the back edge of the large TLC peak, thus resolving it into its two components.

The first derivative curve illustrated in Fig. 1 clearly shows a peak profile sufficiently resolved to allow quantitation of PCA relative to the response of a standard. The enhanced qualitative possibilities of direct derivative TLC are also demonstrated by the detection of an unknown interference on the upward slope of the main peak (labelled by a question mark on Fig. 1), clearly resolved on the first derivative curve. By this procedure the estimated level of PCA in this particular sample could be calculated as 0.43% and the practical limit of detection of PCA was established as 0.05%. Thus, this method effectively competes with the more elaborate procedure described in the pharmacopoeias.

Another interesting application of derivative TLC, in this instance HPTLC, is the quantitative evaluation of hydrochlorothiazide in the presence of nicotinic acid. The commercial product contained xanthinol nicotinate and hydrochlorothiazide, the first of which decomposes on the TLC plates to xanthinol and nicotinic acid. In the solvent system used the nicotinic acid spot (N at $R_F = 0.94$) overlaps the spot of hydrochlorothiazide (HCT at $R_F = 0.90$), as shown in Fig. 2. The xanthinol by-product is well separated with $R_F = 0.49$. The UV absorption spectra taken at 10-nm intervals on the leading and trailing edges of the large unresolved spot are shown at either side of the spectrophotodensitometric trace. The scan of the upward slope of the main peak produces a UV spectral pattern essentially consistent with that of authentic HCT, with a major maximum at 270 nm and a secondary maximum at 320 nm. The spectrum of the downward slope, corresponding mainly to nicotinic acid, is characterized by a simpler envelope from 240 to 290 nm, with a maximum at 270 nm. However, for quantitative purposes significative cross-contributions to both of these UV spectra cannot be ruled out. For this reason, the first and second derivative signals from the zero-order spectrophotodensitometric TLC scan were obtained, as shown in the two lower traces in Fig. 2.

In this case, the two R_F values are so close that the shape of first derivative curve does not indicate the presence of two components in the HPTLC spot. Nevertheless, as shown in Table I, HCT could be quantitated by measuring the distance OB in the first half of the derivative curve. The presence of two components in this spot is more clearly indicated on the second derivative curve. As shown by the second derivative profile, the minimum in this curve is resolved into two adjacent minima, unequivocally pointing to a second component of almost coincidental R_F within the peak of HCT, in this instance nicotinic acid.

Table I gives the values obtained from three different samples of a commercial formulation. In the second derivative mode the OA measure is the more precise relative to the real values, as it corresponds to the part of the curve least affected by the unresolved nicotinic acid (the beginning of the upward slope of the HCT + N peak).

Finally, Fig. 3 illustrates a case of special relevance such as the quantitation of reserpine and rescinamine, two alkaloids of the *Rauwolfia* which, owing to structural similarities, are difficult to resolve. In fact, there has been only one reported separation[11], and we have not been able to reproduce it. After various attempts to

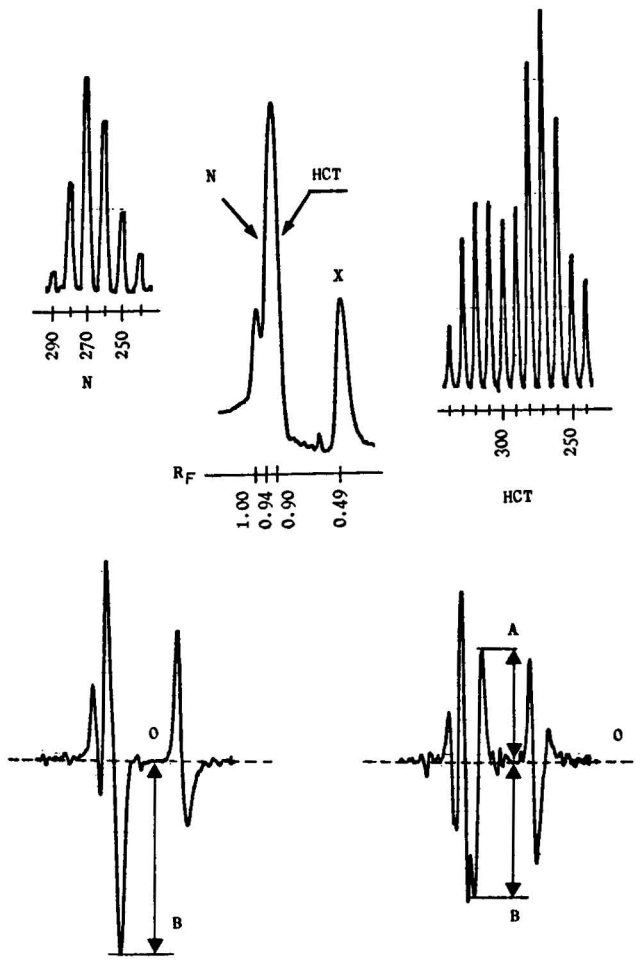


Fig. 2. HPTLC separation of a sample containing xanthinol nicotinate and hydrochlorothiazide (R_F = 0.90). Decomposition of the former yields xanthinol (R_F = 0.49) and nicotinic acid (R_F = 0.94). On the right- and left-hand sides of the chromatogram are shown the responses obtained by scanning at selected wavelength intervals the upward and downwards slopes of the main peaks. Lower left, first derivative; lower right, second derivative.

TABLE I

HPTLC ASSAY OF THE CONTENT OF HYDROCHLOROTHIAZIDE IN A COMMERCIAL FORMULATION

The actual values read at λ = 340 nm (no interference from the spectrum of nicotinic acid at this λ) were 103, 107, 105, respectively. Values represent percentages of the theoretical content (100%); r = correlation coefficient of the calibration curves used.

Derivative	*Sample*			*r*
	A	*B*	*C*	
Second derivative —				
A B	97	91	90	0.9549
O A	103	94	98	0.9916
First derivative —				
O B	102	100	102	0.9945

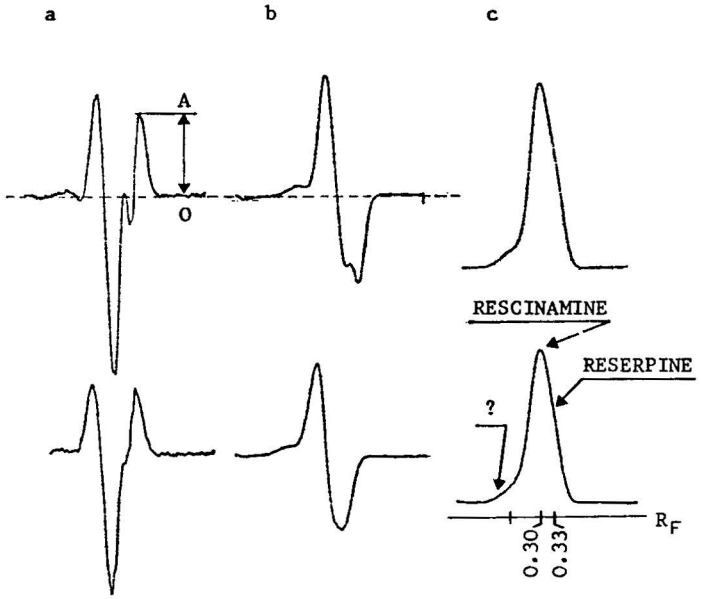


Fig. 3. Spectrophotodensitometric profile (c) of an HPTLC spot (R_F = 0.30) of unresolved reserpine (R_F = 0.33), rescinamine (R_F = 0.30) and an unknown component (?). The corresponding second and first derivatives of this zero-order curve are shown in (a) and (b), respectively. The plate was spotted seven times with the same sample for quantitation according to the data pair procedure[12]. The kind of differences observed in the resolution at various points across the plate is illustrated by the shape of the derivative curves. In contrast, no appreciable difference could be detected by inspection of the chromatographic zero-order profile. Detection was achieved by excitation of the HPTLC spot at 365 nm, reading the fluorescence emission through a Zeiss FL 46 filter.

find a suitable solvent system, we could obtain only a 0.03 difference in R_F values (Fig. 3c), which is not enough to resolve the two compounds. The first and second derivative curves generated from this peak are shown in Fig. 3b and a, respectively. An interesting effect also readily monitored in this fashion is the change in resolving power across an HPTLC plate. For instance, the upper row of zero-order fluorescence and derivative curves in Fig. 3 corresponds to a spot positioned close to the plate border, while those in the lower row correspond to a spot located at the centre of the plate. In this case, the variation of resolution across the TLC plate is of sufficient magnitude to be detected by the first and second derivative curves. There are also indications of an unresolved minor component on the leading edge of the peak (? in Fig. 3c). This component, depending on the prevailing resolution, is occasionally indicated by a second maximum on the last part of the second derivative curve.

The study of the quantitative possibilities of the derivative signals demonstrated that in relation to reserpine, the measure least affected by the overlapping of the rescinamine spot plus the unknown product is the distante AO, corresponding to the first part of the second derivative curve, as illustrated in Fig. 3a.

Regarding the reproducibility in these types of measurements, Table II summarizes the results obtained in replicate determinations of the components in two of the examples discussed here. Considering the difficulties one could foresee when

TABLE II

REPRODUCIBILITY OF THE DERIVATIVE QUANTITATION OF SPECTROPHOTODENSITOMETRIC TLC PROFILES

All determinations were carried out according to the data pair procedure[12].

(A) Determination of traces of PCA in samples of phenacetin. These samples were prepared for assay by addition of 0.2% and 0.05% of PCA to samples of phenacetin.

Parameter	*PCA added (%)*	
	0.2	*0.05*
n	8	8
PCA found ($\bar{x}$) (%)	0.21	0.054
Standard deviation (%)	0.01	0.005
Relative standard deviation (%)	4.5	8.8

(B) Quantitation of reserpine and rescinamine in commercial samples of *Rauwolfia* alkaloids.

Parameter	*Reserpine*	*Rescinamine*
Certified range in commercial samples (%)	7.8–9.6	19.5–24.0
Certified amount in samples analysed (%)	8.7	21.7
Amount found ($\bar{x}$) (%)	8.6	24.5
Standard deviation (%)	0.5	0.6
Relative standard deviation (%)	6.0	2.5
n	16	8

confronted with these totally unresolved TLC profiles (see Figs. 1 and 3), and the speed and simplicity with which the results in Table II can be obtained, a precision of less than 5% and 9% in the determination of 0.2 and 0.05% of PCA, respectively, can be considered as excellent for all practical purposes. The same applies to the example of the two alkaloids, where the precisions can be calculated to be *ca.* 6 and 3% for reserpine and rescinamine, respectively.

In conclusion, derivative TLC provides a rapid, direct, simple and relatively economical means of verifying the homogeneity of any given spot, in many instances enhancing the resolving power of the chromatographic system to a point where the quantitative assay of partially or even totally overlapped components becomes a possibility. Also, the usual changes in resolution across a plate can be readily monitored, resulting in a higher precission of quantitative measurements performed with internal standards applied at different locations on HPTLC or TLC plates.

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CHROM. 13,273

USE OF PLURONIC F68 GLASS CAPILLARY COLUMNS FOR THE ANALYSIS OF ESSENTIAL OIL SAMPLES

J. HLAVAY, Á. BARTHA and Gy. VIGH*
Institute for Analytical Chemistry, University of Chemical Engineering, P.O. Box 28, Veszprém (Hungary)
and
M. GAZDAG and G. SZEPESI
Chemical Works of Gedeon Richter Ltd., Budapest (Hungary)

SUMMARY

The molecular weight distribution (MWD) of the original and heat-treated Carbowax 20M and Pluronic F68 gas chromatographic stationary phases were determined by gel chromatography, and compared. The heat-induced changes in the MWD of Ploronic F68 were negligible compared with those in Carbowax 20M, even though the latter was heated to 30°C higher than Carbowax 20M. This finding supports previous observations concerning the superior stability of Pluronic phases in glass capillary columns. Glass capillaries coated with 0.25 μm Pluronic F68 were successfully used over a long period of time for essential oil analysis.

INTRODUCTION

Effective quality control of batches of essential oils is impossible without high-resolution capillary gas chromatography (GC). Non-polar and medium- and high-polarity stationary phases have all been used for essential oil analysis, polyglycol-type materials, especially Carbowax 20M, being among the most frequently used. They suffer, however, from high bleeding rates and low maximum operating temperatures. Grob and Grob[1] found that Pluronic-type stationary phases were superior to other polyglycol phases with respect to both bleeding rate and maximum operating temperature. Therefore, a comparative investigation of the characteristics of Pluronic F68 and Carbowax 20M was initiated, and glass capillaries for essential oil analysis were prepared.

EXPERIMENTAL

Apparatus

A gel chromatograph consisting of an M6000 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7010 loop injection valve (Rheodyne, Berkeley, CA, U.S.A.), two *ca.* 500 Å and two *ca.* 10,000 Å nominal pore size μStyragel columns

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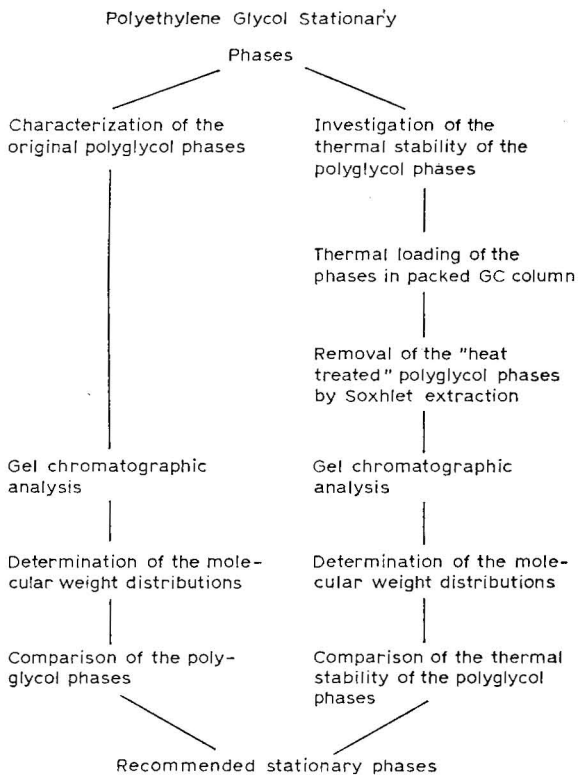


Fig. 1. Schematic diagram of the experimental procedure.

(30 cm × 3/8 in. O.D.) (Waters) connected in series, a Fresnel-type differential refractometer (Varian Aerograph, Walnut Creek, CA, U.S.A.) and a Varian A25 recorder was used. Analytical-reagent grade tetrahydrofuran (THF), filtered through a GF/F glass-fibre filter (Whatman, Clifton, NJ, U.S.A.) was used as the eluent.

GC separations were carried out on a Fractovap 2400P dual-column gas chromatograph (Carlo Erba, Milan, Italy).

Trace amounts of oxygen and water were carefully removed from the carrier gas (nitrogen) by using Hydro-Purge and Dow Oxygen Scrubber gas filters (Applied Science, State College, PA, U.S.A.).

Procedure

The procedure followed is described in detail elsewhere[2,3] and is shown schematically in Fig. 1. Carbowax 20M and Pluronic F68 were dissolved in THF and and analysed by using the gel chromatograph. The molecular weight distributions corrected for band spreading and detector response were determined. Chromosorb W AW DMCS (60–80 mesh) was coated with 5% of Carbowax 20M and Pluronic F68, respectively, and 2 m × 4 mm I.D. glass columns were filled with the packings. After conditioning overnight at 200 and 220°C, respectively, the columns were subjected to 200 temperature programme cycles. The initial temperature and the programming rate were 60°C and 6°C/min, respectively. The upper temperature limit was 220°C

for Carbowax 20M and 250°C for Pluronic F68. After 200 cycles the packing was removed from the column and the stationary phase was removed by Soxhlet extraction with dichloromethane and concentrated under a stream of nitrogen. The stationary phases were redissolved in THF and analysed by gel chromatography. The molecular weight distributions were obtained and compared with each other and with those of the original stationary phases.

Capillary columns were prepared according to Grob *et al.* 's barium carbonate procedure[4]. The capillaries were coated by the static method using dichloromethane solutions of the stationary phases.

Bouquette essential oil samples were dissolved in methanol. The injection splitting ratio was 1:50. The chromatograms were obtained by temperature programming from 50 to 240°C at 3°C/min.

RESULTS AND DISCUSSION

Comparison of the molecular weight distribution of Carbowax 20M and Pluronic F68

The molecular weight distributions of the original stationary phases obtained with a polyethylene glycol calibration graph[2] are compared in Fig. 2. It can be seen that Carbowax 20M has a very wide distribution; the weight-average molecular weight, $\overline{M}_w$, is 14,070, the number-average molecular weight, $\overline{M}_n$, is 4770 and the polydispersity factor, α, the ratio of the weight- and number-average molecular weights, is 2.95. On the other hand, the molecular weight distribution of Pluronic F68 is much narrower, with $\overline{M}_w = 6430$, $\overline{M}_n = 5700$ and $\alpha = 1.13$.

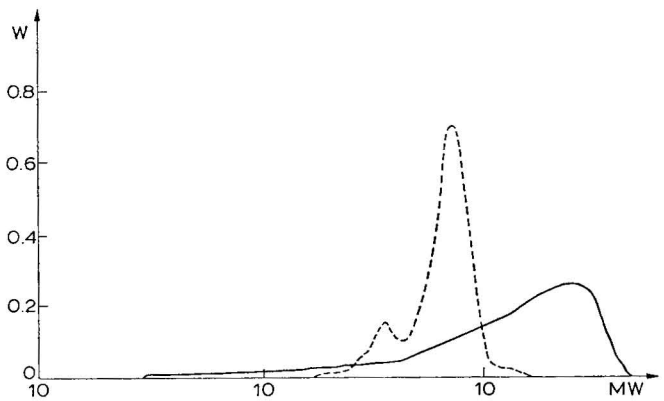


Fig. 2. Molecular weight distribution of the original Carbowax 20M (solid line) and Pluronic F68 (broken line).

These data indicate that Carbowax 20M contains significant amounts of both high- and low-molecular-weight components. It is known that polyethylene glycol molecules of widely different molecular weight have widely different polarity. Therefore, it can be expected that with the preferential loss of the low-molecular-weight components through bleeding during the life of the column, the polarity of the stationary phase will change. Pluronic F68, apart from a small amount of low-molecular-weight oligomers, is essentially a well defined polymer with a narrow molecular weight distribution and constant polarity characteristics. This implies that selective

bleeding, and consequently changes in the polarity of the stationary phase during its use, are less likely with Pluronic F68 than with Carbowax 20M. This indicates that, other factors being equal, Pluronic F68 would be a better stationary phase with respect to both the bleeding rate and the long-term stability of the polarity.

Thermal stability of Carbowax 20M and Pluronic F68

The molecular weight distribution curves of the original stationary phases and the heat-treated phases (after 200 temperature programme cycles, Soxhlet extraction and concentration) are compared in Figs. 3 and 4 for Carbowax 20M and Pluronic F68, respectively. It can be seen that the low-molecular-weight fraction of the heat-treated Carbowax 20M is greater than that of the original stationary phase. The average molecular weights of the heat-treated phase are $\overline{M}_w = 10{,}600$, $\overline{M}_n = 2500$ and $\alpha = 4.24$. Hence, the stationary phase removed from the packing after 200 temperature programme cycles differs significantly from the original one ($\alpha = 4.24$ and 2.95, respectively).

However, Pluronic F68, although programmed to a temperature 30°C higher than Carbowax 20M, suffered a much smaller change. There was a slight increase in both the low- and high-molecular-weight fractions. The average molecular weights of heat-treated Pluronic F68 are $\overline{M}_w = 7250$, $\overline{M}_n = 5560$ and $\alpha = 1.30$. Even heat-

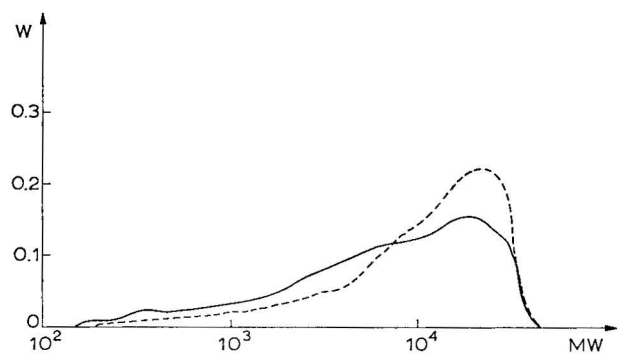


Fig. 3. Molecular weight distribution of Carbowax 20M before (broken line) and after heat treatment (solid line).

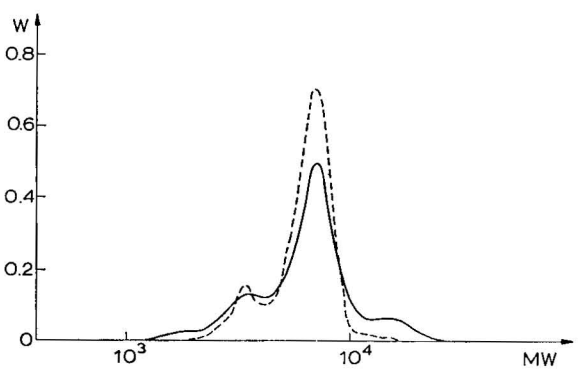


Fig. 4. Molecular weight distribution of Pluronic F68 before (broken line) and after heat treatment (solid line).

treated Pluronic F68 can be considered to be a polymer of narrow molecular weight distribution with well defined polarity characteristics.

On the basis of previous observations regarding the favourable bleeding characteristics of glass capillaries coated with Pluronic stationary phases[1], the higher bleeding rates of Carbowax 20M capillaries[5] and the data presented above, Pluronic F68 is the preferred stationary phase for high-resolution, stable glass capillary columns.

Application of Pluronic F68 capillaries to the analysis of Bouquette essential oil concentrates*

Batches of Bouquette essential oil were analysed on a 0.25-μm Pluronic F68 capillary column, prepared according to Grob *et al.* barium carbonate method[4]. A sample chromatogram is shown in Fig. 5. Using an internal standard technique, five

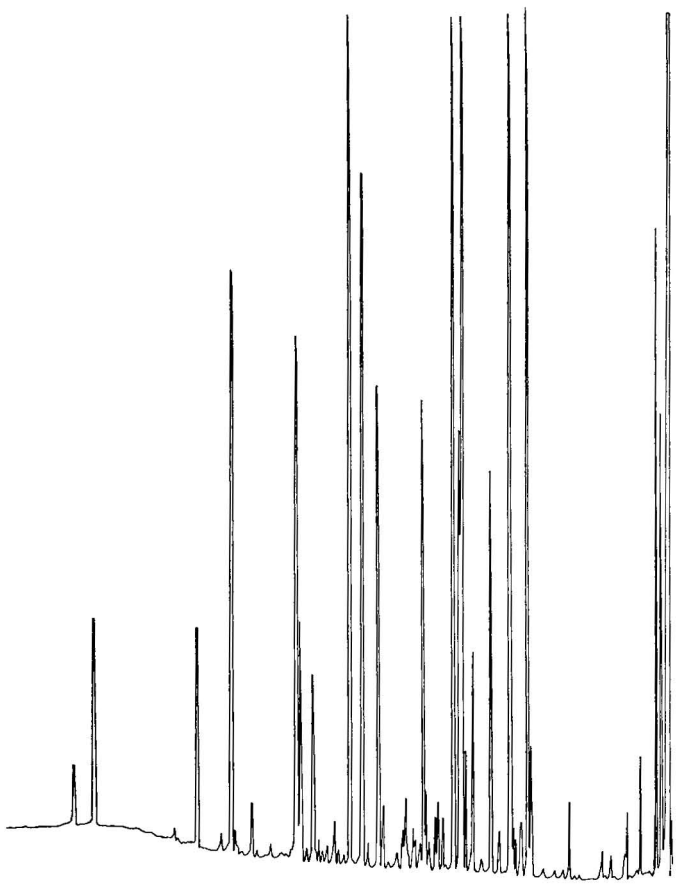


Fig. 5. Chromatogram of a Bouquette essential oil sample (batch No. 6716). Carrier gas, nitrogen; initial temperature 50°C; programming rate, 3°C/min; final temperature, 240°C; column, 40 m × 0.37 mm I.D. $BaCO_3$-treated Pyrex capillary coated with 0.25-μm Pluronic F68; sample volume injected, 1 μl; splitting ratio, 1:50.

* Bouquette is a proprietary material of Manefils (Le Bar sur Loup, France) and is used as a fragrance in cosmetical preparations.

groups of peaks can be identified that show varying peak heights, associated with odour problems in the sample. A detailed multi-dimensional GC and GC–MS study of the five groups of peaks is under way to establish the identity of the components.

The Pluronic F68 capillaries have been used over 18 months for the purpose of quality control of the batches of Bouquette essential oil. During this period no changes in the retention characteristics of the column have been observed.

CONCLUSION

Gel permeation chromatography (GPC) has been used succesfully to select polyglycol-type stationary phases with a narrow molecular weight distribution, *i.e.*, phases that are composed mainly of molecules with closely similar polarities. Changes caused by thermal loading of the stationary phase in a GC column can be followed and compared through the molecular weight distribution determined by GPC. A method based on the determination of the molecular weight distributions of the original and heat-treated stationary phase has been developed to aid the selection of preferred high-performance stationary phases for GC.

The method was used to compare two polyglycol-type stationary phases, Carbowax 20M and Pluronic F68. The original molecular weight distribution of Pluronic F68 is much narrower than that of Carbowax 20M. The heat-induced changes in the molecular weight distribution of Pluronic F68 are negligible compared with those in Carbowax 20M. The polarity of the stationary phase is connected with its molecular weight distribution and therefore, other factors being equal, Pluronic F68 is to be preferred to Carbowax 20M for capillary GC purposes. The conclusions of this study can be used to explain the superiority of the Pluronic capillaries observed by Grob *et al.*[4].

ACKNOWLEDGEMENT

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CHROM. 13,224

APPLICATION OF COLUMN SWITCHING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO ON-LINE SAMPLE PREPARATION FOR COMPLEX SEPARATIONS

F. ERNI*, H. P. KELLER, C. MORIN and M. SCHMITT

Analytical Research and Development and Biopharmaceutical Department, Sandoz Ltd., Basle (Switzerland)

SUMMARY

High-performance liquid chromatography column switching offers interesting possibilities for a powerful combination of simple sample clean-up and efficient separation. A very flexible column-switching set-up using two six-port valves as switching devices is presented. The approach is suitable for automation and can easily be put together from commercially available components. The arrangement can be used for different kinds of "cuts" (front-cut, heart-cut, end-cut), for back-flushing and for on-line concentration. Varying the separation parameters with gradient elution and/or different stationary phases in the sub-separation systems offers many possibilities, including two-dimensional high-performance liquid chromatography. The set-up presented proved to be valuable both during optimisation and for routine work.

Applications of this technique to the analysis of biological samples (animal feed, urine, plasma, etc.) are discussed. They demonstrate that a chromatographic clean-up is very efficient and may be the method of choice when the compounds to be analysed are chemically labile and when there is a high risk of artefact formation with classical clean-up techniques.

INTRODUCTION

High-performance liquid chromatography (HPLC) plays a key role in the determination of pharmaceutically active compounds in biological materials[1]. However, with the new possibilities of automation and data processing[2,3], sample preparation has become a new bottleneck. It often determines the total analysis throughput as well as the analysis costs for assays in complex matrices. The analysis of drugs in biological materials is part of the general problem of determining a single compound or a few specific compounds in *trace* amounts in a very complex mixture. Reversed-phase materials have introduced new possibilities for simple sample preparation and for sample concentration[4–6], but in many cases time-consuming off-line sample clean-up steps, such as liquid–liquid extraction and liquid–solid adsorption, are still necessary and, unlike in capillary gas chromatography, an increase in separa-

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tion performance is still an important goal of modern HPLC. The never-ending fight with interferences in determinations of biological materials can be approached by increasing the performance of the separation (which means more theoretical plates and an exponential increase in the cost in terms of hardware and time[7]) or by achieving greater selectivity in the detection[8–10] and/or the separation, *e.g.* with multidimensional HPLC[11,12].

In the literature, different approaches to column switching have demonstrated its potential as an alternative method for gradient elution[13] and for increased selectivity using mixed columns[14–18]. Recently, column-switching techniques have been used for biological materials[19–21].

In this paper the possibilities of column switching as an on-line method for sample preparation are discussed. On-line sample concentration techniques[4–6], cut-techniques as used in gas chromatography[22,23], and the selectivity increase with mixed columns of different stationary phases, as in multidimensional HPLC[11,12], are presented.

DESCRIPTION OF THE SYSTEM

Goals

In routine determinations in biological materials with HPLC the sample clean-up is critical for the following reasons:

(1) Complicated sample clean-up is time-consuming and expensive;

(2) There is a risk of loss of the interesting compound (recovery);

(3) Sensitive compounds may decompose during the sample treatment.

The goal of all sample preparation systems therefore must be to cope with these problems. The following points have to be considered if the method is to be used for routine determinations:

(1) The total analysis time (sample preparation and chromatography) should be shortened;

(2) To facilitate the operation, the sample preparation should be on-line to the HPLC separation system;

(3) The sample preparation should be automated;

(4) The reproducibility should be good enough to use external standardisation, which eliminates complicated searches for adequate internal standards and the problem of interferences with the internal standard;

(5) The sample preparation should be mild so that sensitive compounds can be handled;

(6) For trace analysis, a concentration step is necessary;

(7) To minimise the problem of interferences no loss of the separation power of the chromatographic system is acceptable: on the contrary, an improvement of the selectivity of the separation is desirable;

(8) To reduce the time for the development and optimisation in a given analytical problem the sample preparation system should be generally applicable, so that the experimental parameters can be changed on a rational basis and with high flexibility.

Basic principles

Some years ago we demonstrated[4–6] that it is possible to inject on to reversed-

phase HPLC columns very large volumes of aqueous sample without loss of separation efficiency. Up to millilitres of urine or other aqueous biological liquid samples can be concentrated on the column and afterwards analysed. This sample concentration is on-line and has been used with success for years. Unfortunately, not only the compounds of interest but also many interferences are concentrated on the column. They elute together with the substance or stick to the top of the column. The use of a precolumn to concentrate the sample makes possible the use of back-flushing[21], which can significantly increase both the life of a separation system and the reproducibility of retention times. As shown in Figs. 1 and 2, if the precolumn and the second column are put in the loops of six-port valves, cutting techniques can be used. With a *front-cut*, only the first part of the chromatogram, including the interesting peak(s), is analysed by columns 1 and 2. After the elution of the last peak of interest from column 1, the precolumn is bypassed by switching valve 1 and can be cleaned by the flow of good eluting solvent using *back-flush* or forward-flush mode. With

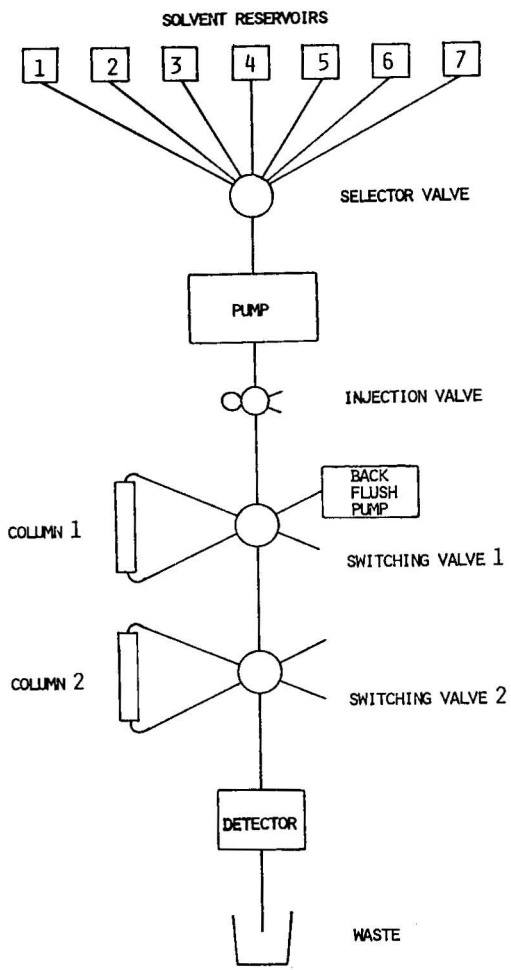


Fig. 1. Diagram of the column-switching system with a step-gradient elution solvent delivery system.

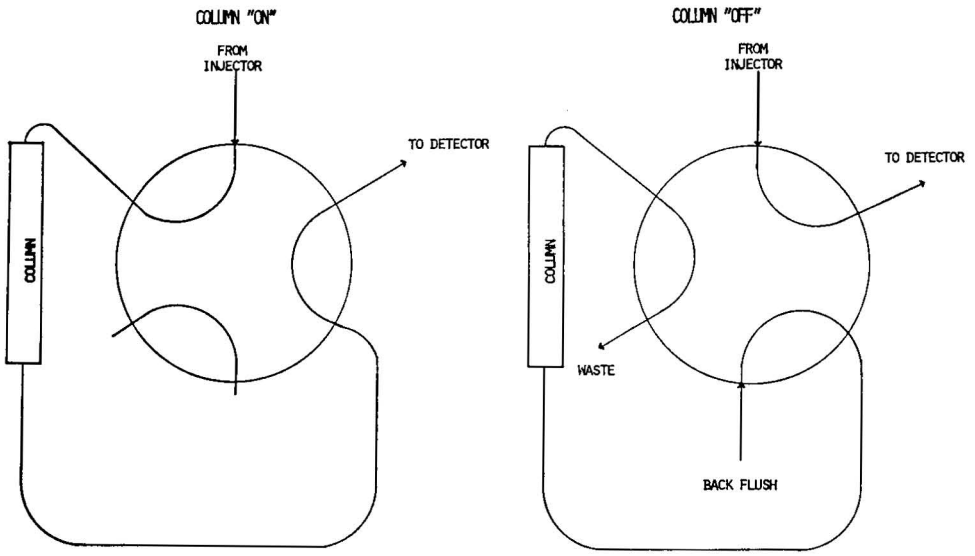


Fig. 2. Flow scheme of the six-port column-switching valves (Rheodyne, Berkeley, CA, U.S.A., type 7010 A, or Valco Instruments, Houston, TX, U.S.A., type ACL-6-UHPa-N60).

this front-cut the analysis time can significantly be shortened because late-eluting background peaks are not analysed on the second column.

Very often the first part of the chromatogram contains a large amount of polar substances. The tailing of these peaks interferes with the compound(s) of interest. It is therefore useful to eliminate these polar interferences using an *end-cut* technique, thus bypassing the separation column 2 during the elution of the first part of the chromatogram. A practical application of this end-cut technique was recently demonstrated by Gfeller[21]. By combining the front-cut and the end-cut, only the relevant part of the chromatogram (*heart-cut*) is analysed by the second column. This minimises the interference of fast-eluting and late-eluting background peaks and also minimises the ballast load on the main separation column that may change the separation characteristics (selectivity and performance) of the column 2 from run to run.

The approach described here (Fig. 1) uses two six-port valves as switching valves (Fig. 2) and therefore has the following advantages:

(1) Front-cut, end-cut and heart-cut techniques are possible;

(2) Cut techniques and back-flushing can be combined;

(3) The combination of hardware shown in Fig. 1 has proved to be optimal for method development and daily checks because each column can be run separately to optimise chromatographic conditions and switching times;

(4) The switching valves can easily be automated.

This system has proved to be an excellent sample-preparation system for different biological samples because only simple clean-up steps, if any, are necessary. It is also very simple and especially suitable for sensitive compounds.

The selectivity of the system can be varied by using different columns in the switching system. If the first column contains a stationary phase with generally

smaller capacity factors (k') than that in the second column (*e.g.* RP-2 in column 1 and RP-18 in column 2), a second concentration on top of column 2 is achieved. This leads to narrower peaks (meaning higher efficiency) of the total separation system. The two different columns also have different separation selectivities, which is especially important for trace analysis. Only a small part of the first column is separated on the second column, with the result that fewer peaks overlap. Information theory[11] shows that with this two-dimensional selectivity system, the information produced by column switching increases with increasing difference between the selectivities of the two columns. Our experience has shown that a combination of Diol phase (column 1) and RP-18 (column 2) gives excellent preconcentration on the second column, together with the advantage of a large selectivity difference. In addition, the combination of column switching with step-gradient elution gives more flexibility: not only can different stationary phases be used in the two columns but it is also possible to use different mobile phases for sample clean-up on the first column and final separation on the second column.

EXPERIMENTAL

Apparatus

The chromatographic system used in this work consists of either a two-pump gradient solvent delivery system or a step-gradient elution system described in an earlier paper[4], an automatic sample injection system[24], six-port switching valves and a variable-wavelength UV detector.

Reagents

All solvents used were HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain) or LiChrosolv grade (E. Merck, Darmstadt, G.F.R.). The water was double-distilled. For the buffer solutions analytical grade reagents were used (E. Merck, and Fluka, Buchs, Switzerland).

The animal feed contains a mixture of cereals, plant and animal proteins, mineral substances, amino acids and vitamins (Type 850 G4, NAFAG, Gossau, Switzerland). The drugs Fluorproquazone and Endralazine were from Sandoz, Basle, Switzerland.

APPLICATIONS

Analysis of medicated feed

During the development of a drug, long-term toxicological studies have to be carried out. For this purpose laboratory animals get a feed spiked with the drug. The homogeneity and the stability of the medicated feed have to be checked to ensure "Good Laboratory and Development Practice" (GLDP). The analysis of the drug in the complex matrix of the feed mixture is often very complicated and time-consuming. A good selectivity of the separation system and an excellent sensitivity for the detection is necessary. Fig. 3 shows the determination of Fluorproquazone[21] in animal feed using the column-switching system described in Fig. 1. The reproducibility of the system is better than 2% relative standard deviation (R.S.D.) at 25 ppm, the calibration curve is linear from 1 to 100 ppm with a correlation coefficient of $r = 0.99998$,

and no systematic error from interferences was detected. Because the sample preparation is simple and the reproducibility good, external standardisation can be used. The total analysis time is only 14 min and the total analysis system operates fully automatically.

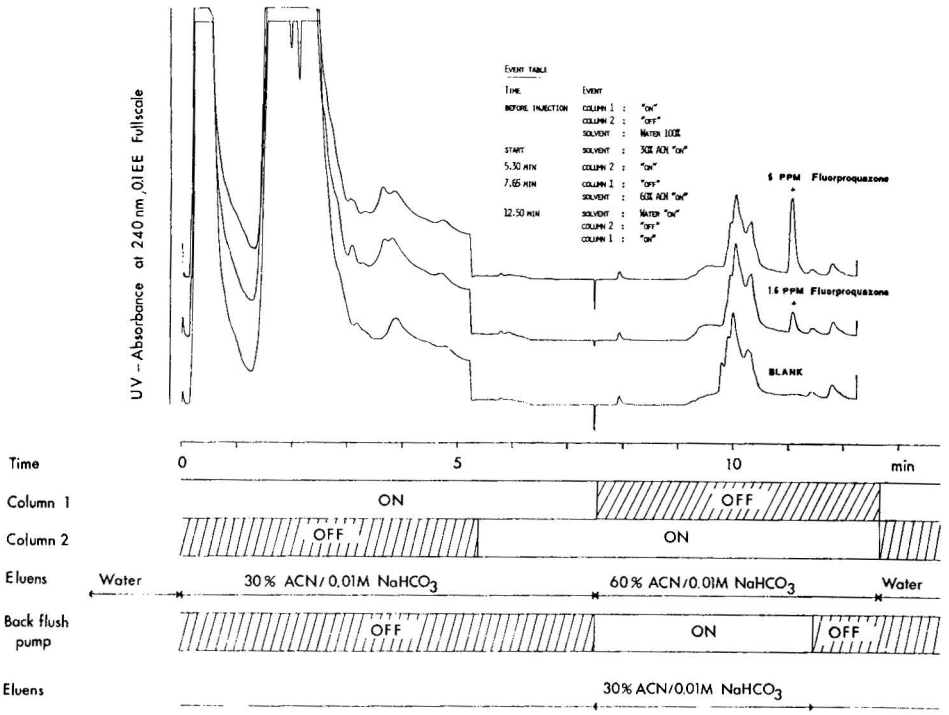


Fig. 3. Analysis of Fluorproquazone in medicated feed with the step-gradient system[4] and column switching (see Fig. 1). Pump: Model 100, Altex Scientific, Berkeley, CA, U.S.A.; automatic sample injector[24]; switching valves: Rheodyne 7010 A; UV detector: Model LC 55 (Perkin-Elmer, Norwalk, CT, U.S.A.); Lab. Data System HP 3354B (Hewlett-Packard, Avondale, PA, U.S.A.). Column 1: RP-2, 5 μm, 3 cm × 4.7 mm I.D. (Knauer, Oberursel, G.F.R.). Column 2: RP-18, 5 μm, 12 cm × 4.7 mm I.D. (Knauer). Sample preparation: stir 100 g of medicated feed with 200 ml of methanol for 10 min, centrifuge, inject 50 μl of the clear solution. ACN = acetonitrile.

The determination of a drug metabolite in plasma and urine samples

The pyruvate of the new antihypertensive drug Endralazine (EP)[25] is a main metabolite in blood and urine of humans and most animals investigated. For pharmacokinetic studies and for the development of dosage forms a bioanalytical method for EP had to be developed with sufficient sensitivity to detect this compound in the lower ng/ml range (ppb) in body fluids. Because of the relatively high solubility in aqueous solution and the sensitivity to hydrolysis in acidic media, classical liquid–liquid clean-up methods could not be used to extract this acidic compound.

From 1 ml of plasma the compound is extracted at neutral pH into chloroform–propan-2-ol as an ion-pair using tetrabutylammonium counter-ions (see Fig. 4 for details). Injection of the extracted residue on to a paired-ion reversed-phase system with UV detection at 280 nm yields a chromatogram where the compound EP elutes

with a k' value of *ca.* 3. The extract contains a large number of coextracted endogenous compounds partly strongly retained. An elution gradient cannot be applied to this HPLC system at the required high detector sensitivity. Therefore the column-switching technique is an excellent alternative way of reducing the analysis time of *ca.* 45 min to *ca.* 15 min (see Fig. 4). The extract is injected on to a short reversed-phase column from which only the front part is transferred on to the second column (Fig. 5), where the separation and detection of the compound of interest occur. The stationary phase and the isocratic mobile phase are the same for both columns. The remaining part of the extract is back-flushed from the first column with the same mobile phase. This simple two-column method was used for several hundred plasma samples from dogs and humans administered with Endralazine. The linearity (r = 0.9995 in the range from 0 to 500 ng/ml) and reproducibility (R.S.D. = 10% for 100 ng/ml standards) are satisfactory with external standardisation.

All liquid–liquid and liquid–solid extraction clean-up techniques tried on urine failed, giving very high blanks. Therefore a column-switching technique with on-line sample clean-up and external standardisation was developed. Urine is buffered

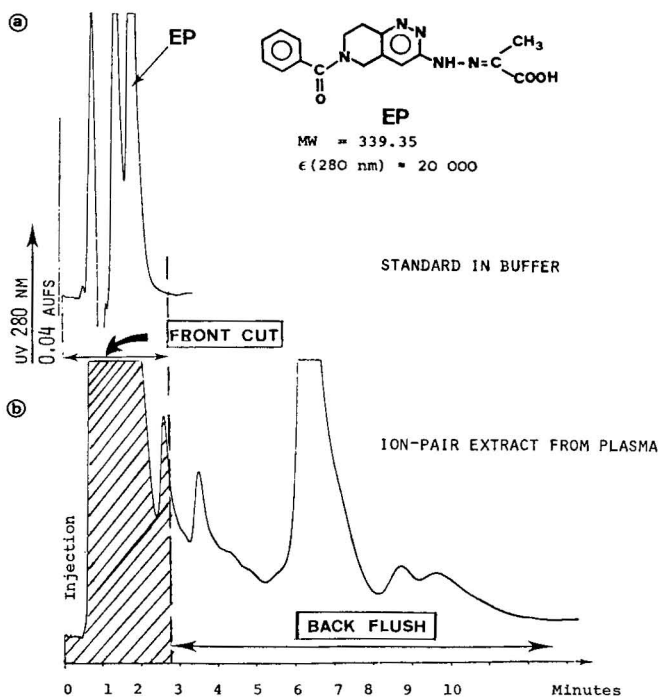


Fig. 4. Analysis of plasma samples: front cut on the first column C_1. (HPLC equipment and chromatographic conditions as in Fig. 5.) Sample (a): 200 ng of pyruvate of Endralazine (EP) dissolved in 150 μl of 0.01 M KH_2PO_4 pH 7.4. Sample (b): extract of 200 ng of EP from 1 ml of heparinised human plasma. Extraction procedure: add 1 ml of plasma to 0.1 M tetrabutylammonium chloride, pH 7.0; extract EP as ion-pair into 10 ml of chloroform–propan-2-ol and evaporate the organic layer to dryness at 40°C under reduced pressure. The residue is reconstituted in 0.01 M KH_2PO_4 pH 7.4 (150 μl). These chromatograms on column C_1 are obtained with the switching valve 1 ON and switching valve 2 OFF (see Figs. 1 and 2).

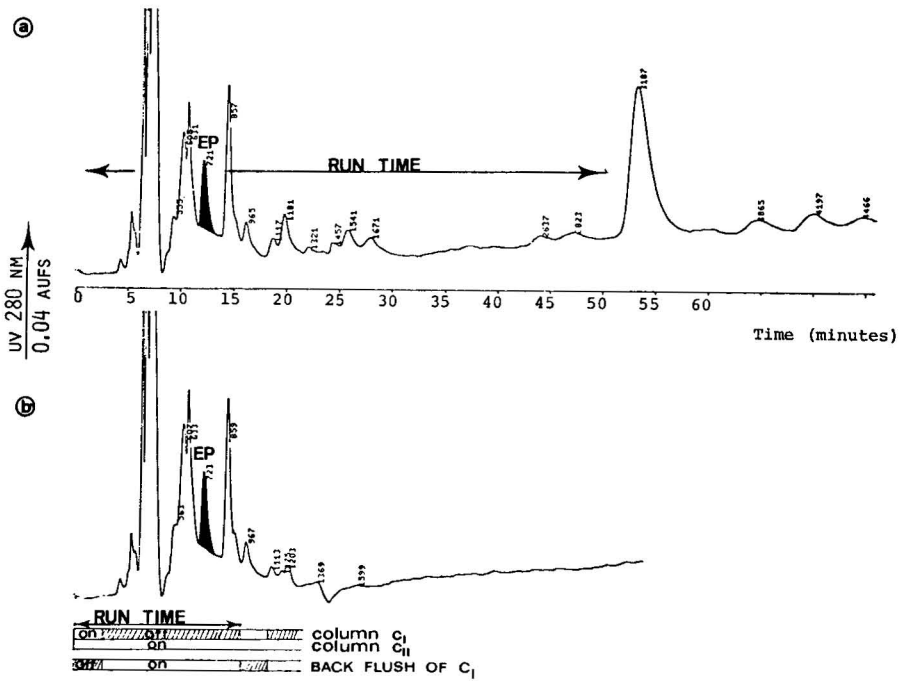


Fig. 5. Analysis of plasma samples: (a) without column switching (switching valves 1 and 2 ON), (b) with column switching (front-cut on column C_I of 165 sec, see Fig. 5). HPLC equipment: pumps (2), Model SP-740 (Spectra-Physics, Santa Clara, CA, U.S.A.); automated sample injector valve, type 7010 (Rheodyne) filled with a 130-μl sample loop; UV detector, Model LC3 (Pye-Unicam, Cambridge, Great Britain); chromatography data system, Model SP-4000 (Spectra-Physics); Valco air-actuated switching-valves type ACV-6-UHPa-N60 (Valco Instruments, Houston, TX, U.S.A.). Column C_I: HPLC-Guard RP-8, 10 μm, 30 × 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.). Column C_{II}: LiChrosorb RP-8, 5 μm, 250 × 4.6 mm I.D. Mobile phase: 0.01 *M* tetrabutylammonium chloride (pH 7.0)–acetonitrile (75:25), flow-rate 0.75 ml/min. (See Fig. 4 for details of sample preparation.)

at pH 8 (see Fig. 6 for details) and injected on to a first column containing LiChrosorb RP-2 and eluted with an almost aqueous phase containing 0–1.5% acetonitrile (Fig. 6). By means of a heart-cut, the fraction containing the compound of interest is transferred on to the second column, filled with LiChrosorb RP-8, and conditioned with the same mobile phase. The strongly retained compound EP on the top of the second column is then eluted with a stronger mobile phase after a very steep gradient step (Fig. 7 and Table I). During the elution of the second column the first column is flushed with a strong mobile phase using an auxiliary pump. At the end of the run both columns are reconditioned with the initial mobile phase. The system proved to be very stable for several weeks, giving a constant retention time of 1682 sec $\pm$1.06 R.S.D. (n = 20 days). The method has a reproducibility for peak areas of better than 5% R.S.D. (n = 6) for a sample concentration of 250 ng/ml. The calibration curve sample concentration range 0–2500 ng/ml gave a correlation coefficient of r = 0.999. With 500-μl injection volumes the limit of detection is *ca.* 20 ng/ml for 1:3 diluted urine, corresponding to *ca.* 150 ng/ml urine.

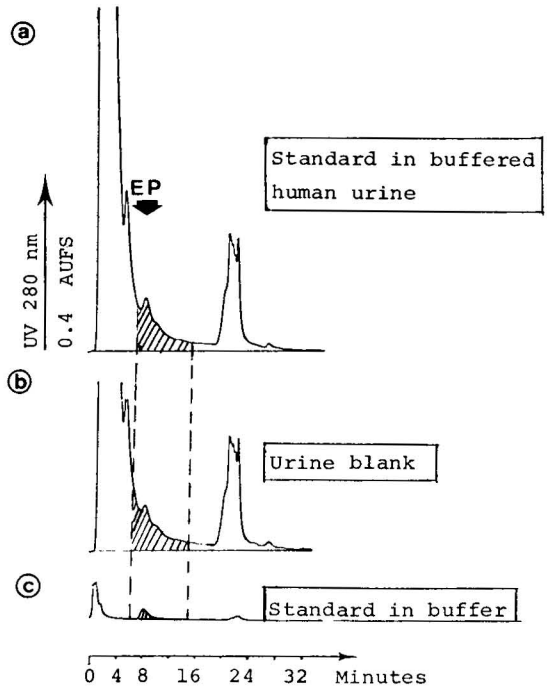


Fig. 6. Analysis of urine samples: heart-cut on the first column C_I. Mobile phase A with a flow-rate of 1.5 ml was used (see Fig. 7). Sample preparation: urine (3 ml) is buffered with 0.2 *M* Na_2HPO_4 pH 8.0 (5 ml) and stabilised with 0.5 *M* Na_2EDTA (0.5 ml) to prevent metal-catalysed oxidation. The pH of the mixture is adjusted to 8.0 by dropwise addition of 1 *N* NaOH, and the volume made up to 10.0 ml with water. Aliquots of 500 μl of this mixture are injected.

DISCUSSION

The three examples show that column switching is an excellent technique for the analysis of complex mixtures. The sample concentration on the first column and the heart-cut to a second independent separation system has proved to be a very efficient sample preparation procedure for medicated feed and urine samples. Preliminary experiments also show that blood plasma samples can be analysed in a similar way to urine samples. The total analysis times are very short and the reproducibilities and recoveries are excellent. The analytical procedure is very mild and therefore well suited to the analysis of sensitive compounds. Because the sample handling is simple and operation automated, accurate determinations at very low concentrations are possible. The two-dimensional HPLC approach minimises interferences and enhances significantly the selectivity. The clean-up effect depends on the quality of separation and efficiency of the first column. The optimisation of the chromatographic conditions, especially for the first column, is very important and demands a careful adjustment of the mobile-phase composition in those cases where large volumes of sample are injected. The on-line concentration between the first and the second column ensures good starting conditions for the separation in the second column.

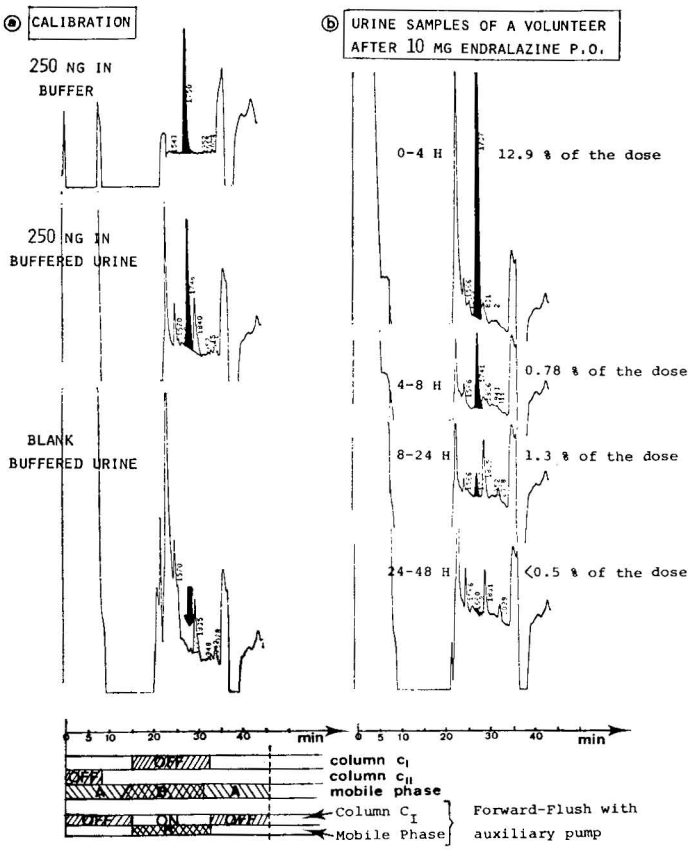


Fig. 7. Analysis of urine samples using column switching and a gradient system. (a) Calibration chromatograms; (b) urine samples of a volunteer who received orally 10 mg of Endralazine. (See Fig. 6 for sample preparation.) HPLC equipment: pumps (2), Model AX-110 (Altex Scientific); gradient controller, Model AX-420 (Altex); sample injector, Model WISP 710A (Waters Assoc., Milford, MA, U.S.A.); switching valves (2), type ACV-6-UHPa-N60 (Valco Instruments); chromatography data system, Model SP-4000 (Spectra-Physics); UV detector, Model LC-55 (Perkin-Elmer). Column C_I: LiChrosorb RP-2, 10 μm, 60 × 4.6 mm I.D. Column C_{II}: LiChrosorb RP-8, 5 μm, 250 × 4.6 mm I.D. Mobile phase A: 0.005 *M* NaH_2PO_4 (pH 5.4)–0.005 *M* Na_2EDTA–acetonitrile (985:3:15). Mobile phase B: water–0.005 *M* Na_2EDTA–H_3PO_4 conc.–acetonitrile (800:2:0.200:200). Injection volume: 500 μl. See Table I for the experimental timing.

The instrumental set-up with two switching valves has proved very efficient for the method development. Routine application of these column-switching techniques showed that the column can be used for several hundred biological samples, indicating that the first column is effectively flushed and regenerated, and that the second column is well protected from column plugging and decomposition when complex samples are injected.

TABLE I

TIMING OF THE AUTOMATED ANALYSIS OF URINE SAMPLES

(1) Programme of the autosampler WISP; (2) programme of the time file of the chromatographic data-system SP-4000; (3) programme of the gradient system.

Time (min)	*Programmation*	*Event*
0–0.08	(1) → (2) → (3)	injection, run start
	(3) flow-rate 1.5 ml/min	
	(3) 0% B	sample clean-up on C_I with mobile phase A
	(2) column C_{II} off	
7.50	(3) flow-rate 0 ml/min	
8.20	(2) column C_{II} on	
8.50–9.50	(3) flow-rate 0–1.2 ml/min linear	transfer of cut from C_I to C_{II} with
14.50	(3) flow-rate 0 ml/min	mobile phase A
14.70	(2) column C_I off	
15.00–16.00	(3) flow-rate 0–1.2 ml/min linear	separation and quantitation
25.00	(2) start integration	on C_{II}
31.67	(2) stop integration	flush of C_I with mobile phase B with
32.00	(3) flow-rate 0 ml/min	auxiliary pump
	(3) 0% B	
32.20	(2) column C_I on	
32.50	(3) flow-rate 1.2 ml/min	reconditioning of C_I and C_{II}
40.00	(1) start injection	
45.20	(2) column C_{II} off	
45.33	(2) print report	
46.00		start new cycle

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PEAK WIDTH IN SOLVENT-PROGRAMMED CHROMATOGRAPHY

I. GENERAL DESCRIPTION OF PEAK BROADENING IN SOLVENT-PROGRAMMED ELUTION

H. POPPE*, J. PAANAKKER and M. BRONCKHORST

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam (The Netherlands)

SUMMARY

An equation describing the peak broadening or peak contraction in liquid chromatography under solvent-programmed or gradient conditions is derived from the mass-transport equation in the dispersion model. The results are applicable to all cases where the retention can be calculated in advance. The predictions obtained with the equation are equal to those obtained by other means, when available. This is the case for step gradients with the assumption of negligible dispersion, and for the linear solvent gradients.

INTRODUCTION

Solvent programming in liquid chromatography is a powerful tool used to resolve some important problems arising in analytical applications. For example, continuous gradients have been employed[1,2] as an answer to the "general elution problem", and step gradients to circumvent poor detection sensitivity in on-column concentration procedures[3–5].

Migration, or retention, of solutes under solvent-programmed conditions is well described. Snyder and Saunders[6] derived the fundamental equation and also gave a numerical treatment of the effect of the solvent program on the peak dispersion, *i.e.*, the contraction effect due to the fact that the peak front moves in a solvent of lesser strength than does the tail of the peak. More recent treatments of solvent-programmed elution[2,7–9], inspired by the increasing popularity of gradients in high-performance liquid chromatography (HPLC), do not differ in essence from the approach employed by Snyder in 1964, although the mathematical tools used are sometimes much more sophisticated. No new treatments of the peak broadening were given. On the other hand, Snyder *et al.*[2] have recently given results of the use of the above numerical procedures for the specific case of the linear solvent strength (LSS) gradients they advocate strongly.

In the present paper a description of band broadening in solvent programming is given which applies to all slow gradients, and the results also seem to be valid for

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step gradients. Experimental verification of the equations as well as a discussion of the implications for optimization will be given in subsequent papers.

THEORETICAL

Mathematical description of the transport of a solute

The differential equation used here to describe mass transport in the column is

$$\frac{\partial c_t}{\partial t} = D \frac{\partial^2 c_m}{\partial z^2} - v \frac{\partial c_m}{\partial z} \tag{1}$$

in which c_t is the "total concentration", defined as

$$c_t = c_m + q\, c_s = c_m(1 + \kappa)$$

c_m is the concentration in the mobile phase, c_s is the concentration in the stationary phase, q is the volume ratio of the stationary and mobile phases, κ is the capacity factor, t is the time, z is the axial coordinate in the column, v is the migration velocity of the mobile phase and D is the dispersion coefficient, equal to $Hv/2$, where H is the plate height. Immediate equilibrium between mobile and stationary phase is assumed described by

$$c_s = K\, c_m \tag{2}$$

in which K is the partition or adsorption constant.

It should be noted that the use of eqns. 1 and 2 introduces certain limitations on the applicability of the results obtained. The most important of these are as follows.

The use of a dispersion model for chromatographic transport[10,11], as implied by eqn. 1 groups together all kinds of dispersion mechanisms (normal and eddy diffusion, resistance to mass transfer in the various phases) into one dispersion coefficient, D. This is an adequate description of the physical reality, if the column length is large enough so that all dispersion mechanisms finally affect the elution function in the same way: a gaussian function results, being an asymptotic solution of the various differential equations, provided the column length (expressed, *e.g.*, as plate number) is large. Our results will therefore be valid only for reasonably large plate numbers (100–1000, depending on the required accuracy).

The capacity ratio, κ, changes with time in gradient elution. It is known[12] that the plate height, H, in LC is a function of κ. We do not include this effect in our treatment because it would lead to unacceptable mathematical complexity. In situations where resistance to mass transfer in the stationary phase plays a predominant rôle (*e.g.*, in classical ion-exchange chromatography) the results would be questionable. However, in most HPLC situations the dependence of H on κ is not so strong as to ruin the practical validity of our treatment.

Changes in κ are accomplished by changes in eluent composition. A number of second-order effects are therefore to be expected: the velocity, v, and the dispersion coefficient D can change because of changes in the viscosity and in the (related) values of the diffusion coefficients. We neglect these effects. In order to avoid complications

in the formulae, we express the influence of solvent strength on the distribution by giving the capacity factor, κ, rather than the distribution constant, K, as a function of z and t:

$$\kappa = \kappa(z,t)$$

Description of the peak width is first carried out by using the second normalized centralized place moment

$$\mu_2 = \frac{\int_{-\infty}^{+\infty} x^2 c_t(x)\,dx}{\int_{-\infty}^{+\infty} c_t(x)\,dx} \tag{3}$$

where $x = z - \mu$, the axial coordinate, relative to the centre of gravity, μ_1, of the $c_t(z)$ function:

$$\mu_1 = \frac{\int_{-\infty}^{+\infty} z c_t(z)\,dz}{\int_{-\infty}^{+\infty} c_t(z)\,dz} \tag{4}$$

Again, some comment is required, in order to indicate the limitations of the treatment. Characterization of local and temporal distribution functions by the use of moments has a number of advantages, among which the most important are the direct relation of the moments with physicochemical parameters in the column, and (connected with this) the mathematical simplification obtained. On the other hand it should be recognized that for odd, non-gaussian peak shapes, expression of the peak width as the second moment, μ_2, gives little information about overlap of adjacent peaks, which is of prime concern to an analytical chemist. Only for known peak shapes, *e.g.*, block functions, first-order responses, gaussian shapes, can we infer from the first and second moments how severe will be the peak overlap. It follows that our treatment will give valuable results for the analytical chemist only for cases where the peak shape is known in advance: gaussians for columns with high plate numbers (>100) and small injection profiles, or, *e.g.*, block functions when these are expected because of the injection conditions. Fortunately these extremes are exactly the cases where solvent programs are important.

A second comment concerns the use of c_t rather than c_m in eqns. 3 and 4. It could be argued that in elution chromatography $c_m(t)$ at the column end is a more relevant function than $c_t(z)$ at a particular moment. The choice of $c_t(z)$ in our treatment is based purely on mathematical reasons. It can be shown that for slowly varying capacity factors (in the case of slow gradients, or in a region of constant κ after a steeper gradient), the second moments based on c_m and c_t are the same. The use of local distributions, prior to the temporal distribution, is widespread in treatments of chromatographic transport. The translation of the results into elution peak widths in time units is straightforward and valid for all columns with large plate numbers.

The denominator of eqns. 3 and 4 is constant and equal to Q/A_m, where Q is the total amount of the solute and A_m is the area of the part of the cross section of the column which is occupied by the mobile phase. Therefore:

$$\mu_2 = \frac{A_m}{Q} \int_{-\infty}^{+\infty} x^2 c_t(x)\, dx \tag{5}$$

and

$$\mu_1 = \frac{A_m}{Q} \int_{-\infty}^{+\infty} z c_t(z)\, dz \tag{6}$$

As we need expressions for the retention parameters for manipulating and interpreting the dispersion formulae, we will repeat known[6–9] derivations of these in our terms. Thus ,differentiating eqn. 6, and interchanging integration and differentiating, one obtains:

$$\frac{d\mu_1}{dt} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} z \frac{\partial c_t}{\partial t}\, dz \tag{7}$$

Substituting from eqn. 1 leads to:

$$\frac{d\mu_1}{dt} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} \left(zD \frac{\partial^2 c_m}{\partial z^2} - zv \frac{\partial c_m}{\partial z} \right) dz \tag{8}$$

Partial integration of eqn. 8 using the physically obvious fact that for $z \to \pm\infty$, $c_m \to 0$, leads to

$$\frac{d\mu_1}{dt} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} v c_m\, dz \tag{9}$$

which indicates that only c_m contributes to migration; c_m is related to c_t via the varying value of $\kappa + 1$ in the column:

$$c_m = c_t \cdot \frac{1}{\kappa + 1}$$

Thus:

$$\frac{d\mu_1}{dt} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} v \frac{c_t}{\kappa + 1}\, dz \tag{10}$$

Assuming that the gradient is flat, *i.e.*, κ changes little within the z region of significant values of c_t, we can treat the κ value as a constant, $\bar{\kappa}$, the value it has for the centre of the peak $z = \mu_1$;

$$\frac{d\mu_1}{dt} = \frac{A_m}{Q} \frac{v}{\bar{\kappa} + 1} \int_{-\infty}^{+\infty} c_t\, dz = \frac{v}{\bar{\kappa}(\mu_1, t) + 1} \tag{11}$$

Eqn. 11 shows the dependence of $\bar{\kappa}$ on t and μ_1. Now, this equation is more easily integrated by replacing the t coordinate by $t - \mu_1/v = \xi$. For gradients *which do not change their shape on migration through the column, i.e.*, those which are not distorted by dispersion or by selective uptake of one of the solvent components by the

stationary phase (solvent demixing), the capacity factor within the peak's centre of gravity, $\bar{\kappa}$, is simply

$$\bar{\kappa} = \kappa(\xi) \tag{12}$$

where ξ is proportional to the volume of solvent passed through the peak centre[6], but expressed as a time coordinate. It can be shown that a linear dependence of κ on z does not impair the validity of this approximation. Expression of eqn. 11 in these new coordinates yields:

$$\frac{\mathrm{d}\mu_1}{\mathrm{d}\xi} = \frac{v}{\kappa(\xi)} \tag{13}$$

This equation, in different forms, has been put forward by Snyder and Saunders[6], and used by Jandera and Churáček[7], Schoenmakers *et al.*[8] and Liteanu and Gocan[9]. The ξ_e for elution ($\mu_1 = L$) is found by

$$\int_0^{\xi_e} \frac{1}{\kappa(\xi)}\,\mathrm{d}\xi = \frac{L}{v} = t_0 \tag{14}$$

Returning to the second moment, we obtain*, when differentiating eqn. 5

$$\frac{\mathrm{d}\mu_2}{\mathrm{d}t} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} x^2 \frac{\partial c_t}{\partial t}\,\mathrm{d}x \tag{15}$$

and substitution of eqn. 1 in eqn. 15 yields:

$$\frac{\mathrm{d}\mu_2}{\mathrm{d}t} = \frac{A_m}{Q} \int_{-\infty}^{+\infty} \left(x^2 D \frac{\partial^2 c_m}{\partial x^2} - v x^2 \frac{\partial c_m}{\partial x}\right) \mathrm{d}x \tag{16}$$

By repeated partial integration of eqn. 16 and using again that for $x \to \pm\infty$, $c_m = 0$, it can be shown that:

$$\frac{\mathrm{d}\mu_2}{\mathrm{d}t} = \frac{A_m}{Q} \left[2D \int_{-\infty}^{+\infty} c_m\,\mathrm{d}x + v \int_{-\infty}^{+\infty} 2x c_m\,\mathrm{d}x\right] \tag{17}$$

Now we assume again that the gradient is flat within the range of x (the total peak width) in which c_m is significantly different from zero. Continuous gradients generally justify this assumption, step gradients obviously do not. However, the curious fact arises that the results obtained with this treatment, mathematically justified only for slowly varying capacity factors, exactly reproduce certain results obtained for transport functions for dispersionless situations and step gradients[3,6]. It therefore seems appropriate to hope for more general validity of the treatment, beyond the range of slow variation of κ with t and z for which the present calculation can be justified mathematically.

* In eqn. 5, c is not the only variable which changes; x, taken relative to the peaks centre of gravity, also changes. It can be shown, however, that the result of a derivation which takes this into account is the same as eqn. 16.

Assuming such a slow variation of κ with x, and again denoting the κ at $x = 0$ (the peak centre of gravity) by $\bar{\kappa}$ (of course a time-dependent value), we can express κ as a function of x by first-order approximation:

$$\kappa(x) = \bar{\kappa}(1 + ax) \tag{18}$$

This leads to:

$$c_m(x) = c_t(x) \frac{1}{1 + \kappa(1 + ax)} \tag{19}$$

With the same assumption of slow variation of κ, this can be set equal to:

$$c_m(x) = c_t(x) \left[\frac{1}{\bar{\kappa} + 1} - \frac{\bar{\kappa}}{(\bar{\kappa} + 1)^2} ax\right] \tag{20}$$

Insertion of this into eqn. 17 yields four terms, two of which vanish because x measures the distance relative to the centre of gravity, *i.e.*:

$$\int_{-\infty}^{+\infty} x c_t(x)\, dx = 0$$

The two remaining terms yield:

$$\begin{aligned}\frac{d\mu_2}{dt} &= \frac{A_m}{Q}\left[2D \frac{1}{\bar{\kappa} + 1} \int_{-\infty}^{+\infty} c_t(x)\, dx - 2v \frac{a\bar{\kappa}}{(\bar{\kappa} + 1)^2} \int_{-\infty}^{+\infty} x^2 c_t(x)\, dx\right] \\ &= \frac{A_m}{Q}\left[2D \frac{1}{\bar{\kappa} + 1} \frac{Q}{A_m} - 2v \frac{\kappa}{(\bar{\kappa} + 1)^2} a\mu_2 \frac{Q}{A_m}\right] \\ &= 2D \frac{1}{\bar{\kappa} + 1} - 2v \frac{\bar{\kappa}}{(\bar{\kappa} + 1)^2} a\mu_2 \end{aligned} \tag{21}$$

Eqn. 21 describes the change in peak width with time. For comparison it should be noted that in the isocratic situation ($a = 0$) the second term on the right-hand side vanishes.

For a number of cases it is easier to look at the change in peak width with the migrated distance, μ_1. By using eqn. 11

$$d\mu_1 = \frac{v}{\bar{\kappa} + 1} dt$$

it follows that:

$$\frac{d\mu_2}{d\mu_1} = \frac{2D}{v} - 2 \frac{\bar{\kappa}}{\bar{\kappa} + 1} \mu_2 = H - 2 \frac{\bar{\kappa}}{\bar{\kappa} + 1} a\mu_2 \tag{22}$$

This equation gives very good insight, as it gives the increase of the second moment with the migrated distance as a sum of the normal dispersion effect (H term) and the band compression effect (last term), proportional to μ_2 and a. However, for a general

solution of eqn. 11 it is convenient to replace the dependent variable μ_2 by $\mu_2[(1 + \bar{\kappa})/\bar{\kappa}]^2 = y$. This gives:

$$\frac{dy}{d\mu_1} = \frac{(1 + \bar{\kappa})^2}{\bar{\kappa}^2} H - \frac{2(\bar{\kappa} + 1)}{\bar{\kappa}^3} \frac{d\bar{\kappa}}{d\mu_1} \mu_2 - 2a \frac{\bar{\kappa} + 1}{\bar{\kappa}} \mu_2$$

$$= \frac{(1 + \bar{\kappa})^2}{\bar{\kappa}^2} H + 2 \frac{\bar{\kappa} + 1}{\bar{\kappa}^3} \mu_2 \left(- \frac{d\bar{\kappa}}{d\mu_1} - a\bar{\kappa}^2 \right) \tag{23}$$

The value of $d\kappa/d\mu_1$ can be derived, again for gradients which *do not change their shape on migration through the column, i.e.*, which are not distorted by dispersion or by selective uptake of one of the solvent components by the stationary phase (solvent demixing). For these cases $\kappa = \kappa(z,t) = \kappa(\xi)$ and

$$\frac{d\bar{\kappa}}{d\mu_1} = \frac{d\bar{\kappa}}{d\xi} \frac{d\xi}{d\mu_1} = \bar{\kappa}\, a\, (-v) \frac{\kappa(\xi)}{v} = - \bar{\kappa}^2 a \tag{24}$$

from eqns. 13 and 18. For those gradients, which are the only ones for which analytical mathematical considerations give insight into transport properties, eqn. 12 becomes simply:

$$\frac{dy}{d\mu_1} = \frac{d\mu_2 \left(\frac{\bar{\kappa} + 1}{\bar{\kappa}} \right)^2}{d\mu_1} = \left(H \frac{\bar{\kappa} + 1}{\bar{\kappa}} \right)^2 \tag{25}$$

As an example, we treat first the LSS gradient. Here

$$\kappa(\xi) = \kappa_0 \, e^{-b\xi} \tag{26}$$

describes the dependence of the capacity factor on time. The result for the net retention time is[1]

$$\xi_e = (1/b) \ln (\kappa_0 b t_0 + 1) \tag{27}$$

and the capacity factor on elution, κ_e, is:

$$\kappa_e = \frac{\kappa_0}{\kappa_0 b t_0 + 1} = \frac{\kappa_0}{\kappa_0 b L/v + 1} \tag{28}$$

This expression can be used to find κ as a function of the peak position, μ_1, in the column. Then the right-hand side of eqn. 25 is known and this equation can be integrated. The result is:

$$y = HL(1 + p + \tfrac{1}{3}p^2) \tag{29}$$

where

$$p = \frac{\kappa_0}{\kappa_0 + 1} b t_0$$

The second place moment is

$$\mu_2 = HL\left[\frac{1 + p + \frac{1}{3}p^2}{(1 + p)^2}\right] \tag{30}$$

in which the bracketed factor in the right-hand side is equal to G^2 as defined by Snyder *et al.*[2]. A plot of G *versus* the slope of the gradient for large κ_0 according to this equation is to be found in ref. 2, and these numerical results are equal to those predicted by eqn. 29.

A second example is the step gradient. The solution of eqn. 24 for a finite H value depends of course on the dependence of κ on μ_1, *i.e.*, on the moment where the peak is overtaken by the step, and a complex expression will result. However, if we take $H = 0$ (ideal, dispersionless chromatography), we should find the same result as that obtained with more elementary reasoning. This is indeed the case. Eqn. 24 predicts that for $H = 0$, y is invariant. Thus the peak widths before (b) and after (a) the passage of the step have a ratio:

$$\frac{\kappa_b + 1}{\kappa_b} \cdot \frac{\kappa_a}{\kappa_a + 1} \tag{31}$$

This is exactly what has been derived[1,3]. The ratio of the concentrations in the mobile phase after and before the passage of the step is:

$$\frac{c_{ma}}{c_{mb}} = \frac{\kappa_b}{\kappa_a} \tag{32}$$

However, with eqn. 25 it is possible to calculate how dispersion affects the validity of eqn. 32, especially when the absolute values of κ are small.

CONCLUSIONS

A general differential equation of relatively simple form can be derived which predicts the peak broadening, or contraction, under solvent-programmed conditions. The predictions coincide with numerical results obtained for LSS gradients, and with derivations for step gradients when dispersion is neglected. A discussion of the effect of the contraction on optimal gradient shape, as well on the choice of optimal conditions for on-column concentration procedures, is now possible. Future papers will be devoted to these subjects.

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"DIRECT" (ON-COLUMN) SAMPLING INTO GLASS CAPILLARY COLUMNS

COMPARATIVE INVESTIGATIONS ON SPLIT, SPLITLESS AND ON-COLUMN SAMPLING

G. SCHOMBURG*, H. HUSMANN and R. RITTMANN
Max-Planck-Institut für Kohlenforschung, D-4330 Mülheim a.d. Ruhr (G.F.R.)

SUMMARY

On-column (direct) sampling is superior to other techniques of sampling into capillary columns, especially for analyses with quantitative objectives, for example the precise and accurate determination of relative response factors. Discrimination by volatility of sample constituents is minimal and only slightly dependent on solvent volatility and column inlet temperature, but is independent of the type of carrier gas. Prior heating of the sample for vaporization before entering the column is avoided and decomposition of labile sample components is reduced to a minimum. The performances of two different on-column sampling techniques for quantitative analysis were compared. A new construction of an on-column sampling device for very low sample volumes (column loads) is described, which allows for the introduction of volumes as low as a few nanolitres of samples into capillary columns.

INTRODUCTION

With "direct" or on-column sampling the liquid sample can be introduced into narrow-bore (0.20–0.35 mm I.D.) capillary columns without any additional heating of either the sampling device or the column inlet itself, thus avoiding thermal or catalytic decomposition of sensitive sample constituents. No volatilization of the sample into the carrier gas before entering the column is carried out and no splitting in the gas phase, which could give rise to changes in composition of that part of the sample which enters the column, is applied. Moreover, compounds of very low volatility and high polarity enter the column directly and without losses or retardation caused by condensation and/or adsorption in insufficiently heated regions such as connecting tubing. For on-column sampling several techniques for sample introduction and/or volume adjustment have been described. Two different techniques have been previously proposed by the present authors for either small (< 100 nl) or large (500–2000 nl) sample volumes[1]. As an alternative, Grob and co-workers[2,3] proposed the use of a special device for on-column introduction through a microsyringe. Temporary additional cooling of the sampling device including the column

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inlet during the introduction of the syringe needle into the column was applied by Galli *et al.*[4] in order to prevent selective vaporization of the sample, *i.e.*, discrimination.

The macroversion of the Max-Planck-Institut (MPI) technique described earlier[1] makes use of a small crucible in which the sample is placed. The column inlet dips into the liquid sample during the sampling procedure, the liquid being transferred into the column by carrier gas pressure. The sample volume can be adjusted with a reproducibility of only about 5% relative. Volumes as low as 500 nl cannot be easily sampled by this technique. In the microversion technique a narrow-bore glass or metallic capillary pipette is introduced into the capillary column in a similar manner as the syringe needle technique proposed by Grob and Grob, Jr.[2]. Small volumes of 5–20 nl can be sampled with this technique, but the sample volume cannot be adjusted as easily as with a syringe. Micropipettes of different volumes have to be used in order to achieve variable sample volumes.

Considering the requirements for the practical analysis of various kinds of samples and the capabilities and features of classical sampling techniques (of both the split and splitless types), it would be desirable if the following requirements for versatile on-column sampling techniques be met:

(1) For the separation of highly dilute solutions of the species of analytical interest in volatile solvents, maximal sample volumes of up to 2 μl have to be introduced into (glass) capillary columns with a diameter between 0.25 and 0.35 mm. The sample volume should be easily adjustable at least in the range between 0.5 and 2 μl.

(2) For less dilute samples the minimal volumes that have to be introduced should be as low as 10–50 nl in order to attain optimal column loads even when using capillary columns with extremely thin films of the liquid stationary phase ($\leqslant$100 nm).

(3) Generally good precision (low standard deviation of repeatability) and accuracy of gas chromatographic (GC) analysis via peak area percentages as well as a reasonable adjustability of the sample volume are desirable.

In this paper we also describe a new construction of the microversion of our on-column injection device that involves splitting of the liquid sample at the column inlet, which allows for the introduction of sample volumes (column loads) of less than 200 μl.

COMPARATIVE MEASUREMENTS OF THE QUANTITATIVE PERFORMANCE OF ON-COLUMN SAMPLING

On-column sampling: crucible technique, MPI versus Grob's syringe technique with additional cooling (Carlo Erba)

We determined response factors of methyl octanoate (b.p. 192.9°C) relative to *n*-dodecane (b.p. 216.3°C) as the standard compound comparing the two on-column sampling techniques. The MPI macroversion (crucible) was coupled with a Varian 2700 instrument, whereas the Carlo Erba (CE), (Milan, Italy) on-column injector was part of a CE 4160 instrument. A 1-μl volume of a mixture of methyl octanoate and *n*-dodecane in cyclohexane was injected in both instances into a 20-m polypropylene glycol column (I.D. 0.28 m) at a column temperature of 80°C, which was increased to 130°C at a programming rate of 10°C/min. The results of these measurements are given in Table I.

Using the crucible technique, a relative standard deviation of the absolute

TABLE I

DETERMINATION OF RELATIVE RESPONSE FACTORS (RRF) OF METHYL OCTANOATE

Determined using on-column (crucible) and on-column (syringe) sampling. Sample: 1 μl of methyl octanoate + *n*-dodecane in cyclohexane; column: PPG, 20 m × 0.28 mm I.D.; temperature: 80–130°C, 10°C/min; carrier gas: N_2, 0.25 bar; instruments: on-column (MPI), Varian 2700; on-column (CE), Carlo Erba 4160.

Material	*Crucible*		*Syringe*	
	Peak area	*Relative S.D. (%)*	*Peak area*	*Relative S.D. (%)*
n-Dodecane	27.747	2.46	27.550	0.56
Methyl octanoate	19.342	2.60	20.293	0.81
RRF	1.551	0.25	1.468	0.14

peak areas of both components of about 2.5% was observed on repetitive injection. The ratio of peak area to grams of substance (relative response factor by weight) for the ester and the standard was found to be 1.55 with a relative standard deviation of 0.25%. For the CE sampling unit (which was equipped with a device for column inlet cooling) the average relative standard deviation of the "absolute" peak areas was 0.7%; the response factor was 1.47 with a relative standard deviation of only 0.14%. The difference in the relative response factors obtained is due to the influence of the different detector types on the response in the instruments used.

Even for the macroversion the standard deviations of the absolute and relative peak areas are surprisingly low. The syringe technique is superior to the technically simple crucible technique with regard to the standard deviation of the absolute peak areas (3–4 times lower) and also with regard to the adjustability of variable sample volumes.

On-column sampling (CE, syringe) versus split sampling using a standard splitter. Influence of parameters with split and splitless sampling

A C_{18}–C_{36} alkane mixture containing the 10 even-numbered alkanes in about the same concentrations, diluted in *n*-octane (b.p. 126°C), was used for discrimination measurements. A 1-μl volume of this sample was injected into the split injector at a splitting ratio of about 1:35, the injector temperature being 300°C. Hydrogen was used as the carrier gas and the column temperature was 120°C in each instance. With sampling parameters such as an injector temperature of 300°C, a boiling point of the solvent (*n*-octane) of 126°C and hydrogen as the carrier gas, strong discrimination is to be expected, as already pointed out previously[1,5].

The mode of injection and the speed of extrusion of the liquid sample from the syringe needle into the vaporization insert also influence the discrimination under the specified conditions (see also Grob, Jr. and Neukom[6]). By using "fast" rather than "slow" injection less discrimination was found with both split and the splitless sampling especially if not too narrow bore insert tubes were used.

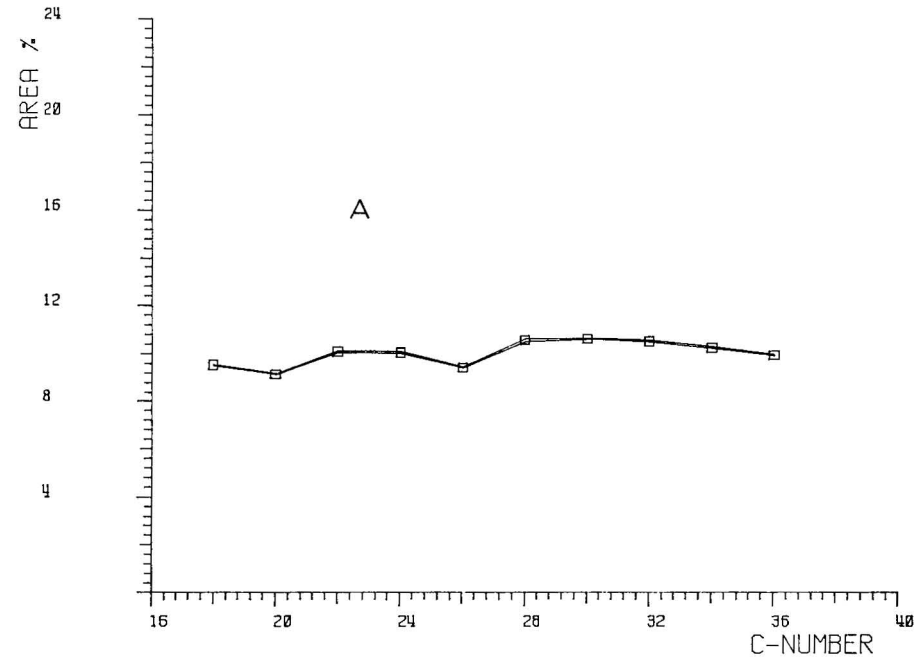
For the experiments on the performance of the on-column sampling the original mixture was diluted a further 35-fold with *n*-octane. Thus the same column load for the C_{18}–C_{36} alkanes as in split sampling at a splitting ratio of 1:35 was achieved. A 1-μl volume was injected in both instances with a 10-μl syringe. The results of these measurements are given in Table II and Fig. 1.

TABLE II

COMPARATIVE MEASUREMENTS OF DISCRIMINATION WITH ON-COLUNN AND SPLIT SAMPLING

Peak areas (%) of C_{18}–C_{36} alkane test mixture.

Sampling	*Parameter*	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}	C_{28}	C_{30}	C_{32}	C_{34}	C_{36}	*Rel. peak area ratio,* C_{18}/C_{36} *(%)*
Split sampling with vaporization insert 80 × 3.9 mm I.D. glass-wool packing	$\bar{M}$	9.760	9.048	9.695	9.427	9.039	10.140	10.658	10.905	10.822	10.507	0.93
	S.D.	0.312	0.251	0.190	0.103	0.120	0.173	0.159	0.141	0.128	0.119	
	Rel. S.D. (%)	3.19	2.77	1.96	1.09	1.32	1.70	1.50	1.29	1.18	1.13	
Split sampling with vaporization insert 80 × 1.7 mm I.D., glass-wool packing	$\bar{M}$	21.068	16.588	14.623	11.351	8.564	7.569	6.351	5.355	4.561	3.971	5.31
	S.D.	1.505	0.997	0.548	0.334	0.408	0.526	0.554	0.563	0.548	0.554	
	Rel. S.D. (%)	7.14	6.01	3.75	2.94	4.76	6.96	8.72	10.52	12.00	13.95	
On-column	$\bar{M}$	9.502	9.115	10.073	10.038	9.408	10.582	10.606	10.519	10.226	9.933	0.96
	S.D.	0.026	0.013	0.022	0.030	0.018	0.063	0.026	0.027	0.039	0.031	
	Rel. S.D. (%)	0.27	0.14	0.22	0.30	0.19	0.60	0.24	0.25	0.38	0.31	



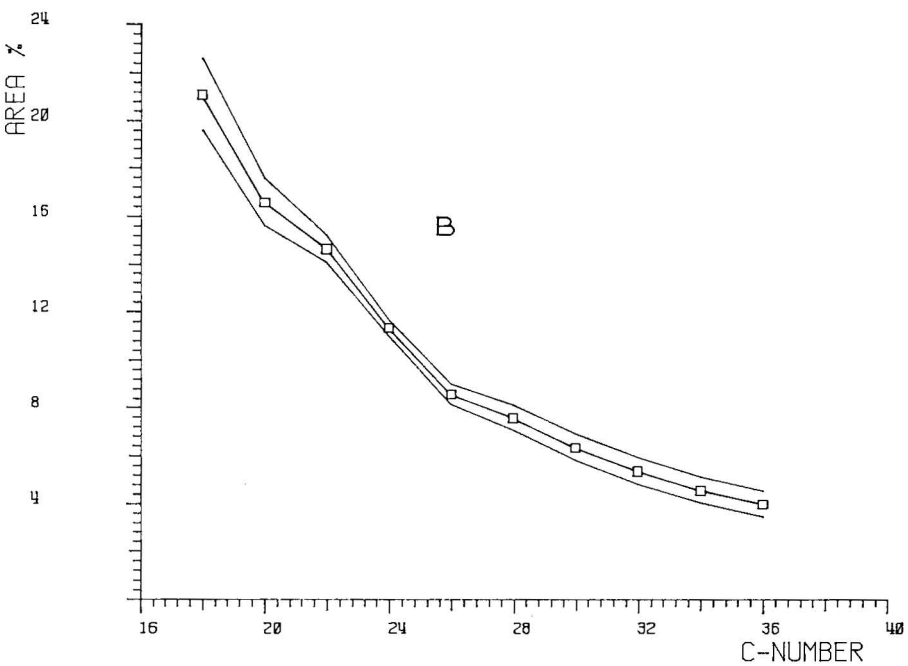


Fig. 1.

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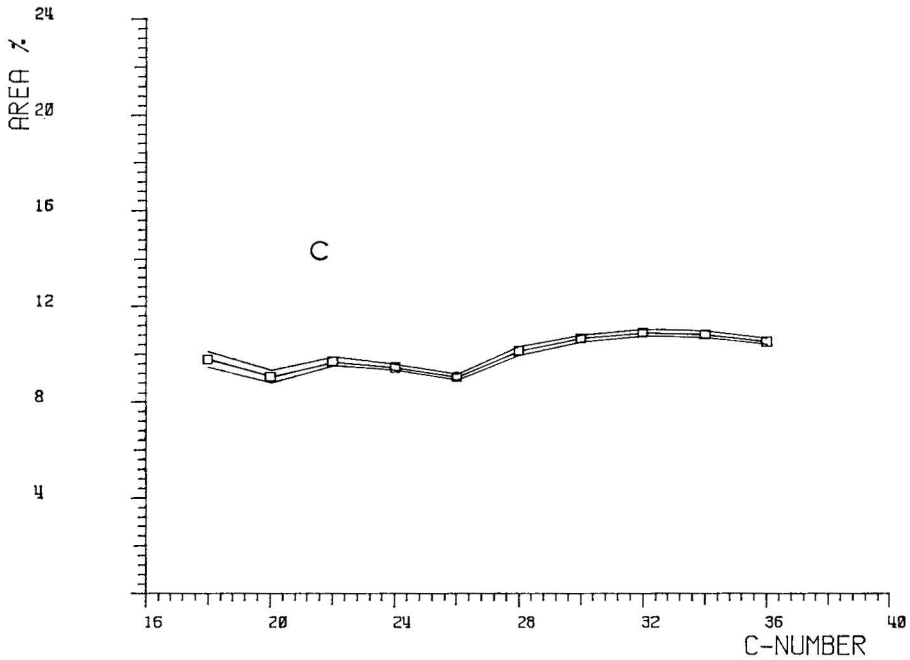


Fig. 1. Comparative measurements of discrimination with on-column and split sampling. (A) On-column (CE) sampling (sampling at column temperature). (B) Split sampling (narrow bore insert, 1.7 mm), glass-wool packing, "fast" injection. (C) Split sampling (wide bore insert, 3.9 mm), "fast" injection. Sample: 1 μl C_{18}–C_{36} alkanes, diluted in *n*-octane (b.p. 126°C); column: methylpolysiloxane (SE-30), I.D. 0.28 mm; temperatures: injector, 300°C; column, 120–300°C, 10°C/min; carrier gas: H_2, 0.65 bar, $\bar{u}$ = 50 cm/sec; split flow-rate: 80 ml/min; column flow-rate: 2.5 ml/min; instrument: Carlo Erba 4160 including sampling device.

The comparison of the relative peak areas normalized to 100% and of the corresponding standard deviations (S.D.) obtained with the different techniques allows the following conclusions to be drawn. With on-column injection of the original sample, the expected normalized peak areas of about 10% for each component were found, the relative standard deviation for repetitive injection being as low as 0.3% on average for the ten components. No appreciable dependence on solvent volatility or type of carrier gas could be observed.

Using the split mode of sampling with a narrow-bore vaporization tube packed with glass-wool, extremely high discrimination was observed under the given conditions, in spite of the fast mode of sample injection that had been applied. The ratio of the C_{18} and C_{36} alkane peak areas was 5.3 compared with 0.96 with on-column injection and the corresponding standard deviations for repetitive sampling were as high as 8% on average. The standard deviations may also be assessed from Fig. 1, in which the parallel lines indicate the positive and negative deviations.

This extremely strong discrimination is due to the high sampling temperature in the injector (300°C), the carrier gas used (hydrogen) and the low boiling point of the solvent, *n*-octane (126°C). In a previous publication[5], we demonstrated that by decreasing the splitter temperature and by increasing the boiling point of the solvent

discrimination can be completely eliminated. We also found that the discrimination in nitrogen as the carrier gas is considerably lower than in hydrogen. Discrimination can therefore also be avoided by using nitrogen in the sample introduction procedure only. After a few minutes hydrogen was used as the carrier gas for the completion of the separation by switching of the carrier gas inlet to a supply of hydrogen. The influence of the various parameters of split sampling can be assessed from the chromatograms in Fig. 2.

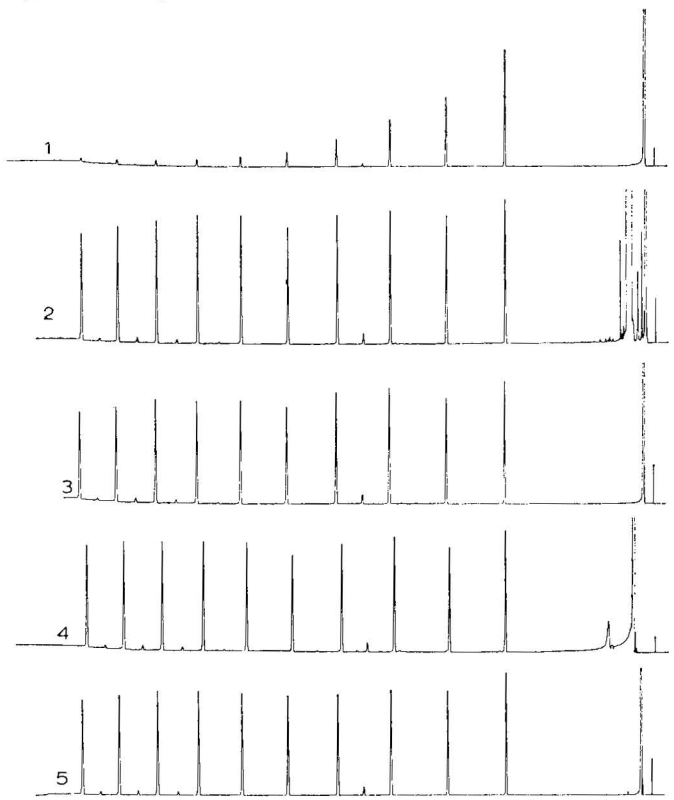


Fig. 2. Discrimination with split sampling of diluted even-numbered C_{18}–C_{36} *n*-alkanes (1). Influence of solvent volatility (2), injection temperature (3), type of carrier gas in sampling (4) and speed of injection (5). Sample: 1 μl of solution in *n*-octane or *n*-dodecane; column: 20-m methylpolysiloxane OV-101, 0.27 mm I.D.; temperature: 120–300°C, 8°C/min.

	1	*2*	*3*	*4*	*5*
Solvent	*n*-Octane	*n*-Dodecane	*n*-Octane	*n*-Octane	*n*-Octane
Injector temperature (°C)	300	300	210	300	300
Injector speed (sec)	2–3	2–3	2–3	2–3	<1
Carrier gas	H_2	H_2	H_2	$N_2 \rightarrow H_2$	H_2

In a second series of measurements using a wide-bore insert tube of I.D. 3.9 mm and length 80 mm we found a significant difference in discrimination when adopting either a fast or a slow mode of injection. With fast injection the discrimination was nearly negligible (compared with the discrimination-free on-column sampling

mode), whereas with slow injection the same poor results as with the narrow-bore insert were obtained. The data in this series of experiments are given in Table II and Fig. 1. The discrimination and the standard deviation of the normalized peak area percentages are much lower than when the narrow-bore insert tube was used. The ratio of the C_{18} and C_{36} peak areas was 0.93, which is similar to that found with on-column (syringe) sampling. The relative standard deviations of the normalized peak area percentages were 1.7% on average for the ten alkanes, which is still about six times higher than with on-column sampling, however.

For comparison we also investigated again the splitless sampling mode under similar conditions, using the same diluted sample as for the on-column sampling. Regarding discrimination, the performance of splitless sampling, applying the same devices (with a wide-bore insert tube) and the fast mode of injection, was higher than with the split mode of sampling but could also be considerably reduced by using a solvent of lower volatility (higher boiling point). No significant improvement could be achieved by changing either the column or injector temperature or by using nitrogen instead of hydrogen as the carrier gas. The results are shown in the chromatograms in Fig. 3.

The conclusion from the results of the two series of experiments on discrimination by volatility with split and splitless sampling is that the main source of error in the introduction of the sample into heated sampling units with a syringe is heat transfer from the vaporization insert on to the syringe needle and the selective vaporization of samples with a wide volatility range from the needle tip. This conclusion is in agreement with the results of Galli *et al.*[4].

According to the results of the experiments, discrimination is increased by the following:

(a) too high a vaporization insert temperature in general;

(b) contact of the needle with the insert walls (especially with too narrow bore insert tubing);

(c) fast heat transfer from the insert walls to the needle by a carrier gas of high thermal conductivity (hydrogen);

(d) too volatile solvents or major constituents of the sample and with samples of high average volatility in general;

(e) too "slow" sample introduction with the syringe (long residence time of the syringe needle within the heated insert).

The most effective means of decreasing the discrimination is to use either a solvent with higher boiling point and/or not too high injector temperatures.

The performance of sample introduction with the on-column (direct) technique is much less influenced by such adverse factors because no additional heating or vaporization of the sample before entering the column is carried out.

On-column (crucible) versus split sampling (standard splitter with narrow-bore glass-wool packed insert) in relative response factor determination using standard compounds with varying boiling points

For methyl pentanoate (b.p. 126.5°C) and heptanoate (b.p. 172°C) relative response factors by weight were determined, using C_8, C_{10}, C_{11} and C_{12} *n*-alkanes as standards (see Fig. 4). The fairly volatile *n*-pentane was used as the solvent.

With on-column injection the relative response factor for methyl pentanoate

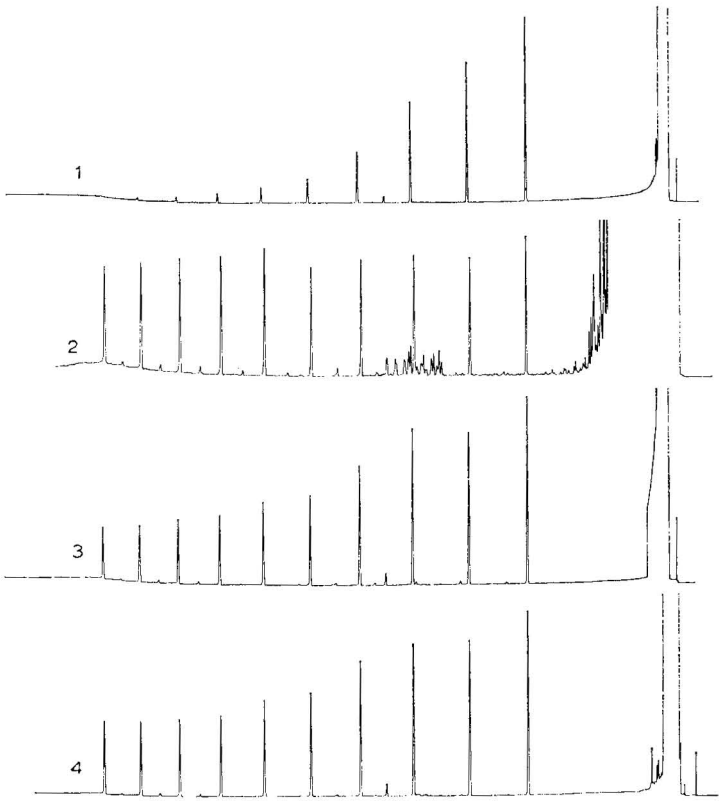


Fig. 3. Discrimination with splitless sampling of diluted even numbered C_{18}–C_{36} *n*-alkanes (1). Influence of: solvent volatility (2), injection temperature (3) and type of carrier gas (4). Sample: 1 μl of solute in *n*-octane or *n*-dodecane; column: 20-m methylpolysiloxane OV-101, 0.27 mm I.D.; temperature: 120–300°C, 8°C/min.

	1	*2*	*3*	*4*
Solvent	*n*-Octane	*n*-Dodecane	*n*-Octane	*n*-Octane
Injection temperature (°C)	210	300	300	300
Carrier gas	H_2	H_2	H_2	$N_2 \rightarrow H_2$

was nearly independent of the volatility of the standards, between 1.78 and 1.80 for all four standard alkanes, although the boiling points of these varied between 126°C (*n*-octane) and 215°C (*n*-dodecane). With split sampling, using a standard splitter with a glass-wool packed narrow-bore (80 × 2.0 mm I.D.) insert, the response factor of methyl pentanoate decreased from 1.80 for *n*-octane as standard to 1.67 for the high-boiling *n*-dodecane as standard. By avoiding the use of a very low-boiling solvent the discrimination at split sampling can be decreased considerably.

The same relative response factors were found with both sampling techniques only if in split sampling a standard *n*-alkane with a boiling point similar to or identical with that of the ester was used.

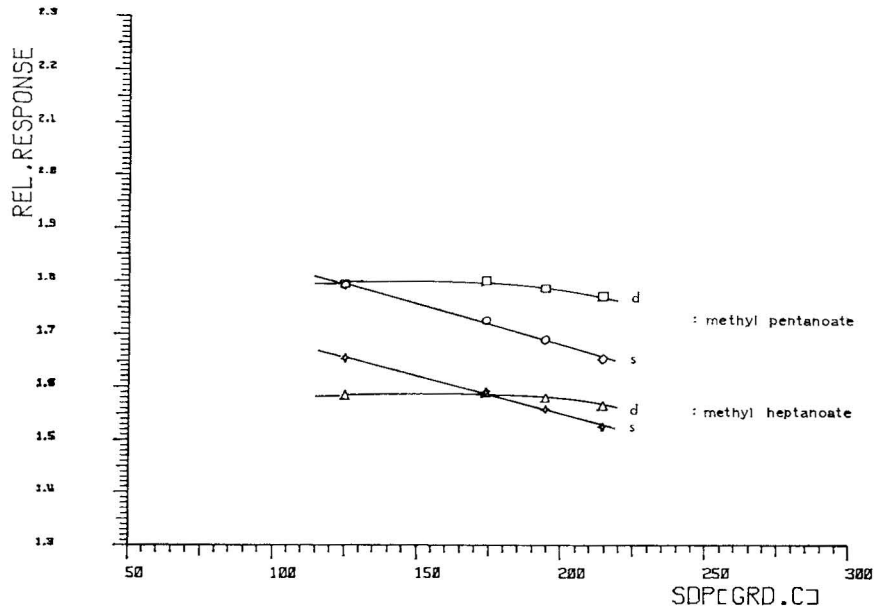


Fig. 4. Influence of sampling technique on discrimination in response factor determination using *n*-alkanes with increasing boiling points as standards. Column: 75-m Emulphor O, I.D. 0.27 mm; sample: C_5, C_7 methyl ester + *n*-C_8, C_{10}, C_{11}, C_{12}; s = split, 1 μl wtih 1:100 splitting ratio; d = direct, 1 μl with diluted sample 1:100 in *n*-pentane; temperatures: split, injector 150°C, column 110°C isothermal; direct, 60°C, column 60 to 120°C at 10°C/min; carrier gas: N_2, 1.1 bar, $\bar{u}$ = 14 cm/sec; instrument: MPI standard splitter (2.0 mm I.D.), glass-wool packing; MPI on-column (crucible) sampling.

CONCLUSION ON COMPARATIVE SAMPLING MEASUREMENTS

For quantitative measurements of mixtures with a wide volatility range and for response factor determinations that require high precision and accuracy, the on-column sampling technique is superior to the classical split and splitless sampling techniques with regard to discrimination and to the standard deviation of the relative peak areas. The performance of any kind of quantitative measurements with the on-column technique is less dependent on the various sampling parameters and on the type of calibration mixtures used regarding volatility range, etc.

The standard compounds used in response factor determinations must have similar volatilities (boiling points) to those of the species to be examined if split sampling is to be applied. It is not important for the standard compound to have the same or a similar retention, however, because the performance of the sampling method determines the accuracy of the calibration.

Comparing the different techniques of on-column injection, we conclude that the proper adjustment of the volume and the reproducibility of its introduction are optimal with the syringe technique. The crucible method is technically simpler and the adjustability of sample volume and the standard deviations of absolute (column load) and relative peak areas are sufficient for practical work. We consider that additional column inlet cooling as used in the CE technique may be of an advantage

to all on-column techniques, but makes the devices more sophisticated. If the solvent volatility and the column inlet temperature are chosen properly, however, additional cooling may not be necessary.

Nevertheless, there is still a need for new devices for on-column sampling techniques that allow especially for the achievement of adequate column loads, as for example in the case of undiluted samples that arise in industrial product control. Further, there is an urgent need for automated techniques of on-column injection.

NEW CONSTRUCTION FOR ON-COLUMN SAMPLING OF LOW VOLUMES

In an earlier paper on on-column sampling[1] with capillary columns, we also described a microversion technique that avoids the use of the septum and also possibly a syringe. The sample is introduced into the capillary column by means of a thin capillary of defined volume, which is directed into the correct position inside the capillary column by a special device; a similar one was used later also by Grob in his syringe technique. The volume of the sample can be varied by the application of micropipettes of different volumes, whereas a syringe can be used more universally for a certain range of variable sample volumes.

The lowest volume that can be sampled with the special syringes used with the CE (Grob) technique may be about 100 nl, which is still too high for the sampling of undiluted samples. With our previously described (micro) technique we have been able to sample volumes of about 20–50 nl reliably.

We have now investigated whether the advantages of the macro version can be combined with those of the micro version and whether it is possible to construct a device that allows for "liquid splitting" of the sample. We used a metallic micropipette newly designed according to the same principles as our previously described micro version device, as can be seen from Fig. 5.

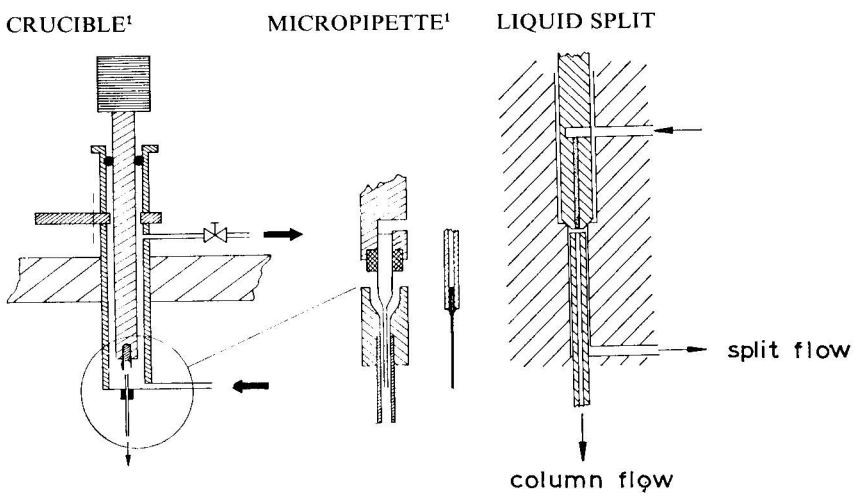


Fig. 5. Three versions of on-column sampling techniques. Liquid split: variable splitting of the liquid sample at the column inlet is used to minimize column load. The splitting ratio is varied with the carrier gas split flow. Discrimination is as low as with other on-column sampling devices.

The column load can be varied by changing the ratio of "split" flow and "column" flow of the carrier gas. Discrimination by volatility is as low as with other constructions of on-column sampling, as could be shown at the separation of various types of samples including the aforementioned discrimination test mixture containing the C_{18}–C_{36} alkanes.

Details of the construction and experimental results for practical applications will be published in the near future.

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CHROM. 13,172

N-ALKYLATION OF TERTIARY ALIPHATIC AMINES BY CHLOROFORM, DICHLOROMETHANE AND 1,2-DICHLOROETHANE

STEEN HONORÉ HANSEN* and LARS NORDHOLM
Department of Organic Chemistry, Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

SUMMARY

The tertiary aliphatic amine imipramine is treated with chloroform, dichloromethane and 1,2-dichloroethane under mild conditions. In chloroform and dichloromethane two quaternary ammonium compounds, N-chloromethylimipraminium chloride and N-methylimipraminium chloride, are formed as well as the secondary amine desmethylimipramine. In 1,2-dichloroethane only two quaternary ammonium compounds are found.

The reaction products are quantified and isolated by means of high-performance liquid chromatography.

Routes of reactions and their importance in relation to analytical chemistry are discussed.

INTRODUCTION

Several observations on the formation of quaternary chloromethylammonium compounds when using dichloromethane as a solvent for tertiary aliphatic amines can be found in the literature[1–9]. Quaternization in chloroform owing to the impurities bromochloromethane and dichloromethane has also been reported[10–13]. The observation of bisquaternization has also been stated[8].

This work has been carried out to investigate the extent to which these reactions take place and to see what influence they have in analytical chemistry.

The three solvents chloroform, dichloromethane and 1,2-dichloroethane, often used for extraction, have been investigated by reaction with the model compound imipramine (IP) —a tertiary amine.

EXPERIMENTAL

Apparatus

A liquid chromatograph consisting of a Waters 6000A pump and a Waters 440 detector (254 nm) connected to an Omniscribe 5117-5 recorder was used. The sample injections were made through a Rheodyne 7120 injection valve.

The thin-layer chromatographic (TLC) and the gas chromatographic (GC)

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systems as well as equipment for ultraviolet absorbance and proton magnetic resonance measurements have been described previously[7].

The apparatus for mass spectrometric (MS) measurements has also been described[7,10]. For the GC–MS determination of desmethylimipramine (DMI) the following chromatographic column was used: 2 m × 2 mm I.D. stainless steel packed with 3% Dexsil 300 on Gas-Chrom Q, 100–120 mesh.

Chemicals

Imipramine hydrochloride was a gift from Ciba-Geigy, Copenhagen, Denmark. N-Chloromethylimipraminium chloride and N-β-chloroethylimipraminium chloride were prepared as described in ref. 7. N-Methylimipraminium iodide was prepared by methylation of IP with methyl iodide dissolved in chloroform. All other chemicals used were of analytical grade.

High-performance liquid chromatography (HPLC)

The column (150 × 4.65 mm I.D.) was packed with LiChrosorb SI 60, 5-μm particles, using a 2% slurry in methanol and a pressure of *ca.* 44 MPa generated by a Haskel pump 28646-4. When operated at ambient temperature with the mobile phase methanol–4 *M* ammonium formate (100:1.3) at a flow-rate of 1.0 ml/min, the column had 6400 theoretical plates, determined with IP, k' (capacity ratio) = 2.0.

RESULTS AND DISCUSSION

To find out to what extent a tertiary amine is transformed into quaternary ammonium compound(s), imipramine was treated with chloroform, dichloromethane and 1,2-dichloroethane. The results are shown in Table I. Chloroform from certain manufacturers contains small amounts of the very reactive bromochloromethane[7]. Therefore pure chloroform previously shown by GC[7] to contain no bromochloro-

TABLE I

PERCENTAGE TRANSFORMATION OF IMIPRAMINE INTO QUATERNARY AMMONIUM COMPOUND(S) WHEN 0.1% SOLUTIONS WERE REFLUXED OR STORED AT AMBIENT TEMPERATURE IN THE DARK

Temperature	*Storage time*	*Solvent*			
		$CHCl_3$	$CHCl_3$ + 0.04% CH_2BrCl	CH_2Cl_2	$ClCH_2CH_2Cl$
Ambient	1 day	—	0.5	10.1	3.9
	3 days	—	—	28.5	8.7
	6 days	—	1.4	58.0	17.9
	9 days	—	—	80.0	26.3
	20 days	1.0	4.0	—	—
	56 days	—	12.5	—	—
	4 years	29.5	—	—	—
Reflux	30 min	0.05	0.1	0.6	—
	60 min	0.1	0.2	1.5	—
	90 min	—	—	2.4	—
	120 min	0.2	0.5	—	—

methane and no dichloromethane was used, as well as chloroform with 0.04% of bromochloromethane added.

The amounts of quaternary compounds were determined by extraction of the organic phase with 0.01 *N* sodium hydroxide. The ultraviolet absorption of the aqueous phases were measured at 250 nm.

The results show that quaternization is fastest in dichloromethane. After *ca.* 45 min 1% of the IP is quaternized in the dichloromethane solution under reflux.

In order to find what products are formed during the reactions, the reaction mixtures were analyzed by TLC and by HPLC (Fig. 1). By analyzing the organic phase prior to and after the extraction with 0.1 *N* sodium hydroxide it was determined what peaks were possibly due to quaternary ammonium compounds, as these would be extracted to the aqueous phase.

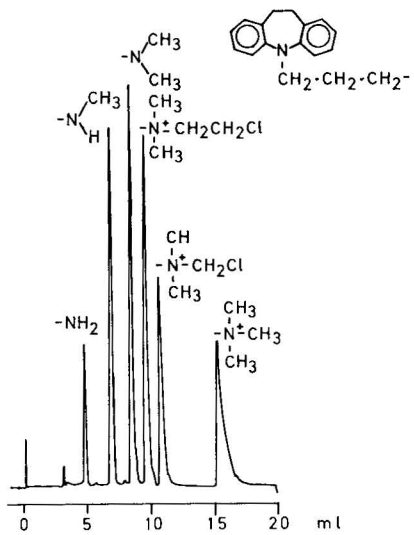


Fig. 1. Separation of imipramine and some of its derivatives on silica with methanol–4 *M* ammonium formate (100:1.3) as the mobile phase.

The reaction mixtures in chloroform and dichloromethane contained the same four components (Table II) (even after storing the chloroform solution for 4 years), and two of these were assumed to be quaternary ammonium compounds.

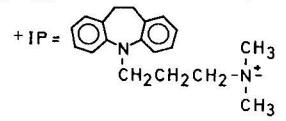
The two lipophilic compounds were found to be DMI and IP by GC–MS analysis. The two hydrophilic compounds were isolated by semi-preparative HPLC using the analytical system. The two components were shown by nuclear magnetic resonance spectroscopy and field desorption MS to be salts of the N-chloromethylimipraminium ion and the N-methylimipraminium ion.

The reaction mixture in 1,2-dichloroethane contained N-β-chloroethylimipraminium ions and a small amount of an unidentified quaternary ammonium compound. The reaction in 1,2-dichloroethane was not investigated any further.

The solvents in the reaction mixtures were analyzed by GC. No changes in the solutions in dichloromethane and 1,2-dichloroethane were seen. The reaction mixture

TABLE II

PRODUCTS OF THE TREATMENT OF IMIPRAMINE WITH DICHLOROMETHANE (CH_2Cl_2), CHLOROFORM ($CHCl_3$) AND 1,2-DICHLOROETHANE ($ClCH_2CH_2Cl$)



Reagent	*Reaction product*				
	DMI	*$ClCH_2$–$^+$IP*	*CH_3–$^+$IP*	*$ClCH_2CH_2$–$^+$IP*	*Other*
CH_2Cl_2	+	+	+	–	–
$CHCl_3$	+	+	+	–	–
$ClCH_2CH_2Cl$	(+)	–	–	+	+

in chloroform showed that dichloromethane is formed when IP is added. To find out whether this was due to the amine or to the sodium hydroxide present during the extraction procedure, 0.1 % solutions of triethylamine in the same three haloalkanes were analyzed similarly. Again formation of dichloromethane in chloroform was seen, while no change in the solutions in dichloromethane and 1,2-dichloroethane could be detected by this method.

The routes of reaction were investigated by heating the different compounds in acetonitrile as shown in Table III. It is seen that DMI is formed by degradation of N-chloromethylimipraminium chloride, and that the N-methylimipraminium salt is formed by methylation of IP with the N-chloromethylimipraminium ion as the methylating reagent.

TABLE III

REACTION PRODUCTS FORMED BY HEATING SOLUTIONS AT 80°C FOR 1 h

P = parent compound.

1% solutions in CH_3CN	*Reaction product*					
	DMI	*IP*	*$ClCH_2$–$^+$IP*	*CH_3–$^+$IP*	*$ClCH_2CH_2$–$^+$IP*	*Other*
IP	–	P	–	–	–	–
$ClCH_2$–$^+$IP, Cl^-	+	–	P	–	–	–
CH_3–$^+$IP, I^-	–	–	–	P	–	–
$ClCH_2CH_2$–$^+$IP, Cl^-	–	–	–	–	P	+
IP + $ClCH_2$–$^+$IP, Cl^-	+	P	P	+	–	–
IP + CH_3–$^+$IP, I^-	–	P	–	P	–	–
IP + $ClCH_2CH_2$–$^+$IP, Cl^-	–	P	–	–	P	+

N-β-Chloroethylimipraminium chloride is unable to methylate IP. The unknown compound formed in this case is also formed by heating N-β-chloroethyl-imipraminium chloride alone.

On basis of this the reaction scheme in Fig. 2 is suggested for the reaction between dichloromethane and IP.

Bisquaternization, which has been reported by some authors[1,8], was not observed in any case.

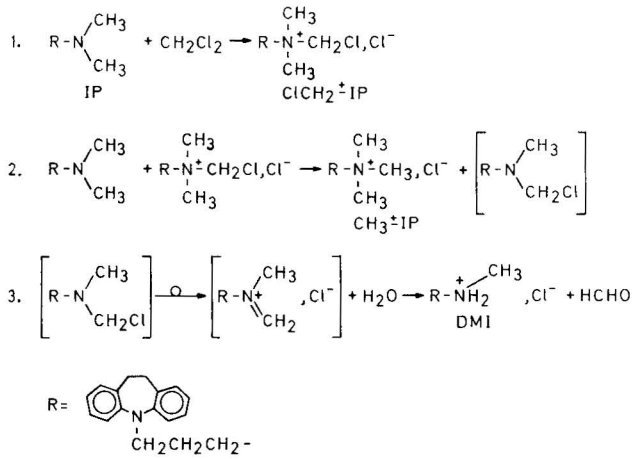


Fig. 2. The suggested reactions of imipramine in dichloromethane.

CONCLUSIONS

Dichloromethane and 1,2-dichloroethane should be used only with due care as solvents and extractants for tertiary aliphatic amines, and solutions should under no circumstances be stored. Solutions of tertiary aliphatic amines in chloroform should not be stored for long periods. The final analytical results may otherwise be invalidated depending of the method of measurement (*e.g.* chromatography).

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CHROM. 13,111

USE OF CIRCULAR DICHROISM AS A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETECTOR

STEVEN A. WESTWOOD, DAVID E. GAMES* and L. SHEEN
Department of Chemistry, University College, P.O. Box 78, Cardiff CF1 1XL (Great Britain)

SUMMARY

A circular dichroism (CD) spectrophotometer has been coupled to a high-performance liquid chromatograph. Natural pyrethrins, tryptophan, rotenoids and Amaryllidaceae alkaloids have been used to evaluate the system. Monitoring at selected wavelengths has enabled detection levels in the low μg range to be obtained. By using stopped-flow techniques, full CD spectra can be obtained, and the system has been used in conjunction with combined high-performance liquid chromatography–mass spectrometry to study an extract of timbo powder.

INTRODUCTION

The combination of high-performance liquid chromatography (HPLC) with some form of detector that is selective for optically active compounds has considerable potential in a wide variety of research areas. Studies aimed at the identification of known natural products or seeking new structural types are examples. A potentially more important area of application is metabolic studies of drugs and pesticides, where detectors of this type could assist in unravelling some of the stereochemistry involved in the metabolic process and provide increased specificity for quantitative and qualitative studies.

We report here the results we have obtained by coupling a circular dichrograph with a high-performance liquid chromatograph. An advantage of using a circular dichrograph is that it not only enables selective detection of optically active compounds with suitable chromophores, but that it enables wavelength selectivity to be utilised in a similar way to that available with a variable-wavelength UV detector. The high degree of selectivity thus obtained enables circular dichroism (CD)-active compounds to be detected in a complex matrix of other compounds such as is encountered in natural-product extracts, or biological fluids.

EXPERIMENTAL

A HPLC system consisting of a Waters M6000 pump connected to columns set up for on-column injection was connected to a Jobin-Yvon circular dichrograph III. Micro HPLC flow-cells of 1 mm and 10 mm path length (Cecil Instruments,

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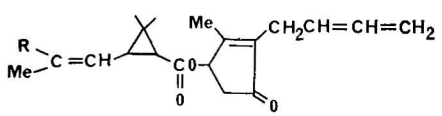
Cambridge, Great Britain) were initially used for fixed-wavelength studies. On-line CD spectra were obtained by using a cell machined from a block of PTFE. The flow channel was 1 mm^2 in area and 10 mm in length, and stress-free silica end windows were used. Solvents were distilled from an all-glass apparatus and were degassed before use.

Combined liquid chromatography–mass spectrometry (LC–MS) was performed on a Finnigan 4000 mass spectrometer equipped with a standard moving-belt interface[1] and coupled to an Incos data system. A source temperature of 240°C and source pressure of 0.2 Torr were used for the methane chemical-ionisation (CI) studies. A Cecil CE272 was used for UV detection in the HPLC studies. The HPLC conditions used are given in the text.

RESULTS AND DISCUSSION

Initial studies to evaluate the system utilised the amino acid L-tryptophan. Standard solutions were injected into the HPLC–CD system A Hypersil ODS (5 μm) column, (100 × 5 mm) was used, with methanol–water (1:1) at a flow-rate of 1 cm^3 min^{-1} as mobile phase, and the CD was monitored at 270 nm. Injection of a solution containing 3 μg of L-tryptophan could be detected with a signal-to-noise ratio greater than 2:1.

In order to assess the possibility of selective detection of CD-active compounds in crude mixtures, an extract of pale pyrethrum was examined. The extract consisted predominantly of pyrethrins I (1a) and II (1b). A column (100 × 5 mm) packed with Spherisorb ODS (5 μm), with methanol–water (4:1) at a flow-rate of 1 cm^3 min^{-1} was used as eluent in this study. By monitoring at 250 nm, both compounds are detected, with pyrethrin II giving the larger signal. None of the other components detected in the UV trace is detected. At 235 nm, the intensity of the signals is reversed, while at 220 nm only pyrethrin I can be detected.



1a R = Me
1b R = CO_2Me

Fig. 1 shows a comparison of the traces obtained from HPLC with UV detection, HPLC–CD and the reconstructed total-ion-current trace obtained during methane CI LC–MS of a chloroform extract of timbo powder. The features of the methane CI spectra are given in Table I. The extract consists of four main components. As can be seen from Table I, components A and B gave virtually identical methane CI spectra, indicating molecules of MW 410; components C and D also gave very similar spectra and had MW 394. The HPLC–CD trace enabled some differentiation to be made of the pairs of isomeric compounds, and HPLC–CD and LC–MS examination of a commercially available sample of rotenone (3) (BDH, Poole, Great Britain) showed that component C is rotenone. Although it might be expected from the HPLC–CD trace that component D might have the opposite

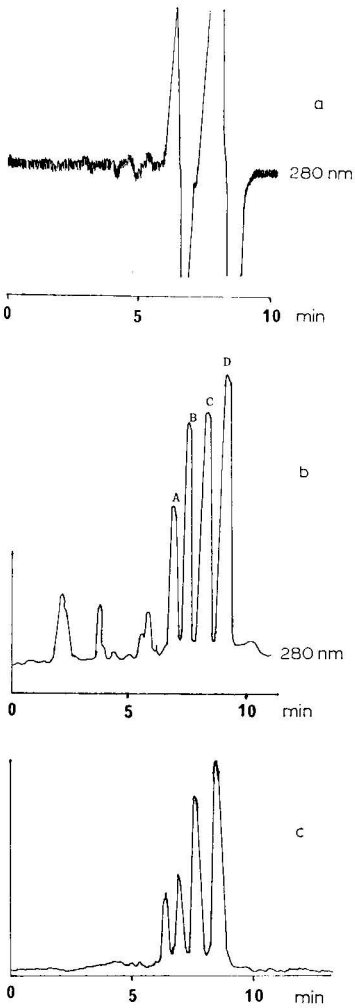
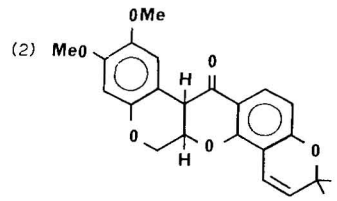


Fig. 1. Chomatograms of timbo powder extract. Column: 250 × 4.6 mm; Hypersil C_{22} (5 μm). Mobile phase: acetonitrile–water (4:1); flow-rate, 1 $cm^3\ min^{-1}$. (a) HPLC–CD; (b) HPLC–UV; (c) LC–MS.

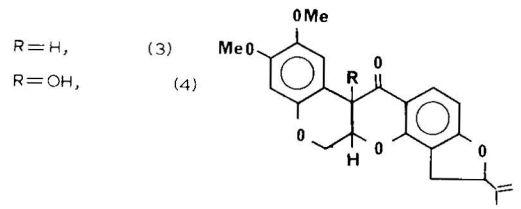
TABLE I

METHANE CI SPECTRA OBTAINED DURING LC–MS OF TIMBO POWDER EXTRACT

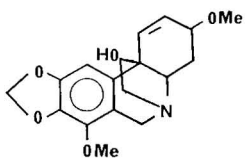
Peak	*m/z (% relative intensity)*
A	411(23), 410(16), 393(100), 323(10), 208(13)
B	411(30), 410(19), 393(100), 208(15)
C	395(100), 192(10)
D	395(100), 192(8)



(2)



R=H, (3)
R=OH, (4)



(5)

configuration to rotenone, an alternative explanation could be that the compound is isomeric, with a different chromophore. This could be resolved by obtaining an on-line CD spectrum. Comparison of mass spectra, HPLC–CD and HPLC retention times in fact showed the latter explanation to be correct, and component D is deguelin (2). The identity of components A and B has not yet been satisfactorily resolved, but rotenalone (4) has a similar retention to component A.

In order to ascertain the possibility of obtaining on-line CD spectra, the base extract of bulbs of *Crinum glaucum* was examined. Preliminary examination of this extract showed the presence of a number of known Amaryllidaceae alkaloids, together with other alkaloids of unknown structure[2]. We have reported LC–MS studies on this extract elsewhere[3]. The extract is a complex mixture of alkaloids, and one of the major components is the alkaloid ambelline (5), the CD spectrum of which has been reported[4]. Fig. 2a shows the background-subtracted on-line CD spectrum of ambelline obtained during HPLC–CD of the extract, together with the CD spectrum obtained under standard conditions (Fig. 2b); as can be seen, the CD spectra are in good agreement.

CONCLUSIONS

HPLC–CD shows promise as a selective detection system for compounds that are CD-active. At present, detection is in the low μg range. An ability to obtain on-line CD spectra during HPLC–CD of complex mixtures has additional utility, particularly when used in combination with LC–MS in ascertaining the structures of components of natural extracts.

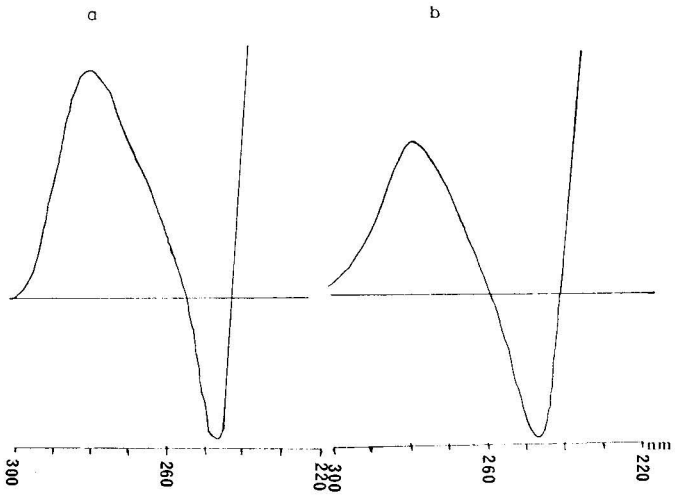


Fig. 2. (a) On-line CD spectrum: time constant, 10 sec; sensitivity, 1×10^{-6}. Separation was carried out on a column (150 × 5 mm) of C_8 bonded phase, with methanol–water (3:2) containing a trace of ammonia as eluent (1 cm^3 min^{-1}). (b), CD spectrum of ambelline.

ACKNOWLEDGEMENTS

We thank the ARC for financial support, Dr. D. Whiting (University of Nottingham) for samples of deguelin and rotenalone and Professor D. A. H. Taylor (University of Natal) for the *Crinum glaucum* extract. We are indebted to Professor R. D. Gillard (University College, Cardiff) for allowing us to use his circular dichrograph for this study, and to Dr. P. A. Williams (University College, Cardiff) for his advice and encouragement during this investigation. Finally, we thank the SRC for funds for the purchase of a data system for our Finnigan 4000.

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CHROM. 13,183

SEPARATION AND DETERMINATION OF ALIPHATIC AND AROMATIC AMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

BÖRJE BJÖRKQVIST
Kemira Oy, Espoo Research Centre, P.O. Box 44, SF-02271 Espoo 27 (Finland)

SUMMARY

Aliphatic and aromatic primary and secondary amines react with phenyl isocyanate to yield N,N′-disubstituted ureas, very stable and highly UV-absorbing compounds. The reaction is quantitative and is complete in a few minutes.

The derivatives are easily chromatographed on a reversed-phase high-performance liquid chromatographic system. UV-monitoring allows detection down to the 1-ng level. No pH suppression of the eluent is needed.

INTRODUCTION

Underivatized volatile amines can usually be separated by gas–liquid chromatography (GLC) on a polar stationary phase. Derivatization, such as silylation and reaction with aldehydes followed by GLC on a non-polar stationary phase, is also sometimes used. Incomplete derivatization and thermal lability of some of these derivatives can make quantitation difficult.

Amines have also been separated by high performance liquid chromatography (HPLC) using different derivatives, both UV-absorbing and fluorescent, by ion-pair chromatography and cation-exchange chromatography. The HPLC separation of free amines usually requires pH suppression, and this again tends to destroy modern silica-based stationary phases. Poor UV-absorptivity of most aliphatic amines also makes the detection of small amounts difficult[1–3].

Phenyl isocyanate has proved to be an excellent derivatizing reagent for compounds with active hydrogen atoms such as alcohols and water[4–6]. The corresponding urethanes and diphenylurea are easy to chromatograph on a reversed-phase system with UV detection. This method is also well suited for primary and secondary amines, both aliphatic and aromatic. They react with phenyl isocyanate to yield N,N′-disubstituted ureas, as shown in Fig. 1. A single and very stable derivative is obtained even with primary amines. It is easily chromatographed on a reversed-phase system and requires no pH suppression. UV-monitoring at 240–260 nm allows detection of the derivatives down to the 1–10-ng level.

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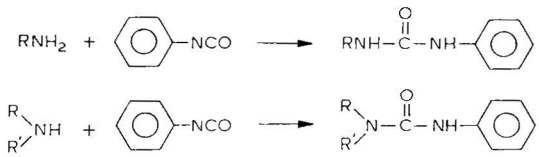


Fig. 1. General reactions of primary and secondary amines with phenyl isocyanate.

EXPERIMENTAL

Apparatus

A Varian 5000 Series liquid chromatograph (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a Perkin-Elmer LC-55 variable-wavelength UV-VIS detector (Perkin-Elmer, Oak Brook, IL, U.S.A.), a Goerz Servogor 541 recorder (Goerz Electro, Vienna, Austria) and a self-packed Spherisorb 10 ODS reversed-phase column (250 × 4.6 mm I.D.) were used for the chromatographic separation and detection of the products. Injections were made by a pressure-tight 10-μl SGE syringe (Scientific Glass Engineering, North Melbourne, Australia) into a Hewlett-Packard septumless on-column injector (Hewlett-Packard, Karlsruhe, G.F.R.).

A Perkin-Elmer 402 scanning spectrophotometer (Perkin-Elmer, Beaconsfield, Great Britain), a Jeol FX-60 Fourier-transform ^{1}H and ^{13}C NMR spectrometer (Jeol, Tokyo, Japan) and a Büchi melting-point apparatus (Büchi, Flavil, Switzerland), together with the HPLC apparatus, were used to check the purity and structure of the amine derivatives.

Reagents

The eluent was a mixture of acetonitrile (ACN; HPLC-grade, Rathburn Chemicals, Walkerburrn, Great Britain) and water, twice distilled. Phenyl isocyanate (PHI) and N,N-dimethylformamide (DMF), distilled under vacuum, as well as all the standard amines used in this study were of analytical grade.

Caution: It is best to handle phenyl isocyanate in a fume cupboard because it is an eye and respiratory irritant.

Standards

N-Phenyl-N′-alkyl(aryl)urea standards were prepared by mixing each amine with PHI in an equivalent ratio of 1:1.1. Because the reaction is very vigorous it is advisable to keep the reaction vessel in an ice-bath and also add the PHI dropwise. After completion of the reaction the precipitate was collected by suction filtration, washed with cold hexane and recrystallized from chloroform until a sharp melting point was obtained.

Note: Avoid water during the first step because it forms N,N′-diphenylurea[5], a product similar to those obtained by the amines. Aniline also forms N,N′-diphenylurea (Fig. 2).

Sample derivatization procedure

It is preferable to dissolve the samples in DMF, because it both seems to catalyze the reaction and also serves as a good solvent for the disubstituted ureas.

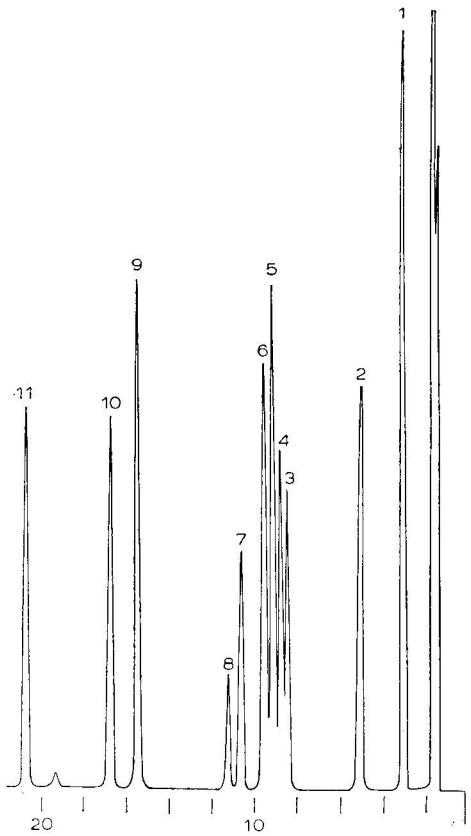


Fig. 2. Gradient run of 10 amines and water derivatized with PHI. Chromatographic conditions: column, Spherisorb 10 ODS, 25 cm × 4.6 mm I.D.; UV monitoring at 240 nm, 1.0 a.u.f.s.; linear gradient from 20% ACN to 70% ACN in 20 min. The peaks correspond to the following amines (*ca.* 1 mg/ml of the phenylurea of each in DMF): 1, ammonia; 2, dimethylamine; 3, diethylamine; 4, 2-butylamine; 5, isobutylamine; 6, *n*-butylamine; 7, benzylamine; 8, water; 9, diphenylamine; 10, dibutylamine; 11, decylamine.

A 0.5-ml volume of the amine (usually less than 10 mg) dissolved in DMF was pipetted into a glass-stoppered test tube containing a short magnetic rod. Then 0.5 ml of PHI was added and the tube placed on a magnetic stirrer for 5 min. In order to destroy the excess of PHI, 0.5 ml of an aliphatic alcohol, *e.g.* methanol, was added. The "destroyer" must be chosen so that it doesn't interfere with the peaks to be determined.

Before injecting the sample, an appropriate amount of internal standard, usually another amine derivative or an O-alkyl-N-phenylurethane[4], was added.

It is advisable to prepare a blank consisting of "derivatized" reagents.

Liquid chromatographic separation and quantitation

Derivatized samples in a DMF solution free of particulate matter were injected (1–10 μl) into the chromatographic system with a pressure-tight syringe. The Spherisorb 10 ODS column was at ambient temperature. The eluent, consisting of

ACN and distilled water, was pumped (2 ml/min) either isocratically or using different gradients depending on the amount or quality of amines to be determined. The peaks were usually monitored at 240 nm.

A 20-cm "plastic integrator", *i.e.* a ruler, was used for both qualitative identification and quantitative determinations. Qualitatively the retention times were compared to those of known standards, and quantitatively the peak height measurement with the internal standard method was used[5].

RESULTS AND DISCUSSION

Amount of phenyl isocyanate, reaction time and temperature. Destruction of excess phenyl isocyanate

As mentioned earlier, alcohols, water, phenols and carboxylic acids, besides amines, react with PHI. Therefore it is advisable to use a large excess of the derivatizing agent. With very diluted samples it is recommended that the water be evaporated or the amines be extracted before derivatization. Otherwise each water molecule consumes two PHI molecules and causes a huge diphenylurea peak to appear in the chromatogram.

Of the compounds mentioned above, amines react the fastest. With simple monoamines the reaction is complete in a few seconds at room temperature, but to ensure complete derivatization in samples, a reaction time of 5–15 min was used.

No thermal treatment is usually needed, at least not elevated temperatures. During the destruction of the excess of PHI the temperature rises. With thermally labile compounds the reaction vessel can be placed in an ice-bath at this step.

If not destroyed before injection, PHI reacts in the column with the water that is part of the eluent.

Aliphatic alcohols are good destroying agents. The chain length must be chosen so that the corresponding alkylphenylurethane doesn't interfere with the peaks to be determined. As a rule, it can be said that methylphenylurethane elutes at *ca.* V_0 with an eluent consisting of equal amounts of ACN and water.

Stability of N,N'-disubstituted ureas

No noticeable changes in the chromatograms of the ureas dissolved in DMF appeared during several days' storage at ambient temperature. This enables samples to be prepared long before the chromatographic run.

Detection and linearity

N-Alkyl-N'-phenylureas show a very strong UV maximum at *ca.* 240 nm. The molar coefficient is *ca.* 20,000 l mol^{-1} cm^{-1}, which allows detection down to the 1–5-ng level.

N-Aryl-N'-phenylureas also show a strong UV maximum at *ca.* 255 nm. The molar coefficient is usually greater than 25,000 l mol^{-1} cm^{-1}, which allows detection even below 1 ng.

The reaction is quantitative, as was shown by letting different amounts of *n*-butylamine react with PHI according to the method described under *Sample derivatization procedure*. The excess of PHI was destroyed with *n*-propanol, which elutes short after N-(*n*-butyl)-N'-phenylurea. N-Dimethyl-N'-phenylurea was used

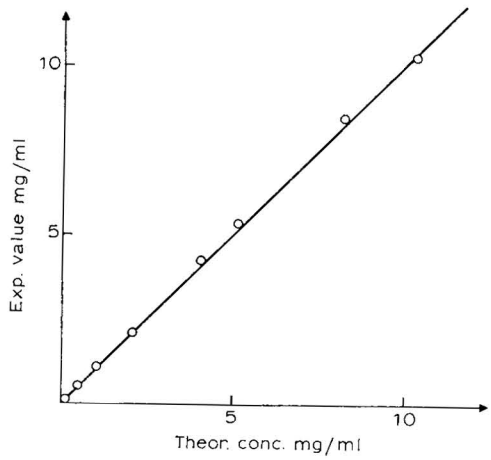


Fig. 3. Linearity curve for N-(*n*-butyl)-N′-phenylurea. For details, see text.

as internal standard. The corresponding purified crystalline ureas were used to calibrate the method. The curve, experimental *vs.* theoretical value, is shown in Fig. 3.

Advantage and disadvantages of the method

Advantages

(1) The derivatives are not pH-sensitive and need no suppression of the eluent.
(2) The derivatives are stable even in solution for days.
(3) Volatile amines can be "caught" and made non-volatile in a few seconds.
(4) Standards are easy to prepare.
(5) The method also works in acidic media.
(6) The method is sensitive.
(7) No catalysts are needed.

Disadvantages

(1) PHI is an eye and respiratory irritant.
(2) Water forms the same derivative as aniline and each water molecule (MW 18) consumes two PHI molecules (MW 120).
(3) Tertiary amines do not form these derivatives.

Applications

To test the usefulness of reversed-phase HPLC together with a gradient system and sensitive UV detection, a series of derivatized amines was chromatographed on the 25-cm 10-μm ODS column using a 20-min linear ACN–water gradient and UV-monitoring at 240 nm. As can be seen in Fig. 2, both aliphatic and aromatic primary and secondary amines form a pure single derivative that is not pH-sensitive and is very stable.

The method was also used to determine a mixture of C_{16}–C_{18} amines used as a coating in fertilizers.

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CHROM. 13,174

SOLVENT DEGASSING AND OTHER FACTORS AFFECTING LIQUID CHROMATOGRAPHIC DETECTOR STABILITY

JANET N. BROWN, MICHAEL HEWINS*, JOOP H. M. VAN DER LINDEN and RODERICK J. LYNCH

Development Laboratories, Pye Unicam Limited, York Street, Cambridge (Great Britain)

SUMMARY

We have measured the absorbance of methanol in the region 200–300 nm as a function of dissolved helium, nitrogen and air, and shown that a 1 % change in oxygen level can cause a $4 \cdot 10^{-3}$ a.u. change at 210 nm. We have also measured the amount of oxygen that can be removed from different solvents by the degassing techniques of refluxing, helium sparging, vacuum degassing and ultrasonic agitation. Using these results we have determined the operating conditions for minimum baseline drift in high-performance liquid chromatographic detection at 0.005 a.u.f.s. sensitivity in the range 190–220 nm. We observed a residual change in the absorbance of methanol, related to temperature, even after removal of oxygen.

INTRODUCTION

The increasing importance of high sensitivity in high-performance liquid chromatography (HPLC) systems, particularly at low UV wavelengths, makes it necessary to look at all factors affecting total system stability. A major group of such factors is that concerned with gases dissolved in solvents which affects not only flow stability but also the UV absorption, causing baseline drifts and random noise in UV detector outputs. These arise not only from actual absorbance changes but also from the low energy through-put of detectors operating with highly absorbing solvents.

Dissolved gases are usually present in mobile phases, and Bakalyar *et al.*[1] and others[2] have written about the effects of dissolved oxygen on UV detectors. Most published results have been in the region of 254 nm, however, despite the fact that many workers routinely use methanol-based mobile phases at 220 nm and below. We have measured the absorbance of methanol over the range 200–300 nm when saturated with helium, nitrogen air and carbon dioxide, and the efficiency of various techniques for removing dissolve ' oxygen. Using these results we have determined the operating conditions for minimu n baseline drift at 0.005 a.u.f.s. sensitivity in the wavelength range 190–220 nm.

Although most ' the results relate to methanol the efficiency of oxygen removal has been measured for other solvents.

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EXPERIMENTAL

Equipment

A Pye Unicam LC-UV detector, XPD pump and Partisil 5 ODS column (25 cm × 4.6 mm I.D.) were used for all HPLC experiments. A Pye Unicam SP8-250 UV–visible spectrophotometer equipped with a thermostated (0.05°C) 1-cm cuvette was used for absorbance against temperature measurements. Oxygen levels were measured on a Pye Unicam GCD gas chromatograph fitted with an electron-capture detector and a back-flush system.

Solvents

All solvents were "HPLC Grade" from Rathburn Chemicals, Walkerburn, Great Britain.

Gases

Helium, air and nitrogen (white spot quality) were from British Oxygen Co. (London, Great Britain).

High-performance liquid chromatography

Solvents were contained in 2.5-l glass containers having a single opening of 20 mm diameter through which all connecting tubing passed. In some experiments this opening was sealed and vented through a separate Dreschel gas wash bottle of solvent to eliminate any back diffusion of air. Solvents were saturated with the appropriate gas by bubbling it through a 15-μm sinter into the solvent for 10–15 min at a flow-rate of 300–600 ml min^{-1}. Subsequently a continuous purge of gas was maintained at the flow-rate appropriate to the experiment.

Flow-rates were between 1 and 2 ml min^{-1} and column pressures 30–130 bar. Temperature measurements were made using a platinum resistance thermometer in a Wheatstone bridge configuration.

Oxygen removal

Oxygen was displaced by another gas as described above or removed by one of the following methods:

(1) Boiling the solvent in a glass vessel equipped with a reflux condenser;
(2) Evacuation of the air above the liquid in the solvent contained by a vacuum pump;
(3) Immersing the solvent container in an ultrasonic bath (10 W).

Oxygen was measured by injecting 10-μl of liquid directly into a Porapak T column (17 cm × 0.4 cm I.D.) at 110°C which was connected via a magnesium perchlorate guard column (10 cm × 0.4 cm I.D.) to a 5A molecular sieve column (90 cm × 0.2 cm I.D.). The latter two columns were operated at 20–25°C. The sample was allowed to flush through all the columns to an electron capture detector operated in the pulsed mode and subsequently the Porapak was back flushed for 4 min.

RESULTS AND DISCUSSION

Absorbance of methanol saturated with various gases

Table I shows the absorbance of methanol measured over the range 200–300 nm when saturated with helium, nitrogen, air and carbon dioxide. It shows not just that oxygen is the main cause of higher absorbance but that the effect is very large indeed at wavelengths below 230 nm. Either helium or nitrogen replacement of the oxygen cuts the absorbance by half and extends the useable wavelength range 5 or 10 nm by significantly increasing the energy through-put of the detector, effectively extending the UV window of the methanol. It is interesting that CO_2 gives similar results.

The measured difference in absorbance at 210 nm between air-saturated and nitrogen-saturated methanol corresponds to a drift of $4 \cdot 10^{-3}$ a.u. for every 1 % change in dissolved oxygen. The significance of this in chromatographic terms is shown in Fig. 1, where displacement of oxygen by nitrogen causes a reversible 0.41 a.u. fall in absorbance. Significantly the trace shows helium is less efficient than nitrogen at replacing oxygen in this experiment and this was traced to air back-diffusing against the lighter helium.

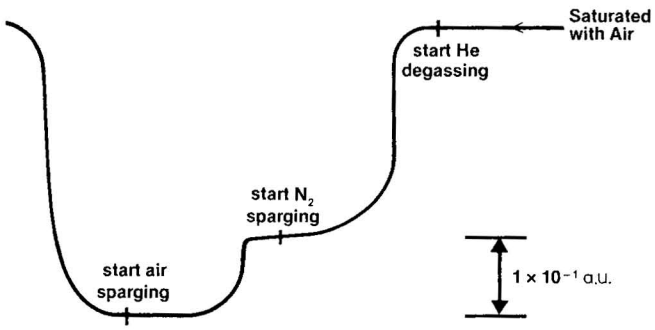


Fig. 1. Baseline changes in methanol at 210 nm and 2 ml min^{-1} on removing oxygen. Replacement of oxygen by nitrogen decreases the absorbance by 0.407 a.u. The effect is reversible.

Baseline stability under helium sparging

It is clear that temperature changes of the mobile phase will cause changes in dissolved gas content. If oxygen has not been removed from a methanol mobile phase, for instance, a 1.0°C rise will cause a 0.3 % decrease in oxygen level at 300°K and a drift of $1.2 \cdot 10^{-3}$ a.u. will result. Fig. 1 suggests that complete oxygen replacement by helium is not always achieved and this probably accounts for the random drifts reported by workers using helium sparging. Incomplete replacement of the oxygen by helium appears to be a serious problem that has not been reported before, and we found replacement was variable and incomplete even when large amounts of methanol in narrow-necked containers were sparged. The problem was traced to back-diffusion of air against the lighter helium gas.

Chromatographic results with methanol at 210 nm show random variations of $2 \cdot 10^{-3}$ a.u. for such a system, but closing the solvent container through a Dreschel bottle to prevent back-diffusion improved the baseline stability ten-fold (Fig. 2). This reduction was achieved at helium sparging rates of only 4 ml min^{-1}, a considerable

TABLE I

ABSORBANCE OF METHANOL SATURATED WITH DISSOLVED GASES AT 20°C

Methanol was saturated with helium, nitrogen, air or carbon dioxide at 20°C and pumped through the flow cell of a Pye Unicam LC-UV detector at 2ml min^{-1}. The absorbance was recorded, in a.u., at various wavelengths in the range 200–300 nm.

Gas	*Wavelength (nm)*											
	300	*280*	*270*	*260*	*250*	*240*	*230*	*220*	*215*	*210*	*205*	*200*
Helium	0.058	0.074	0.091	0.100	0.141	0.154	0.179	0.202	0.243	0.416	0.896	>1.28
Nitrogen	0.054	0.074	0.091	0.111	0.141	0.154	0.178	0.195	0.230	0.387	0.909	>1.28
Air	0.055	0.076	0.096	0.116	0.157	0.202	0.288	0.429	0.547	0.794	>1.28	>1.28
CO_2	0.051	0.073	0.091	0.111	0.141	0.166	0.229	0.278	0.352	0.448	0.896	>1.28

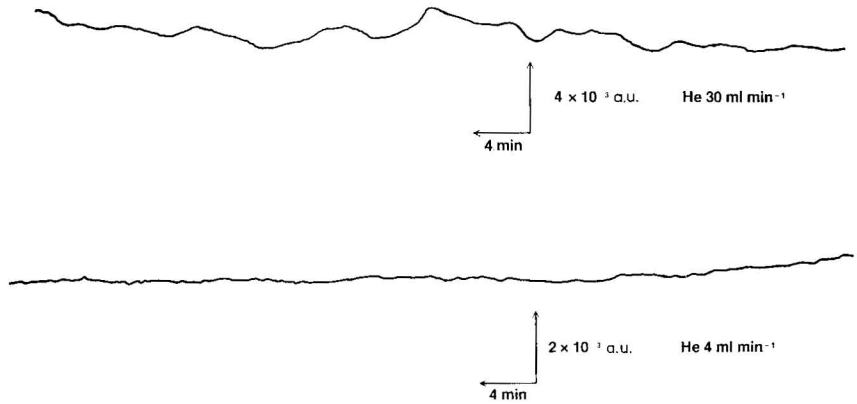


Fig. 2. Baseline drifts with helium sparging (210 nm). Above: random drifts due to air back-diffusing through 20-mm hole. Below: ten-fold improvement in random drifts when container closed with bubble trap.

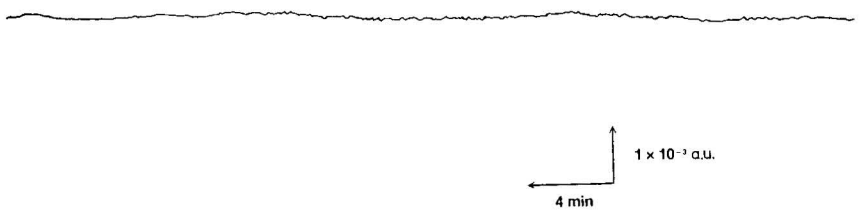


Fig. 3. Marginal improvement of fast sparging. Increasing the sparging rate to 120 ml min^{-1} in a closed container gives a two-fold improvement.

saving in helium gas. In fact increasing the sparging rate from 4 to 120 ml min^{-1} with a closed container only improved the stability to $1 \cdot 10^{-4}$ a.u. (Fig. 3).

It is clear therefore that if helium sparging is to give a stable baseline at high sensitivity and low wavelength the solvent container must be closed to a narrow opening of perhaps 1 mm or less cross-section to eliminate back-diffusion of air.

Temperature dependence of methanol absorbance

Further studies of absorbance changes showed that even when oxygen was apparently completely removed from the mobile phase the baseline still drifted in phase with temperature change (Fig. 4) to the order of $+3 \cdot 10^{-3}$ a.u. deg^{-1} for methanol at 10 nm. We have not yet established the cause of this drift but it is not due to residual oxygen since increasing temperature would decrease the oxygen level and

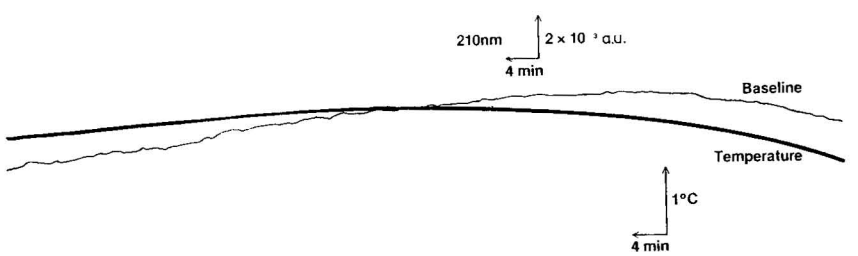


Fig. 4. Detector drift following ambient temperature.

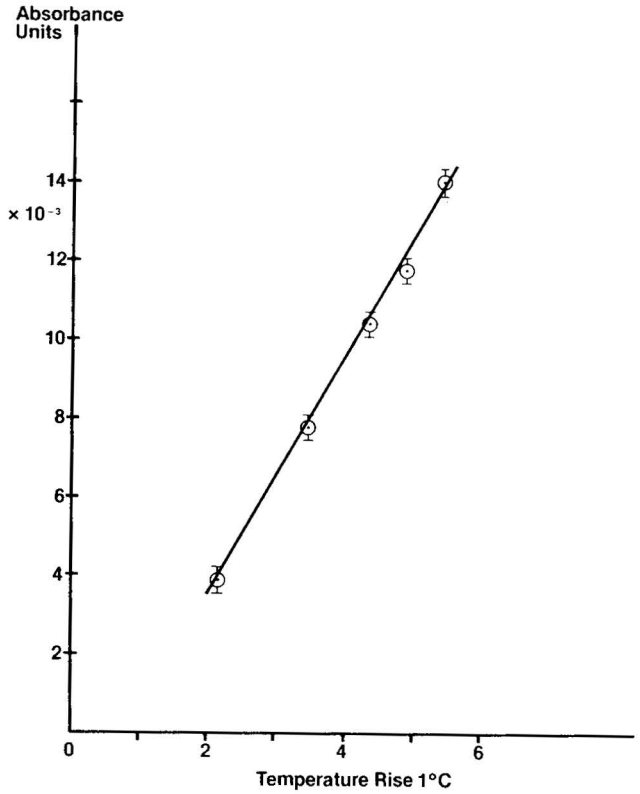


Fig. 5. Absorbance as function of temperature. A plot showing the residual change in absorbance with temperature of methanol, after removing oxygen.

hence decrease the absorbance. Spectrophotometric measurement of this apparent temperature effect confirmed its presence (Fig. 5) and work continues.

Various methods of oxygen removal

In view of the importance of removing dissolved oxygen from mobile phases we compared the efficiency of solvent refluxing, helium replacement of oxygen, ultrasonic degassing and vacuum degassing. Solvent refluxing completely removed all detectable dissolved oxygen in any solvent. The efficiency of the other methods were dependent on the solvent used, water and methanol being the most difficult to degas, hexane and acetonitrile less so. Ultrasonic degassing was particularly disappointing and on occasions actually increased oxygen levels. Fig. 6 and 7 show the comparative efficiencies of the various methods for water and methanol.

Nitrogen was not included in these early studies but its effect is identical to helium without the disadvantage of back-diffusion problems.

Obviously refluxing is an inconvenient and potentially hazardous technique and helium or vacuum degassing is often the best compromise. Nitrogen sparging will replace oxygen and is more generally available than helium, but it may cause gas bubbles in pump inlets when used in conjunction with low pressure solvent mixing or similar flow restrictions.

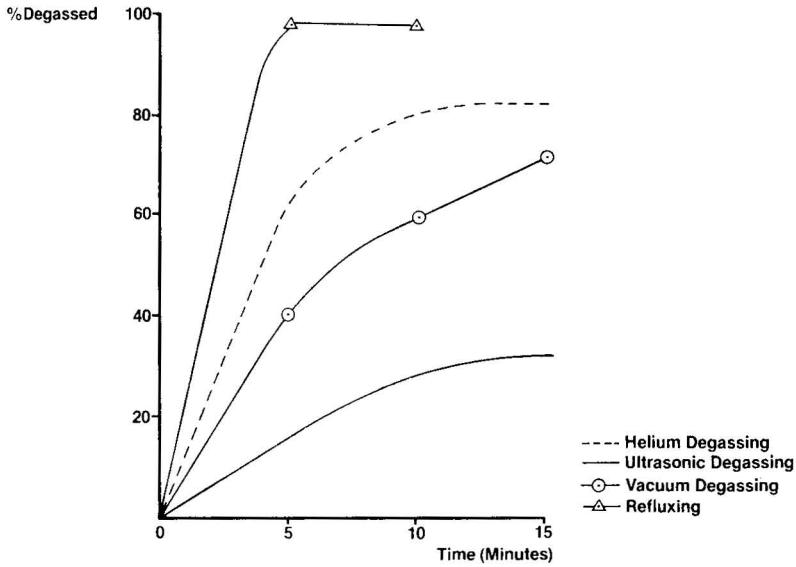


Fig. 6. Oxygen replacement in methanol.

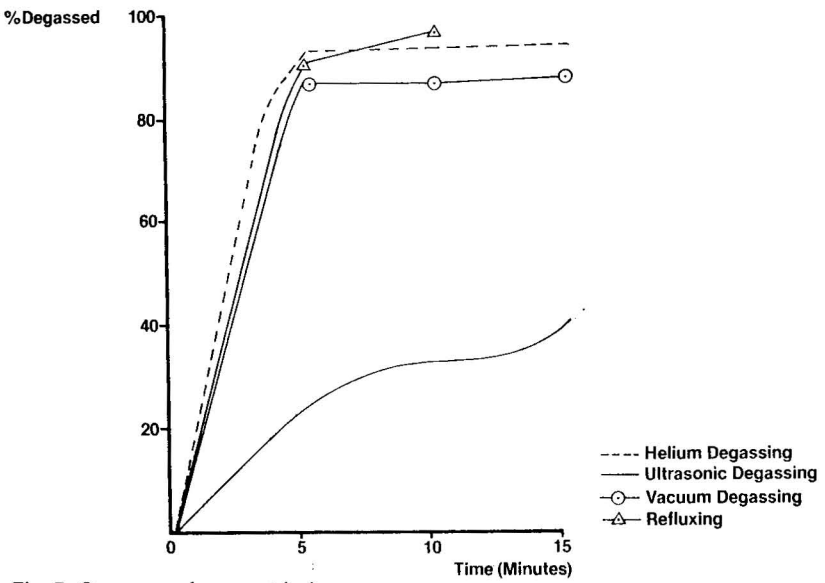


Fig. 7. Oxygen replacement in hexane.

In all cases it is necessary to either keep the mobile phase continuously sparged, or continuously heated, to prevent re-absorption and variable oxygen levels.

CONCLUSIONS

(1) The absolute absorbance of a methanol mobile phase is critically dependent on its dissolved oxygen content. At low wavelengths the effect of oxygen is very much larger than might be inferred from previous papers[1].

(2) *Any* change in oxygen content will cause detector baseline drifts; for example, at 210 nm a 1% change in dissolved oxygen level will cause a $4 \cdot 10^{-3}$ a.u. change in absorbance in methanol.

(3) Such changes can be brought about by changes in the ambient temperature of the mobile phase. A 1°C rise in temperature will cause a 0.3% fall in dissolved oxygen in methanol, leading to a $1.2 \cdot 10^{-3}$ a.u. change. They can also be caused by variations in oxygen level resulting from air back-diffusing against a helium sparge.

(4) Nitrogen will effectively replace oxygen from a mobile phase and may be used to extend the UV window of methanol downwards providing gas bubble formation in the pump or detector is not a problem.

(5) Refluxing is completely effective in removing oxygen from all solvents studied but it is necessary to keep the solvent warm to prevent subsequent re-adsorption. Gas bubble formation will not be a problem since all other gases are also removed by refluxing.

(6) Vacuum degassing and helium replacement are moderately effective in removing oxygen from solvents such as water and very effective with hexane. Methanol and acetonitrile fall mid-way between the two. With such solvents the amount of oxygen (and nitrogen) removed is adequate to reduce bubble formation at the inlet to a pump, but without special care it is insufficient to achieve the lowest mobile phase UV absorption. In all cases continuous sparging, or warming, is necessary to prevent re-adsorption.

(7) Ultrasonic degassing is ineffective and can lead to increased oxygen levels undef some conditions.

(8) After complete removal of oxygen from methanol we measured a reversible absorbance change of $3 \cdot 10^{-3}$ a.u. deg $^{-1}$ at 210 nm. Work is continuing to discover the cause of this effect.

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NEW ELECTROCHEMICAL CELL FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RAYMOND BEAUCHAMP* and PIERRE BOINAY
Rhône-Poulenc, Centre de Recherches, 24 Avenue Jean-Jaurès, 69150 Décines (France)
JEAN-JACQUES FOMBON and JACQUES TACUSSEL
Centre de Recherche CERAC, 72 à 78 Rue d'Alsace, 69100 Villeurbanne (France)
and
MYLENE BREANT, JOSEPH GEORGES, MAURICE PORTHAULT and OLIVIER VITTORI
Université Claude Bernard, Laboratoire de Chimie Analytique III, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne (France)

SUMMARY

A new electrochemical detector of low dead volume allowing the use of various solid electrodes as well as a dropping mercury electrode has been developed for high-performance liquid chromatography. The electroactivity range has been determined in typical chromatographic effluents so as to select the appropriate working electrodes, and the main parameters of the detector (linear range, detection limits, reproducibility) examined using oxidizable or reducible organic molecules. Some applications involving the different chromatographic modes are presented. This new detector is very simple to use and gives satisfactory results with all kinds of chromatographic modes.

INTRODUCTION

Nowadays, electrochemical detection is a well-known detection mode in liquid chromatography. As early as 1950, the first studies were published on the use of the combination of polarography and liquid chromatography for separating and determining organic and inorganic compounds[1,2]. In 1969, a spectacular new development appeared in liquid chromatography: high-performance liquid chromatography (HPLC). This technique quickly became very successful owing to its high efficiency, and stimulated the development of improved detection methods[3–19].

In this paper we introduce a new type of cell which offers a unique flexibility, as it can be used with both solid electrodes and with dropping mercury electrodes. In the first case, one can use the "longitudinal" or the "wall jet" operating mode. Also it is possible to employ simultaneously two working electrodes and carry out a double detection for a particular solute. This cell can also be operated with a low dead volume mixer if it is desired to add a supporting electrolyte or a reagent at the column output.

We believe that thanks to this flow-through detector, using a single cell, it is

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possible to benefit from the many advantages of amperometric detection, in modern HPLC.

EXPERIMENTAL

Description of the measurement cell

The amperometric detection cell is based on a three-electrode system. A cross section can be seen in Fig. 1. The parallelepiped cell body is made of polychlorotrifluoroethylene (PCTFFE), and the electrodes are housed in PCTFE or PTFE stems, threated for ease of removal and interchangeability; fluorinated polymer O-rings ensure watertightness. The thin liquid layer circulating inside the cell is geometrically defined in a precise and reproducible way, by means of a 250-μm-thick PTFE gasket, sandwiched between the two half-bodies of the cell, and with a channel accurately positioned in its centre.

According to the way the electrodes are installed, the detection cell can operate in the "thin-layer" mode or in the "wall-jet" mode. Some other inlets are available either for another working electrode (bipotentiostatic configuration) or for simultaneous conductimetric detection. The inner and active element of the miniature reference electrode is Ag/AgCl/saturated KCl. The electrolyte junction, made of a porous ceramic plug, is very close to the working electrode in order to make measurements easier in low conductivity media. When using the dropping mercury electrode, the central PTFE gasket is replaced by a thicker one, adapted to the capillary shape and size.

The dead volume is variable, depending on the selected configuration (Table I).

Operation of the detection cell

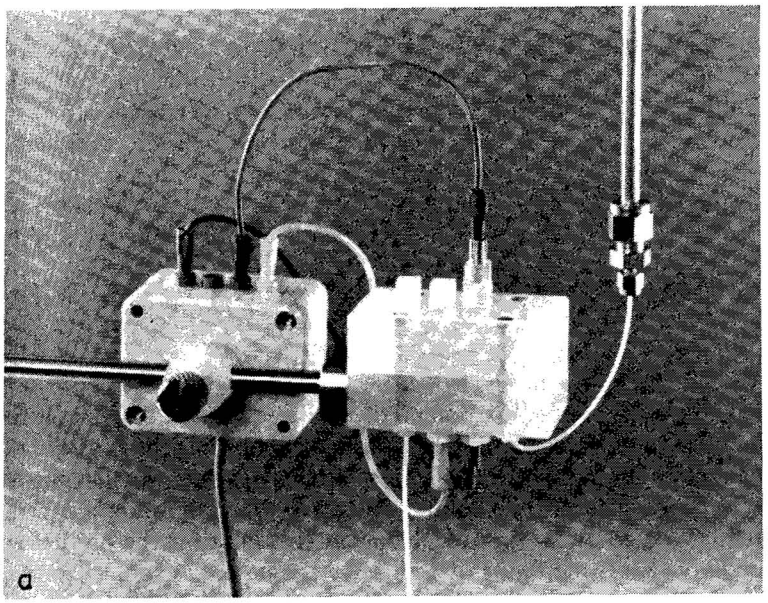
This detection cell can be used with any HPLC system. We have carried out a large number of tests with the following equipment: Orlita AE 104 or Chromatem 380 pumps, fitted with a pulse damping system; Rheodyne or Valco injection valve; stainless-steel columns (1/4 in. I.D.) of various lengths. The associated electronic instruments were: P.A.R. 174 polarographic analyser; Tacussel PRG 5, PRG-MOD and PRG-E polarographic analyser; Leeds and Northrup recorder; Tacussel EPL 1/TV 11 GD recorder. Chemicals were of analytical grade from Prolabo (Paris, France) and E. Merck (Darmstadt, G.F.R.).

Selection of working electrode

The tests of the various materials available for the working electrode were conducted under the usual voltametric conditions with rotating disc electrodes (Tacussel Type EDI). Boron carbide, tungsten, molybdenum, zinc and nickel were tested, in addition to the usual materials, platinum, glassy carbon and mercury.

The determination of the electroactivity ranges in usual chromatographic solvents (water, methanol and water–acetonitrile mixtures containing 20% or 80% of water) indicated the possibility of using different electrodes. Since it is necessary to add a supporting electrolyte in electrochemical methods, all the tests were carried out with 0.1 *M* $LiClO_4$. The reference electrode was Ag/AgCl in aqueous saturated KCl.

A number of trials were carried out, first without then with nitrogen bubbling through the eluting solvent. This confirmed that oxygen, the presence of which is of



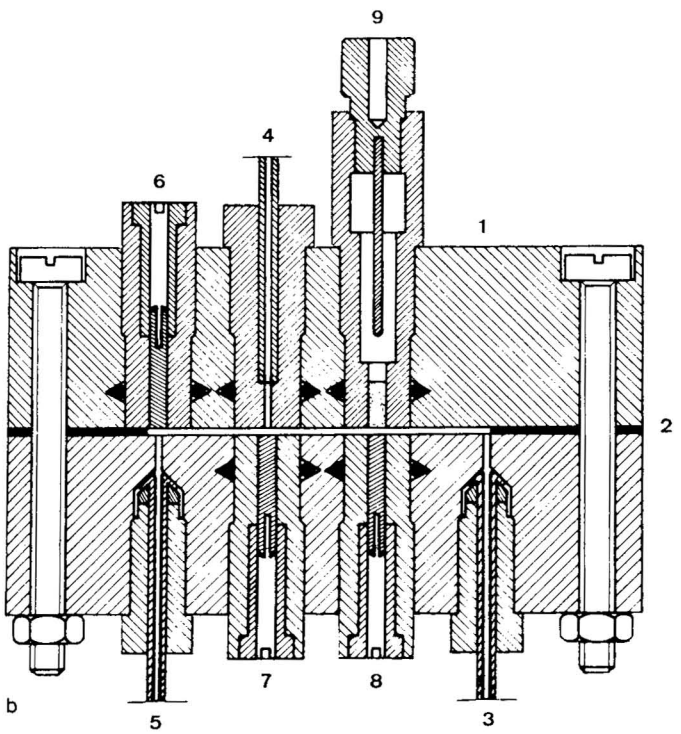


Fig. 1. (a), The new electrochemical cell for HPLC, where the operating volume is delimited by an opening in the central part of a PTFE sealing foil sandwiched between two PCTFE half-bodies. The electrodes are interchangeable by means of threaded PCTFE stems. (b), Cross-section of the cell: 1 = PCTFE cell body; 2 = PTFE (100–250 μm) sealing foil; 3 = solute input ("thin layer" mode); 4 = solute input ("wall jet" mode); 5 = solute output; 6–8 = solid electrodes (working, auxiliary); 9 = reference electrode.

TABLE I

EFFECT OF CONFIGURATION ON DEAD VOLUME

Working electrode		*Hydrodynamic configuration*	*Dead volume (μl)*
Number	*Nature*		
One	Pt, glass carbon, etc.	"Thin layer"	*ca.* 4
		"Wall jet"	*ca.* 0.2
	Mercury droplets	"Thin layer"	*ca.* 4
Two	Pt, glassy carbon, etc.	"Thin layer"	*ca.* 8

little importance in the positive range of potentials, drastically limits the negative range, whatever the working electrode; as soon as the applied potential becomes smaller than −0.1 V (platinum) or −0.25 V (other electrodes) one must eliminate oxygen from the solvent at the top of the column as well as from the sample before injection. Consequently, oxygen was removed from all the solutions before determining the electroactivity range, the limits of which are the potential values (anodic and cathodic) corresponding to a current density of 1 μA mm^{-2}. Table II shows the results obtained in a water–acetonitrile mixture: the larger ranges correspond to the use of a mercury electrode in the cathodic rangs, and nickel, platinum, boron carbide or glassy carbon (GC) electrodes in the anodic range.

TABLE II

ELECTROACTIVITY RANGE IN 80% ACETONITRILE–20% WATER AT DIFFERENT WORKING ELECTRODES

Units are volts *versus* the Ag/AgCl/KCl electrode.

Limits	*Electrode*							
	Pt	*GC*	*B_4C*	*Hg*	*Ni*	*W*	*Mo*	*Zn*
Anodic	1.3	1.1_5	1.3	0.4_5	1.2	0.8	0.1	0.4
Cathodic	-0.6_5	−0.9	-1.3_5	−1.9	−0.8	−0.9	-0.9_5	−0.6

Initially, a rather large number of materials seemed to be suitable, but a second series of tests reduced the available options. Thus, using 4-nitrophenol as solute, the stability of the diffusion current, its reproducibility and its dependence on the concentration of the solute were examined: only glassy carbon, platinum and mercury showed satisfactory results.

RESULTS AND DISCUSSION

Use of the cell with a glassy carbon electrode

Electrolysis output. Potassium ferrocyanide was selected a solute. The mobile phase was 0.05 *M* KNO_3, flow-rate 1 ml min^{-1}. The chromatographic column (200 × 4.6 mm) was filled with glass beads (100 μm). The applied potential was

+0.9 V *vs.* (Ag/AgCl/saturated KCl) and 10^{-7} mol was injected at the top of the column with a loop of 10 μl. The variation of the output with the flow-rate was normal and for 1 ml min^{-1} we obtained a yield of 2%.

Dynamic range. Three different compounds were used, two of which are oxidizable (aniline and phenol), and the other is reducible (nitrobenzene). The dynamic range is five decades in oxidation (10^{-12}–10^{-7} mol injected) and 3.5 decades in reduction ($5 \cdot 10^{-11}$–10^{-7} mol). The smaller dynamic range in reduction is probably due to O_2, H^+ and/or metallic compounds present as impurities.

Reproducibility of the detector response. The reproducibility is good, *ca.* 2–3%, as can be seen in Table III.

TABLE III

REPRODUCIBILITY OF THE DETECTOR RESPONSE WITH GLASSY CARBON

Compounds introduced	*Mean peak height (nA)*	*S.D.*		*No. of measurements*
		nA	%	
Aniline, 10^{-8} mol	2583	26.09	1	20
Nitrobenzene, 10^{-8} mol	5534	193.7	3.5	10
Mixture of aniline and chloroaniline	1st peak 766.4	17	2.22	
	2nd peak 221.6	4.03	1.82	10
	3rd peak 200.4	2.94	1.47	
Mixture of two phenolic compounds	1st peak 1185	57.37	4.84	58
	2nd peak 738	34.75	4.7	

Detection limits. With well defined chromatographic conditions (pump, solvent, flow-rate), the results are 10^{-12} mol (93 pg) for aniline and $5 \cdot 10^{-11}$ mol (6 ng) for nitrobenzene.

Applications in organic analysis. The performance of the detector was tested by injecting a mixture of chlorophenols. Fig. 2a shows the reversed-phase chromatographic separation of five phenols. This type of chromatography is well adapted to electrochemical detection. Indeed, the eluents are made up of a mixture of water, with different kinds of solvents, *e.g.*, methanol, acetonitrile, tetrahydrofuran. We had to add a supporting electrolyte to conduct the current, and the pH and ionic strength were adjusted to obtain the best conditions for electrochemical reactions. Most of the separations were carried out by this mode of chromatography. However, adsorption chromatography is still used in many cases and we tried to work with this mode of separation for the analysis of a mixture of chloroanilines on a column of Partisil (5 μm). The electrochemical detection is inoperative in solvents of low dielectric constant such as dichloromethane or hexane, even when they contain a few percent of isopropanol or acetic acid. We added a supporting electrolyte, either directly to the eluent (for example 0.1 *M* $(C_4H_9)_4NClO_4$), or by mixing at the output of the column; in the last case, a new solvent containing the supporting electrolyte (methanol + 0.2 *M* $LiClO_4$) was added to the eluent. The two flow-rates were adjusted to about the same value. The introduction of the mixture did not disturb the separation.

Glassy carbon seems to be fully satisfactory in all solvent mixtures.

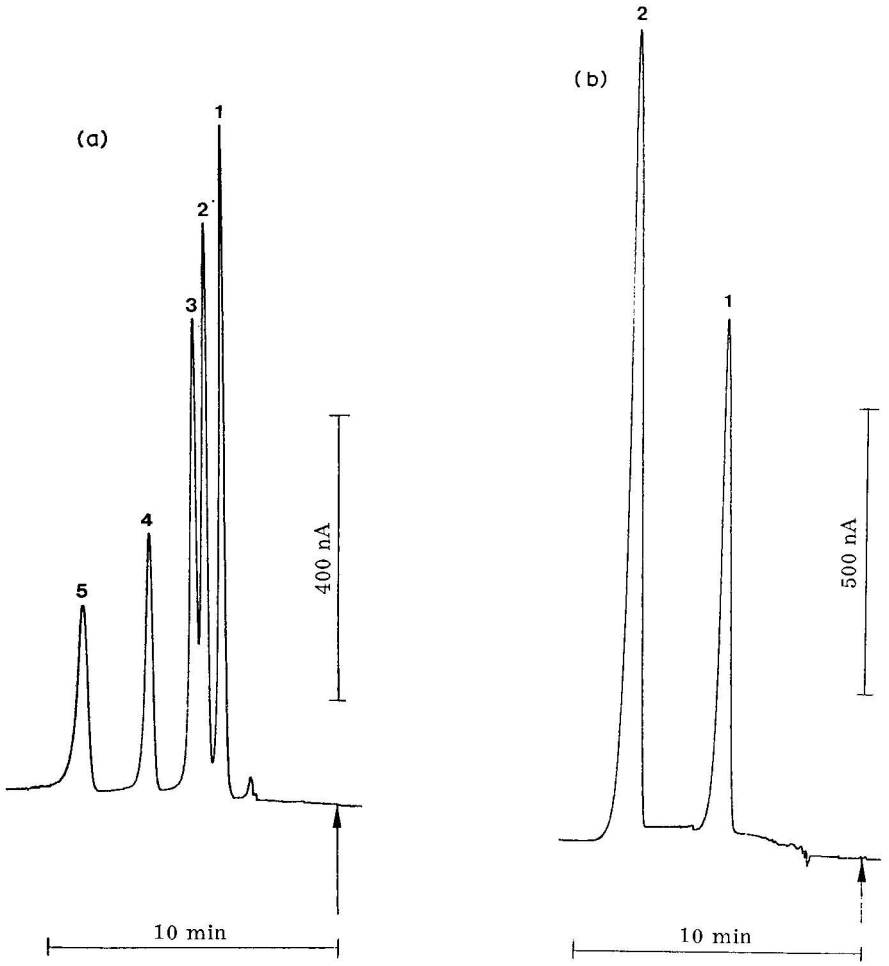


Fig. 2. (a), Analysis of mixtures by reversed-phase chromatography: 1 = phenol (200 ng); 2 = *o*-chlorophenol (200 ng); 3 = *p*-chlorophenol; 4 = dichlorophenol (200 ng); 5 = trichlorophenol (400 ng). Column: LiChrosorb RP-8, 5 μm, 20 cm. Eluent: methanol–water (70:30) + 0.05 *M* $LiClO_4$; flow-rate, 0.8 ml min^{-1}. Injection: 10 μl. Cell: Tacussel DELC. Polarographic analyzer: Tacussel PRG-E. Electrode: glassy carbon. Applied potential: + 1.20 V (Ag/AgCl/saturated KCl). (b), Separation of two nitrophenols at the dropping mercury electrode (DME): 1 = *p*-nitrophenol (10^{-8} *M*); 2 = *o*-nitrophenol (10^{-8} *M*). Column: Normatom C 18, 10 cm × 1/4 in. I.D. Eluent (deaerated): methanol–water (60:40) + 0.01 *M* H_3PO_4; flow-rate, 1 ml min^{-1}. Injection: 10 μl. Cell: Tacussel DELC. Polarographic analyzer: Tacussel PRG-E. Electrode: DME; drop time, 80 msec. Applied potential: −0.8 V (Ag/AgCl/saturated KCl).

Use of the cell with the dropping mercury electrode

The same cell may be used with a dropping mercury electrode after changing the joint and replacement of the glassy carbon. In this mode the background current decreases with the drop time while the peak current increases. The best compromise is reached with a drop time of *ca.* 0.08–0.1 sec.

Dynamic range. The dynamic range for nitrobenzene is three decades (10^{-10}–10^{-7} mol injected).

Detection limits. For the same compound, the detection limit was 10^{-10} mol injected under well defined chromatographic conditions.

Reproducibility. For the same compound, the following results were obtained: amount injected, 10^{-8} mol; mean peak height, 2250 nA; S.D. 130 nA (5.7%); number of measurements, 13.

Applications. Fig. 2b shows the separation of a mixture of nitrophenols (*o* and *p* isomers, 10^{-8} mol) after deoxygenation of the supporting electrolyte and of the sample. The oxygen peak, which is not present after deoxygenation, is confused with the peak of *p*-nitrophenol.

Use of the cell in flow injection analysis

In this case, separation was not necessary. This technique was used for measuring a series of the same samples with different concentrations. We used the same chromatographic system with a small column of glass beads: the eluent flowed continuously in the cell and from time to time the sample was injected by means of the 10-μl loop. The analysis of a phenolic compound is possible at a rate of twenty samples per hour.

Broading of chromatographic peaks

The response time of the working electrodes and of the associated electronics is less than 0.5 sec; however, the dead volume is lower, especially in the "wall jet" mode, than that of most other types of HPLC detectors. For different reasons, care must be taken that the band broadening due to the detector itself is very small; we have checked this experimentally by using alternately a UV detector (Varian 4, 300–254 nm) and an electrochemical detector for separating a mixture of phenol (0.94 μg), cresol (1.08 μg) and *tert.*-butyl-4-catechol (1.6 μg); the two detectors have also been set up in series for certain trials.

Concerning band broadening, the results obtained are exactly the same for the two detectors; a greatly increased sensitivity, especially for *tert.*-butyl-4-catechol, was noticed in the electrochemical detection.

Pulse voltametry

When a solid electrode is used (platinum, glassy carbon, etc.) the flow can be either perpendicular (wall-jet) or tangential to the conducting surface. The electroactive material is brought to the electrode by convection, and if a laminar flow is assumed, this convection increases when the flow-rate is increased. One of the major points of interest in pulse polarography, for motionless solutions, is the importance of an instantaneous faradaic current following pulse application, due to the greatly concentrated gradient present in the electrode vicinity during the first few milliseconds. In the present case, the importance of this gradient depends on the flow-rate: the greater is the flow, the thinner is the hydrodynamic layer, δ, as pointed out by Levich[20].

It appears, therefore, that the pulse method will give higher currents for lower flow-rates than direct current methods. However, both methods will be equivalent when the flow-rate is increased because the hydrodynamic layers will become the same size. In spite of these remarks, the pulse method may prove of value for simultaneously eluted electroactive material, the redox potentials of which are quite different.

CONCLUSION

Simple in design but flexible in its use, the amperometric detection cell described can operate freely in the "thin layer" or "wall jet" mode. Many uses are offered by the possibility of rapidly modifying the number and types of electrodes, whether solid or dropping mercury electrodes. After thorough experimentation in the different modes of liquid chromatography, the reproducibility is excellent, the linearity range broad and the detection limits very satisfactory.

Operating in the pulse mode results in an improvement which is more appreciable in the selectivity than in sensitivity.

We think that this new cell can make a perceptible contribution to the progress of electrochemical detection, and constitute a valuable alternative to the conventional optical techniques primarily used in chromatographic analytical laboratories.

ACKNOWLEDGEMENT

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ALTERNATING VOLTAGE POLAROGRAPHIC DETECTION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS EVALUATION FOR THE ANALYSIS OF BILE ACIDS

WIKTOR KEMULA and WŁODZIMIERZ KUTNER*
Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44, 01-224 Warsaw (Poland)

SUMMARY

The first attempt to apply alternating voltage (a.v.) polarography for detection in high-performance liquid chromatography is presented. The performance of a flow-through polarographic detector in the a.v. polarographic mode is illustrated with the separation of a mixture of bile acids. Generally, the retention time of a substance is found to be proportional to its extent of adsorption at the electrode at the applied potential. Thus, the more the substance is retained, the easier is the detection of its peak. The observed noise level is close to 0.024 μF^{-1}. The linear dynamic range for cholic acid determination extends from 1.25 nmol to over 50 nmol with a correlation coefficient of 0.998. The sensitivity is close to $8 \cdot 10^4$ sec l μF^{-1} mol^{-1} and the detector response index is 0.98. The standard deviation of the peak area from nine consecutive injections was 4.5%. The detection limit was close to 1 nmol per injection, almost one order of magnitude larger than that for a UV detector operated at 210 nm, but still one order of magnitude lower than that for a refractive index detector.

INTRODUCTION

The first attempt at applying alternating voltage (a.v.) polarographic detection[1,2] in high-performance liquid chromatography (HPLC) is presented in this paper.

Many organic and inorganic substances present in electrolyte solutions result in a change in the double layer capacity at the dropping mercury electrode (DME) due to their specific adsorption. This effect was extensively exploited for the determination of many organic compounds using alternating current (a.c.) polarography and the adsorption data for many systems are available[3]. The effect was also applied for detection in liquid chromatography, but until now only a.c. polarography has been used, in both total current[4–6] and phase sensitive[7] modes of operation. We found that a.v. polarography is also useful for monitoring HPLC signals.

EXPERIMENTAL

A Radelkis OH-105 universal polarograph (Hungary), operating at $\omega = 60$ Hz

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and amplitude of alternating voltage $\Delta E_{\sim} = 20$ mV, was used in a.c. polarographic measurements.

For a.v. polarographic experiments the home-made[2] PZN 71 a.v. polarograph was used which recorded the $1/C$ *vs.* E curves. This apparatus operated at 240 Hz in a "tast" mode in order to eliminate the oscillations due to the dropping mercury. The signal was sampled for 0.2 sec, after 1 sec of mercury drop age. A simple resistance–capacitance damping circuit having a time constant of 3 sec was also used.

The home-made HPLC apparatus with 5-μl injection valve[8] and the flow-through polarographic detector[8,9] used were described previously. The detector operated in the three-electrode mode with two 470-μF condensers connected together, with opposite signs between the auxiliary and reference electrodes. To verify the accuracy of experiments the resistance of the detector filled with the working solution was measured and compared with the highest acceptable resistance. For a capacity, C, of ≈ 0.2 μF the error in the measured $1/C$ values at $\omega = 240$ Hz was less than 10% for an electrolyte resistance, R, of $\leqslant 30$ kΩ as measured using the equivalent circuit, somposed of capacitor and resistor in series; the resistance of the detector, when filled with a solution containing 60% methanol and 40% 1/15 M phosphate buffer, pH 6, according to Michaelis, was *ca.* 5.5 kΩ, for a drop time, t_1, of 1.6 sec, measured with the a.c. bridge[10].

The characteristics of the DME capillaries were as follows: (i) in steady-state a.v. and a.c. polarographic measurements (Fig. 1), $t_1 = 2$ sec, $m = 2.966$ mg sec^{-1}, $h_{Hg} = 117$ cm; (ii) in HPLC experiments (Fig. 2), $t_1 = 1.4$ sec, $m = 2.375$ mg sec^{-1}, $h_{Hg} = 40$ cm.

Analytical reagent grade chemicals and doubly distilled water were used. Bile acids were generously supplied by the Institute of Pharmaceutical Industry, Warsaw.

A 250 × 4 mm I.D. stainless-steel column was slurry packed at 435 kg m^{-2} with 10-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.). The chromatographic procedure was as described previously[8].

RESULTS AND CONCLUSIONS

The steady-state experiments were performed with both a.c. and a.v. techniques.

The performance of the detector in a.v. polarographic mode may be illustrated with the separation of a bile acid mixture. The addition of bile acids to a methanol–water mixture resulted in an increase of the broad maximum in the $1/C$ *vs.* E a.v. polarographic curve (Fig. 1) due to specific adsorption. The potential of the maximum was practically independent of the nature and concentration of the added bile acid. This is why this potential is useful for analytical detection in HPLC.

As the detection limit of this method is dependent on the difference in double layer impedance, $1/C$, at the chosen potential in the absence and the presence of the studied substance, a suitable eluate for a.v. polarographic detection should reveal the lowest possible impedance value at this potential.

a.v. polarography appears to be advantageous for HPLC detection for several reasons. The equivalent circuit of the electrolytic cell in a.v. polarography comprises a capacitor and riesstance in series which corresponds to the scheme of connections

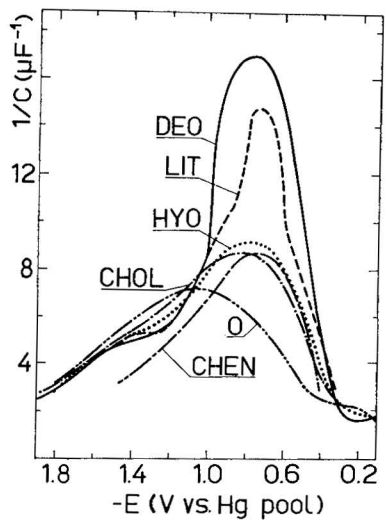


Fig. 1. Reciprocal capacity curves as recorded by a.v. polarography for 10^{-3} *M* bile acids in 0.1 *M* $NaClO_4$ solution containing 80% methanol and 20% 0.2 *M* acetate buffer, pH 4, according to Walpole. Names of steroids as in Fig. 2; O = no steroids.

in the electrochemical cell in the absence of faradaic reaction, contrary to a.c. polarography for which the equivalent circuit is a capacitor and resistance in parallel, which has no physical meaning. a.v. polarography is especially useful for measurements in electrolytes of low conductivity[2] and is the most sensitive for low capacities. The a.v. amplitude adjusts naturally to capacity variations, since the a.c. amplitude is kept constant. Finally, the calibration curve of $1/C$ a.v. polarographic output signal *vs.* concentration measured at the detection potential is more suitable for quantitative determinations than that of condenser current, i_C, output signal *vs.* concentration as recorded in a.c. polarography[11].

The HPLC separation of bile acids in 70% methanol–30% 0.2 *M* acetate buffer, pH 4 is presented in Fig. 2. Generally, the retention time of a substance was found to be proportional to the extent of its adsorption at the DME at the applied potential. Thus, the more retained the substance, the easier its peak detection. Until now, bile

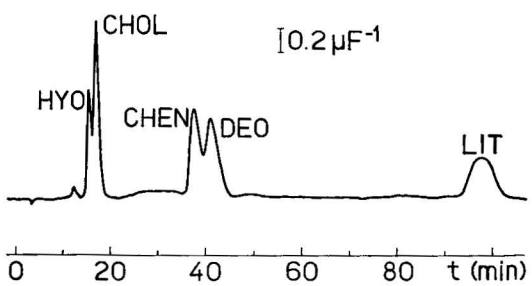


Fig. 2. HPLC chromatogram of 10^{-2} *M* bile acid mixture with a.v. polarographic detection. Separation conditions: stationary phase, 10-μm LiChrosorb RP-18; mobile phase, 0.1 *M* $NaClO_4$ solution containing 70% methanol and 30% 0.2 *M* acetate buffer, pH 4, deaerated with argon; mobile phase flow-rate, 1 ml min^{-1}; potential, $E = -0.5$ V *vs.* Ag/AgCl electrode. Peaks: HYO = hyodeoxycholic acid; CHOL = cholic acid; CHEN = chenodeoxycholic acid; DEO = deoxycholic acid; LIT = litocholic acid. Sample size, 5 μl.

acids which do not absorb UV-light at 254 nm were analysed in HPLC either with a UV detector operated at 210 nm[12] or a refractive index (RI) detector[13–15]. The present approach offers a new and simple alternative mode of detection.

The conditions imposed by HPLC facilitate the use of detection based on measurements of changes in the double layer capacity. The high value of the solution flow-rate, which increases the rate of transport of a substance to the DME surface due to convection, especially at long drop times[8], enables the determination of lower concentrations than in the similar system under steady-state conditions. The curve of $1/C$ *vs.* E for a solution containing $5 \cdot 10^{-5}$ M of cholic acid (CHOL), and at steady-state conditions was almost indistinguishable from the curve in the absence of CHOL. However, it was possible to detect CHOL in 5 μl of $2.5 \cdot 10^{-4}$ M CHOL injected in the column where it was diluted approximately 400 times by the 1 ml min^{-1} flow-rate. So, the limit of detection of CHOL, determined by the transport rate of the substance to the DME surface, is close to 1 nmol per injection. This value is almost one order of magnitude larger than that for a UV detector operated at 210 nm[12], but still one order of magnitude lower than that for a RI detector[15]. The observed noise level is close to 0.024 μF^{-1}. The linear dynamic range for CHOL determination extends from 1.25 nmol to at least 50 nmol per injection, with a correlation coefficient of 0.998. The sensitivity is close to $8 \cdot 10^4$ sec 1 μF^{-1} mol^{-1} and detector response index is 0.98. The standard deviation of the peak area from nine consecutive injections was 4.5%.

The results obtained enable us to state that a.v. polarographic detection based on the changes in double layer impedance at the DME is a new useful electrochemical method, especially in comparison to other electroanalytical detectors based on measurements of faradaic currents. The limitation of the method can depend on the rate of attaining the adsorption equilibrium at the DME. Also two-dimensional condensation of the adsorbed substance at the DME should be absent; this is normally fulfilled in water–organic solvent mixtures.

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CHROM. 13,225

QUANTITATIVE TRACE ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND LOW-TEMPERATURE FLUORIMETRY IN SHPOLSKII MATRICES AT 4.2°K

APPLICATION TO MEDICINAL WHITE OILS

J. M. COLIN* and G. VION

Total–Compagnie Française de Raffinage, Centre de Recherches, Boîte Postale 27, 76700 Harfleur (France)

and

M. LAMOTTE and J. JOUSSOT-DUBIEN

Université de Bordeaux 1, Laboratoire de Chimie Physique A, ERA 167, 351 Cours de la Libération, 33405 Talence (France)

SUMMARY

A scheme of analysis for polycyclic aromatic hydrocarbons (PAHs) in medicinal white oils was developed; this was made possible by carrying out three initial chromatographic stages to isolate the PAHs and following this by a combination of high-performance liquid chromatography and spectrofluorimetry at room temperature. The identification of the PAHs was confirmed by trapping the chromatographic peaks and analyzing them by Shpolskii fluorimetry at 4.2°K.

INTRODUCTION

Although high-performance liquid chromatography (HPLC) has largely been developed for the analysis of polycyclic aromatic hydrocarbons (PAHs) in, *e.g.*, air, water and automobile exhaust, this technique has seldom been applied for PAH analysis in white oils. The very low amounts of PAHs in such oils (of the order of ppt*) makes such analysis particularly difficult and requires unusually good selectivity as well as a high level of sensitivity for detection and identification.

Because of their high sensitivity, fluorescence methods appear to fulfil such requirements and to be particularly convenient for the detection of very small amounts of PAHs (which are known to be efficient fluorescent compounds). In this work, HPLC separation and quantitative analysis of selected aromatic compounds in white oils has been achieved by using a Jobin–Yvon fluorimeter (JY 3) as a detector; this instrument allows wavelength selection for both excitation and emission[1]. Moreover,

* Throughout this article, the American trillion (10^{12}) is meant.

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an individual PAH can be characterized by its fluorescence spectrum even at very low levels of concentration.

Spectral resolution, however, depends considerably on the experimental conditions. When in solution, at room temperature, fluorescence spectra are usually broad, the linewidth for the observed vibronic bands being typically of the order of 100 to 500 cm^{-1} (Fig. 1). In fact, the specific vibrational structure that contains most of the characteristic information on a particular PAH is lost unless good purification or complete separation is achieved (which is never the case with the HPLC separation of complex mixtures). Identification of PAHs from fluorescence spectra at room temperature is difficult to establish conclusively.

A notable improvement in the specificity of PAH fluorescence spectra can be obtained by application of the so-called Shpolskii effect[2]. This effect is observed when

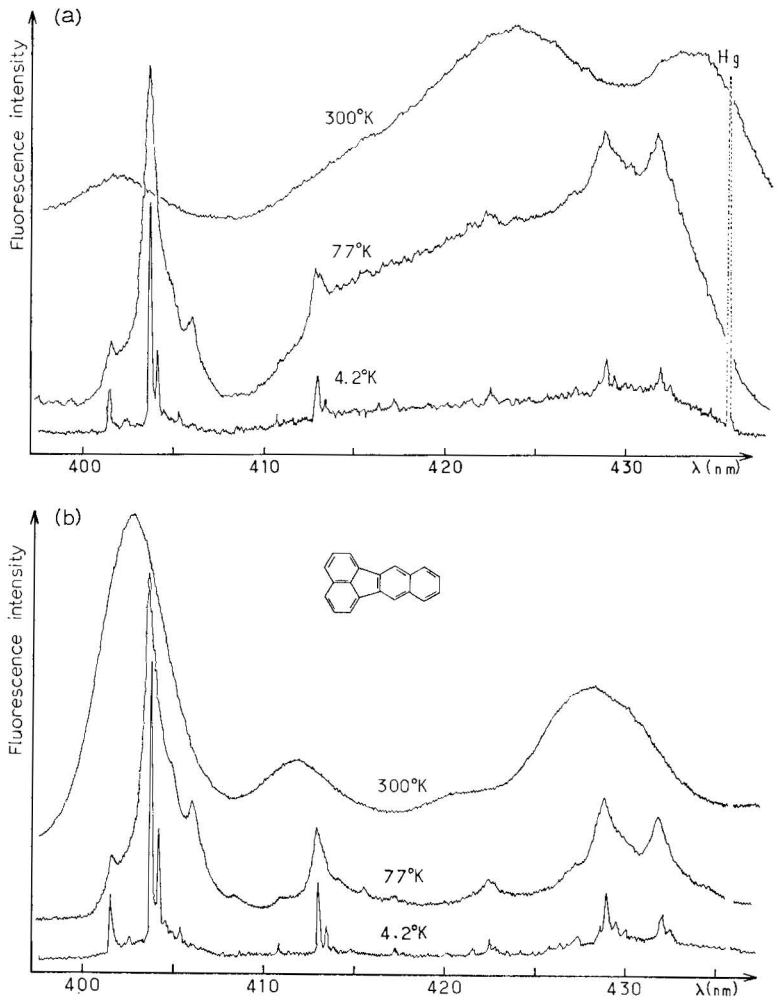


Fig. 1. Effect of temperature on fluorescence spectra. (a) Spectra from the fraction in which benzo[*k*]-fluoranthene has been identified ($\lambda_{ex.}$ = 310 nm). (b) Fluorescence spectra of benzo[*k*]fluoranthene.

aromatic compounds are dissolved in a suitable *n*-alkane matrix at low temperature. The resulting spectra exhibit well-resolved vibrational structure, and at 77°K the linewidths are reduced to *ca.* 10 to 50 cm^{-1}, which usually permits PAH identification with good confidence[3,4]. Even so, at this temperature the lines are still broadened by a temperature-dependent phonon interaction with the alkane lattice, but a further decrease in the linewidth to a few wavenumbers can be gained by lowering the temperature to 4.2°K (Fig. 1). At this temperature, the spectra are perfectly resolved and practically all the discrete vibronic transitions are observed. Further, the lines are split into multiplets due to various insertion sites in the crystalline matrices. The structure of the multiplet is very sensitive to the molecular shape, and hence reliable identification of a given PAH can be made even when the solution contains impurities, other aromatic compounds or isomers[5].

We have used this technique in order to confirm peak identification based on retention times and room-temperature fluorescence of fractions collected from HPLC separations of white oils. Moreover, we have taken advantage of the fact that the fluorescence due to aromatic compounds present in such a fraction can easily be separated from the diffuse emission due to other compounds to estimate the "purity" of the fraction and subsequently to correct quantitative estimations based on HPLC peak intensity.

EXPERIMENTAL

Analysis by liquid chromatography

The stages necessary for the isolation and determination of the amount of PAHs have already been described in detail[1]. Let us reiterate the main points:

(1) Frontal elution on silica gel gives a separation of all the aromatic compounds[6].

(2) By using adsorption chromatography (alumina as adsorbent), these aromatic compounds are separated into two fractions, one of which contains the PAHs.

(3) Partition chromatography on Sephadex LH-20 impregnated with dimethylformamide and water[7,8] separates this polyaromatic fraction into five sub-fractions, of which only three contain PAHs.

(4) By HPLC (Varian 5020 chromatograph) with an octadecyl-grafted silica support, coupled directly with a spectrofluorimetric detector operated at room temperature, isolation and determination of the amount of PAH in each of the three sub-fractions can be achieved. The spectrofluorimeter (Jobin–Yvon JY 3) is equipped with two concave holographic gratings (excitation and emission) and not with only filters (as is generally used in liquid chromatographic detection).

The wavelengths used for the detection of the PAHs are shown in Table I.

For the measurement of the individual PAHs, a standard solution containing the PAH to be measured is injected in the chromatograph just before the HPLC analysis. This procedure is the only one possible, because of the specificity of the detector, which does not permit use of the internal-standard procedure.

Identification by low-temperature fluorimetry

Sample preparation. The PAHs were collected from the HPLC column as solutions (25 ml) in a mixture of water and methanol. In order to obtain high-

TABLE I

WAVELENGTHS USED FOR DETERMINING PAHs

Compound	*Excitation wavelength (nm)*	*Emission wavelength (nm)*
Fluoranthene	365	500
Pyrene	338	394
Chrysene	268	410
Benz[*a*]anthracene	290	410
Benzo[*a*]pyrene	390 365	405
Benzo[*k*]fluoranthene	297 390	405
Benzo[*b*]fluoranthene	365	500
Perylene	403	440
Anthanthrene	403	440
Benzo[*ghi*]perylene	297	405
Indeno[1,2,3,*cd*]pyrene	365	500

resolution Shpolskii fluorescence spectra, it was necessary to transfer the solutes into *n*-alkane solutions. This was accomplished by evaporating the first solvent under vacuum at reduced temperature in an ice bath. We verified that this process gave a recovery of better than 80% for most aromatic compounds. The fractions were then taken up in *ca.* 2 ml of *n*-heptane, which was used in all experiments at 4.2°K.

Fluorescence spectrometry at 4.2°K. Fluorescence spectra at 4.2°K have been obtained with use of a laboratory-made fluorimeter. Excitation was provided by a xenon lamp (Osram 450 W), the light from which was filtered through a Jobin–Yvon HRS grating monochromator (1200 lines/mm). For most measurements, bandwidths for excitation were set at *ca.* 3 nm.

The fluorescent radiation was observed at 90° through a Jobin–Yvon HRP monochromator equipped with the same grating as for excitation. The bandwidth for analysis of the fluorescence was set so as to give a resolution of between 0.1 and 0.06 nm.

An EMI photomultiplier 9789 CXB was used as detector, and the spectra were recorded on a Servotrace recorder at a rate of 5 nm/min.

The sample cell (a 4-mm O.D. silica tube) containing the solution was introduced, after a preliminary rapid cooling to liquid-nitrogen temperature, into a liquid helium Meric cryostat.

The *n*-heptane (Fluka, Buchs, Switzerland; purissimum grade) used was dried and kept over molecular sieves (5 and 10 Å); it was verified to be free of fluorescence with use of a MPF 3 Perkin-Elmer fluorimeter.

RESULTS AND COMMENTS

The method outlined above was applied to the analysis of two medicinal white oils. The results are shown in Table II.

The chromatogram (Fig. 2) obtained by the direct coupling of HPLC and spectrofluorimetry at room temperature show that, by choosing the appropriate excitation and emission wavelengths, it is possible to detect the PAHs specifically

TABLE II

ANALYSES OF TWO WHITE OILS

Compound	*Oil 1 (ppt)*	*Oil 2 (ppt)*
Fluoranthene	645	830
Pyrene	610	734
Chrysene	235	265
Benz[*a*]anthracene	130	103
Benzo[*a*]pyrene	72	55
Benzo[*k*]fluoranthene	65	62
Benzo[*b*]fluoranthene	146	142
Perylene	17	13
Anthanthrene	22	12
Benzo[*ghi*]perylene	224	180
Indeno[1,2,3,*cd*]pyrene	72	80

and hence measure their concentrations. The results presented are for eleven PAHs, but the method could be applied to many more compounds, provided that reference specimens were obtainable.

Because of the high specificity of fluorescence spectra in Shpolskii matrices at 4.2°K, analysis of the PAHs trapped at the exit of the HPLC column by low-temperature fluorimetry enables identification to be made with very good confidence. It con-

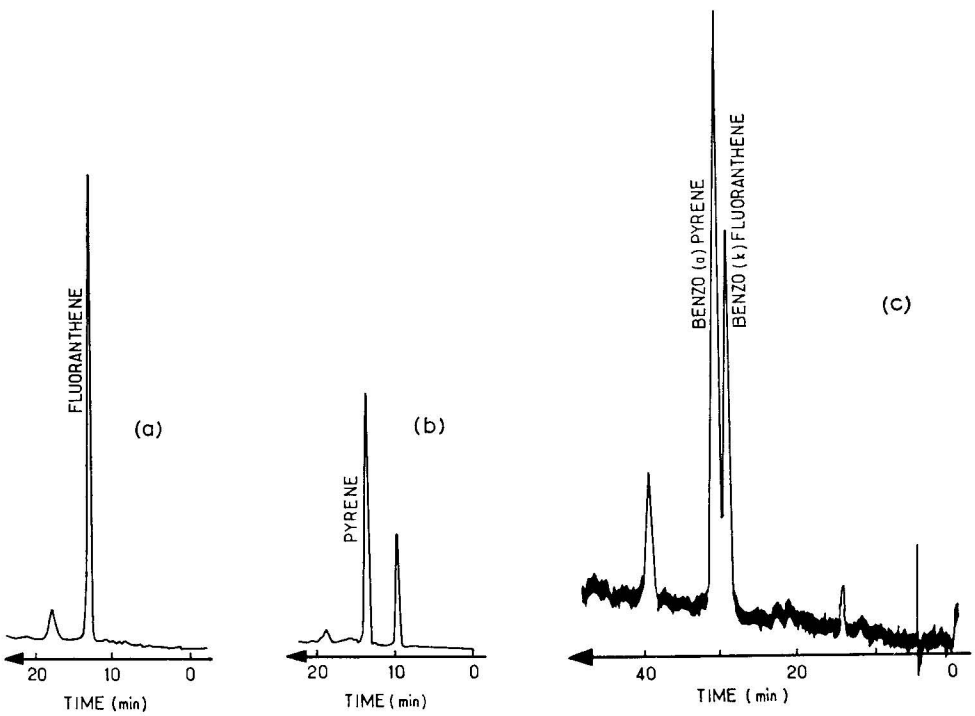


Fig. 2.

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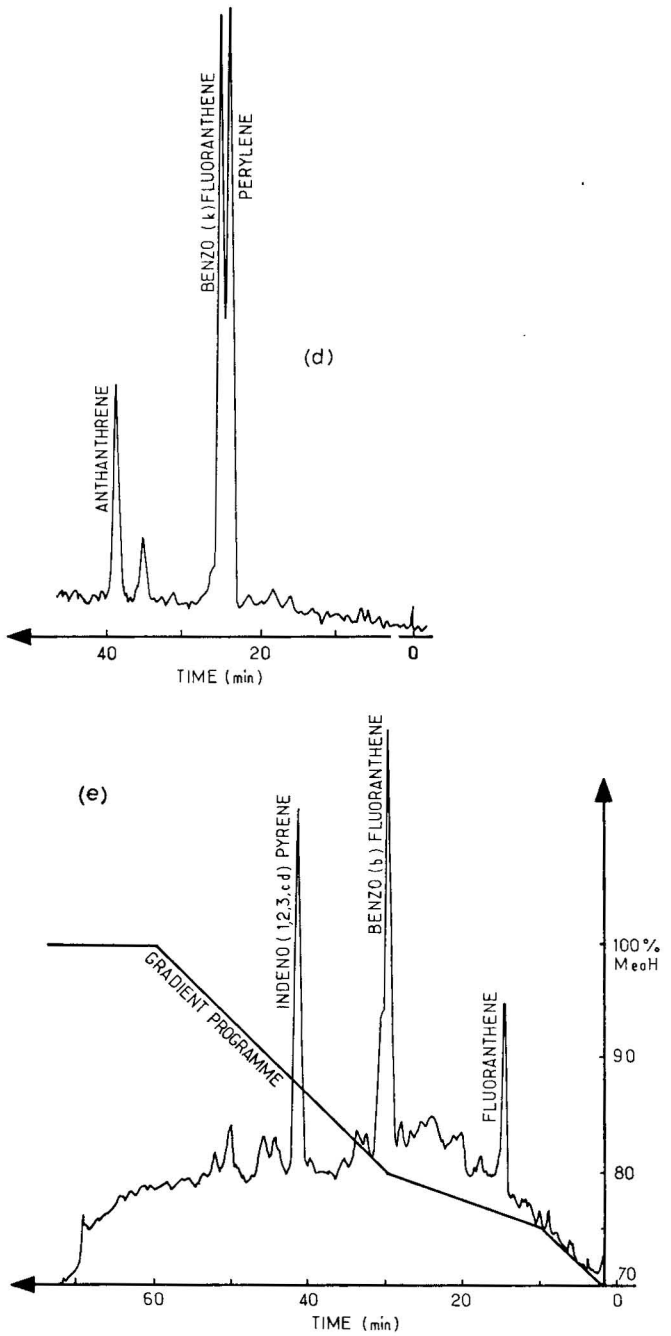


Fig. 2. HPLC separations using the Varian 5020 chromatograph with a column (30 cm × 4 mm I.D.) of Varian MicroPak MCH 10. Mobile phase, water–methanol; flow-rate, 2 ml/min; gradient programme used in all experiments shown in Fig. 2e. (a), $\lambda_{ex.} = 365$ nm, $\lambda_{cm.} = 500$ nm; (b), $\lambda_{ex.} = 338$ nm, $\lambda_{em.} = 394$ nm; (c), $\lambda_{ex.} = 390$ nm, $\lambda_{em.} = 405$ nm; (d), $\lambda_{ex.} = 403$ nm, $\lambda_{em.} = 440$ nm; (e), $\lambda_{ex.} = 365$ nm, $\lambda_{em.} = 500$ nm.

firms the sometimes ambiguous identification made by traditional methods (*e.g.* retention times and fluorescence spectra at room temperature). Two examples are shown in Figs. 1 and 3.

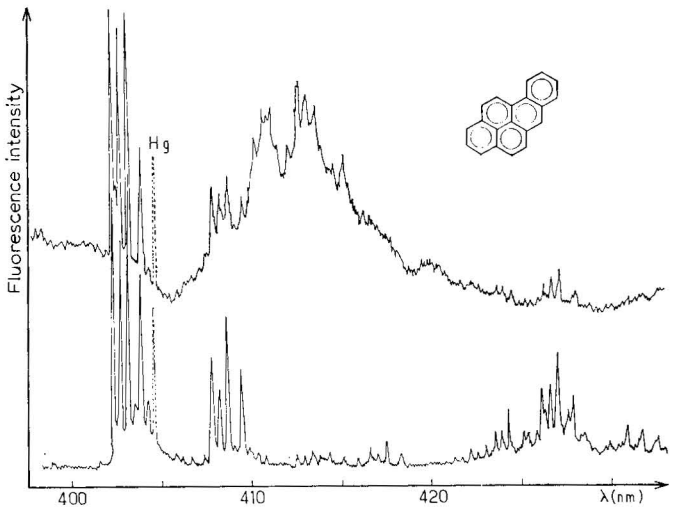


Fig. 3. Top: Spectrum from the fraction in which benzo[*a*]pyrene has been identified. Bottom: Fluorescence spectrum of benzo[*a*]pyrene.

The comparison of the spectra shows conclusively that the trapped peaks contain benzo[*k*]fluoranthene (Fig. 1) and benzo[*a*]pyrene (Fig. 3), respectively. However, in both instances, the presence of background fluorescence indicates the presence of other species, which is normal considering the complexity of the original mixture. This is particularly clear with benzo[*a*]pyrene, since the important part of the spectrum between 410 and 420 nm cannot be attributed solely to benzo[*a*]pyrene. Measurement must therefore be made between 400 and 410 nm, and great care must be taken in the region 410 to 420 nm, where other species fluoresce. In other words, one is unable to obtain precise quantitative measurements by direct coupling of HPLC and fluorimetry unless the detector used is equipped with an emission monochromator having a sufficiently narrow bandwidth ($\leqslant$10 nm).

Although Fig. 2 shows only two examples of low-temperature fluorimetry, the technique was used to confirm the identification of all the PAHs analyzed by direct HPLC–fluorimetry coupling.

The analysis being very long (*ca.* 1 month each run), we have not measured the standard deviation of the data until now, but each oil was analysed twice, the results shown in Table II being the average of two measurements. For each PAH, the difference between the two measurements is less than 20%, which seems satisfactory at such a level of concentration (10 ppt).

The lower limit of detection is between 2 ppt (for perylene or anthanthrene) and 50 ppt (for fluoranthene, pyrene or chrysene).

CONCLUSION

The coupling of HPLC with room-temperature fluorimetry permits the measurement of traces of PAHs in medicinal white oils. Analysis by low-temperature fluorimetry of the chromatographic peaks collected from the column confirms their identification and verifies the absence of interference in the measurement.

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CHROM. 13,182

NEW DETECTION AND SEPARATION METHOD FOR AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Y. ISHIDA*, T. FUJITA and K. ASAI

R/D Engineering Department, Analytical Instrument Division, Shimadzu Corporation, 1 Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604 (Japan)

SUMMARY

A simple system for simultaneous fluorescence detection of primary and secondary amines has been developed. The optimum conditions for fluorescing secondary amines in the two-stage reaction technique using sodium hypochlorite and *o*-phthalaldehyde have been studied, and then the switching flow method (sodium hypochlorite is added only to secondary amines) and the non-switching flow method (sodium hypochlorite is added to all the amino acids) have been compared. It was concluded that the non-switching flow method ensures high efficiency and reliability. This newly-developed system was applied to the detection of amino acids and gave satisfactory results.

INTRODUCTION

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of 2-mercaptoethanol, to form fluorescent substances. This reaction is often utilized for the post-column derivatization in trace analysis of amino acids by high-performance liquid chromatography (HPLC). It has recently been effectively used for close examination for inborn errors of amino acid metabolism. However, OPA does not react with amino acids such as proline and hydroxyproline to form fluorescent compounds.

Roth[1] reported, in his paper concerning the fluorescing reaction of primary amines, that proline can be derivatized by treating it with sodium hypochlorite (NaClO) to give a compound that reacts with OPA.

Then, St. John[2] tried fluorescence detection of proline and other amino acids, using a two-stage reaction coil system, as shown in Fig. 1A. In the first reaction coil, the sample was made to react with NaClO, while in the second reaction coil, the reaction with OPA was made. He used the switching flow method so that NaClO was added only to the part of the effluent that contained proline or hydroxyproline. As for the other part of the effluent, some buffer solution was added so that the flow-rate of the mobile phase was kept constant in the second reaction coil. Such a complicated method was adopted in order to prevent the trouble that might be caused if NaClO had been added to the primary amines. This switching method is costly and not very easy to operate.

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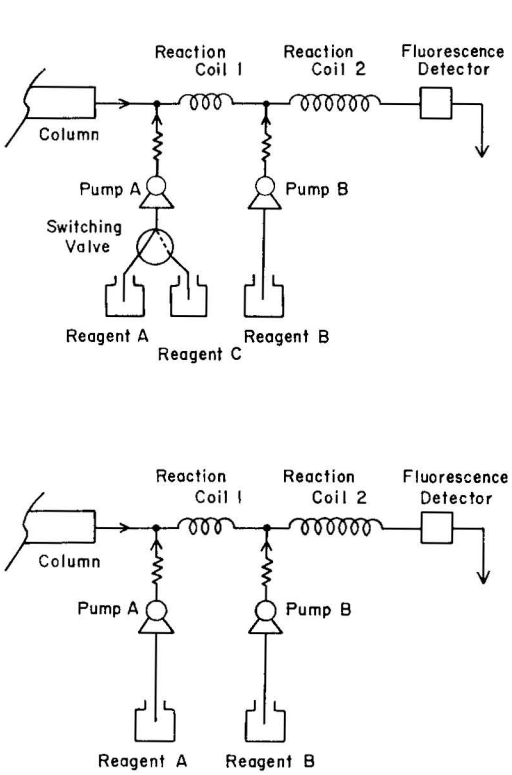


Fig. 1. Diagrams of (A) the switching flow method and (B) the non-switching flow method. Reagent A, buffer with sodium hypochlorite; reagent B, OPA reagent; reagent C, buffer without sodium hypochlorite.

We tested the non-switching flow method in analysis of amino acids, in which NaClO is added to all the effluent, and found it practically useful.

EXPERIMENTAL

Equipment

The HPLC system used was the Shimadzu LC-3A which incorporates a liquid pump of CDQR system[3]. The column was housed in the Shimadzu CTO-2A column oven. The flow of the mobile phase was changed by means of the Shimadzu SGR-1A step gradient elution unit. In the SGR-1A, the mobile phase changeover is performed with a rotary six-port valve which is operated according to the program set on digital timers. Samples were introduced by the Shimadzu SIL-1A variable injector.

As for the detector, the Shimadzu CRD-5A[4] chemical reaction detector was used. The CRD-5A consists of a chemical reaction bath, a pump for reaction reagent, and a filter-type fluorometric detector. The chemical reaction bath consists of a temperature controller and a thermostatted box containing a tin block in which a stainless steel reaction coil, a heater, and a temperature sensor are embedded. The

temperature of the reaction coil is controlled at any temperature between ambient temperature plus 15°C and 150°C, to a control accuracy of ±0.2°C.

A commercial, low-cost reciprocating plunger pump was used for supplying reaction reagents. The Shimadzu FLD-1 filter-type fluorometric detector was used in the CRD-5A.

A mercury lamp generating a light of 300–400 nm wavelength and having the peak intensity at 360 nm was used as the excitation lamp. A fluorescent filter that cuts off the wavelengths shorter than 400 nm was used for the fluorometry.

The switching between the buffer solution that contained NaClO and the buffer solution that did not contain NaClO was carried out by a three-port valve which was driven manually or automatically by a motor according to a preset program.

Column

Amino acids were separated on a Shimadzu LC column ISC-07/S1504. It is a strongly acidic cation-exchange resin of styrene–divinyl benzene copolymer, 10% in cross-linkage and 7.5 μm in the average particle diameter, and is packed in a stainless-steel tube (150 × 4.0 mm I.D.).

Reagents

Sodium hypochlorite (NaClO) was purchased from Shimakyu Yakuhin, Osaka, Japan, and its chlorine concentration was 10%; *o*-phthalaldehyde (OPA) was purchased from Wako, Osaka, Japan. Sodium citrate, sodium hydroxide, perchloric acid, boric acid, 2-mercaptoethanol and the standard amino acid samples, were also purchased from Wako. Grape juice and wine samples were kindly offered by Research Institute of Fermentation, Yamanashi University (Yamanashi, Japan).

RESULTS AND DISCUSSION

Fluorescing reaction of proline

Length and temperature of reaction coil. Using the flow system shown in Fig. 1B, we investigated the influence of the dimensions of the reaction coil 1 (*i.e.* the length of reaction time) and its temperature, upon the fluorescence intensity of the derivative of proline produced. The reagent A was 0.1% NaClO solution in 0.3 *M* borate buffer (pH 10.5); the reagent B was OPA reagent (400 mg of OPA and 1 ml of 2-mercaptoethanol in 500 ml of 0.3 *M* borate buffer (pH 10.5). Distilled water was used as the mobile phase, its flow-rate adjusted to 0.5 ml/min. The flow-rates of the reagents A and B were adjusted to 0.75 ml/min. The reaction coil 2 was 1000 × 0.3 mm I.D., and was kept at 50°C.

Our experiments showed that the optimum size of the reaction coil 1 at temperatures 50–60°C was 1000–2000 × 0.3 mm I.D. The same results were obtained when different buffers, phosphate buffer for example, were used in place of borate buffer. It was found that use of a longer reaction coil 2 resulted in a higher fluorescence intensity of the proline derivative. But use of a longer reaction coil resulted in dispersion of the sample bands, hence broadened peaks. In our experiments, we used the reaction coil of the Shimadzu CRD-1D chemical reaction detector system which is now effectively used in various fields. It was also found that the optimum length of the reaction coil 1 was not dependent on the length of the reaction coil 2.

Concentration of reagent. The optimum content of NaClO in the reagent A depends on the amount of 2-mercaptoethanol in the reagent B. When the amount of 2-mercaptoethanol in the OPA reagent is adjusted to 0.2%, the optimum concentration of NaClO is 0.1–0.2%. When the NaClO content is equal, a higher concentration of 2-mercaptoethanol will result in lower fluorescence intensity of the proline derivative. (The reaction coil 1 is 1000 × 0.3 mm I.D., and the other conditions are the same as described under *Length and temperature of reaction coil.*

Switching flow method

In the switching flow method, shown in Fig. 1A, the reagent C, which is borate buffer containing no NaClO, is allowed to flow till the glutamic acid fraction is eluted out, after which the switching valve is turned to the position of solid line to allow the reagent A to flow. Thus, the proline is detected. After the proline has been detected, the switching valve is turned back to the position of dotted line, to allow the reagent C to flow again. This valve-switching step had been thought necessary because, if the reagent A is added to the entire effluent, the fluorescing reaction of the amino acids having primary amine radicals might be hindered. The switching of the valve, however, inevitably causes fluctuation of the baseline, because of the different fluorescent background between the reagent A and the reagent C. In order to prevent the elution of proline during the baseline fluctuation, it is necessary to adjust the operational conditions so that glutamic acid and proline are separated to an otherwise unnecessary degree.

The biggest disadvantage of the switching flow method is the high cost of the switching valve and the programmer for driving it. Thus we proceeded to investigate the practicality of the non-switching flow method.

Non-switching flow method

We investigated how the primary amines are affected when the non-switching flow method shown in Fig. 1B is used. Tables I and II show the influences of the reaction temperature and the amount of NaClO on the fluorescence intensities of the four amino acids. These data show that the influences are negligible if the reaction

TABLE I

INFLUENCE OF THE REACTION COIL TEMPERATURE ON THE DETECTION OF AMINO ACIDS

Sample solution: Asp, 2.4 mg; Ser, 2.1 mg; Glu, 2.1 mg; Gly, 2.5 mg; in 50 ml of mobile phase. Injection volume: 5 μl. Detector attenuation: × 32. Separation conditions: column, LC column ISC-07/S1504; mobile phase, 0.2 *N* sodium citrate (pH 3.20); flow-rate, 0.5 ml/min; column temperature, 55°C.

Other conditions are the same as in Table III. Except coil temperature and NaClO concentration.

Amino acids	*Peak height (relative response)*								
	Reaction coil temperature (°C)								
	16	*30*	*40*	*50*	*55*	*60*	*65*	*70*	*80*
Asp	8.0	21.7	30.0	37.5	35.0	31.0	22.5	13.0	4.0
Ser	28.2	49.0	52.0	53.5	49.0	44.5	35.0	24.0	7.0
Glu	8.0	18.0	22.0	25.0	23.5	20.0	14.5	9.0	2.0
Gly	65.0	74.0	69.5	70.0	68.0	69.0	—	65.5	58.5

TABLE II

INFLUENCE OF THE CONCENTRATION OF SODIUM HYPOCHLORITE ON THE DETECTION OF AMINO ACIDS

For conditions, see Table I.

NaClO concn. (μl/ml buffer)	*Peak height (relative response)*			
	Asp	*Ser*	*Glu*	*Gly*
0.5	44	61	29	86
1.0	44	60	28	81
2.0	42	57	26	77
4.0	37	52	23	66
8.0	30	40	17	47

TABLE III

COMPARISON OF THE SENSITIVITY OF VARIOUS AMINO ACIDS IN THE SWITCHING AND THE NON-SWITCHING FLOW METHOD

Separation conditions: column: LC column ISC-07/S1504, strong acidic cation-exchange resin, 7.5 μm, 150 × 4.0 mm I.D.; mobile phase: A, 6% ethanol in 0.2 *N* sodium citrate (pH 3.20); B, 0.2 *N* sodium citrate (pH 4.25); C, 0.6 *N* sodium citrate (pH 8.0); D, 0.6 *N* sodium citrate (pH 10.0); E, 0.2 *N* NaOH; stepwise gradient program of mobile phase: A, 14 min; B, 16 min; C, 5 min; D, 30 min; E, 3 min; flow-rate: 0.5 ml/min; column temperature: 55°C.
Reaction conditions: reagent: A, 0.1% sodium hypochlorite in 0.4 *M* borate buffer (pH 10.0), flow-rate: 0.75 ml/min; B, 400 mg of *o*-phthalaldehyde and 2 ml of mercaptoethanol in 500 ml 0.3 *M* borate buffer (pH 10.0), flow-rate: 0.75 ml/min; reaction coil: 1, 1000 × 0.3 mm I.D.; 2, 2000 × 0.3 mm I.D.; reaction temperature: 55°C.
R = (response in non-switching flow method)/(response in switching flow method).

Amino acid	*R*	*Amino acid*	*R*
Asp	0.63	Met	0.66
Thr	0.95	Leu	0.93
Ser	0.83	Ileu	0.87
Glu	0.74	Tyr	0.86
Pro	1.00	Phe	0.97
Gly	0.83	His	0.75
Ala	0.81	Lys	0.94
Cys	1.88	Arg	0.99
Val	0.60		

temperature is within 50–60°C and the NaClO concentration is 0.1–0.2%, though it is possible that the results may depend on the reaction time in the reaction coil 2. We determined the optimum reaction conditions and LC conditions as shown in Table III, for analysis of amino acids.

The switching flow method and the non-switching flow method were compared under these operational conditions, and the results are shown in Table III. The data show that, contrary to the reports presented before, the non-switching flow method ensures satisfactory results provided that the operational conditions are appropriate. The data for the primary amino acids are not noticeably affected by the continuous supply of NaClO.

The calibration curve for each amino acid is satisfactorily linear from a few picomole level to a few tens of nanomole level. This shows that the primary amino acids undergo quite stable reaction when the non-switching flow method is used. The

detection limit for proline is a few tens of picomole level. If the Shimadzu RF-500 LC fluorescence spectromonitor, which employs a xenon lamp, is used as the detector, the sensitivity can be enhanced by five times for most amico acids.

Applications

From the results of our experiments described above, we have come to a conclusion that the non-switching flow method is quite simple to perform and provides satisfactory performance, and we have developed an amino acid analysis system which utilizes the non-switching flow method. The system was successfully applied to various analyses. Fig. 2 shows an example of analyses.

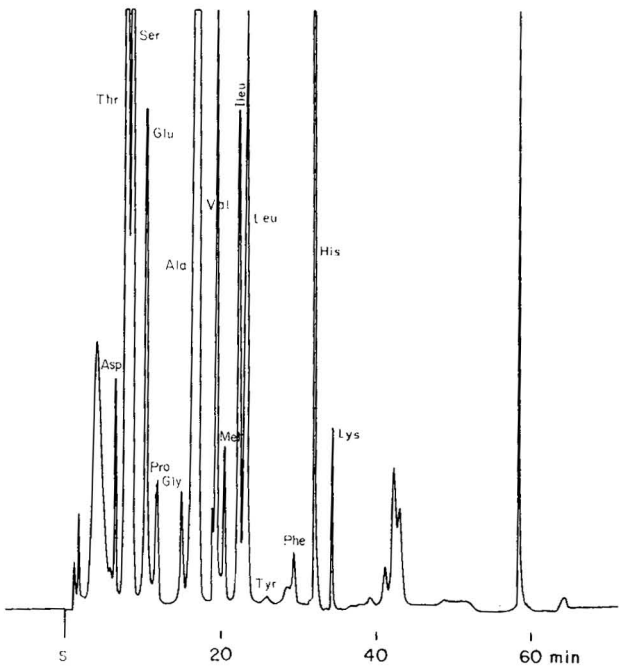


Fig. 2. An analysis of the amino acids in grape juice. Operational conditions are the same as in Table III.

CONCLUSION

The switching flow method has been so far used for adding sodium hypochlorite (NaClO) to detect secondary amino acids in the post-column derivatization for amino acids with *o*-phthalaldehyde.

The non-switching flow method was tested for the same purpose and has proved to provide satisfactory results, using a much simpler system.

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CHROM. 13,303

NUMERICAL SPECTROSCOPY: ABSORBANCE INDEX TECHNIQUE AND ALGORITHM FOR QUALITATIVE ANALYSIS IN LIQUID CHROMATOGRAPHY

A. F. POILE* and R. D. CONLON
The Perkin-Elmer Corporation, Chromatography Division, Norwalk, CT 06856 (U.S.A.)

SUMMARY

This work was undertaken as part of an effort to provide efficient numerical techniques for utilizing spectroscopic data in high-performance liquid chromatography. Such techniques are necessary because retention time is not sufficient confirmation of peak identity, nor is symmetry proof of peak purity. Several numerical techniques for comparing spectral data exist which range from simple absorbance ratios through spectral deconvolution.

This paper presents a new algorithm called absorbance index which has the calculation simplicity of absorbance ratios but makes more efficient use of the spectral data. The data obtained from this algorithm are used to perform qualitative tasks in liquid chromatographic analysis such as determination of peak identity and peak purity.

INTRODUCTION

Chromatographers are increasingly aware of the importance of spectroscopy to the practice of liquid chromatography. Many papers in the current literature are appearing which present numerical procedures for performing the tasks of:

detection of impurities in an apparently pure chromatographic peak;

confirmation of the identity of a peak when paired with retention time data;

identification of an unknown compound by reference to a user-built library of spectral data;

qualitative and quantitative analysis of a mixed compound peak not separable by high-performance liquid chromatography (HPLC).

Techniques currently being used to perform these tasks include factor analysis in the form of matrix rank analysis[1] and principal components analysis[2], least means squares[3] and absorbance ratios[4,5]. The majority of these techniques involve complex calculations often involving matrix manipulations, and are efficiently performed only with the aid of a computer.

The occasional user who does not have computer capability readily available, however, needs a technique which is suitable for manual calculation. The only technique which we have found in the literature which meets this requirement is the

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absorbance ratio, which is obtained through simple division. While attractive from a computational point of view, however, the absorbance ratio technique has a serious limitation for the liquid chromatographer since it requires that the user has some prior knowledge not only of the spectrum of the compound of interest, but also of the spectra of all the compounds that might be present and to which it is to be compared. In the course of investigating this limitation, a new numerical technique, absorbance index, was developed. While related to absorbance ratio, this new technique requires no prior knowledge of the spectra of the compounds to be compared.

EXPERIMENTAL

The spectral data were taken from peaks eluted from the column by stopping the flow and spectrally examining the captive peak. The procedure has been described previously[5]. For our studies, we used corrected absorbance values from the nine wavelengths from 210 nm to 300 nm taken at 10-nm intervals, which is the default program for the Perkin-Elmer LC-75 and Autocontrol detector system and from spectra sampled at 1-nm intervals.

RESULTS AND DISCUSSION

The tasks listed in the Introduction (above) require that we compare the spectrum of a standard to the spectrum of the sample. Thus, to check the purity or homogeneity of a chromatographic peak, the data points of one spectrum are taken on the leading edge and the data points of the other are taken on the trailing edge. For compound confirmation, the data points of the spectrum of the unknown sample are compared to those for the spectrum of the known compound.

In the absorbance index technique, essentially we divide one of the spectra into the other. If the graphed result of this division is a straight horizontal line (constant), then the spectra, and, by inference, the two compounds are the same (Fig. 1A). If the graphed result of this division is not a straight line, then the spectra are different (Fig. 1B). The results can be obtained from as many data points across the spectra as desired. Our experiments indicate, however, that a minimum of nine data points should be used. We call the values which make up this line the absorbance indices (Q). The degree of variation of the line is tested with the discriminator (D), which is the quotient of the largest to the smallest value of Q obtained. For identical compounds, and under ideal conditions, D would be equal to unity. To accomodate instrumental and environmental variations, a value greater than unity is taken as a practical threshold. Normally we set a maximum limit for D at 1.5 for accepting the two spectra as the same if the signal-to-noise ratio is greater than about 10:1.

To put the new method into context, we shall compare the absorbance index technique to the established technique of absorbance ratios. In the absorbance ratio technique, a spectrum is characterized by dividing its absorbance measured at one wavelength by its absorbance measured at some other wavelength. Other spectra can then be compared to this spectrum by measuring the absorbance ratio of the sample spectra at the same two wavelengths. The spectra can be considered identical if the absorbance ratios are the same (within the experimental error of the measuring system). When the sample spectrum is different from the standard spectrum, a pair of

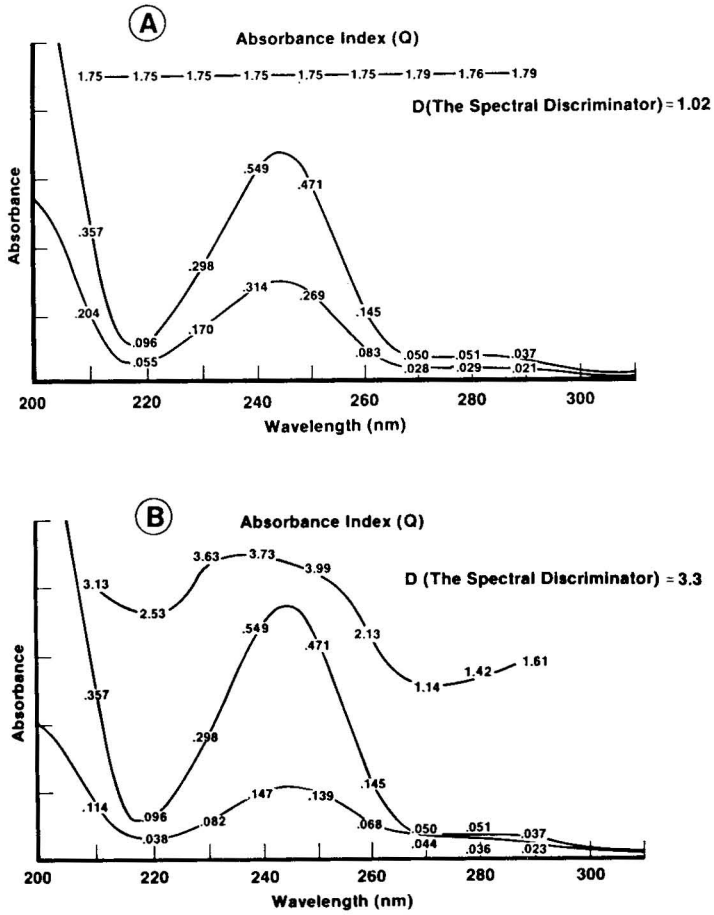


Fig. 1. Comparison of two spectra: A, the same compound at two different concentrations; B, two different compounds at different concentrations.

wavelengths can be found which best indicate this difference. This "best" wavelength pair make the largest quotient when their absorbance ratios are divided. This pair of wavelengths will change depending on the sample spectrum.

An inherent shortcoming of the absorbance ratio technique, however, is that it is always possible to find a sample compound which will give the same ratio as the standard at any given preselected pair of wavelengths. To avoid a false assumption that they are identical, we must perform a second absorbance ratio measurement at a different pair of wavelengths. Even this second measurement may be inconclusive if the difference between the two ratios is within the experimental error of the system. We must, therefore, continue to calculate absorbance ratio measurements at different pairs of wavelengths until a value is obtained which is significantly different from the standard or until no further ratios can be calculated. If none of the ratios are significantly different, then we can make the assumption that the two spectra, and hence, the two compounds are the same.

If we had obtained absorbance measurements at nine wavelengths for both the

standard and the sample, then we could calculate 36 different ratios on both spectra, a total of 72 separate calculations. The best pair of wavelengths would then be those which gave the largest quotient of the respective ratios on the sample and the standard if the two were different.

The calculation of this quotient would require an additional 36 calculations for a total of 108 calculations. This quotient can be expressed as

$$\frac{A_i/A_j}{B_i/B_j}$$

where A_i and A_j are the absorbance values of the standard at wavelengths i and j and B_i and B_j are the absorbance values of the sample at the same two wavelengths. This expression can be rearranged to give

$$\frac{A_i/B_i}{A_j/B_j}$$

which will have its maximum value when the denominator A_j/B_j is a minimum, and when the numerator A_i/B_i is a maximum.

These two expressions are both absorbance index values. By definition, the absorbance index discriminator is the quotient of the maximum and minimum absorbance index obtained. Hence the absorbance index technique will automatically find the best two wavelengths at which to compare the two spectra, if they are different. The absorbance index technique using the same nine wavelengths for both the sample and the standard, only requires ten calculations to obtain information equivalent to that which results from the absorbance ratio technique.

CONCLUSION

The absorbance index technique is a simple, sensitive test for comparison of spectra. The test can be performed manually from discrete absorbance values and the entire process can be accomplished in only a few minutes on peaks eluted by HPLC. The usefulness of this numerical spectroscopy procedure for determining peak purity and for peak confirmation is not only in the simplicity and utility with which the two spectra can be tested for likeness, but also in the consistency and confidence level of the results.

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CHROM. 13,268

DETERMINATION OF THE TOTAL SULPHUR CONTENT IN GAS OILS WITH A FLAME PHOTOMETRIC DETECTOR

RICHARD VISSER* and ADRIE HOVESTAD

Department of Chemical Technology, Twente University of Technology, P.O. Box 217, 7500 AE Enschede (The Netherlands)

SUMMARY

A method is described for the quantitative determination of the total sulphur content of gas oils, using a gas chromatograph with an empty glass column and a dual-flame flame photometric detector. The method can also be used for the analysis of the volatile sulphur content in aqueous solutions.

INTRODUCTION

During a study of catalytic hydrodesulphurization of gas oils[1] a method was needed not only for the determination of the distribution of sulphur compounds, but also, more importantly, for the determination of total sulphur content. Methods have been published for the determination of the distribution of sulphur compounds in gas oils using gas chromatography (GC) with a coulometric detector[2,3]. The flame photometric detector (FPD) has proved its usefulness for the analysis of sulphur compounds. The response of the FPD for nanogram amounts of sulphur is comparable with that of the microcoulometric detector[4].

The dual-flame concept was introduced to improve the FPD performance[5,6]. The work of Rupprecht and Phillips[6] is of particular interest, since the system was used not only as a GC detector but also for the continuous monitoring of the odorant sulphur content in hydrocarbon gases and the determination of sulphur content in volatile liquids. Difficult operation and somewhat poor reproducibility apparently prohibited a more widespread use. These problems seem to be greatly reduced in a more recent design[7].

In this paper a simple method is described for the quantitative determination of the total sulphur content in oil fractions, using a gas chromatograph with an empty glass column and a dual-flame FPD. The method was extended to the determination of the total (volatile) sulphur content of aqueous solutions. This was applied to a scrubber for gasifier effluents.

EXPERIMENTAL

Because of the non-linear response of the FPD, the simplest approach was to

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elute all sulphur compounds in one peak, preferably with reproducible peak width. The samples were rapidly evaporated in an injector at relatively high temperature. The vapour was then led through an empty glass column of length 3 m before reaching the detector, to prevent a sudden pressure rise in the detector, extinction of the flames, and errors caused by too low response of the detector system.

The usual injection method yielded non-reproducible results. However, when only the needle volume of a standard Hamilton 10-μl syringe (701N) was injected during exactly 5 sec, a reproducible injection volume of *ca.* 0.5 μl was obtained.

The non-linearity of the FPD made careful calibration necessary. For gas oils calibration was performed in two ways:

(1) A standard reference oil containing sulphur was diluted (w/w) with the same type of oil, but absolutely free from sulphur;

(2) A series of reference oil samples was measured independently.

The sulphur amounts of reference samples were checked by X-ray fluorescence, using the method of standard additions.

For aqueous solutions calibration was performed by injection of solutions of known concentration of thioacetamide in water. The detector responses were compared for a series of six compounds dissolved in water.

Conditions

A gas chromatograph Varian 3700 with a dual-flame FPD was used under the following conditions: column, empty glass column, 3 m $\times$ 0.25 in. O.D.; injector temperature, 320°C; column oven temperature, 300°C; FPD block temperature, 240°C; injection, 0.5 μl; carrier gas, helium, at a flow-rate of 3 ml/min for oil, 20 ml/min for aqueous solutions; characteristic retention time, 250 sec for oil, 40 sec for aqueous solutions; integrator, Infotronics 304.

RESULTS

The detector responses for two series of oil samples are presented in Fig. 1. The two series coincided on the same calibration curve. In this case the detector electronics were operated in the "root" mode. The curve shows an exponential relationship; the concentration dependence exponent is 0.82, corresponding to $n = 1.64$ in the normal mode. The relative accuracy was 2% at the 1% level and 10% at the 100 ppm level of sulphur percentage.

The results of the analysis of a characteristic series of oil samples (before and after desulphurization) are listed in Table I. For these samples the sulphur contents were checked by X-ray fluorescence. Reference samples were injected between the regular samples.

Table II summarizes the detector responses for a series of aqueous solutions. In this case the detector electronics were operated in the "direct" mode. Again an exponential relationship was found with $n = 1.76 \pm 0.05$.

In a separate experiment a mixture was prepared of 15 ml of the aqueous thioacetamide solution and 1 ml of the aqueous solutions of the other compounds listed in Table II, all solutions containing 800 mg/l. Analysis of this mixture as described above, using the thioacetamide calibration curve, yielded a sulphur peak area of $190 \cdot 10^5$ counts/sec, corresponding to a sulphur concentration of *ca.* 815 mg/l.

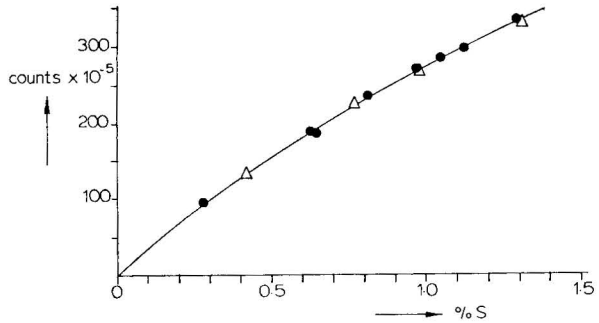


Fig. 1. Calibration curve for the determination of total sulphur content in oils (△ = diluted sample, ● = independent sample).

TABLE I

DETERMINATION OF TOTAL SULPHUR CONTENT IN GAS OILS

Samples 1–5 are distillation fractions of a virgin oil.

Sample	*Sulphur (%, w/w)*	
	GC	*X-ray*
1, Untreated	1.03	1.02
2, Untreated	1.35	1.31
3, Untreated	1.44	1.40
4, Untreated	1.80	1.75
5, Untreated	2.09	2.11
1, Desulphurized	0.015	0.014
2, Desulphurized	0.013	0.013
3, Desulphurized	0.021	0.024
4, Desulphurized	0.011	0.021
5, Desulphurized	0.084	0.082
Virgin oil, original	1.54	1.55
Virgin oil, desulfurized	0.046	0.044

TABLE II

DETECTOR RESPONSES (PEAK AREA) FOR SIX COMPOUNDS IN WATER (VALUES IN INTEGRATOR COUNTS × 10^{-5})

Compound	*Concentration of sulphur (mg/l)*			*n*
	200	*400*	*800*	
Thioacetamide	16.7	53.1	183	1.73
Dimethyl sulphoxide	15.0	52.4	186	1.82
Sulpholane	16.0	55.8	181	1.75
Dimethylsulpholane	16.9	55.1	177	1.69
3-Methylsulpholene	16.3	63.4	192	1.78
3,3-Thiodipropionitrile	15.6	49.2	192	1.81
Mean	16.1	54.8	185.2	1.76
S.D.	0.7	4.8	6.0	0.05

DISCUSSION

Several authors[1,8–10] have found different relative response orders of sulphur compounds when using a single-flame FPD. However, Burnett *et al.*[11] showed that the actual FPD response order of those compounds depends on the concentrations or ranges of concentrations being examined. For a series of eleven sulphur compounds they found exponential proportionality constants n between 1.48 and 2.00. Maruyama and Kakemoto[12] found that the response to sulphur compounds (containing C_1–C_{12}) was dependent only on the number of sulphur atoms in a molecule and not on its structure.

The dual-flame FPD seems to have overcome many of these problems[7]. Patterson[13] found a pure square-law dependence for thirteen compounds, including methyl mercaptan, hydrogen sulphide, sulphur dioxide and parathion, and an equal response per nanogram of sulphur for methyl parathion and hexanethiol. Gangwal and Wagoner[14] slightly amended these results. They plotted the value area·height$^{-\frac{1}{2}}$ *versus* sulphur content, and found slopes of 0.92 for H_2S, COS and SO_2, 0.93 for methyl mercaptan and thiophene, and 0.85 for CS_2, corresponding to $n = 1.84$, 1.86 and 1.70, respectively. Equal responses were found for *ca.* 5 ng of each compound.

Despite the fact that in our work all sulphur compounds are coeluted on top of the bulk of the organic material (oil) or water, the constant n is of the order of 1.64 for oil and 1.7–1.8 for aqueous solutions; the absolute responses are highly comparable in the latter case. This proves that even under our rather extreme conditions the detector is still working properly. Deviations from the square-law dependence may be explained by deviation of the Gaussian shape, tailing and change in peak width with sulphur mass[14], or change in flame temperature or composition and concentration of free-radical species in the flame[11].

CONCLUSION

It is possible to determine total (volatile) sulphur contents in oil and water, using GC with an empty glass column and dual-flame FPD. The method is simple and can be performed by the same person carrying out other GC analyses, without any further special experience or apparatus, thus avoiding laborious micro-elemental analysis.

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CHROM. 13,280

MONITORING OF AGGRESSIVE COMPONENTS BY MEANS OF A SIMPLE CORROSION-RESISTANT ALL-GLASS PROCESS GAS CHROMATOGRAPH WITH AN ARGON PLASMA DETECTOR

A. KUESSNER
BASF AG, Ludwigshafen (G.F.R.)

SUMMARY

A corrosion-resistant gas chromatograph is described for the analysis of aggressive gaseous compounds (HCl, Cl_2, $COCl_2$, NO_2, etc.) in any gas mixtures or of inert compounds (as vinyl chloride) in aggressive media, even in the presence of water. The detection is based on an electrodeless plasma discharge in argon as the carrier gas. As all surfaces in contact with the sample gas consist of glass or PTFE, stability against corrosion could be achieved.

Disadvantages of this detector are the limited dynamic range of approximately three decades and the individuality of each discharge tube; individual calibration graphs are required. Advantages are the corrosion resistance and consequently the constancy of sensitivity over several years of maintainance-free process operation.

INTRODUCTION

For the determination of aggressive gases such as HCl, Cl_2, $COCl_2$ and NO_2, normally chemical or physico-chemical methods are used. Gas chromatography (GC) has not been applied very widely, and the literature is sparse[1–4]. The problem is mainly the corrosion that occurs on all metallic surfaces that come into contact with the sample gases, especially if in a prolonged process operation traces of water cannot be excluded.

For operation in chemical plants working with corrosive gases such as HCl and $COCl_2$ some simple and rugged and maintenance-free gas chromatographs were desired. A requirement was that no metallic surfaces should come into contact with the sample gas.

For the gas inlet commercial magnetic valves are readily available, the tightening parts of which consist of PTFE (Convac, Serres, G.F.R.). Separation columns made of glass with inert supports and stationary phases also are well known (*e.g.*, Fluorolube on Chromosorb T). The real problem was to obtain a corrosion-resistant detector.

A GC detector that really avoids metallic surfaces coming into contact with the sample gas is the plasma discharge detector with electrodeless excitation. In contrast to all molecular gases, the monatomic noble gases can be excited by electric fields to give plasma discharges even at ambient pressure. This excitation can be

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effected by the capacitative transfer of a sufficiently high and sufficiently high-frequency alternating voltage through the isolating wall of an appropriate discharge tube. If pure argon is used as the carrier gas in a gas chromatograph, it can be excited to give a plasma discharge when leaving the separation column. Any concentration profile in this argon will influence this plasma, and any method for measuring the extent of this influence is a method for quantitative detection.

Plasma discharge detectors based on this principle, using frequencies between 100 and 2500 MHz, are well known[5–7]. Their electronics are expensive and this precludes their application for the desired simple and cheap surveillance instruments. However, another approach seemed feasible. The capacitive charge transfer is proportional to both frequency and voltage and higher voltages allow lower frequencies, so that the electronics should be simpler and cheaper.

The transfer of the signal to the recording system also must be effected by capacitive charge transfer. The compounds of interest will be completely ionized in the plasma and so increase its conductivity. Variations in the voltage drop along the plasma discharge should be transferred to a coil around the discharge tube.

EXPERIMENTAL

Fig. 1 shows the assembly developed. The output of a 10-kHz frequency generator circuit was amplified by an HC 1000 high-performance transistor and fed into an ordinary ignition coil of a car engine. The resulting voltage of *ca.* 10 kV was sufficient to excite a plasma discharge in argon after an electrodeless charge transfer. Owing to the high inductance of the ignition coil it is unimportant whether the original alternating voltage has a sine, rectangular or triangular shape.

The length of the discharge tube is 160 mm, and in the centre of this quartz tube a supplementary electrode is attached. This is a metal wire (*ca.* 30 mm long, 0.1 mm diameter, preferably steel), protected by a thin quartz capillary which is closed at the front end. It is positioned in the centre by a glass plunger and is displaceable

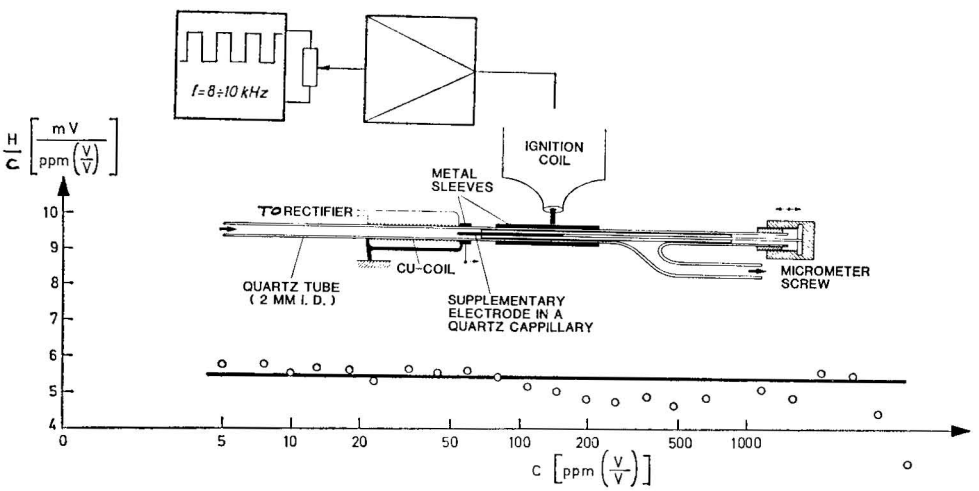


Fig. 1. Discharge tube and sensitivity diagram.

in the axial direction by a micrometer screw. The correct position of this supplementary electrode relative to the outer coverings is essential for the detector performance, and must be optimized once at the beginning of the operation.

The signal coil consists of *ca.* 100 turnings of an isolated copper wire of diameter 0.17 mm, fixed by a thin layer of two-component resin. The transferred signal voltage is rectified by an ordinary measuring rectifier of an adjustable coil a.c. meter [copper(I) oxide diodes in a Graetz circuit] or by a specially constructed circuit in order to avoid non-linearity at low values. The first method is simpler, but requires a non-linear calibration graph. The signal voltage output goes up to 100–200 mV.

The electronics consist of semiconductor elements, the performance of which depends on temperature. To make use of the stability of the sensitivity the whole assembly has to be kept at constant temperature. The chromatograph with all supplementary devices (inlet valves, timer, column, detector) is housed in a plastic box (40 × 50 × 60 cm), the interior of which is maintained at *ca.* 45°C. The stability of the sensitivity allows the application of a bar graph technique for recording the results (see Fig. 2).

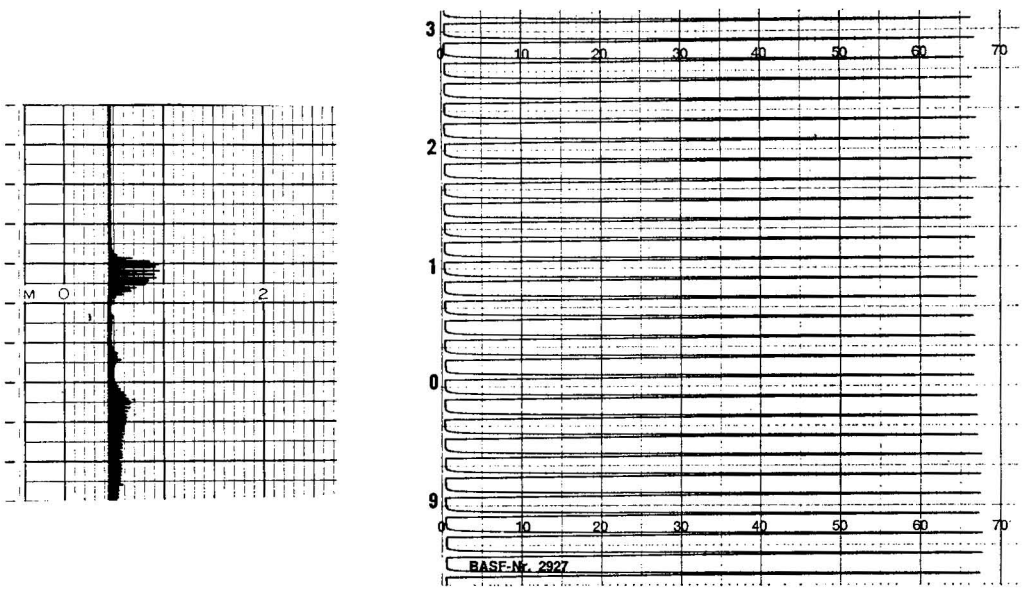


Fig. 2. Example of bar graph and reproducibility test.

RESULTS

After separation in an appropriate separation column, all of the aggressive gases mentioned give rise to ordinary GC peaks. The reproducibility is *ca.* 5% relative. The dynamic range extends over approximately three decades of concentration. The calibration graph in Fig. 1 was obtained by an automatic logarithmic dilution procedure. Each discharge tube has to be calibrated individually. Differences may result from different distances of the covering electrodes and the shape of the closing tip of the capillary of the supplementary electrode. Deviations from linearity

of 20–30% may occur as can be seen in Fig. 1), but these are reproducible, which means that they can be corrected by using an appropriate calibration graph. For most industrial monitoring purposes, especially regarding exhaust gases, they are unimportant.

The detection limit corresponds to *ca.* $5 \cdot 10^{-10}$ g sec^{-1} of chlorine.

The advantage of this detector is it resistance to chemical attack. The mentioned reproducibility has been observed over a period of 2 years of continuous process operation.

DISCUSSION

In the well known noble gas detector[8], ions are formed by the interaction of excited metastable noble gas atoms with the neutral molecules to be detected. This mechanism cannot be valid in the present instance. The highest excited metastable state of argon has an energy of 11.72 eV, while we find carbon monoxide (14.01 eV) and carbon dioxide (12.34 eV) still detected.

The ionization energy of argon is 15.76 eV. Up to this limit, ionization processes by particle collisions or by internal photoionization processes should be possible. On the other hand, methane (12.98 eV) and nitrogen (15.51 eV) are not detected. A possible explanation of this apparent contradiction might be to accept primary energy transfer processes by collisions with high-energy particles in competition with bimolecular deactivation processes[9]. The failure to detect nitrogen and methane may be the result of a longer lifetime of activated but not yet ionized molecules and atoms (according to the Lindemann–Hinshelwood mechanism). This concept is supported by the observation that at decreasing pressures in the discharge tube ($p \leqslant 300$ mbar) signals for nitrogen and methane begin to appear. These considerations are equally applicable to the assumption of an internal photoionization process[10].

A detailed investigation has not been carried out ,but it would be of only theoretical interest. The use of a vacuum pump would be an unacceptable complication in the described simple unit. Futher, all of the compounds of interest can be easily detected at ambient pressure.

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CHROM. 13,332

DETECTION OF SMALL VARIATIONS IN SHAPE BETWEEN TWO CHROMATOGRAPHIC PEAKS

H. RIX*

Laboratoire d'Informatique, Département IMAN, Institut de Mathématiques et Sciences Physiques, Université de Nice, Parc Valrose, 06034 Nice Cedex (France)

SUMMARY

The detection of an impurity in a visually single peak by means of the "Distribution Function Method" necessitates a thorough control of every parameter which influences the shape of a peak. In particular, the heights of the peaks compared must be as equal as possible when the chromatographic response is not a linear function of the injected concentration.

The Distribution Function Method can also be used for the determination of the region in which the chromatographic response is linear.

INTRODUCTION

From the recording of a chromatographic peak, one can estimate characteristic quantities such as retention time, peak width or peak area. The shape of the profile, independent of the value of the parameters of position (time base) or scale (peak height, peak width) may also be worth considering. Then it is important to have a criterion that makes it possible to decide whether two peak profiles that are visually similar have the same shape or not. The distribution function method (DFM)[1] gives such a criterion: its mathematical treatment[2], simulated results[3,4] and experimental results[5] have been published. These results concern the main application of the method: the detection, under a visually single peak, of a secondary peak that corresponds to contamination of a few percent of the main peak and that constitutes with the main peak a strongly overlapped doublet (resolution *ca.* 0.25).

The DFM is particularly suitable for studying the problem of strong overlapping, to which the classical methods of deconvolution[6,7], slope analysis[8] and moments[9] are inapplicable. In this application, the detection of an impurity is based on the difference in shape observed between a peak produced by a pure sample and another peak produced by an impure sample; of course, the detection is valid if the only cause of the variation in shape is the presence of an impurity. In particular, the heights of two peaks to be compared were always equal. In fact, for certain chromatographic measurements, the response of the chromatograph can be assumed to be a

* Present adress: LASSY ERA 835, IUT, 41 Boulevard Napoléon III, 06041 Nice Cedex, France.

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linear function of the concentration injected over a fairly wide range. However, in the problem of detecting very small variations in shape, this range may be very much reduced. The aim of this paper is to point out the influence of variations in peak height on the peak shape by means of some experimental results.

THE DFM PRINCIPLE

The comparison of the shapes of two peak profiles $f(t)$ and $g(t)$ is made by a comparison of their normalized integrals (distribution functions) $F(t)$ and $G(t)$. Resolving the equation

$$F(t) = G(t')$$

for a series of points (t', t) gives a curve $t = \varphi(t')$; if f and g have the same shape, *i.e.*, if there is a relationship such as $g(t) = kf[(t-d)/a]$, the curve $t = \varphi(t')$ is a straight line. The mean deviation, Δ, of the curve from linearity is a measure of the difference in shape between the two profiles.

DETECTION OF DOUBLE PEAKS

Fig. 1 gives an example of the difference in shape, Δ, created by the 1 and 2% contamination of *n*-hexane with *n*-heptane (resolution *ca.* 0.25) for a signal-to-noise ratio of *ca.* 300. Each point represents the mean of ten measurements of the shape difference between a pure peak and another pure peak or a contaminated peak. The standard deviation of Δ calculated from the ten measurements is represented by the length of an upwards or downwards arrow. In this example, the detection of an impurity of 1% is obvious, assuming that the only cause of the variation in shape is such a contamination. In particular, the heights of all the peaks were equal. The following results emphasize this important point.

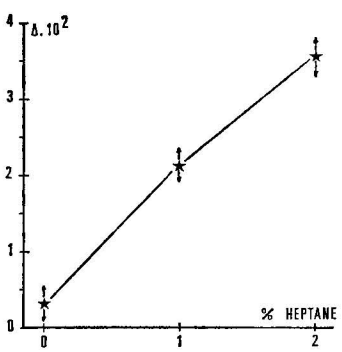


Fig. 1. Effect of contamination of *n*-hexane by *n*-heptane on peak shape. •, Mean value of 10 determinations; ↑, standard deviation. Unit of Δ: half width of the pure peak.

LINEARITY OF THE MEASUREMENT DEVICE

Ideally, the response of a chromatograph is proportional to the concentration of a component in the sample. In order to check this hypothesis, Fig. 2 was constructed. The ordinates represent the difference in shape, Δ, between two pure peaks of *n*-hexane eluted under the same conditions; one has a height H (as in the preceding example) and the other a height $H+\Delta H$. The difference in shape is plotted against the relative varation of height, $\Delta H/H$. From Fig. 2, one can make two observations:

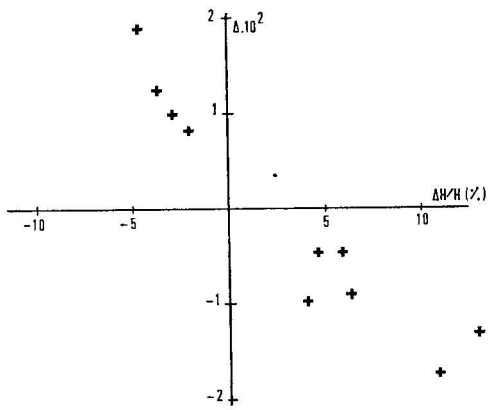


Fig. 2. Differences in shape between a peak of *n*-hexane with height H and other peaks of *n*-hexane with height $H + \Delta H$ *versus* the relative difference $\Delta H/H$. H corresponds to an injection of 0.5 μl.

(1) In comparison with Fig. 1, a slight variation in height (of a few pecent) may produce a shape difference masking a contamination of 1 or 2%.

(2) The DFM can be used to check the linearity of the response of the instrument around a given value of the concentration injected. In a more precise manner, the maximum variation of height (*i.e.*, concentration) consistent with an admissible variation in shape may be determinated by a statistical study using the DFM.

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CHROM. 13,138

DESIGN OF AND EXPERIENCE IN OPERATING TECHNOLOGICAL PREPARATIVE INSTALLATIONS

K. I. SAKODYNSKII*, S. A. VOLKOV, Yu. A. KOVAN'KO, V. Yu. ZEL'VENSKII, V. I. REZNIKOV and V. A. AVERIN

Karpov Institute of Physical Chemistry, Ul. Obukha 10, 107120 Moscow B-120 (U.S.S.R.)

SUMMARY

Examples are given of the use of gas chromatography under industrial conditions for producing thiophene, indole and xylene on columns 500 and 1200 mm in diameter with annual capacities of from 100 to 1000 tons. Some problems associated with designing industrial columns are considered, including optimization of temperature conditions.

INTRODUCTION

During the past decade, gas chromatography (GC) has been adopted in industry as a method for separating mixtures. The efficiency of chromatography can be greater than that of distillation if a highly selective sorbent is used. This has been observed for such mixtures as benzene–thiophene, benzene–cyclohexane and α- and β-pinenes. The possibility of separating multicomponent mixtures in one cycle, small losses of the compounds being separated with almost 100% extraction, the absence of toxic discharge and the high degree of automation are among the advantages of chromatography.

In this paper several examples of industrial application of chromatography in the U.S.S.R. and some aspects of designing columns and selecting appropriate thermal conditions for their operation are discussed.

INDUSTRIAL APPLICATION OF CHROMATOGRAPHY

In the U.S.S.R. chromatography was first introduced under industrial conditions in 1970 when an installation for producing high-purity reagents was put into service[1]. About 30% of the operations performed were by GC; seven Etalon-2 chromatographs with columns up to 100 mm in diameter were being used. More than 100 reagents were produced (99.8% pure) and the total output capacity was 500 kg per year.

This example illustrates one of the current trends in the industrial application of chromatography: a great variety of reagents is produced in relatively small amounts

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on columns with relatively small diameters (100–200 mm). The technology of the process is close to that of ordinary laboratory chromatography. Since several mixtures are separated in the same installation, it must be flexible to ensure easy change-over of the operating conditions. Similar experience in designing and operating such installations has been gained by French researchers[2].

A second trend is the production of large amounts (several tons) of compounds in installations designed for separating one particular mixture. A number of such installations have been built and operated for a long time[3]. The first was designed for separating commercial 95% thiophene from a benzene fraction containing 10–12% of thiophene. The column (5 m × 250 mm I.D.) had an annual throughput capacity of 120 tons. The separation factor, α, of the mixture was 1.4, the volume of a single sample was 2.5–3.5 l and the cycle duration was 12–15 min. Maximum throughput capacity is attained when the process is performed in two stages: preparation of a concentrated thiophene fraction (70–75%) and its subsequent purification to give the final product. The efficiency of separation and main parameters of the column remained virtually unchanged over an operating period of two years.

Later an installation with columns 1.2 m in diameter and 2.4 m in length was put into service for producing 99.8% toluene, its annual throughput capacity being 1200 tons. The content of toluene in the raw material was 99%, the volume of a single samples was 30 l and the carrier gas flow-rate was 240 m^3/h. The column operated for 1.5 years with the same sorbent. The height equivalent to a theoretical plate (HETP, H) at a dose of 0.04 kg/m^2 was 8–12 mm.

At present an installation is in service in which indole is separated from an indole fraction containing about 4% of the desired product. The column is 520 mm in diameter and 1.5 m in length, single batches amount to 1.5–2.0 kg and the annual throughput capacity is 100 tons. Separation is performed in two stages: preparation of crude indole (*ca.* 75% pure) and subsequent purification to commercial indole (92% pure). The columns operate at 220°C. The HETP of the column, determined under the same conditions as for the column 1200 mm in diameter, was 7–10 mm.

Operating experience on the above installations has demonstrated the potential of chromatography under industrial conditions. Such application create a number of engineering problems related to evaporation of the sample, detection, automation of the process, collecting of the fractions and circulation of the carrier gas.

We shall discuss two aspects closely connected with column operation: construction of a column, and reduction of column efficiency caused by heating of the sorbent bed by the gas flow from the evaporator.

CONSTRUCTION OF INDUSTRIAL COLUMNS

In designing industrial columns one must optimize the length of sections, as well as the shape of the bottom and cover plates for each section. Usually sections are interconnected by means of transition pipes of small diameter.

Giddings and co-workers[4,5] have shown that on increasing the length of a column the HETP first rises and then attains a constant value. From this it was concluded that it was reasonable to use short sections so that the limiting value of HETP could not be attained. The theory presented in ref. 4 was based on the assumption that in large-diameter columns the velocity profile of the carrier gas is parabolic. It was of

interest to verify the applicability of this theory to those cases in which such a profile does not exist.

The presence or absence of the velocity profile is determined by the method of filling the column. Earlier[6], it had been shown by means of a laser anemometry technique that if the packing is compacted by tapping the column walls a velocity profile is always formed with an enhanced velocity near the walls. This method (No. 1) was adopted as a reference. We usually pack large-diameter columns by loading in portions and compacting with a plunger with simultaneous pumping of the column with a vacuum pump (No. 2). In this case (see ref. 6) there is virtually no velocity profile along a cross section of the column.

A chromatographic column 100 mm in diameter was assembled from sections 400 and 750 mm in length connected through flanges without any transition pipes or partitions. A sample of *n*-pentane was introduced through an evaporator at 70°C; the column temperature was 25°C. The carrier gas flow-rate was constant at the column exit (10 l/min). Spherochrom-1 (grain size 0.5–1.0 mm) with 20% of dinonyl phthalate was used as sorbent. Upon lengthening the column the sorbent in the lower sections was not re-packed: after filling with sorbent and measuring the HETP of one section, we removed the cover plate, levelled the sorbent bed and lengthened the column by connecting the next section filled with the sorbent.

For comparison, experiments were also performed with columns consisting of sections 750 and 400 mm in length connected by transition pipes (150 × 6 mm O.D. × 4 mm I.D.). Each section was filled separately with a sorbent by method 2.

The dependence of H on the column length for both filling methods is shown in Fig. 1. As is expected from the theory[4], in the case of method 1, H first increases with increasing column length and then attains a constant value. In method 2, H

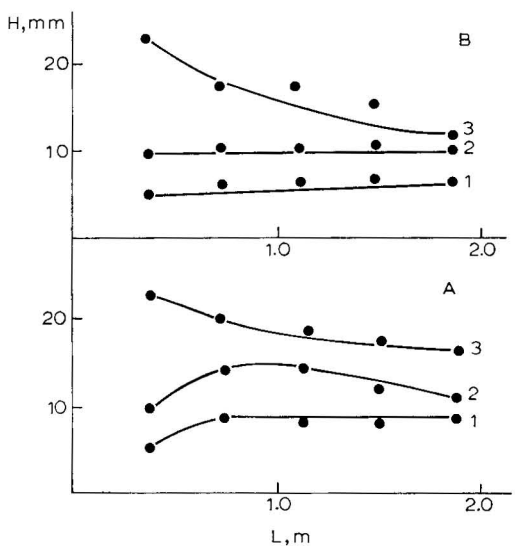


Fig. 1. Relationship between HETP and column length when packing is compacted by tapping the column walls (A) or by a plunger with vacuum pumping (B). Sample volumes of *n*-pentane: 0.5 ml (1); 5 ml (2); 10 ml (3).

undergoes only a slight linear increase with increasing column length. This may be due to the fact that as the bed becomes longer the rarefaction created by the pump diminishes and the packing becomes looser. Separate experiments have shown that compaction without pumping lowers the efficiency.

Introduction of large amounts of sample does not affect the character of the dependence of H on column length.

The data obtained show that sections 1.0–1.5 m in length can be used for industrial columns. The HETP values for continuous columns and for columns assembled from short sections are almost the same:

Column length (mm)	1150	1500	1900
H of a continuous column (mm)	6.2	7.1	6.7
H of a sectional column (mm)	6.9	5.7	6.5

These results mean that it is possible to simplify considerably the construction of industrial columns. Such columns are usually heated with the aid of jackets.

The shape of cover and bottom plates of columns also deserves attention. Usually, preparative columns have conical covers and bottoms, the height of the cone increasing considerably with the column diameter. As a result, the overall dimensions of the entire installation grow. The use of flat or frustoconical covers and bottoms would substantially reduce the overall dimensions of the installation and simplify the technology of manufacturing the columns.

Investigations were carried out on a column (100 cm × 140 mm I.D.) with the same sorbent as in the previous experiment. The column had a welded conical bottom and a removable cover fixed with flanges. A 100 mm high cone (cone angle 60°), a 50 mm high frustum of a cone (diameter of the upper base 80 mm), a 30 mm high spherical segment (radius of curvature 100 mm) and a flat cover were studied. The diameter of the inlet pipe was 6 mm in all cases; the gas flow could be switched so that the removable cover was either at the inlet or at the outlet of the column. The flow-rate of the carrier gas was varied from 4 to 10 l/min, and the volume of pentane as sample was 1.5 and 10 ml. Table I lists typical data for a 1 ml sample volume and various gas velocities.

TABLE I

EFFECT OF COVER CONSTRUCTION ON HETP OF THE COLUMN

Flow-rate of carrier gas (l/min)	*Flat*		*Spherical segment*		*Frustum of a cone*		*Cone*	
	Inlet	*Outlet*	*Inlet*	*Outlet*	*Inlet*	*Outlet*	*Inlet*	*Outlet*
4	2.8	3.1	3.7	3.8	3.8	3.9	3.1	3.1
6	3.4	3.3	4.2	4.1	3.9	4.6	3.4	3.5
8	3.9	3.4	4.3	4.3	3.9	4.8	4.0	3.8
10	4.3	3.5	4.5	4.6	4.1	5.3	4.1	4.4

As can be seen from the Table, each type of cover gave approximately identical HETP values; in some cases the use of the flat cover somewhat increases the column efficiency, which may be due to difficulties encountered when filling the covers and to their enhanced hydraulic resistance.

A disc-shaped reflector (diameter 70 mm) arranged in front of the hole in the flat cover and an air gap between the cover and the packing surface exerted almost no effect on the efficiency. This was the case for a high-quality column packing when HETP was 4.0 mm. At a higher HETP value caused by poor-quality packing both the reflector and the gap improved the efficiency.

In all the above examples of industrial application of chromatography, columns with flat covers were employed.

TEMPERATURE GRADIENTS IN LARGE-DIAMETER COLUMNS CAUSED BY GAS FLOW FROM EVAPORATOR

Under the usual operating conditions of a preparative installation the evaporator temperature (T_{ev}) exceeds the column temperature (T_c) by about 50°C. In the case of direct connection of the evaporator to the column the temperature of the gas entering the column also exceeds T_c which, in its turn, causes heating of the initial sorbent zone. The poor conductivity of the sorbent results in temperature gradients in the column, which decrease the column efficiency. Experiments have confirmed that the greater the column diameter the greater is this effect. This might be one of the reasons for the difficulties encountered when we tried to obtain reproducible HETP values for the large-diameter columns.

When designing industrial installations[7] it was proposed that an additional heat exchanger be placed between the evaporator and the column to act as a heat compensator. To measure the temperature distribution in a sorbent bed, an installation was assembled with a column (750 × 100 mm) fitted with eleven thermocouples as shown in Fig. 2. The experimental conditions were as follows: Spherochrom-1 (0.5–1.0 mm) with 20% dinonyl phthalate; $T_c = 20°C$; $T_{ev} = 70°C$; carrier gas (nitrogen) flow-rate 10 l/min. Fig. 3 shows temperature *vs.* time curves where $\Delta T = T_\tau - T_0$ and T_τ is the current temperature and T_0 the initial temperature. Zero time was adopted as the moment the carrier gas entered the heated evaporator. It follows from the curves that stationary temperature conditions characterized by both the longitudinal (ΔT_l) and transverse (ΔT_a) gradients are established in the column after

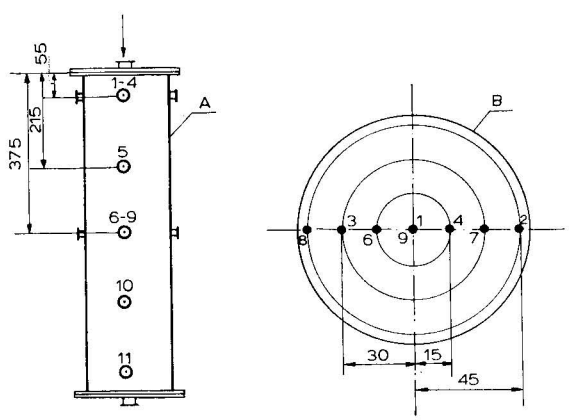


Fig. 2. Location of thermocouples along the length (A) and cross section (B) of the column.

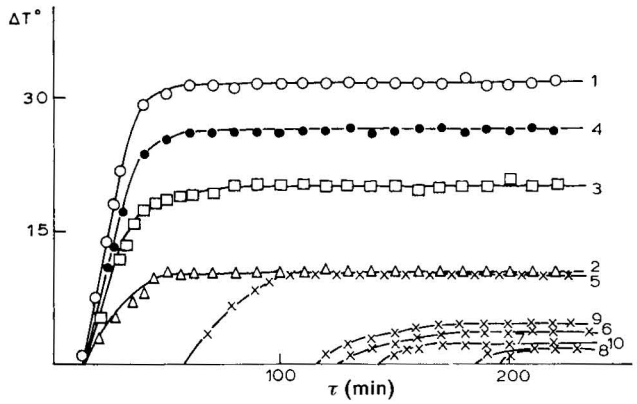


Fig. 3. Curves of temperature *vs.* time. Number on the curves correspond to those of the thermocouples (see Fig. 2).

a certain time (in our case, 220–250 min). It should be noted that the eleventh thermocouple did not record any deviation from T_0 during the whole period of measurement (*ca.* 300 min). Along the column the temperature varied approximately exponentially (Fig. 4). The shape of the transverse profile (for the first cross section) is shown in Fig. 5. A change in the carrier gas flow-rate, liquid sample volume (10 ml of *n*-pentane every 10 min) or in the shape of the inlet cover (flat or conical) affected the time of attainment of the stationary state but did not change the type of temperature profiles. The time dependence of HETP (Fig. 6) is in fair agreement with the temperature curves. The HETP value almost doubles up to 250 min, after which a thermal equilibrium is attained in the column and HETP remains constant.

Let us now evaluate the contributions of longitudinal (ΔH_l) and transverse (ΔH_a) temperature gradients to the HETP value. Evidently, the additional dispersion of a band because of the transverse temperature gradient is due to the different velocities of zones which move along the cross sectional areas at different temperatures.

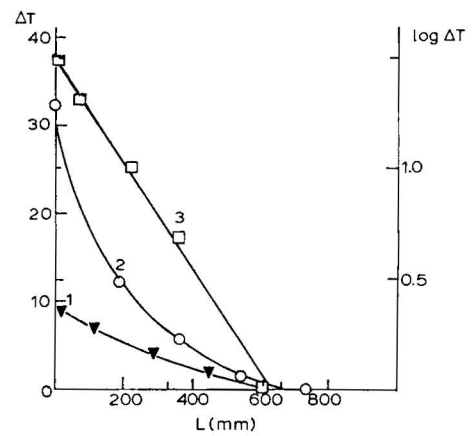


Fig. 4. Dependence of ΔT on L for column wall (1) and centre of the column (2) and dependence of log ΔT on L for centre of the column (3).

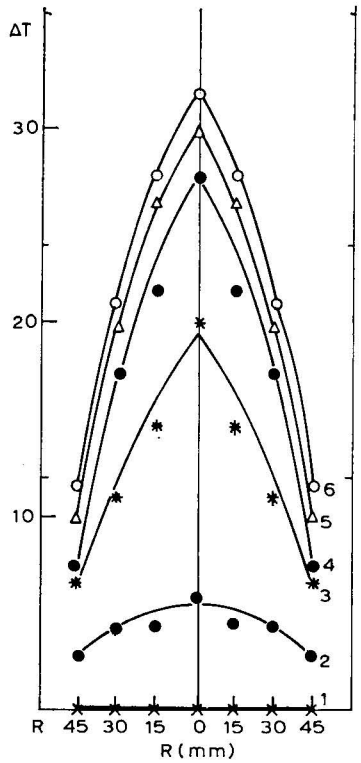


Fig. 5. Change in temperature profile along the column cross section (first section) as a function of time: 0 (1); 20 (2); 30 (3); 40 (4); 50 (5); 70 (6) min. R = Column radius.

The value of this contribution depends on the shape of the temperature profile. Giddings[8,9] has derived an equation for calculating ΔH_a

$$\Delta H_a = \frac{q^2}{(2+q)^2} \cdot \frac{\bar{v} r_0^2}{24 D_g} \tag{1}$$

where $\bar{v}$ is the mean velocity of the carrier gas, r_0 is the column radius, D_g is the coefficient of gas diffusion and q is the term characterizing the temperature profile

$$q = (1 - R_w)(\Delta Q / R T_w^2) \Delta T \tag{2}$$

where $R_w = \bar{u}/\bar{v}$, $\bar{u}$ is the mean velocity of the component, ΔQ is the heat of vaporization of the sorbate, R is the gas constant and ΔT is the difference between the temperatures of the centre and wall (T_w) of the column.

From the logarithmic dependence of ΔT on column length, L (Fig. 4), we calculated the mean value, $\Delta \bar{T}$, as a logarithmic ($\Delta T_{lg} = 7.7°$) and $\bar{T}_w$ (linear dependence on L) as an arithmetic mean ($\bar{T}_w = 296°$K). A value of $\Delta H_a = 4.1$ mm was obtained from the following values of the parameters in eqns. 1 and 2: $\bar{v} = 3.02$ cm/sec; $\bar{u} = 0.333$ cm/sec; $D_g = 0.087$ cm^2/sec (n-pentane in nitrogen at 20°C); $\Delta Q = 6.165$ kcal/mol of n-pentane and $r_0 = 5$ cm.

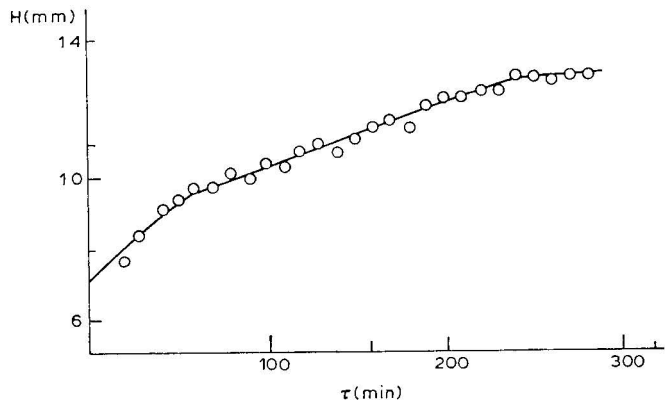


Fig. 6. Dependence of HETP on time. Sample: 0.5 ml *n*-pentane. Nitrogen flow-rate 10 l/min.

The longitudinal temperature gradient gives rise to a velocity gradient, Δu, of the component along the column. Giddings[9] has developed a theory of dispersion in a column with a longitudinal gradient, according to which band dispersion in a gradient column is always greater than in a non-gradient column

$$\bar{H} = L \int_0^L \frac{H}{R_w^2 v^2} \mathrm{d}z \Big/ \left(\int_0^L \frac{\mathrm{d}z}{R_w v} \right)^2 \tag{3}$$

where z is a longitudinal coordinate.

In our case, when the temperature change along the column length is close to exponential and the temperature dependence of u is exponential ($u = v/Ae^{Q/RT}$, where A is an entropy factor and Q is the heat of sorption), integration of eqn. 3 presents certain difficulties. To simplify the evaluation of ΔH_1, we divide the column into two equal parts, for each of which dispersions and peak retention times are σ_1^2, σ_2^2 and t_1, t_2, respectively.

Then:

$$\bar{H} = H_0 \, 2(t_1^2 + t_2^2)/(t_1 + t_2)^2 = H_0 f \tag{4}$$

When $t_1 = t_2$, *i.e.*, in the absence of a gradient, $f = 1$ and $\bar{H} = H_0$. In all other cases $f > 1$ and $\bar{H} > H_0$.

Let us assume that the temperature along each part is constant and can be regarded as an arithmetic mean of the temperatures at the ends of the parts. For the first part we have initial temperature 325°K, final temperature 300°K and $\bar{T}_1 = 311$°K; for the second part initial temperature 300°K, final temperature 293°K and $T_2 = 296$°K. Using the ratios of the component velocities, u_0/u_1 and u_0/u_2, and the experimental values $Q = 6500$ kcal/mol and $u_0 = 0.254$ cm/sec ($T_c = 293$°K), we can calculate u_1 and u_2 and t_1 and t_2 ($t_1 = 112$ sec, $t_2 = 119$ sec). Taking into account $H_0 = 7.1$ mm (Fig. 6), we have $\bar{H} = 8.2$ mm, *i.e.*, $\Delta H_1 = 1.1$ mm. Thus, the calculated value of the total increase of HETP due to the transverse and longitudinal temperature gradients will be $\Delta H_T = 5.2$ mm and the experimental $\Delta H_T = 5.9$ mm, *i.e.*, the difference between these values is about 12% which is quite satisfactory. The evaluation of ΔH_1 can be made more accurate if the column is divided into a greater number of parts.

It has already been noted that the stationary temperature gradient is established over a definite length of the sorbent bed (*ca.* 600 mm in our case) and, therefore, with increasing length the relative contribution of ΔH_T to diffusion of the zones must diminish. This is confirmed by comparison of the time dependence of H for columns of different lengths (Fig. 7): $\Delta H_T = 21$, 15 and 7 mm for $L = 400$, 750 and 1500 mm. Linear extrapolation of the dependence of H on L shows that when $L \approx 2$ m, ΔH_T tends to 0, *i.e.*, for columns longer than 2 m the contribution of ΔH_T to H becomes negligible. However, these data refer to columns 100 mm in diameter, and with increasing diameter the length at which ΔH_T will not affect the column efficiency will exceed practical values.

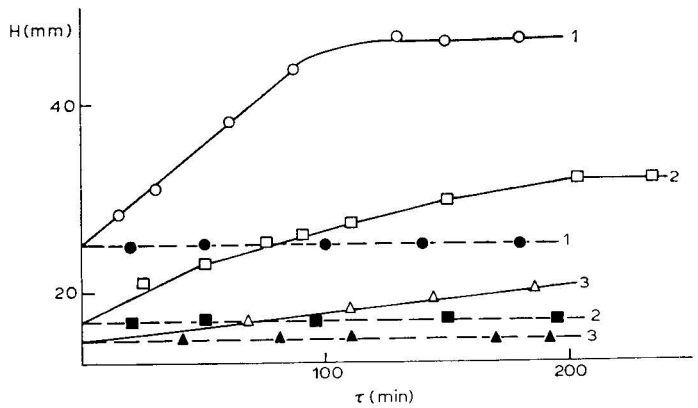


Fig. 7. Dependence of HETP on time for columns 400 (1), 750 (2) and 1500 (3) mm in length with a heat compensator (— — —) and without it. Sample: 10 ml *n*-pentane. Nitrogen flow-rate 10 l/min.

Therefore, when the column diameter is more than 50 mm it is desirable to add a heat compensator to a conventional preparative chromatograph in order to cool the gas flow leaving the evaporator down to the column temperature. The effect of a heat compensator on HETP is shown in Fig. 7. When a copper-coil heat exchanger cooled with water at a temperature of 18–22°C was placed between the evaporator and the column the HETP was independent of the length and remained constant during the whole observation period.

It should be noted that there may be operating conditions under which cooling of the gas flow from the evaporator will result in partial condensation of the mixture being introduced in the heat compensator. In this case the evaporator must operate only during sample introduction, and in periods between introductions, the gas flow bypass the evaporator.

CONCLUSION

Present-day engineering advances allow the construction of industrial gas chromatographic installations with capacities of several tons of product per year. The main problem is to find mixtures which could be economically separated by this method. Another problem consists of developing methods of calculation for such installations and in finding ways of enhancing their efficiency and capacity.

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APPLICATIONS IN BIOCHEMICAL ANALYSIS

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CHROM. 13,298

SEPARATION OF ENANTIOMERIC AMINES BY ION-PAIR CHROMATOGRAPHY

CURT PETTERSSON* and GÖRAN SCHILL

Department of Analytical Pharmaceutical Chemistry, University of Uppsala, Biomedical Centre, Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

A high-performance liquid chromatographic method for the separation of optical isomers (enantiomers) of amines is described. It is based on ion-pair chromatography with a chiral counter ion in a system with an organic mobile phase and an adsorbing stationary phase. The method has been applied to enantiomers of 1-aryloxy-3-isopropylamino-2-propanol derivatives (alprenolol, metoprolol, propranolol) which are completely resolved with (+)-10-camphorsulphonate as the counter ion. Studies of the influence of the counter-ion structure and the mobile phase composition are presented.

INTRODUCTION

Many studies on methods for the separation and determination of enantiomeric organic compounds have been made in recent years. Methods for the transfer of enantiomers to diastereomeric derivatives that can be separated by liquid and gas chromatography have attracted much interest[1,2]. Many investigations have also been focused on the preparation of chiral solid phases for gas or liquid chromatography that can bind the enantiomeric forms with different strengths[3–5]. It has recently been shown that separations can be effected by liquid chromatography with chiral eluents. The investigations have so far been concentrated on the use of ligand exchange in metal complexes[6–8]. Interactions between ions of opposite charge have been used for the purpose of separating enantiomers. Optically active cations have been used as liquid ion exchangers in Craig counter-current extraction for the resolution of sodium *dl*-mandelate, but attempts to use these cations in reversed-phase liquid chromatography have failed[9,10]. Paper chromatographic studies with (+)-10-camphorsulphonic acid or (+)-tartaric acid in the stationary or mobile phase have in some instances resolved a few amines[11–13]. The anion of (+)-tartaric acid has also been used for the resolution of optically active octahedral metal complexes by formation of diastereomeric charged ion pairs in paper electrophoresis and in ion-exchange chromatography[14–16].

In this study, stereoselective association between ions has been used to resolve enantiomeric amines. Diastereomeric ion pairs between the amines and the optically

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active (+)-camphorsulphonic acid are formed and they can have such structural differences that they show different distributions between the phases in the chromatographic system. As the separation is based on the properties of the ion pairs, only such phase systems have been used that favour a high degree of ion-pair formation, *i.e.*, mobile phases of low polarity with solid adsorbents as stationary phases. The investigations have been concentrated on studies of the influence of sample and counter-ion structure and phase composition on selectivity.

EXPERIMENTAL

Apparatus

The detector was a Model 440 UV monitor (Waters Assoc., Milford, MA, U.S.A.) with a 12.5-μl cell, measuring at 254 nm. The pump was an LDC Model 711-26 solvent delivery system. The injector was a Rheodyne Model 7120 with a 20-μl loop. The columns were made of stainless steel with a polished surface, length 100 or 150 mm, I.D. 3.2 mm. The column and injector were thermostated at 25.0 ± 0.1°C using a water-bath.

Chemical and reagents

LiChrospher SI 1000 (10 μm), LiChrosorb-DIOL (10 μm) and LiChrosorb-DIOL (5 μm) were obtained from E. Merck (Darmstadt, G.F.R.).

Methylene chloride (p.a. grade, Merck) was freed from water before use by passage through a molecular sieve column (4 Å). The water content was less than 0.001%. (+)-10-Camphorsulphonic acid, (+)-3-bromo-10-camphorsulphonic acid and (+)-3-bromo-8-camphorsulphonic acid were obtained from Merck. Racemic alprenolol chloride, (+)-alprenolol chloride, (−)-alprenolol tartrate and racemic metoprolol tartrate were supplied by Hässle (Mölndal, Sweden), racemic propranolol chloride by ICI (Macclesfield, Great Britain) and racemic oxprenolol chloride by Ciba-Geigy (Basle, Switzerland). All other substances and solvents were of analytical or reagent grade and were used without further purification.

Chromatographic technique

The columns were packed with a slurry technique using chloroform as the slurry medium. They were washed with methanol, methylene chloride and finally *n*-hexane before use. The mobile phase containing the counter ion in acidic form was recirculated through the column and constant retention times were reached after about 2 days.

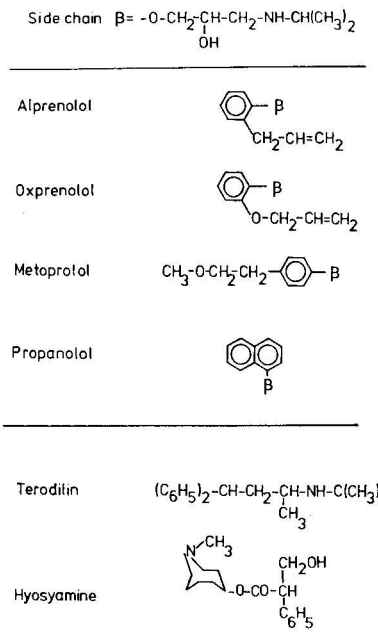
The samples were introduced as bases or salts dissolved in the mobile phase.

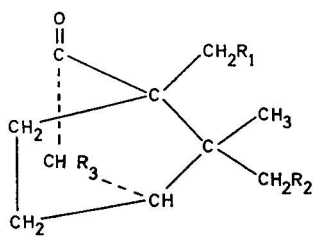
RESULTS AND DISCUSSION

The proposed method for the separation of enantiomeric amines as ion pairs with a chiral counter ion is based on the assumption that diastereomeric ion pairs can have different distributions between an organic mobile phase and a stationary adsorbent. However, differences cannot be expected if the ion pair components are bound only by electrostatic attraction between the charged groups and it is, as a rule, assumed that interaction at three points in the vicinity of the chiral carbon atom is

necessary to give chiral ion pairs with different mobile phase solvation or stationary phase adsorption (*cf.* Dalgliesh's "three-point" rule[17]).

The main group of enantiomeric amines used in this study contained a so-called β-chain (Fig. 1). The chain carries, in addition to the secondary amino group, a hydroxyl group at the chiral carbon in position 2. (+)-10-Camphorsulphonate (Fig. 2) was used as the counter ion in the main part of the studies. It is an aprotic anion with a rigid structure with an oxo group in position 2.





	R_1	R_2	R_3
(+) -10-Camphorsulphon. ac.	SO_3H	H	H
(+) -3-Br-10-Camphorsulphon. ac.	SO_3H	H	Br
(+)-3-Br-8-Camphorsulphon. ac.	H	SO_3H	Br

Fig. 1. Sample structures.

Fig. 2. Counter-ion structures.

Ion pairs between these components can be bound not only by electrostatic attraction but also by hydrogen bonding between the hydroxyl and the oxo group as well as by hydrophobic interaction between the ring systems. Three interaction points, *i.e.*, the basic prerequisites for separation, seem to exist.

Influence of phase composition

The separations were usually performed with LiChrosorb-DIOL as stationary phase and methylene chloride + 1-pentanol as mobile phase. Test runs with more hydrophobic phases (Nucleosil CN and LiChrosorb RP-2) gave highly asymmetric peaks. The more hydrophilic LiChrospher SI 1000 gave too high a retention.

It is of vital importance for the separation selectivity to keep the content of polar components in the mobile phase as low as possible. Water, even in very low concentrations, seems to have a very adverse influence on the separation and no separation of the enantiomers could be obtained if the chromatographic system contained a stationary aqueous phase. The influence of other less strongly hydrogen bonding

agents is illustrated in Table I for alprenolol. Lipophilic alcohols of different structures (1-pentanol, and isopropanol) seem to give about the same selectivity. Hydrogen-donating agents such as acetonitrile, tetrahydrofuran and ethyl acetate might give slightly higher separation factors, when used at the same concentration as the alcohols. However, the peak asymmetry is significantly greater, which makes them less suitable than, *e.g.*, 1-pentanol. An increase in the 1-pentanol content from 0.5% to 5% decreased the separation factors from 1.08 to 1.02.

TABLE I

INFLUENCE OF POLAR COMPONENTS IN THE MOBILE PHASE

Mobile phase: (+)-10-camphorsulphonate, $2.1 \cdot 10^{-3}$ *M* in methylene chloride–polar solvent (flow-rate, 3.2 mm/sec). Sample: alprenolol (+ and − forms).

Polar solvent	*Content (%)*	*k' (+)*	*k' (−)*	*Separation factor (α)*	*H (mm)*	*Asf**
Pentanol	0.5	20.3	22.0	1.08	0.15	1.5
	1	10.8	11.5	1.06	0.15	1.1
	5	2.02	2.07	1.02	0.15	1.1
Isopropanol	0.5	13.5	14.5	1.07	0.18	2.6
Acetonitrile	1	25.1	27.3	1.09	0.20	2.2
Tetrahydrofuran	1	11.9	13.1	1.10	—	3.7
Ethyl acetate	1	29.0	31.8	1.10	—	3.6

* Asf = back part of the peak/front part of the peak.

The negative influence of the hydrogen-bonding agents is probably due to their interaction with hydrogen-bonding groups of the ion-pair components, which decreases the bonding strength within the ion pair.

All of the chromatographic systems had a fairly low separation efficiency. The 1-pentanol-containing systems, which were the most efficient, showed a reduced plate height of 12–15 even though testing of the column packing before conditioning with camphorsulphonate gave a reduced plate height of 4.

Molecular structure and chiral selectivity

The influence of the sample structure on the separation factor in a system with (+)-10-camphorsulphonate as counter ion is illustrated in Table II. The results are in agreement with the assumptions regarding the interactions between the ion-pair

TABLE II

SEPARATION OF ENANTIOMERS

Mobile phase: (+)-10-camphorsulphonate, $2.2 \cdot 10^{-3}$ *M* in methylene chloride–1-pentanol (199:1).

Substance	*k' (+)*	*k' (−)*	*Separation factor*
Alprenolol	24.9	27.4	1.10
Metropolol	34.9	38.7	1.11
Oxprenolol	15.0	15.0	1.00
Propranolol	40.9	46.0	1.12
Terodiline	4.9	4.9	1.00
Hyoscyamine	7.3	7.3	1.00

components. Fairly high separation factors are obtained for most of the 1-aryloxy-3-isopropylamine-2-propanol derivatives and complete resolution of the enantiomers is possible on a 400-mm column.

The enantiomers of oxprenolol are not separated, probably owing to the presence of a hydrogen-accepting 2-allyloxy group in such a position in the ring that internal hydrogen bonding with the hydroxyl in the side-chain is possible. Terodilin, which lacks the hydroxyl group, and hyoscyamine, which has the hydroxyl group too distant from the amino group, show no separation into enantiomers.

A further illustration of the importance of a good fit between the binding groups in the ion-pair components is given by a study of the separation of the enantiomers of alprenolol with three different camphorsulphonates (Fig. 2) as counter ions. Introduction of a 3-bromo substituent in 10-camphorsulphonate brings about a complete loss of stereoselectivity, possibly owing to steric repulsion by the bromo atom preventing a close three-point contact between the ion-pair components. A slight stereoselectivity ($\alpha = 1.02$) remains, however, when the sulphonate group is situated in position 8, *i.e.*, more distant from the oxo group.

Retention principles

The retention decreases with increasing hydrophobicity of the ion-pair components, which shows that the solid adsorbent, as expected, is the more polar phase. An increase in the concentration of camphorsulphonic acid in the mobile phase also gives rise to a decrease in the retention. This might indicate some kind of ion-exchange mechanism with camphorsulphonic acid acting as stationary phase, but other retention models are also possible.

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DIRECT RESOLUTION OF α-AMINO ACID ENANTIOMERS BY LIGAND EXCHANGE: STEREOSELECTION MECHANISM ON SILICA PACKINGS COATED WITH A CHIRAL POLYMER

J. BOUÉ, R. AUDEBERT* and C. QUIVORON

Laboratoire de Physico-Chimie Macromoléculaire de l'Université P. et M. Curie, E.S.P.C.I., 10 Rue Vauquelin, 75231 Paris Cedex 05 (France)

SUMMARY

A chiral polymer easily obtained by treatment of a linear polyacrylamide with L-proline was adsorbed on a silica support treatment of a linear polyacrylamide with L-proline. After complexation with copper(II), and in spite of a rather low efficiency due to slow ligand exchange, such packings are very convenient for the resolution of amino acid enantiomers eluted in water (relative retentions, k'_L/k'_D, higher than 2 may be observed). Other eluents may also be used. The chiral polymer was studied in solution by potentiometry and nuclear magnetic resonance spectroscopy (^{13}C relaxation measurements). A model of the complex involved in the stereoselection is proposed which accounts for the chromatographic results obtained.

INTRODUCTION

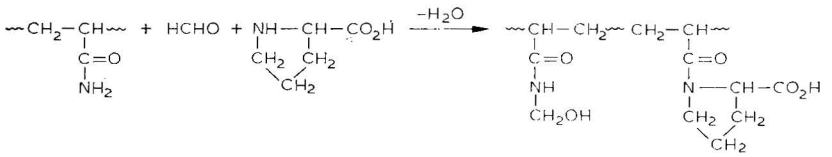
Direct resolution of α-amino acids by column liquid chromatography has been progressively improved during the last twelve years. Some experiments have recently been performed with a chiral eluent, but most studies employ a chiral stationary phase, generally a cross-linked macromolecular compound or sometimes a grafted phase (see reviews[1,2]). The few packings which are efficient in high-performance liquid chromatography (HPLC) (see refs. 1 and 2) are difficult to prepare and are expensive. Moreover organic gels, because of their swelling ability, may be damaged under high pressure and are not compatible with every type of eluent.

We describe in this paper an inexpensive, versatile silica-modified phase which shows good selectivity for α-amino acids. We have used this packing to explain the separation mechanism of enantiomers by ligand-exchange chromatography.

Previously[3–6] we showed that an acrylamide type gel, when first treated with L-proline and then complexed by cupric ions, can give a complete resolution of about ten α-amino acids in less than 1 h (for a column length of 25 cm or less). We then prepared another stationary phase from linear polyacrylamide and L-proline. The packing is obtained by simple coating of silica beads with this chiral polymer.

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The reaction of polyacrylamide and L-proline is carried out in the presence of formaldehyde:



The polymer is characterized by the mean number, p, of proline residues per monomer unit or by its capacity, x, in amino acid (expressed in mequiv./g of dry polymer). The chiral macromolecule is used after complexation by cupric ions and the extent of uptake, r, of cupric ion by the proline graft in the stationary phase ($0 < r < 0.5$) has to be taken in account.

EXPERIMENTAL

Stationary phase

To 7 g of polyacrylamide ($\overline{M}_n = 23{,}000$) dissolved in 50 ml of water, were added successively the following reagents: 0.68 g of potassium hydroxide (in 10 ml of water); 4.9 g of formaldehyde (in the form of polyoxymethylene) and 2.38 g of L-proline. The mixture was stirred for 1.5 h at room temperature and finally neutralized by hydrochloric acid. The capacity in proline was obtained by titration of this purified polymer. We think that the crude reaction mixture may be used directly (after neutralization) for the coating of the silica beads.

These conditions yield a capacity for proline of $x = 0.86$ mequiv./g. By increasing the concentration of the reactants and the reaction time, polymers with capacities in the range of 0–4.2 mequiv./g may be obtained (*i.e.*, p values between 0 and 0.90)[6].

Packing material

The polymers obtained were adsorbed on mineral beads. LiChrosorb (E. Marck, Darmstadt, G.F.R.) and Spherosil (Rhône Poulenc, Paris, France) were tested. The best results were obtained with silica Spherosil XOA 600 (particle diameter 5–7 μm).

For a 15-cm column, 400 mg of polymer (dissolved in 15 ml of water) were used. To this solution were added 2 g of silica beads (in suspension in 10 ml of water) and the mixture was stirred for 15 min. The unadsorbed polymer was removed by filtration of the beads, and by washing them with water on the filter. The packing was then complexed by stirring with a large excess of cupric ions in a buffer (generally acetate). After filtration and washing with water (pH 6–7) the stationary phase is ready to be packed.

The degree of loading of the beads can be deduced from a titration of the non-adsorbed polymer or from the capacity for cupric ions. Saturation in Cu(II) ($r = 0.5$) was obtained with a buffer of pH > 5[5], typically, after contact with Cu(II) and glutarate buffer (pH 5.4). The beads were washed with water (pH = 6–7), the Cu^{2+} was displaced in acid medium and titrated (spectrophotometric titration with diethyldithiocarbamate at 450 nm gives good results).

Packing and chromatographic apparatus

The columns were packed in water (2 g of packing per 20 cm^3 of water) under pressure (400 bars) with an Haskel MCP 110 pump. A Waters ALC 201 chromatograph with an U6 K injector, R 401 refractometer, a MC 440 spectrophotometer and a Perkin-Elmer 241 MC polarimeter was used.

RESULTS

Extent of loading

The weight of polymer adsorbed per gram of silica (τ, expressed in percent) increases with the polymer concentration in the solution and becomes almost constant (τ = 6–7%) when the concentration is greater than 10% (20% was used for the preparation of our packings).

Column efficiency

Spherosil (5–7 μm) is said to give an efficiency of about 7000–8000 plates for a 10 cm column[7]. After coating with polymer, the efficiency strongly decreases: we found about 1200 plates with naphthalene (eluted in *n*-hexane); for the elution of α-amino acids in water, the efficiency is unfortunately very low (about 100 plates for 10 cm). This behaviour is not due to the packing conditions but is a consequence of the slowness of the ligand-exchange kinetics, as pointed out previously[4,8,9].

Influence of the amount of sample

Although the loading of stationary phase is higher than for classical grafted packings, the k' values are very sensitive to the amount of the injected sample. Typically, for D,L-valine eluted in pure water (1 ml/min) on a 10 × 0.48 cm I.D. column, the capacity factor of the D isomer shifts from 3 to 5.5 when the amount of injected sample decreases from 1 mg to 0.15 mg. On the other hand, the selectivity factor, α (ratio of the capacity factors fro the two isomers, k'_L/k'_D), is practically constant when the column is not overloaded.

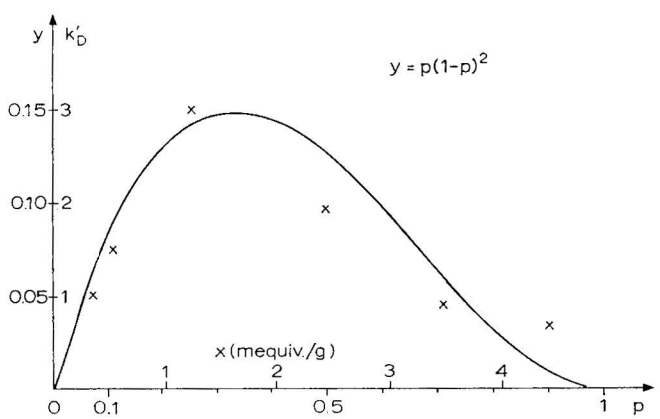


Fig. 1. Dependence of capacity factor, k'_D, of D-valine *versus* the capacity, x, or the extent of substitution, p, of various stationary phases. Eluent: 0.1 M KNO_3; flow-rate, 1 ml/min. Column: 10 × 0.48 cm I.D. ×, Experimental points; ——, the curve $y = p(1-p)^2$, arbitrary units on y axis.

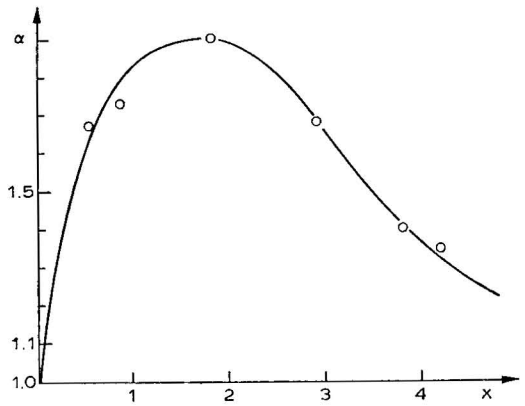


Fig. 2. Dependence of selectivity factor, α, *versus* the graft capacity, x, of the packings. Solute: D,L-valine. Other details as in Fig. 1.

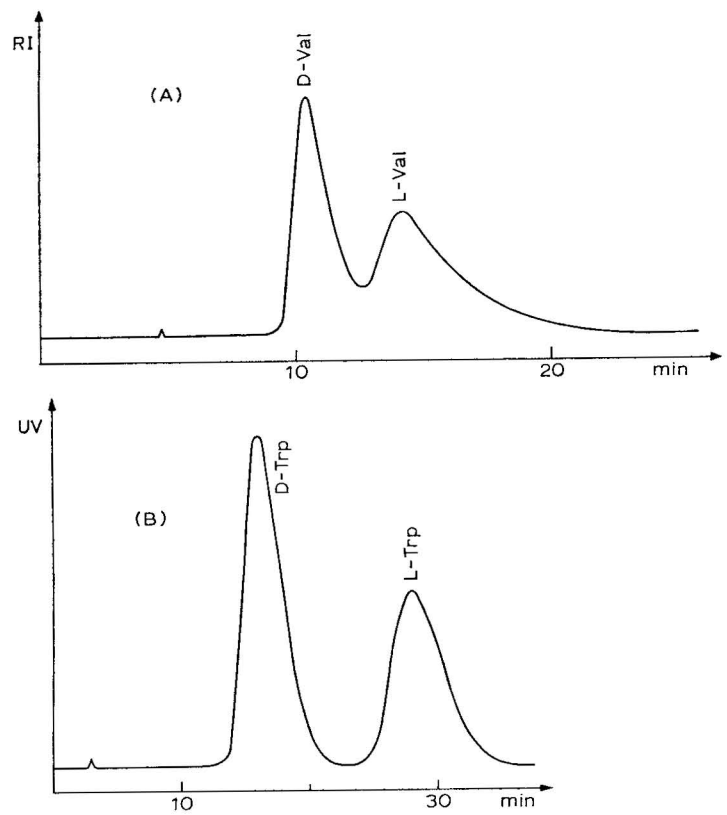


Fig. 3. Chromatograms of amino acids. (A), Column 10 × 0.48 cm I.D., eluent 0.5 ml/min water, $r = 0.3$, $x = 1.8$ mequiv./g, injection of 3 μl of a saturated water solution of D,L-valine, refractive index (RI) detection; (B), column as in (A), eluent 0.1 M KNO_3 (1 ml/min), $r = 0.4$, $x = 0.56$ mequiv./g, injection of 0.1 μl of a saturated water solution of D,L-tryptophan, UV detection.

Influence of the degree of complexation

As previously described[5], the retention of amino acid solutes considerably decreases with the mean number, r, of cupric ions per graft.

Influence of the degree of grafting

Curiously, the capacity factor of a given solute does not continuously increase with the number of sites for complexing in the column, *i.e.*, with the capacity, x, of the polymer. A maximum is observed for $x \approx 2$ mequiv./g (Fig. 1). The same behaviour is observed for α as a function of x (Fig. 2).

Influence of the solute

From our results on the effect of the sample size on capacity factors, it is difficult to compare k' values of different solutes (the amount of the sample is probably not the most important parameter, and the number of injected molecules should also be considered). α values are practically independent of the sample size. The results concerning thirteen amino acids are given in Table I and two examples of chromatograms are shown in Fig. 3.

TABLE I

CAPACITY FACTORS, k', AND SELECTIVITY FACTORS, α, FOR SOME SOLUTES

Column: 25 × 0.48 cm I.D.; $r = 0.4$; $x = 0.86$ mequiv./g. Eluent: 0.1 M KNO_3 with $0.2 \cdot 10^{-4} M$ Cu(II); flow-rate, 0.4 ml/min.

Solute (D,L form)	*Amount injected**	k'_D	k'_L	α
Alanine	$\approx 10^{-4}$g	≈ 1.80	≈ 1.80	≈ 1 (but > 1)
Aminobutyric acid	$\approx 10^{-3}$g	1.49	1.94	1.25
Norvaline	10 µl	1.80	2.34	1.30
Norleucine	5 µl	3.94	4.81	1.20
Valine	10 µl ≈ 10^{-3}g	1.76	3.09	1.75
Isoleucine	5 µl	2.84	4.37	1.55
Proline	$\approx 10^{-3}$g	3.20	2.06	0.70
Phenylalanine	10 µl ≈ 10^{-4}g	6.10	12.35	2.00
Tyrosine	10 µl	16.31	36.27	2.20
Tryptophan	50 µl	15.73	38.28	2.45
Asparagine	10 µl	3.29	4.16	1.25
Serine	10 µl ≈ $5 \cdot 10^{-4}$g	2.27	4.20	1.85
Threonine	3 µl	2.80	5.31	1.90

* All volumes were of saturated solutions.

DISCUSSION

Stereoselection mechanism

The complexation properties of our grafted linear polymers and of corresponding low-molecular-weight models were studied by potentiometry and NMR spectroscopy[6,10]. In solution, at pH > 5.5, the main complexed species are the GMG, SMS and GMS forms where G is the graft (L-proline), M the metal ion (Cu^{2+}) and S the solute. The equilibrium involved in the stereoselection is the exchange between a graft and a solute molecule:

$$GMG + S \overset{K}{\rightleftharpoons} GMS + G$$

The constant K varies according to the solute form (D or L). For instance with S = valine and a polymer for which $x = 0.86$ mequiv./g, in solution in 0.1 M KNO_3, at 25°C, $K_L/K_D = 1.5$ (error in each pK value is estimated as $\pm$ 0.05 pK unit); with S = proline, $K_L/K_D = 0.8$. Although these differences are small (but significant) they are consistent with the chromatographic results: the more strongly complexed is the isomer, the larger is the k' value[6,10].

Effect of the degree of grafting

It also appears that the stability of the GMG complex increases with the degree of grafting of the polymer; at the same times the difference between K_L and K_D decreases. For instance, regarding the complexation constants, a polymer with $x = 3.83$ forms a complex with copper which is about 1000 times more stable than that formed by a polymer with $x = 0.86$ mequiv./g; at the same time the values of K_L/K_D are 1.35 and 1.5 for valine and 0.9 and 0.8 for proline respectively. This suggests that different types of grafts are formed in the stationary phase (see Fig. 4): if in the GMG complex the two grafts are located on vicinal sites, the complex is very stable, more stable than a complex with no vicinal grafts.

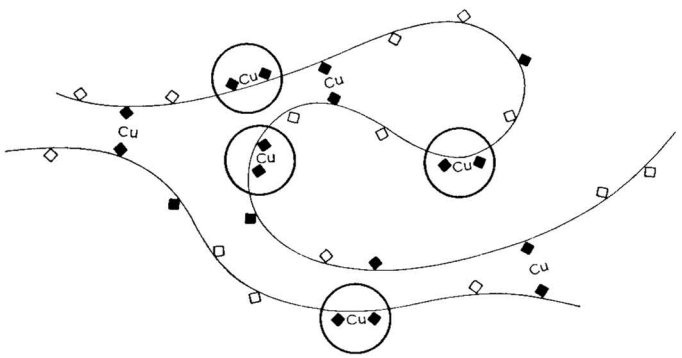


Fig. 4. The different kinds of copper complexes involved in the grafted polymer chains. ♦, L-proline graft; ◇, ungrafted site. The circles represent complexes involving vicinal grafts.

The mean number, p, of grafts per monomer unit is also the probability of finding a graft on a given repeat unit[11], and $(1 - p)$ is the probability of finding an ungrafted unit. Consequently, the probability of finding a grafted unit with free sites in vicinal position (neighbouring units being free) is $p(1 - p)^2$.

If we assume that the "vicinal" GMG complexes are too stable to participate in the ligand exchange, only the complexes involving isolated grafts are efficient for the retention and the separation on the chromatographic column. Hence, the k' values would be described by an equation of the $p\ (1 - p)^2$ form (which can be expressed in terms of x by taking in account the molecular weights of grafted and free sites). This is fairly well verified (see Fig. 1). The maximum value of k' is observed for $p = 0.33$, *i.e.*, $x \approx 2$ mequiv./g.

Calculation of k'

The value of k' was determined[6] by taking in account the various equilibria (dissociation, complexation, partition) involved

$$k'_D = (V_S/V_0)\, k\, (1 + K_D Z) \qquad k'_L = (V_S/V_0)\, k\, (1 + K_L Z)$$

where $Z = [r/(1 - 2r)]p(1 - p)^2$, and V_s and V_0 are respectively the volume of the stationary phase and the column void volume. k is the distribution coefficient by Donnan effect, *i.e.*, the concentration of uncomplexed solute in the stationary phase divided by the concentration of the solute in the mobile phase. This relationship not only describes k' as a function of p but also accounts for the strong increase in retention when r tends to 0.5, and gives a simple expression of α:

$$\alpha = k'_L/k'_D = (1 + K_L Z)/(1 + K_D Z)$$

α is close to 1 (no separation) when $K_L Z$ and $K_D Z < 1$, that is when there is no copper ($r = 0$) or when the extent of grafting is very low ($p \approx 0$) or, on the contrary, almost) complete ($p \approx 1$). On the other hand, if $K \gg 1$, α is virtually constant ($\alpha_{max.} = K_L/K_D$) over a large range of p and r. Such behaviour is observed (see Fig. 2: α tends to zero for extreme values of the extent of grafting and the curve has a flat maximum).

The maximum resolution between two isomers, $R_{max.}$, may easily be predicted by using the basic relationship

$$R = \left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)N^{1/2}$$

and the above expressions for k'_L and α yield:

$$R_{max.} = \frac{N^{1/2}}{4}\left(1 - \frac{1}{\alpha_{max.}}\right)$$

The unusually strong dependence of k' on the amount of injected sample may also be derived from the k' expression:

$$k' = \frac{V_S}{V_0} k \left(1 + K \frac{[GMG]}{[G]}\right)$$

In the ligand-exchange reaction, an increase of the solute concentration leads to a decrease in [GMG] and an increase in [G] that is, as observed, a decrease of k'. Consequently the peaks are not gaussian, but exhibit tailing (see Fig. 3).

Structure of the stereocomplexes

Potentiometric and nuclear magnetic resonance (NMR) spectroscopic studies[10] suggest a structure for the GMG complexes. In particular, the study of the relaxation time, T_1 in ^{13}C NMR gives an indication of the relative distance between each carbon atom and the copper ion.

The structure with S = valine is shown in Fig. 5. The amide function of the

polymer is in an apical position in the complex. Our results are therefore different from those[12–15] for chiral packings obtained with the same graft and the same complexing ion but with a styrene-type polymer. However, they are similar to those published on another kind of hydrophilic stationary phase[16]. As suggested by the NMR results and supported by molecular models, there is a steric hindrance between the amide group and the isopropyl substituent of the D-valine (see Fig. 5A). This effect does not exist with L-valine because these two groups are not on the same side of the main coordination plane (Fig. 5B). Consequently, L-valine is the most strongly complexed and shows the greatest retention volume.

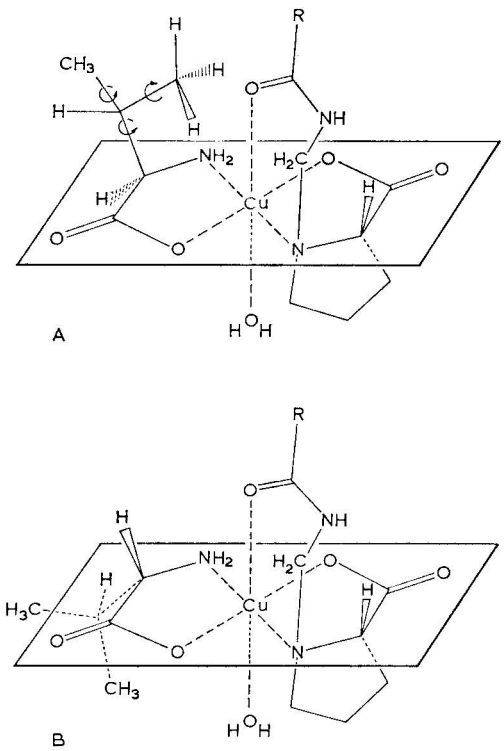


Fig. 5. Structures of the GMG complexes: A = L-proline–Cu^{2+}–D-valine (R is the macromolecular chain); B = L-proline–Cu^{2+}–L-valine.

More generally, this structure may be adopted for all amino acids having low polarity substituents. It may be noted that the larger are the substituents R which may interfere with the carbonyl group the stronger is the hindrance to rotation of R and the higher is the separation factor: for instance (see Table I), better separations are obtained for valine [R = $CH(CH_3)_2$; $\alpha = 1.75$] and serine ($R = CH_2OH$; $\alpha = 1.85$) than for the *n*-alkyl parent compounds norvaline (R = $CH_2CH_2CH_3$, $\alpha = 1.3$) and alanine (R = CH_3, $\alpha \approx 1$). In the same way, threonine [R = $CH(CH_3OH$; $\alpha = 1.90$] may be separated more readely than valine ($\alpha = 1.75$). As a result, the best resolutions are obtained for amino acids with bulky aromatic substituents: phenylalanine ($\alpha = 2.0$); tyrosine ($\alpha = 2.2$) and tryptophan ($\alpha = 2.45$).

In the series of amino acids with R = *n*-alkyl (alanine, $n = 1$, $\alpha \approx 1$; aminobutyric acid, $n = 2$, $\alpha = 1.25$; norvaline, $n = 3$, $\alpha = 1.30$; norleucine, $n = 4$, $\alpha = 1.20$), the selectivity first increases, as expected, with the size of the substituent and then remains constant or slightly decreases: it should be borne in mind that for such complexes the long substituent tends to be in an equatorial position (in the main complexation plane) and cannot greatly interfere with the carbonyl group in the apical position.

For D,L-proline as solute, a hindrance to rotation of R cannot be invoked because in this case the cyclic substituent is blocked. The separation mechanism is probably different and for this solute the L isomer is eluted first.

CONCLUSION

Chromatographic experiments and physico-chemical studies in solution with a chiral linear polymer, used as stationary phase, explain the difficulties generally encountered in separation of optical isomers by ligand-exchange chromatography: the necessity for similar stabilities of the complex formed by the graft and by the solutes; the absence of universality because the stereocomplex is formed only with a family of solutes, and even in this series the effect of the substituents might be predominant.

The proposed packing has some benefits: it is not expensive and is easily prepared. It yields high separation factors and the k' values can readily be adjusted, according to the solute to be separated, by selecting the complexation level of the ion fixed on the packing. Moreover, the use of a mineral support permits variation of the solvent. The last advantage has not yet been explored and may provide a way[17] of improving the low plate number.

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SEPARATION OF THE DIASTEREOISOMERS OF PYROGLUTAMYL-HISTIDYL-3,3-DIMETHYLPROLINEAMIDE BY LIGAND-EXCHANGE CHROMATOGRAPHY

K. SUGDEN*, C. HUNTER and J. G. LLOYD-JONES
Reckitt and Colman, Pharmaceutical Division, Dansom Lane, Hull (Great Britain)

SUMMARY

A novel high-performance ligand-exchange technique based upon complexation with Cu^{2+} for the rapid and direct separation of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide from its D-L-L, L-D-L and L-L-D isomers is described and the effect of various chromatographic parameters on retention and on bonding of copper to peptide is discussed.

INTRODUCTION

The conventional method for the determination of the chiral integrity of a peptide involves conversion of the acid hydrolysate amino acid mixture into diastereomeric dipeptides by reaction with the N-carboxyanhydride of an optically pure amino acid, usually L-leucine or L-glutamic acid, with subsequent analysis on an amino acid analyzer[1]. This method has several disadvantages in that it can lead to erroneous results via either racemisation of the amino acids on hydrolysis or by the production of interfering artefacts on derivatisation. Moreover, a single analysis on the amino acid analyzer can take many hours to complete and total analysis time may be of the order of several days.

Other more recent methods also require hydrolysis prior to analysis and involve the direct separation of the optical isomers of amino acids by liquid chromatography on optically active metal chelate stationary phases[2–5].

This report describes how sample pretreatment can be avoided to directly resolve the diastereoisomers of the tripeptide pyroglutamylhistidyl-3,3-dimethylprolineamide by use of a simple and rapid ligand-exchange technique.

EXPERIMENTAL

A Waters 6000A constant flow pump (Waters Assoc., Northwich, Great Britain) was used to provide mobile phase flow and either a Pye LC3 (Pye Unicam, Cambridge, Great Britain) or an Applied Chromatography Systems 750/11 (Applied Chromatography Systems, Luton, Great Britain) UV detector was used to monitor column eluent.

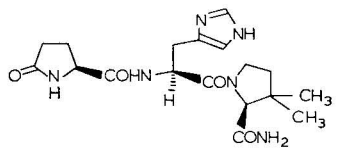
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The chromatographic columns were prepared by packing either Partisil–ODS (10 μm) (Whatman, Maidstone, Great Britain) or Spherisorb-CN (5 μm) (Phase Separations, Queensferry, Great Britain) into a 25 cm $\times$ 0.46 cm I.D. stainless-steel tube via a methanol slurry and a Magnus P5000 slurry packing unit (Magnus Scientific, Sandbach, Great Britain). The temperature of the column was controlled by a surrounding water-jacket thermostatically heated and fed by a Shandon water pump (Shandon, London, Great Britain).

Sample injection was achieved via a Rheodyne 7120 valve fitted with a 20-μl loop.

The isomers of pyroglutamylhistidyl-3,3-dimethylprolineamide were synthesised in the Medicinal Chemistry Laboratory of the Pharmaceutical Division of Reckitt and Colman. All solutions of the peptides used in this work were prepared in water.

RESULTS AND DISCUSSION



Pyroglutamylhistidyl-3,3-dimethylprolineamide

Previous work in this laboratory[6] and elsewhere[7] has shown that the diastereoisomers of certain enkephalins can be resolved on either 3-cyanopropyl- or octadecylsilylated high-performance silica gel. However, a preliminary investigation of the liquid chromatographic properties of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its D-L-L, L-D-L and L-L-D isomers on both of these columns showed some of the diastereosiomers to co-chromatograph to give partial resolution only (Figs. 1 and 2). To achieve the desired separation it was necessary to develop a ligand-exchange technique based upon a cyano phase modified by complexation with Cu^{2+} added to the mobile phase. The elemental composition of such a phase (Table I) indicates that few of the surface cyano sites are available for complexation, but nonetheless by careful control of the various parameters it was possible to resolve all four isomers within 25 min (Fig. 3), with a detection limit of approximately 60 ng on column when the eluent is monitored at 210 nm. The effect of the concentration of Cu^{2+} in the mobile phase on the retention of the four isomers is given in Fig. 4. The retention of all the isomers increases as the Cu^{2+} ionic strength is increased from $1 \cdot 10^{-3}$ mM to $5 \cdot 10^{-2}$ mM but above this concentration the retention time is seen to

TABLE I

ELEMENTAL ANALYSIS OF THE Cu^{2+}-LOADED CYANO PHASE

Carbon (μmoles/g)	Hydrogen (μmoles/g)	Nitrogen (μmoles/g)	Copper (μmoles/g)
480	440	440	5.2

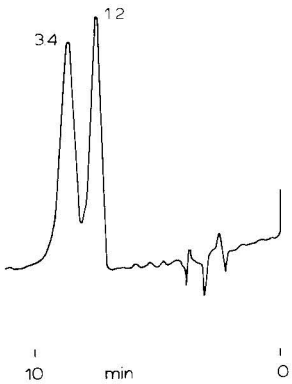


Fig. 1. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Partisil-ODS (10 μm), 25 $\times$ 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.1 mM sodium acetate (30:70), flow-rate 1 ml/min; detection, UV absorption at 210 nm.

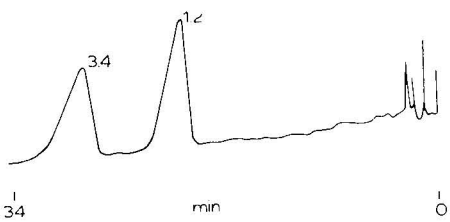


Fig. 2. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Spherisorb-CN (5 μm), 25 $\times$ 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.01 mM sodium acetate (30:70), flow-rate 2 ml/min; detection, UV absorption at 210 nm.

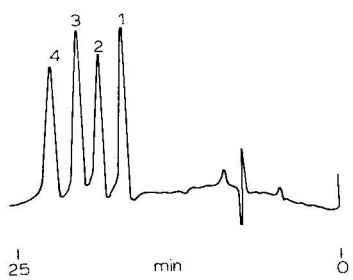
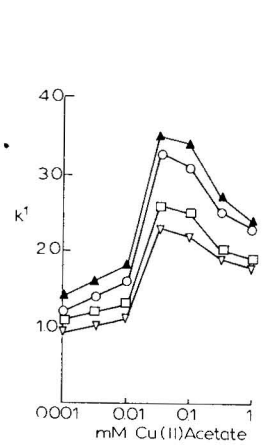


Fig. 3. Chromatogram of L-pyroglutamyl-L-histidyl-L-3,3-dimethylprolineamide and its isomers. Peaks: 1, L-L-D isomer; 2, L-D-L isomer; 3, L-L-L isomer; 4, D-L-L isomer. Conditions: column, Spherisorb-CN (5 μm), 25 $\times$ 0.46 cm I.D. at 87°C; mobile phase, acetonitrile–0.01 mM copper(II) acetate (30:70), flow-rate 0.5 ml/min, detection, UV absorption at 210 nm.

decrease. Cooke *et al.*[8] have noticed similar trends in the retention of sulpha drugs on a diamine phase modified by the presence of Cd^{2+} in the mobile phase. When the concentration of Cu^{2+} in the mobile phase is kept low (0.01 mM), to ensure that all active sites remain complexed, and ionic strength is adjusted by the addition of a non-complexing ion, such as Na^{+}, then trends in retention similar to those observed in the



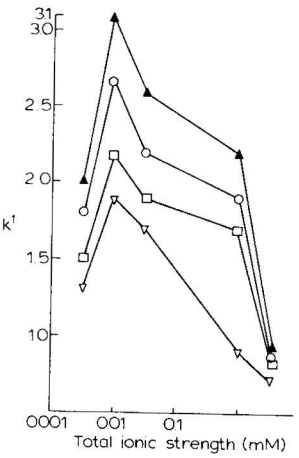
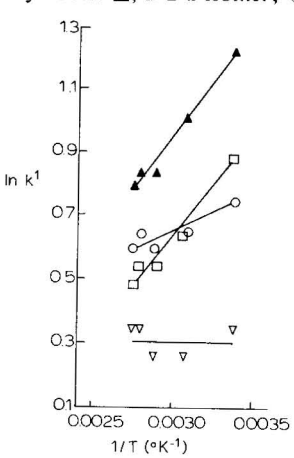


Fig. 4. Effect of Cu^{2+} concentration (mM) in the mobile phase on capacity ratio (k') on the cyano column. Conditions: mobile phase, acetonitrile–copper(II) acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

Fig. 5. Effect of Na^+ concentration (mM) in the mobile phase on the capacity ratio (k') on the cyano column modified by complexation with Cu^{2+}. Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate + sodium acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.



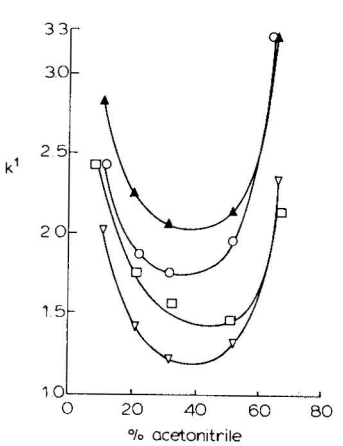


Fig. 6. Effect of column temperature (T) on capacity ratio (k'). Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate (30:70); column, Spherisorb-CN, 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

Fig. 7. Effect of acetonitrile concentration of the mobile phase on capacity ratio (k'). Conditions: mobile phase, acetonitrile–0.01 mM copper(II) acetate (30:70); column, Spherisorb-CN (5 μm), 25 × 0.46 cm I.D. at 87°C. Symbols: ▲, D-L-L isomer; ○, L-L-L isomer; □, L-D-L isomer; ▽, L-L-D isomer.

presence of varying concentrations of Cu^{2+} only are observed (Fig. 5). Although with the presence of Na^+ the total ionic strength required to achieve maximum retention time is shifted from $5 \cdot 10^{-2}$ M to $1 \cdot 10^{-2}$ M, total removal of Cu^{2+} from the mobile phase results in slow deterioration in column performance, due to sequestration of

the copper from its binding site, and it appears that the significant role of Cu^{2+} is to initially modify and then maintain the copper loading on the column.

Increasing the temperature of the column greatly reduces the retention times of the D-L-L and L-D-L isomers but has less effect on L-L-L and negligible effect on L-L-D (Fig. 6). This suggests that the rate of ligand exchange is more sensitive to changes in the chirality of the glutamyl and histidyl moieties than in the 3,3-dimethylprolineamide moiety. Hence the role of the latter in the separation mechanism is less significant. Initial Cu^{2+}–peptide binding studies using proton NMR tentatively indicate by proton broadening that it is the histidyl moiety which bonds predominantly to Cu^{2+}. Other workers[9] have confirmed this observation by similar studies with thyrotropin releasing hormone (TRH). From a practical viewpoint control of the temperature is very advantageous since it enables both the absolute and relative retention of the diastereoisomers to be changed, and provides an improvement in the resolution.

The acetonitrile concentration of the mobile phase has a significant effect on both the absolute and relative retention of the isomers (Fig. 7). The role of the solvent is unclear but it does have a more marked effect upon a conformational change in the histidyl moiety than on any other. A similar study carried out with the L-L-D isomer only in systems free of Cu^{2+} but containing Na^{+} shows similar trends in retention behaviour and suggested that a mixed chromatographic mechanism is involved. At low acetonitrile concentrations, such that the polarity of the mobile phase is greater than that of the stationary phase, reversed-phase partition occurs and at high acetonitrile concentration an absorption mechanism characteristic of conventional silica gel chromatography seems to be involved.

CONCLUSION

The chirality of pyroglutamylhistidyl-3,3-dimethylprolineamide can be determined by high-performance ligand-exchange chromatography. The technique overcomes the problems inherent in the conventional method in that it is simple to perform, rapid and minimises the possibility of racemisation.

Various chromatographic parameters have been shown to have a significant effect on the relative retention of the diastereoisomers and it is foreseen that the technique will prove to be useful to resolve the isomers of other peptides capable of association with metals.

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QUANTITATIVE ANALYSIS OF DIASTEREOISOMERIC ALKANOLAMINES AS CYCLIC BORONIC ACID DERIVATIVES

G. MUNRO* and L. R. ROWE
Glaxo Group Research Ltd., Ware, Herts. (Great Britain)
and
M. B. EVANS
The Hatfield Polytechnic, Hatfield, Herts. (Great Britain)

SUMMARY

Cyclic boronic derivatives formed by the interaction of bi-functional compounds and organo-boronic acids are commonly used to facilitate the gas–liquid chromatographic (GLC) analysis of diols, amino-alcohols and hydroxy compounds with adjacent primary amide moieties. When the phenylalkanolamines salmefamol and labetalol were converted to their bis-boronate derivatives irreproducible quantitative results were obtained by GLC. Because these results raise serious doubts concerning the suitability of boronic acid derivatives for quantitative analysis the factors affecting the reproducibility of the quantitative data have been investigated.

INTRODUCTION

Salmefamol, a long acting bronchodilator[1], and labetalol, a new type of anti-hypertensive drug[2] which both possess in their molecules two asymmetric centres, normally exist as diastereoisomeric mixtures, consequently a sensitive method for the determination of the isomeric composition of both drugs was required for quality control purposes.

The separation of diastereoisomers is highly dependent upon molecular structure[3,4], in particular it has been shown that separation can be enhanced by incorporation of part of the molecule into a cyclic system or the introduction of bulky groups on or near to asymmetric centres.

Cyclic boronic esters formed by interaction of bi-functional compounds and organo-boronic acids are commonly used to facilitate the gas–liquid chromatographic (GLC) analysis of diols[5], amino alcohols[6] and hydroxy compounds with adjacent primary amide moities[7]. The present communication is concerned with the conversion of both salmefamol and labetalol into rigid five-membered rings containing boron.

EXPERIMENTAL

Reagents

Organo-boronic acids and organo-boron dihalides were obtained from commercial suppliers and used as supplied.

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Equipment

Gas chromatograms were obtained using a Perkin-Elmer F-30 gas chromatograph equipped with glass columns and flame ionisation detection. The columns, 2 or 4 m × 1.75 mm I.D., were packed with mixtures of stationary phase and support phase (for details see the text). Nitrogen was used as carrier gas. Solutes for analysis were introduced as dilute solutions by means of SGE micro-syringes via a serum cap.

Preparation of derivatives

Compound (10 mg) and methyl, *n*-butyl or phenyl boronic acid (100 mg) in anhydrous dimethylformamide (0.5 cm^3) were allowed to stand at approximately 20°C for 20 min. Aliquots of 1.0 mm^3 were taken for GLC analysis.

RESULTS AND DISCUSSION

When salmefamol and labetalol were converted to rigid five-membered rings containing boron, excellent separation of the individual diastereoisomers was observed. However the reproducibility of the isomer ratios was found to be poor, as illustrated by the repetitive analysis in Table I, and the total yield of derivative was inconsistent[8]. As these results raise serious doubts concerning the suitability of boronic acid derivatives for quantitative analysis, factors affecting the reproducibility of quantitative data have been investigated.

Possible causes of the irreproducibility of boronate analyses could include:

(1) Different rates of reaction between the individual diastereoisomers and the boronic acid.

(2) Thermal instability of one or both of the derivatives.

(3) Hydrolysis of one or both of the derivatives prior to or during chromatography.

(4) Reaction with excess reagent to form involatile products.

(5) Chemisorption of the solute due to reaction with organo-boronic acid bound to the stationary phase.

Differential reaction rates, thermal instability and hydrolysis were shown to be unlikely because satisfactory quantitative results were obtained on freshly packed columns (see Table II), furthermore heating derivatives in solution provided no evidence of decomposition and the addition of the water scavenger, isopropenylacetate, produced no improvement of results obtained from serial injection.

The possible effects of excess boronic acid deposited onto the column packing upon the chromatography of boronate esters were investigated by the use of:

(1) Cyclic boron derivatives synthesised by alternative routes.

(2) Columns prepared from diatomaceous supports, deactivated diatomaceous supports and non-diatomaceous support phases with a range of liquid phases.

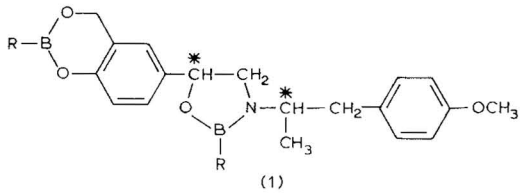
(3) Columns equipped with *n*-butyl boronic acid pre-columns.

The attempt to study cyclic boron derivatives synthesised by alternative routes which would ensure that there was no excess of reagent present, was made by utilising three boron dihalides, methyl boron dichloride, *n*-butyl boron dibromide or phenyl boron dibromide. Such boron halides are known to be more reactive than organo-boronic acids[9]. Unfortunately despite the use of a wide range of anhydrous solvents such as pyridine, dimethylformamide, acetonitrile, acetone, chloroform, methylene

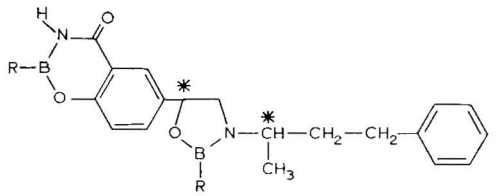
TABLE I

THE RESULTS OF SERIAL INJECTIONS OF THE BIS-BORONATE DERIVATIVES OF SALMEFAMOL (1) AND LABETALOL (2)

Column: 2 m × 1.75 mm I.D. glass column packed with 5% OV-101 coated onto 100–120 mesh Gas-Chrom Q AW HMDS.



(1)



(2)

The ratio is defined as the % (w/w) of isomer 1 : % (w/w) of isomer 2 where % (w/w) isomer 1 (I_1) =

$$100 \left[\frac{\text{integrated area of the peak due to isomer 1}}{\text{integrated area of the peaks due to both isomers 1 and 2}} \right]$$

and % (w/w) isomer 2 (I_2) =

$$100 \left[\frac{\text{integrated area of the peak due to isomer 2}}{\text{integrated area of the peaks due to both isomers 1 and 2}} \right].$$

Isomer 1 = the first eluted isomer; isomer 2 = the second eluted isomer.

Bis-boronate derivative	*Ratio of % (w/w) I_1:% (w/w) I_2*		
	Injection A	*Injection B*	*Injection C*
Salmefamol	57.5:42.5	62.7:37.3	68.8:31.2
Labetalol	50.6:49.4	53.1:46.9	56.7:43.3

dichloride and toluene, no stable derivatives were formed even when the reaction vessel was placed in an ice-bath. Solutions of sample and reagent quickly discoloured and the reaction vessel gaskets and teflon seals were rapidly decomposed, although initially the gas chromatograms of these solutions exhibited two peaks corresponding to those in the satisfactory chromatograms obtained following reaction between salmefamol or labetalol with the appropriate organo-boronic acid. The derivatives giving rise to these peaks were clearly unstable. Subsequent injections were devoid of any peak except that associated with the reagents used. Confirmation that the column was still performing satisfactorily was obtained by making an injection of a bis-boronate of salmefamol which had been prepared by the usual procedure. Good results were initially obtained, thus the use of boron dihalides was discontinued but the fact that the GLC columns did not appear to deteriorate as rapidly when the boron dihalides were used instead of the organo-boronic acids is circumstantial

TABLE II

COMPARISON OF THE ISOMER RATIOS* FOR SALMEFAMOL AND LABETALOL

Obtained by using freshly prepared *n*-butyl boronic acid derivatives injected onto a freshly prepared GLC column (5% w/w, OV-101 coated on 100–120 mesh Gas-Chrom Q AW HMDS), and the silylation–acylation procedure described by Munro and co-workers[8,10,11]

Compound	*n-Butyl boronic acid derivative* I_1:I_2	*Silylation–acylation* I_1:I_2
Salmefamol (1; R = *n*-butyl)	51.5:48.5	50.7:49.3
Labetalol (2; R = *n*-butyl)	51.5:48.5	51.2:48.8

* The ratio of % (w/w) of isomer 1 to % (w/w) of isomer 2 as defined in Table I.

evidence to implicate the organo-boronic acids in the anomolous quantitative results which had been obtained previously.

To further test the effects of excess boronic acid deposited onto the column packing upon the chromatography of boronic acid esters, fresh columns were prepared. Two of these contained 5% (w/w) OV-101 on 100–120 mesh Gas-Chrom Q AW HMDS. The third contained 5% OV-101 coated onto an unsilanised 80–120 mesh Diatomite S support. A sample of the *n*-butyl boronate of salmefamol was prepared and extracted into a methylene dichloride solution containing *n*-triacontane and *n*-tetratriacontane as internal standards. This solution was examined using a deactivated Gas-Chrom Q column and peak area ratios for the boronate ester diastereoisomers determined relative to the *n*-alkane standards. The column was then removed from the gas chromatograph and 5 mg of *n*-butyl boronic acid was added as a pre-column. Analysis of the boronate *n*-alkane calibration solution was repeated using identical instrumental conditions.

Whereas the deactivated OV-101 column gave satisfactory results, the *n*-butyl boronic acid contaminated and the diatomite S columns gave chromatograms with

TABLE III

THE RESULTS OF INJECTING THE *n*-BUTYLBORONATE DERIVATIVES OF SALMEFAMOL AND LABETALOL

I_1 and I_2 are the first and second eluted isomers respectively C_A and C_B are the preceding and succeeding hydrocarbons. Columns: glass columns packed with 5% w/w OV-101 coated onto 100–120 mesh Gas-Chrom Q AW HMDS (column 1); unsilanised, non-acid-washed 80–100 mesh Diatomite S (column 2); 100–120 mesh Gas-Chrom Q AW HMDS with 5 mg *n*-butyl-boronic acid added at the injection port end (column 3).

Compound	*Ratio of peak areas*								
	Column 1			*Column 2*			*Column 3*		
	$\frac{I_1}{C_A}$	$\frac{I_2}{C_A}$	$\frac{C_A}{C_B}$	$\frac{I_1}{C_A}$	$\frac{I_2}{C_A}$	$\frac{C_A}{C_B}$	$\frac{I_1}{C_A}$	$\frac{I_2}{C_A}$	$\frac{C_A}{C_B}$
Salmefamol	4.49	4.74	1.1	No isomers eluted		1.1	No isomers eluted		1.1
Labetalol	2.70	2.79	0.71	0.2	0.21	0.79	No isomers eluted		0.76

alkane but no boron derivative peaks (see Table III). These results suggest that the presence of either excess reagent or free surface silanols is sufficient to render unsatisfactory the gas chromatographic examination of the *n*-butyl boronic acid derivative of salmefamol. Subsequent experiments revealed that the methyl and phenyl boronic acid derivatives of salmefamol and the *n*-butyl methyl and phenyl boronic acid derivatives of labetalol all behaved similarly.

As the elution of the *n*-alkanes would not be expected to be effected by the change of support or by the presence of *n*-butyl boronic acid/anhydride these results clearly demonstrate the occurrence of on-column reaction.

Further studies to elucidate the nature and mechanism of the on-column reaction which occurs between cyclic boron derivatives and gas chromatography columns are currently in progress and will be reported elsewhere.

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CHROM. 13,322

SYNTHÈSE DE GLYCOSIDES

DOSAGE DIRECT DES ANOMÈRES PERBENZYLÉS INTERMÉDIAIRES PAR CHROMATOGRAPHIE EN PHASE LIQUIDE À HAUTE PERFORMANCE

M. DREUX* et M. LAFOSSE

Laboratoire de Chimie Organique Physique et Chromatographie, U.E.R. de Sciences Fondamentales et Appliquées, 45046 Orléans Cedex (France)

et

P. H. AMVAM ZOLLO, J. R. POUGNY et P. SINAŸ

Laboratoire de Biochimie Structurale, E.R.A. 739, U.E.R. de Sciences Fondamentales et Appliquées, 45046 Orléans Cedex (France)

SUMMARY

Synthesis of glycosides. Direct high-performance liquid chromatographic determination of intermediate perbenzylated anomers

High-performance liquid chromatography has been used to determine the stereospecificity of the synthesis of α or β glycosides. The protecting groups are benzylic ethers, therefore we have employed reversed-phase liquid chromatography to separate α and β anomers. Some octadecyl supports have been compared, in order to optimize the separation for several glycosides.

INTRODUCTION

La synthèse stéréospécifique de glycosides α ou β est un problème classique en chimie des sucres[1]. Il existe de nombreux groupes protecteurs temporaires et récemment nous avons proposé un procédé très stéréosélectif d'accès aux glycosides α[2,3] qui utilise les éthers benzyliques comme groupement protecteur non participant et les imidates comme groupe activant du carbone anomère.

Par exemple la synthèse de l'α-méthylmaltoside s'effectue avec un excellent rendement selon le schéma en trois étapes de la Fig. 1.

L'intérêt de cette synthèse est multiple:

(a) le passage des glycosides benzylés aux glycosides libres s'effectue sans racémisation et avec de très bons rendements (étape 3, Fig. 1); (b) la présence des noyaux aromatiques permet une détection UV facile et sensible des glycosides benzylés; (c) l'imidate permet l'obtention stéréospécifique des anomères α avec de bons rendements; toutefois, la recherche d'une éventuelle contamination par l'anomère β même en faible proportion est un problème d'importance.

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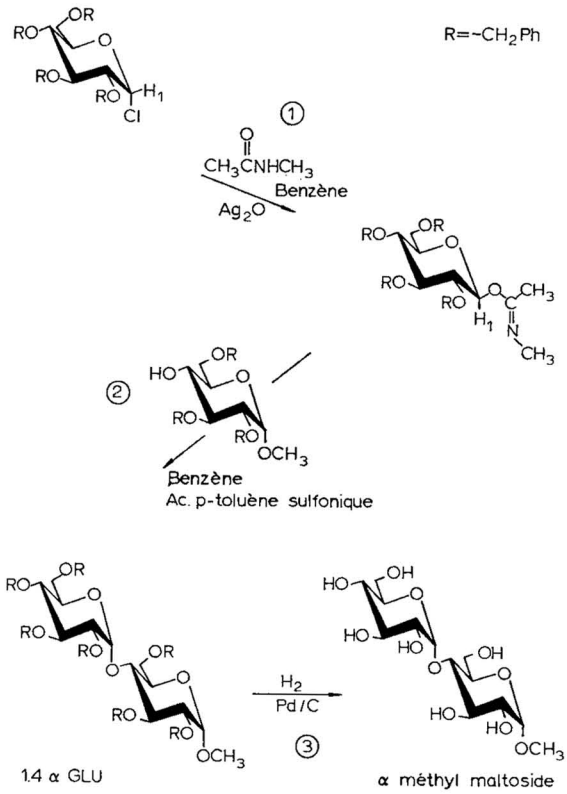


Fig. 1. Schéma de principe de la synthèse de l'α-méthyl-maltoside.

La RMN du proton, le pouvoir rotatoire, la chromatographie sur couche mince ne sont à cet égard pas toujours exploitables. La chromatographie en phase gazeuse permet, au contraire, des dosages précis de faibles quantités d'isomères, de diastéréoisomères et d'isomères optiques[4]. Dans le cas de ces molécules possédant plusieurs noyaux aromatiques, il est alors nécessaire d'effectuer une transformation en produits débenzylés que l'on silyle. Ces manipulations intermédiaires peuvent ne pas être quantitatives ce qui hypothèque alors sur la précision du dosage[5].

Nous proposons ici un dosage rapide, direct et précis des anomères α et β par chromatographie en phase liquide à haute performance (HPLC) en phases inversées (phases greffées apolaires). Le dosage de glycosides a déjà été proposé par HPLC[6,7]: il utilise, compte tenu de la difficulté de détection des sucres libres, la formation de dérivés esters benzoïques facilement détectables en UV et des phases polaires (silice, alumine).

Depuis l'apparition des phases apolaires ce système est de choix pour la chromatographie de molécules possédant des noyaux aromatiques[8]; il a l'avantage d'une reproductibilité et d'une stabilité des supports supérieures aux phases polaires précédemment citées.

PARTIE EXPERIMENTALE

L'appareil utilisé est un chromatographe Waters (Waters Assoc., Milford, MA

U.S.A.) équipé d'une pompe 6000 A, d'un détecteur UV à filtre modèle 440. Pour réaliser une double détection nous avons employé un réfractomètre différentiel Waters R.401, placé après le détecteur UV, l'enregistrement est réalisé sur un appareil à double voie Vitatron 2001 (Vitatron, Malakoff, France).

Les colonnes (15 cm × 6.4 mm O.D.) sont remplies avec des silices greffées de granulométrie 5 et 7 μm: (a) LiChrosorb RP-18, 5 μm (Merck, Darmstadt, R.F.A.); (b) Nucleosil 5 C_{18}, 5 μm (Macherey Nagel, Düren, R.F.A.); (c) Spherosil XOA-600 C_{18}, 7 μm (Prolabo, Paris, France); et (d) Zorbax ODS 7 μm (DuPont, Wilmington, DE, U.S.A.). Le remplissage s'effectue à l'aide d'une suspension de la phase greffée dans le tétrachlorure de carbone puis percolation sous 400 bars avec le triméthyl-2,2, 4 pentane.

Les éluants sont constitués de mélange d'eau distillée et de méthanol (Rathburn Chemicals, Walkerburn, Grande-Bretagne). Les proportions sont indiquées en volume.

Les produits analysés ont été synthétisés au laboratoire[2,3] et mis en solution dans le chlorure de méthylène.

Méthyl-2,3,6-tri-O-benzyl-4-O-(2,3,4,6-tétra-O-benzyl-α-D-glucopyranosyl)-α-D-glucopyranoside = 1,4 α Glu; méthyl-2,3,6-tri-O-benzyl-4-O-(2,3,4,6-tétra-O-benzyl-β-D-glucopyranosyl)-α-D-glucopyranoside = 1,4 β Glu; méthyl-2,3,4-tri-O-benzyl-6-O-(2,3,4,6-tétra-O-benzyl-α-D-glucopyranosyl)-α-D-glucopyranoside = 1,6 α Glu; méthyl-2,3,4-tri-O-benzyl-6-O-(2,3,4,6-tétra-O-benzyl-β-D-glucopyranosyl)-α-D-glucopyranoside = 1,6 β Glu; (2,3,4,6-tétra-O-benzyl-α-D-glucopyranosyl)-2,3,4,6-tétra-O-benzyl-β-D-glucopyranoside = 1,1 $\alpha\beta$ Glu; (2,3,4,6-tétra-O-benzyl-α-D-glucopyranosyl)-2,3,4,6-tétra-O-benzyl-α-D-glucopyranoside = 1,1 $\alpha\alpha$ Glu; (2,3,4,6-tétra-O-benzyl-β-D-glucopyranosyl)-2,3,4,6-tétra-O-benzyl-β-D-glucopyranoside = 1,1 $\beta\beta$ Glu.

RESULTATS ET DISCUSSION

Les isomères, quelles que soient les phases greffées, sont facilement séparés (Tableau I). Les anomères quant à eux nécessitent parfois le choix approprié du support octadécyl et la composition de la phase éluante.

TABLEAU I

RÉTENTION RELATIVE D'ANOMÈRES α ET β EN CHROMATOGRAPHIE EN PHASES INVERSÉES (ÉLUANT MÉTHANOL–EAU, 95:5) SUR DIFFÉRENTS SUPPORTS GREFFÉS OCTADÉCYL

Solutés	*LiChrosorb RP-18*	*Nucléosil C_{18}*	*Sphérosil C_{18}*	*Zorbax ODS*
1,4 α Glu	1	1	1	1
1,4 β Glu	1.15	1.07	1.07	1.13
1,6 α Glu	1.20	1.10	1.07	1.16
1,6 β Glu	1.20	1.10	1.07	1.16
1,1 $\alpha\alpha$ Glu	1.78	1.46	1.56	1.89
1,1 $\alpha\beta$ Glu	2.12	1.63	1.86	2.29
1,1 $\beta\beta$ Glu	2.27	1.80	2.02	2.43
Volume de rétention (ml) réduit de 1,4 α Glu	8.20	8.20	8.60	11.20

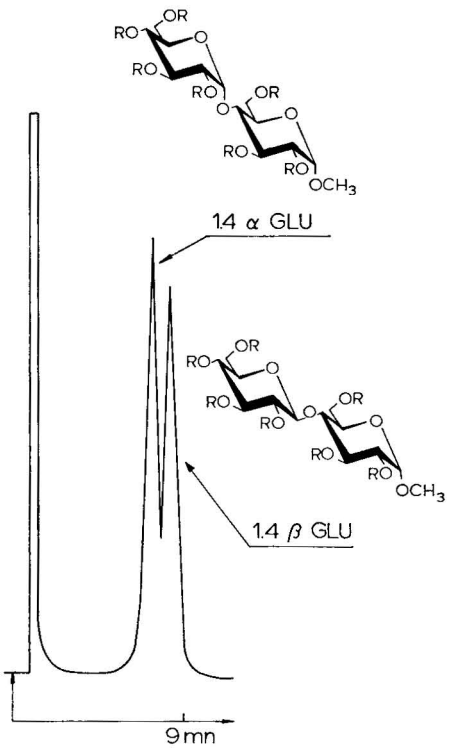


Fig. 2. Séparation du couple d'anomères 1,4α Glu/1,4β Glu sur Zorbax C_{18}. Éluant, méthanol–eau (95:5). Débit 2 ml/mn. (R = CH_2–C_6H_5).

Par exemple alors que le couple 1,4α Glu/1,4β Glu est facilement séparé (Fig. 2) (méthanol–eau; 95:5) et dosable en quelques minutes seulement (Tableau I), le couple 1,6α Glu/1,6β Glu nécessite la phase greffée Zorbax ODS et un éluent méthanol–eau (88:12) pour une analyse beaucoup plus longue (Tableaux I et II). Un même greffon octadécyl sur des silices différentes (sphériques et non sphériques) montre, sur ces exemples, des rétentions et des sélectivités différentes.

TABLEAU II

VOLUME DE RÉTENTION (ml) D'ANOMÈRES α ET β EN CHROMATOGRAPHIE EN PHASES INVERSÉES

Éluant méthanol–eau	*Solutés*	*LiChrosorb RP-18*	*Zorbax ODS*
88:12	1,6 α Glu	65	126
	1,6 β Glu	65	116
91:9	1,1 $\alpha\beta$ Glu	77.6	172
	1,1 $\beta\beta$ Glu	68.8	153

Des trois supports octadécyl à grains de silice sphérique (Nucléosil, Sphérosil et Zorbax), le Zorbax offre la rétention la plus grande. A rétention pratiquement égale (Zorbax et LiChrosorb), nous trouvons une meilleure sélectivité du grain de forme sphérique (Zorbax) par rapport au grain de forme non sphérique (LiChrosorb).

Quant à la précision du dosage par UV il est lié, la séparation réalisée, à l'hypothèse de l'égalité des coefficients d'extinction moléculaire pour deux anoméres à la longueur d'onde de détection 254 nm. Une détection réfractométrique différentielle est également possible et sa sensibilité est bonne: ceci provient sûrement de la présence des groupes benzyles dans les molécules. Si la réponse n'est pas la même pour les deux anomères la précision s'en trouve affectée et c'est pourquoi une double détection UV/réfractométrie différentielle permet un dosage plus précis.

CONCLUSION

La stéréospécificité de cette synthèse, qui donne accès aux glycosides α, est facilement suivie sur les intermédiaires par HPLC en phases inversées.

RÉSUMÉ

La chromatographie en phase liquide à haute performance a été utilisée pour déterminer la stéreospécificité de la synthèse des glycosides α ou β. Comme les groupes protecteurs sont les éthers benzyliques, nous avons employé la chromatographie en phases inversées pour séparer les anomères α et β. Différents supports greffés octadécyl ont été comparées, pour optimiser la séparation de certains glycosides.

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CHROM. 13,327

SEPARATION OF 5-ALKYLURACILS AND PURINE BASES IN HYDROLYSATES OF ENZYMATICALLY SYNTHESIZED NUCLEIC ACIDS BY HIGH-PERFORMANCE ION-PAIR LIQUID CHROMATOGRAPHY

Á. H. CSÁRNYI, M. VAJDA* and J. SÁGI

Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O. Box 17, H-1525 Budapest (Hungary)

SUMMARY

Reversed-phase and reversed-phase ion-pair chromatographic methods were used to determine 5-alkyluracils in the presence of purine bases (mainly adenine) in the hydrolysates of enzymatically synthesized DNA. The effect of ionic strength, pH, concentration of the ion-pairing agent and methanol on the selectivity between alkyluracils and purine bases was examined in order to simplify the routine work and to reduce the time necessary for analysis. Optimal conditions could be developed for the isocratic separation of the various mixtures obtained by hydrolysis of the products of enzymatic synthesis.

INTRODUCTION

Studies of the incorporation of 5-alkyluracils into polydeoxynucleotides produced during *E. coli* DNA polymerase I catalysed synthesis from dATP and 5-alkyl-dUTP in our Institute[1–4] made it necessary to develop a rapid, routine method for the determination of alkyluracils in the presence of adenine (Ade) and other purine bases in hydrolysates. The hydrolysates always contain a purine base, usually Ade and two alkyluracils. The 5-alkyl substituents on the uracils investigated were hydrogen, methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl and *n*-hexyl. The results obtained in the separation of the α- and β-anomers of 5-alkyl-2′-deoxyuridines, the phosphate of which we used in the synthesis, were published in a previous paper[5]. In earlier work ion exchange was used in the separation by high-performance liquid chromatography (HPLC) of naturally occurring[6–8] and modified[9,10] bases by various workers. In our hands the ion-exchange method was only moderately successful, because an impurity peak could not be separated from thymine (Thy) and the time necessary for gradient separation and re-equilibration was unacceptably long.

The development of reversed-phase HPLC opened up new possibilities for the analysis of nucleic acid constituents[11–13]. Ion-pair chromatography has also been used for the separation of the naturally occurring bases[14,15].

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EXPERIMENTAL

Reversed-phase and ion-pair chromatographic work was performed on a Perkin-Elmer Series 3 system with an LC 55 detector at 260 nm. A Sigma 10 Data System was used for peak integration and for calculations of response factors. Separations were achieved with a Hypersil ODS (5 μm) column (25 cm $\times$ 4 mm I.D.), and a pre-injector guard column (5 cm $\times$ 4 mm I.D.) packed with LiChroprep RP-18 was used. Injections were made with a Rheodyne Model 7120 injector with a 20-μl loop. The mobile phase was degassed ultrasonically. The compounds were dissolved in 0.1 *M* hydrochloric acid.

For the reversed-phase separations the mobile phase was a mixture of 0.01 *M* potassium dihydrogen orthophosphate (pH 5.5) and methanol–water (80:20) solution at a flow-rate of 1.0 ml/min. Gradient elution was necessary. The slope of the gradient was chosen according to the composition of the samples. The analyses were performed at ambient temperature.

In ion-pair chromatography the mobile phase was a potassium dihydrogen orthophosphate buffer–methanol mixture with octyl sulphate as counter ion; pH values were measured and adjusted after methanol had been added. The flow-rate was 1.0 ml/min. The solvent composition and pH were varied (see Results and Discussion). The columns and solvent were thermostated at 25°C with a Knauer water-jacket and an MLW ultrathermostat.

Generally, the samples contained Ade and one or more alkyluracils. All of the 5-alkyluracils tested were prepared and identified in our laboratory.

RESULTS AND DISCUSSION

Reversed-phase chromatography

Chromatography on a Hypersil ODS column was successful (Fig. 1). Satisfactory separations were achieved, using a two-step gradient, as shown in Fig. 1. This gradient caused a slight drift in the baseline and the peak shapes were not fully symmetrical, and Ade showed pronounced tailing. Re-equilibration after the gradient was time consuming (15–20 min). With the usual three-component mixtures steeper gradients were tried but, as the baseline drift became larger, the results became less accurate.

pH values between 2.5 and 6.5 were examined and the peak shape of Ade and its separation from Thy were found to be optimal at pH 5.5. The effect of methanol concentration on the k' values is shown in Table I.

Ion-pair chromatography

In this study guanine (Gua) and cytosine (Cyt) were also investigated, as enzymatic experimentx with Gua and Cyt as bases are also planned.

Effect of concentration of ion-pairing agent on capacity factor. Varying the concentration of the ion-pairing agent from 0.001 to 0.01 *M* and the methanol concentration from 10% to 60% (v/v) gave the results for Gua, Ade and Cyt shown in Fig. 2. The capacity factors of the alkyluracils are not influenced by the ion-pairing agent.

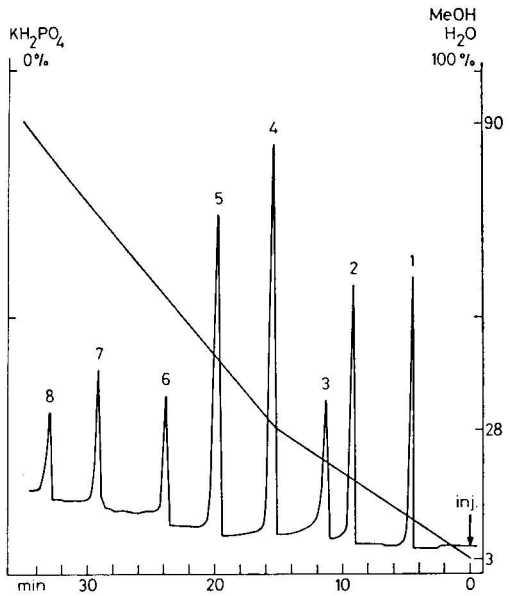


Fig. 1. Gradient separation of adenine and 5-alkyluracils. Peaks: 1 = uracil; 2 = thymine; 3 = adenine; 4 = 5-ethyluracil; 5 = 5-propyluracil; 6 = 5-butyluracil; 7 = 5-pentyluracil; 8 = 5-hexyluracil. The gradient curve is shown. Components: A, 0.01 *M* KH_2PO_4 in water (pH = 5.5); B, methanol–water (80:20). Flow-rate: 1 ml/min.

TABLE I

EFFECT OF METHANOL CONCENTRATION ON CAPACITY FACTORS WITH AND WITHOUT ION-PAIRING AGENT

Mobile phase: 0.01 *M* KH_2PO_4 solution. Concentration of ion-pairing agent: 0 or $5 \cdot 10^{-3}$ *M*.

Compound	*k'*					
	20% Methanol		*40% Methanol*		*60% Methanol*	
	*0**	*$5 \cdot 10^{-3}$**	*0**	*$5 \cdot 10^{-3}$**	*0**	*$5 \cdot 10^{-3}$**
Uracil	0.21	0.27	0.11	0.13	0.09	0.11
Thymine	0.50	0.55	0.20	0.21	0.12	0.14
5-Ethyluracil	1.21	1.27	0.41	0.39	0.20	0.21
5-Propyluracil	3.15	3.29	0.84	0.79	0.31	0.34
5-Butyluracil	9.43	9.36	1.96	1.88	0.60	0.63
5-Pentyluracil	32.96	31.85	4.71	4.57	1.08	1.12
5-Hexyluracil	—	—	12.11	11.44	2.46	2.67
Adenine	0.81	6.75	0.19	1.19	0.22	0.41

* Concentration of ion-pairing agent (*M*).

As alkyluracils show no basic dissociation, whereas the purine bases and Cyt have pK_a values of 3.2–4.6, it is probable that alkyluracils do not form ion pairs in the pH range studied. The capacity factors of Ade, Gua and Cyt increase with increasing concentration of ion-pairing agent, except in solutions containing 60% of methanol. The retention order of Gua and Cyt is reversed if the methanol concen-

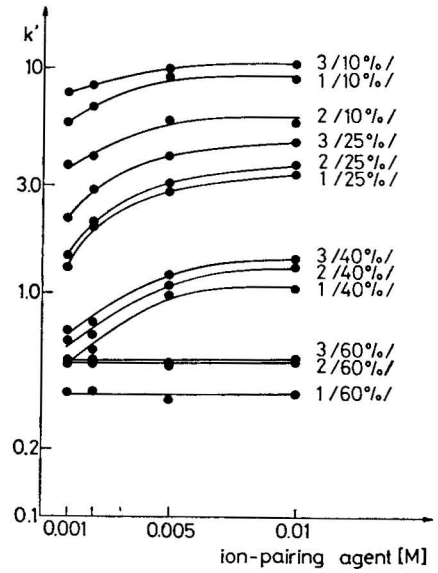


Fig. 2. Influence of concentration of ion-pairing agent at different methanol concentration (in parentheses). Mobile phase: 0.01 *M* KH_2PO_4 (pH = 2.5). Compounds: 1 = guanine; 2 = cytosine; 3 = adenine.

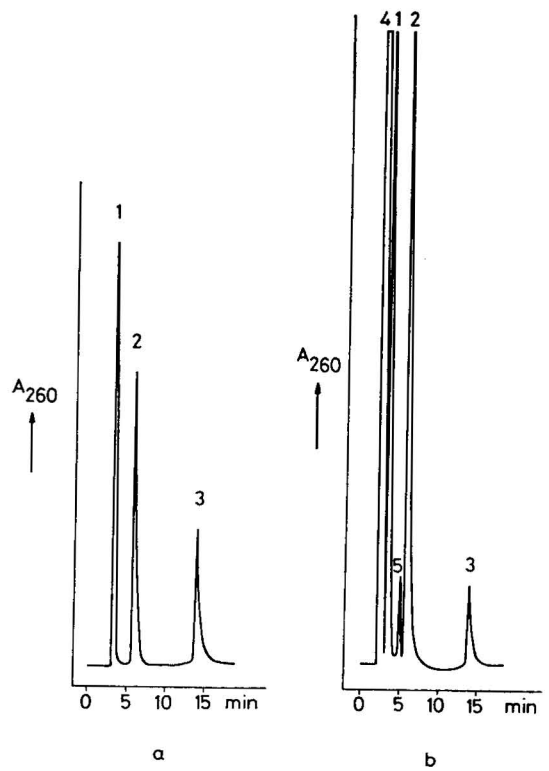


Fig. 3. Chromatogram of standard mixture (a) and hydrolysate (b). Peaks: 1 = thymine; 2 = adenine; 3 = pentyluracil; 4 and 5 = impurities in hydrolysis mixture. Solvent: 0.01 *M* KH_2PO_4 buffer–methanol (60:40) containing 0.005 *M* octyl sulphate (pH = 2.5); flow-rate, 1 ml/min.

tration is changed from 10% to 60%. This difference in solvent selectivity between Gua and Cyt is obviously due to the difference in the two ring systems.

Influence of methanol concentration. As expected from the results of the reversed-phase experiments, the methanol concentration in the mobile phase has a significant effect on the capacity factors, which increase with the number of methylene groups in the aliphatic chains (Table I). If no ion-pairing agent is present, the effect of methanol concentration is significantly different on Ade, Cyt, Gua and alkyluracils. When no ion-pairing agent is added, the variation of k' values of alkyluracils with methanol concentration is the same as in the presence of an ion-pairing agent, whereas the retention of Ade, Cyt and Gua is influenced to a lesser extent than in the presence of ion-pairing agent and the k' values show minima at a methanol concentration of 40%.

Effect of pH. Variation of the pH from 2.5 to 5.0 affects the k' values only of purine bases and Cyt (Table II). Alkyluracils have constant k' values in the pH range studied.

TABLE II

EFFECT OF pH ON CAPACITY FACTORS

Mobile phase: 0.01 *M* KH_2PO_4 solution. Methanol concentration: 25%. Concentration of ion pairing agent 0.001 *M*.

Compound	k'			
	pH 2.5	*pH 3.3*	*pH 4.1*	*pH 5.0*
Thymine	0.52	0.55	0.52	0.57
5-Ethyluracil	1.06	1.09	1.09	1.13
Adenine	2.92	3.19	1.34	0.94
Cytosine	2.30	2.90	0.96	0.41
Guanine	1.44	1.60	1.51	0.43

Effects of ionic strength. Variation of the concentration of the buffer from 0.01 to 0.2 *M* has a small effect of the capacity factors. The k' values are slightly lowered in the case of Ade, Cyt and Gua (*e.g.*, for Ade from 1.4 to 0.4) and there is almost no effect with alkyluracils.

The complete selectivity of the ion-pairing agent towards the purine bases and Cyt indicated the possibility of influencing the k' values of these compounds. We could shift their peaks to appropriate positions on the chromatogram relative to the other bases occurring in the hydrolysis mixture. The use of different methanol concentrations for the various mixtures gave the possibility of optimizing the separation times. (Compare Fig. 1 with Fig. 3).

We consider that similar selective ion pairing can also be used in other analytical problems.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE PIGMENTS

APPLICATION TO THE DETERMINATION OF UNCONJUGATED BILIRUBIN IN SERUM

C. K. LIM*, ROSALIND V. A. BULL and J. M. RIDEOUT

Division of Clinical Chemistry, MRC, Clinical Research Centre, Harrow, Middlesex HA1 3UJ (Great Britain)

SUMMARY

A rapid, sensitive and specific method for the determination of unconjugated bilirubin in serum is described. Reversed-phase chromatography on short-chain alkylsilica (C_2) or octadecylsilica (C_{18}) with acetonitrile–dimethylsulphoxide–water as eluent is used for the separation of bilirubin. The method is applied to the analysis of bilirubin in patients with disorders associated with jaundice.

INTRODUCTION

Bile pigments are tetrapyrroles derived from oxidative fission of porphyrin macrocycles[1]. Most natural bile pigments have the IXα configuration of side chains, arising by fission at the α-bridge of protoporphyrin IX. The compounds are classified primarily according to the extent of conjugation, ranging from the green or blue biliverdins to the colourless urobilinogens with no conjugation. The most important bile pigment is the yellow pigment bilirubin IXα.

The estimation of unconjugated bilirubin concentration in serum is important in the diagnosis, investigation and control of treatment of haemolytic anaemias or prehepatic jaundice[2,3], although in these conditions concentrations of total bilirubin are adequate. However, in haemolytic anaemia, if increased haemopoiesis fails to maintain adequate concentrations of haemoglobin, the anaemia may become sufficiently severe that anoxia causes liver damage and an increased concentration of conjugated bilirubin in the serum.

In posthepatic (obstructive) jaundice, serum bilirubin is almost wholly conjugated, as it may also be in the cholestatic jaundice, common in many patients with severe hepatitis. In hepatitis with less cholestasis, increased concentrations of unconjugated bilirubin appear to accompany the conjugated bilirubin although this may be due to the inadequacy of existing methods for the estimations. Although the proportions of conjugated and unconjugated bilirubin are probably of little significance in hepatitis, their estimation is necessary to distinguish the Dubin–Johnson and

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Rotor's syndrome from the pure unconjugated hyperbilirubinaemia of Gilbert's syndrome due to a defect in transport of unconjugated bilirubin and of the Crigler–Najjar syndrome in which there is a defect in the enzymic mechanism of conjugation. In the first two conditions the serum contains both conjugated and unconjugated bilirubin, the Dubin–Johnson syndrome being due to a defect in the excretion of conjugated bilirubin with no impairment of the conjugation mechanism while in Rotor's syndrome the abnormality is failure of uptake and storage of unconjugated bilirubin[2,3]. In the Dubin–Johnson syndrome haem granules are deposited in the liver.

In neonates, unconjugated hyperbilirubinaemia must not become high enough to permit entry of unconjugated bilirubin into the basal ganglia causing kernicterus. The need for urgent treatment must depend upon the concentration of non-protein bound unconjugated bilirubin in the serum. Although methods have been proposed for the approximate measurement of this bilirubin fraction, an accurate method for the estimation of unconjugated bilirubin with an estimation of the albumin concentration could be of great importance in assessing the need for urgent treatment.

Existing methods of estimating unconjugated bilirubin depend either upon the extent of the direct and indirect diazo reaction under specific conditions[4,5] or on solvent partition by which the unconjugated bilirubin passes into an organic solvent leaving the conjugated bilirubin in an aqueous layer[6]. These methods give conflicting results[7] and a more specific and reliable method is therefore needed.

Although high-performance liquid chromatography (HPLC) separations of bilirubin have been reported[8–10] its application to the analysis of bilirubin in serum has not been described. This paper describes a rapid, sensitive and specific method for the determination of unconjugated bilirubin in serum.

EXPERIMENTAL

Materials and reagents

Bilirubin was from Sigma (London, Great Britain). Acetonitrile was HPLC grade from Rathburn (Walkerburn, Great Britain). Tetraethylenepentamine (TEPA) was technical grade and dimethylsulphoxide (DMSO) was AnalaR grade from BDH (Poole, Great Britain).

Apparatus

Hypersil, Hypersil-SAS and Hypersil-ODS (Shandon Southern Products, Runcorn, Great Britain) were slurry packed. Except for the Hypersil column which was 250 mm × 5 mm I.D., the column dimensions were 100 m × 5 mm I.D. A Pye Unicam (Cambridge, Great Britain) LC3-XP liquid chromatograph with a LC3-UV detector was used. Injection was via a loop-value injector (Rheodyne 7210) fitted with a 20-μl loop.

HPLC

The mobile phases were thoroughly de-gassed (ultrasonication) before use. The separation of bilirubin isomers was performed on Hypersil-SAS with acetonitrile–DMSO–water (30:30:40) as the mobile phase and on Hypersil eluted with acetonitrile–water–TEPA (90:10:0.05). The IXα isomer was collected and used for the construction of the calibration curve.

The analysis of bilirubin in serum was carried out on Hypersil-ODS with acetonitrile–DMSO–water (40:40:20) as the eluent. A small pre-column packed with Hypersil-ODS was used to protect the analytical column. The flow-rate for all the separations was set at 1 ml/min and detection was at 450 nm. Serum (20 μl) or standard bilirubin solution in DMSO were injected directly. Due to the instability of bilirubin to oxygen and light, standard solutions (1–10 mg/dl) were freshly prepared and stored in the dark.

RESULTS AND DISCUSSION

Bilirubin occurs in nature as the IXα isomer and since commercial preparations contain small quantities of IIIα and XIIIα isomers the purification of the IXα isomer before the construction of a calibration curve is essential.

Separations of bilirubin isomers have been reported[9,10] but these required gradient elution and long analysis time. Fig. 1a shows the separation of bilirubin IIIα, IXα and XIIIα isomers by reversed-phase chromatography on Hypersil-SAS with acetonitrile–DMSO–water (30:30:40) as the eluent. The same separation is also obtainable on Hypersil-ODS with acetonitrile–DMSO–water (38:38:24) as the solvent. Alternatively a silica column can be used with acetonitrile–water–TEPA (90:10:0.05) as the mobile phase (Fig. 1b). The separation mechanism here is uncertain, but probably involves adsorption and partition as well as ion exchange. By careful adjustment of the acetonitrile–water ratio, the system is found to be useful for the separation of other bile pigments such as bilirubin conjugates, biliverdins, biliviolins, stercobilins, half-stercobilin and urobilins[11].

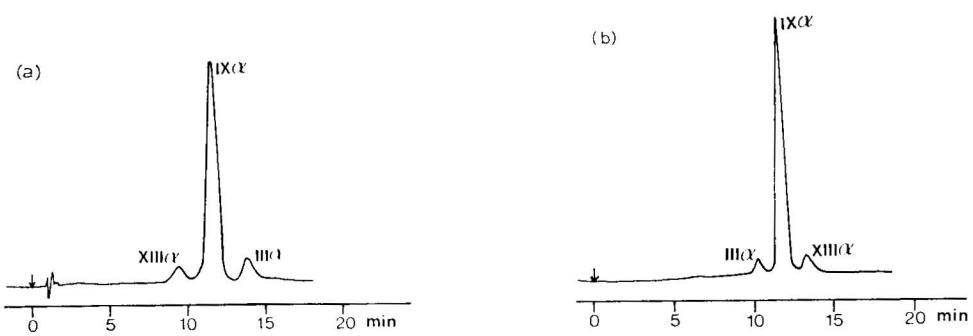


Fig. 1. HPLC separation of bilirubin isomers; (a) Column, Hypersil-SAS; mobile phase, acetonitrile–DMSO–water (30:30:40); flow-rate, 1 ml/min; detection, 450 nm; (b) Column, Hypersil; mobile phase, acetonitrile–water–tetraethylenepentamine (90:10:0.05); flow-rate, 1 ml/min; detection, 450 nm.

For the determination of bilirubin in serum, Hypersil-ODS was used. The mobile phase, acetonitrile–DMSO–water (40:40:20) was chosen to give fast analysis time (5 min) without the loss of resolution. Bilirubin is unstable in solution, being easily oxidised and is sensitive to light. Sample preparation by protein precipitation and solvent extraction was therefore avoided. Serum samples were injected directly and a pre-column was used to protect the analytical column from irreversible adsorption of serum proteins and other impurities.

Fig. 2a and b show the separation of bilirubin from standard solution and from serum of a patient with jaundice due to unconjugated hyperbilirunaemia respectively. Background contamination in the analysis of serum specimens from patients was never a problem as few compounds absorbed at 450 nm, the wavelength used for the detection of bilirubin.

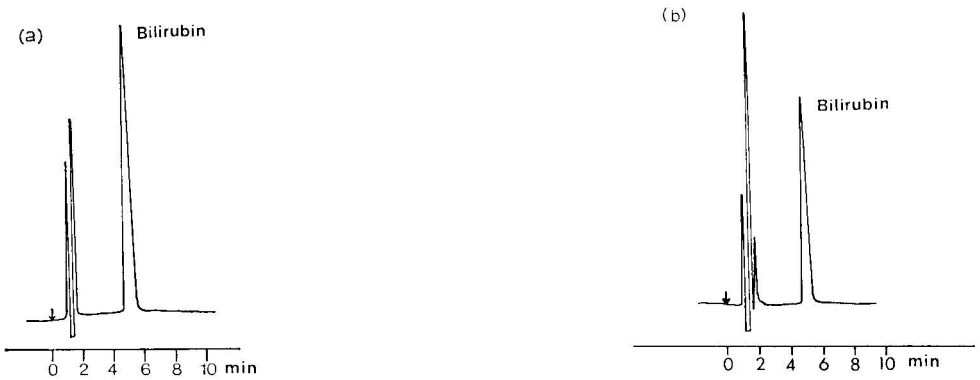


Fig. 2. HPLC separation of bilirubin IXα; (a) Standard; (b) in serum of a patient with unconjugated hyperbilirubinaemia: column, Hypersil-ODS; mobile phase, acetonitrile–DMSO–water (40:40:20); flow-rate, 1 ml/min; detection, 450 nm.

The recovery of bilirubin from a pooled serum sample was calculated by chromatographing the sample before and after adding a known amount of bilirubin. The recoveries (98–99%) were identical for standard bilirubin solutions prepared in DMSO and in a protein matrix[4]. The calibration curve over the range of 1–10 mg/dl was linear. From ten replicate analyses of a serum specimen the coefficient of variation was 2.0%.

The analysis of unconjugated bilirubin in serum of neonates treated with phototherapy[10] by this method always gave results which were lower than the diazo reaction[5] or direct spectrophotometric method. This is not surprising since photobilirubin present in these sera gives positive diazo reaction and spectrophotometry does not differentiate bilirubin from photobilirubin. The HPLC systems described separate bilirubin from photobilirubin.

In sera of patients not undergoing phototherapy and containing both unconjugated and conjugated bilirubin, the HPLC method often gave results for unconjugated bilirubin which were 2–3% higher than the diazo reaction method[5]. Unconjugated bilirubin is known to react with diazotised ethyl anthranilate[7]. This may account for the lower values obtained by the diazo reaction method.

Direct spectrometry measures only total bilirubin. It therefore cannot be used to determine unconjugated bilirubin in samples containing both conjugated and unconjugated bilirubin.

CONCLUSION

The simple reversed-phase procedure described for the rapid and specific determination of unconjugated bilirubin in serum should be of particular value in the

diagnosis and treatment of neonatal jaundice, especially in assessing the effectiveness of phototherapy in reducing serum unconjugated bilirubin.

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DETERMINATION OF 5-HYDROXYINDOLEACETIC ACID IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: A FULLY AUTOMATED METHOD

J. P. GARNIER, B. BOUSQUET* and C. DREUX
Hôpital Saint-Louis, Laboratoire Central de Biochimie, 2 Place du Docteur Fournier, 75010 Paris (France)

SUMMARY

High-performance liquid anion-exchange chromatography is combined with continuous flow spectrofluorometry at an optimalized pH for the determination of 5-hydroxyindoleacetic acid (5-HIAA) in urine. Its chief advantages are speed (7 min per determination), enabling measurement of large series of samples, precision (coefficient of variation = 0.72%), sensitivity (detection limit: 1 pmol) and specificity (no interference with 5-HIAA precursors or with the drugs tested). Reference values ($n = 112$) are 25 ± 20 μmol per 24 h. Comparison with a reference spectrofluorometric technique shows a correlation coefficient of 0.96.

INTRODUCTION

5-Hydroxyindoleacetic acid (5-HIAA), a major serotonin catabolite, is eliminated in the urine. In carcinoid tumours, 95% of which occur in the small intestine, very large quantities of serotonin are secreted by the enterochromaffin cells. Early detection of these tumours is essential since unless surgically treated in the initial stages, their prognosis is serious. Such detection is ensured by determination of blood serotonin and urinary 5-HIAA[1].

Systematic measurement of urinary 5-HIAA calls for a quick, reliable and automated technique. For this purpose, the α-nitrosonaphthol colorimetric method lacks specificity[2] and spectrofluorometry after ethyl acetate extraction is time-consuming and tricky[1,3]. Both these methods are manual. Low pressure chromatography techniques are time-consuming (5-HIAA retention time >30 min) and either insensitive or unreliable[4–6]. Reversed-phase separation chromatography also lacks sensitivity and specificity[7], except if preceded by manual extraction which is often time-consuming and reduces the overall precision. Recently, a new electrochemical detection method[8] has been described for tryptophan metabolites at the picomole level. However, 5-HIAA in urine must initially be isolated on extraction columns, eluted with a suitable solvent and then injected into a liquid chromatograph connected to an amperometric detector. This general approach might be of great interest in a research laboratory but not in routine clinical investigation. On the other hand, high-

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performance liquid chromatography (HPLC) allows finer separation but, for the principal methods described[9,10], prior extraction is always required especially when these methods are applied to 5-HIAA determination in urine. One technique[11] also uses UV detection which is not highly specific for 5-HIAA measurement.

We propose a fully automated method in which 5-HIAA is separated from the other metabolites by anion-exchange HPLC at pH 2.30; this permits fast, efficient separation. Continuous flow spectrofluorometric detection is carried out at pH 7.0, at excitation and fluorescence wavelengths of 301 and 338 nm respectively. Chromatography and detection are continuously coupled, in accordance with a process which was developed in our laboratory[12,13] and permits optimization of 5-HIAA pH readings. The use of an automated injector increases precision and allows large numbers of samples to be handled.

MATERIALS AND METHODS

Apparatus

A Chromatem 38 (Touzart Matignon) high-performance liquid chromatograph was connected to an AutoAnalyzer II peristaltic pump, fluorometer and recorder (Technicon) and Micromeritics 725 automated sampler and injector (Coultronics). A Whatman 10-μm Partisil SAX, column (250 $\times$ 4.7 mm I.D.) was used with a precolumn (150 $\times$ 4.7 mm).

Reagents and standard

The mobile phase, $5 \cdot 10^{-3}$ *M* $NH_4H_2PO_4$ adjusted to pH 2.30 with concentrated H_3PO_4, and the phosphate buffer solution, pH 7.0, 0.1 *M* (8.7 g/l $Na_2HPO_4 \cdot 2\,H_2O$ + 5.6 g/l KH_2PO_4), are stable for 3 months at +4°C. A sulphosalicylic acid solution (500 g/l) was prepared in distilled water. The 5-HIAA standard, 250 μM in 0.01 *M* HCl, is stable in a brown flask for 2 weeks at +4°C. It was prepared from a stock solution containing 2.5 m*M* 5-HIAA in 0.01 *M* HCl which is stable for 3 months at +4°C in a dark bottle. The internal standard, 2 m*M* N-acetyltryptophan (NAT) solution in 0.01 *M* HCl, may be kept in a brown flask for 3 months at +4°C.

Preparation and analysis of urine samples

Urine was collected over a 24 h period in 5 ml HCl (10 *M*). During the 24 h preceding this collection, the subjects were asked to not to eat foodstuffs such as

TABLE I

SAMPLING CONDITIONS FOR AN AUTOMATIC INJECTOR

Vortex stir for 15 sec.

	Volume (µl)			
	External standard	*Urine*	*Urine + 25 µM 5-HIAA*	*Urine + 50 µM 5-HIAA*
NAT (2 m*M*) (internal standard)	100	100	100	100
5-HIAA (250 μM)	50	—	50	100
Mobile phase	550	100	50	—
Acidified urine	—	500	500	500

bananas and grapefruits and to avoid drugs such as reserpine, fluorouracil, heparin, isoniazide, methyldopa, monoamine oxydase inhibitors, corticotrophin and metamphetamine, which affect the *in vivo* urinary concentration of 5-HIAA. A 5-ml volume of urine was acidified by adding 50 μl of sulphosalicylic acid solution. The preparation was then vortexstirred for 15 sec, centrifuged when necessary and filtered.

Determinations were made as follows. For standardization, each subject's urine was submitted to double overloading with known amounts of 5-HIAA in the presence of the internal standard (NAT). An external standard was used for each series of determinations for the purpose of verifying the fluorescence yield of overloading. When an automatic injector is available, the amounts indicated in Table I can be introduced directly into the sampling vials. A 50-μl volume of each sample was automatically injected every 7 min. The apparatus is represented in Fig. 1. The flow-rate was 1.50 ml/min, and an example of a urinary chromatogram is given in Fig. 2.

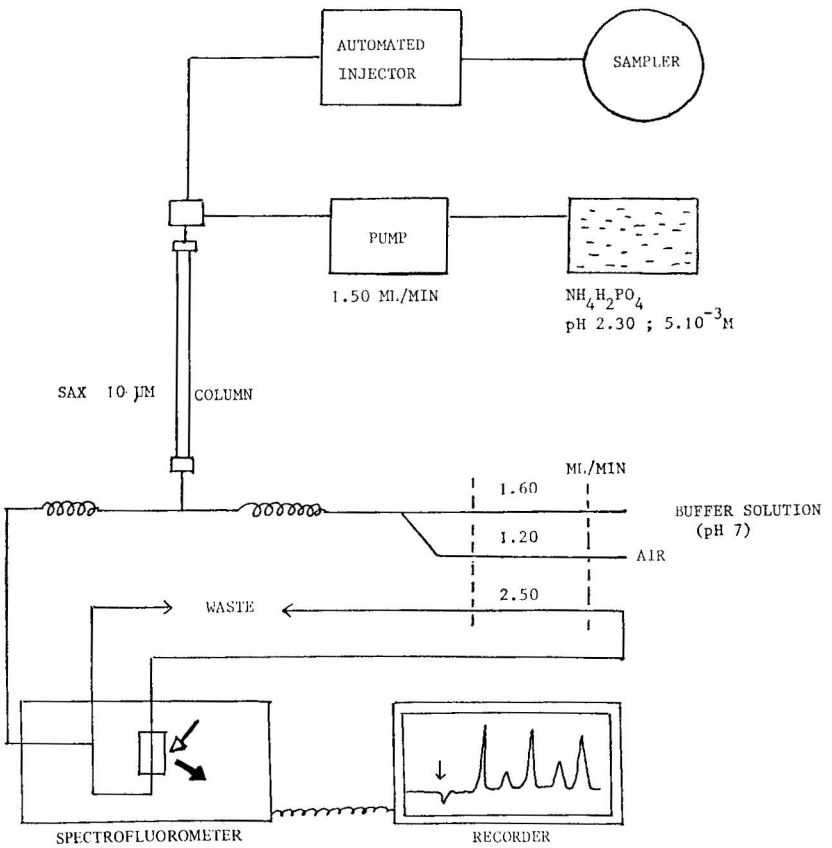


Fig. 1. Chromatographic and analytical system for fully automated determination of 5-HIAA, $\lambda_{ex.} = 301$ nm; $\lambda_{em.} = 338$ nm.

RESULTS AND DISCUSSION

Choice of experimental conditions

The pH and the concentration of the mobile phase were varied within limits

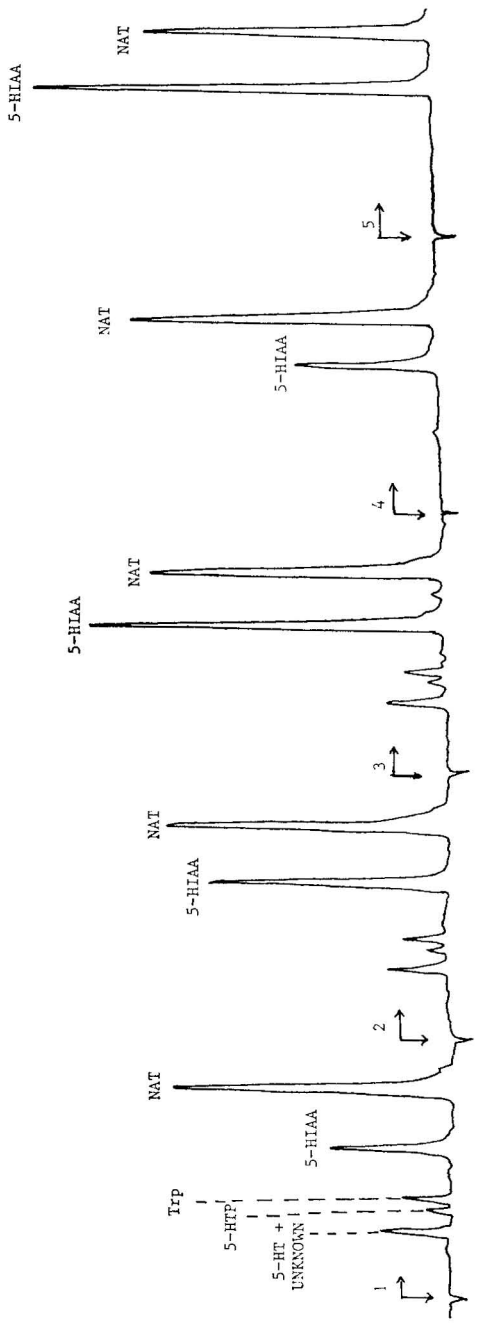


Fig. 2. Chromatograms of urine (1), urine + 25 μM 5-HIAA (2), urine + 25 μM 5-HIAA (3), 25 μM 5-HIAA standard (4) and 1 μl of urine from subject with carcinoid tumour (5).

compatible with the stationary phase, *i.e.*, from pH 1.50 to 7.00 and from 10^{-4} *M* to 1 *M*. Under the optimal conditions of pH 2.30 for a concentration of $5 \cdot 10^{-3}$ *M* 5-HIAA was well separated from its metabolites (tryptophan Trp; hydroxy-5-tryptamine 5-HT; hydroxy-5-tryptophan, 5-HTP) (Fig. 2).

The fluoroscence spectrum of 5-HIAA was verified with our apparatus and the excitation and emission maxima were found to be 301 and 338 nm respectively. The effect of pH was studied between 2 and 10, and the optimal pH was found to be 7.0, at which the fluorescence intensity of 5-HIAA was four times that at pH 2.30.

Chromatography requires the use of an internal standard to eliminate uncertainty regarding the volumes injected. The spectrofluorometric characteristics of NAT enable satisfactory detection under the conditions described above. Its retention time is 1 min 30 sec longer than that of 5-HIAA.

Fluorometric detection requires standardization by overloading. The overloading yield (R) was established for each determination. It enabled us to determine the presence of either a fluorescence activator ($R > 100\%$) or inhibitor ($R < 100\%$). In 99% of cases, the overload yield was between 90 and 105%. Minor interferences were then automatically corrected by calculating the overload.

Analytical results

The detection limit was 1 pmol, in accord with published results[6,7,9]. Linearity was satisfactory up to 250 μM, *i.e.*, about ten times the usual mean value. As regards within-run precision, 36 successive measurements of the same sample (28.5 μM) yielded the following coefficients of variation (C.V.): 2.90% for manual injection; 1.30% for automatic injection with no internal standard and 0.72% for automatic injection with an internal standard.

Sample preparation is simple and quick (a few minutes) and one sample can be injected every 7 min. Since injection is automatic, the apparatus requires no supervision.

The absence of contamination was verified by measuring low standards (5 μM) and high standards (100 μM). There was no significant difference ($p < 0.05$) between the low standard values, whether they preceded or followed the high concentrations.

The specificity of the 5-HIAA determinations was ensured by combining chromatographic separation with spectrofluorometric detection: chromatography permitted separation of 5-HIAA from its metabolic precursors present in urine, *i.e.*, Trp, 5-HTP and 5-HT (Fig. 2); spectrofluorometry was carried out at the wavelengths of the maxima and at the optimum pH for 5-HIAA.

The extent of interference was tested on certain drugs, particularly those interfering in the α-nitrosonaphthol technique. In each test the drug was added to urine at a concentration corresponding to the maximum therapeutic dose; 6 g/l acetylsalicylic acid; 150 mg/l promethazine; 200 mg/l chlortetracycline; 2.5 g/l sulfamethoxazole; 150 mg/l chlorpromazine and 100 mg/l prochlorperazine. At these concentrations, no significant interference was shown, unlike that observed with the colorimetric method.

In order to compare our direct method with a spectrofluorometric reference method, 68 determinations of urinary 5-HIAA were made by a manual spectrofluorometric technique used in our laboratory[3], preceded by extraction. A statistical

study showed a good correlation: correlation line, $y = 0.89\,x + 1.88$; correlation coefficient, $r = 0.96$; t (Student) = 38.9; theoretical $t = 3.44$ ($p < 0.001$). Reference values, determined in 112 healthy subjects, were 25 ± 20 μmol/24 h (mean $\pm$ 2 σ, $p < 0.005$), in accord with published values[1,2,4,5,14–17].

Urinary 5-HIAA was also determined in nineteen cases of carcinoid tumours. Values ranged from 94 to 4350 μmol per 24 h. A typical chromatogram is shown in Fig. 2. Results were excellently correlated with the reference technique[3] and agreed with clinical and anatomopathological observations.

CONCLUSION

High-performance liquid chromatography combined with continuous flow fluorometric detection at an optimized pH permits quick and specific measurement of urinary 5-HIAA. Since the method is both completely automated and simple, it can be used for systematic determinations in carcinoid tumour detection. Its specificity enables ethyl acetate extraction to be avoided and its precision and sensitivity make it a particularly reliable method for use in clinical biochemical laboratories.

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METHOD FOR QUANTITATIVE DETERMINATION OF THE FOUR KNOWN JUVENILE HORMONES IN INSECT TISSUE USING GAS CHROMATOGRAPHY–MASS SPECTROSCOPY

B. JOHN BERGOT*, MILTON RATCLIFF**, and DAVID A. SCHOOLEY*
Zoecon Corporation, 975 California Avenue, Palo Alto, CA 94304 (U.S.A.)

SUMMARY

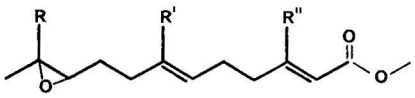
A rapid, specific, and sensitive method is described for the extraction, purification, and quantitative determination of the three known juvenile hormones in insect tissue, using combined gas chromatography–mass spectroscopy (GC–MS) in the electron impact mode with selected ion-monitoring (SIM) for detection. A fourth, new, naturally occurring juvenile hormone was detected (and subsequently identified by other means) in this way. After initial extraction of biological material, a fast and efficient purification sequence utilizing non-aqueous reversed-phase and alumina mini-column chromatography achieves quickly a 10^3–10^4 purification factor. Derivatization for analysis by acid-catalyzed methan-d_3-olysis of the 10,11-epoxide moiety, followed by liquid chromatography separation of the resultant 11-methoxy-d_3-10-hydroxy (methoxyhydrin) derivatives, completes the sample workup. The methoxyhydrins are then directly analyzed by GC–MS (SIM). A tritium-labeled analogue is added during the extraction as an internal standard, which allows determination of recovery for each sample and simultaneously assures quantitative accuracy. Recoveries are 75–80%, and detection limits of 10–40 pg per gram of tissue are normally achievable.

INTRODUCTION

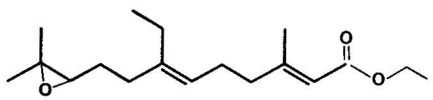
Several chemical or spectral methods currently exist for the isolation and simultaneous quantification of the insect juvenile hormones (JH) 2–4. Using gas chromatography–electron capture detection (GC–ECD) Bergot *et al.*[1] and Peter *et al.*[2] were able to identify the JHs from insect tissue after appropriate derivatization to electrophoric aromatic esters. More recently, Hagenguth and Rembold[3], and Huibregtse-Minderhoud *et al.*[4] employed much the same methodology, using a heptafluorobutyryl derivative for ECD. Earlier, Van Broekhoven *et al.*[5] developed an ECD method, applicable to JH III only, employing a bis-heptafluorobutyryl ester derivative of the JH diol for detection. Although these methods offer excellent absolute sensitivities, sample preparation is lengthy and tedious, with moderately low

* Present address: International Plant Research Institute, San Carlos, CA, U.S.A.
** Present address: Nelson Analytical, Cupertino, CA, U.S.A.

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1 $R = R' = R'' = C_2H_5$ (JH 0)
2 $R = R' = C_2H_5$, $R'' = CH_3$ (JH I)
3 $R = C_2H_5$, $R' = R'' = CH_3$ (JH II)
4 $R = R' = R'' = CH_3$ (JH III)



5 (IS)

recoveries. More importantly, the lack of sufficient specificity of detection inherent in electrophoric derivatization schemes necessitates analysis on several packed (or capillary) columns before a final quantitative and qualitative determination of JH can be made with any degree of confidence.

JH determination by GC–MS, on the other hand, offers better selectivity of detection and requires less derivatization and cleanup. The first such method reported by Bieber *et al.*[6] suffered from low sensitivity and was applicable to JH I only. Subsequently, Trautmann *et al.*[7] developed a method of analysis for JH I, II, and III with a good limit of detection (0.1 ng/g), but requiring kilogram samples of insect tissue. Recently Mauchamp *et al.*[8] described a method of analysis for JH I with good sensitivity, but requiring selected ion-monitoring (SIM) of three ions for JH I due to a relatively low degree of sample cleanup. Lanzrein *et al.*[9] reported an isolation procedure for JH I–III from cockroach hemolymph using chemical ionization (CI) GC–MS for qualitative identification. Recently Rembold *et al.*[10] reported a procedure using glass capillary GC–MS to detect JH derivatives formed by solvolysis of the epoxide moieties with nonafluorohexanol; the procedure affords excellent sensitivities with small samples and apparently requires rather few purification steps, though full experimental details were absent. We now wish to report full procedural details of an improved assay using GC–MS, offering a high degree of specificity and sensitivity, for the simultaneous identification and quantification of JH I, II, III, and the recently discovered[11] JH 0, on the sub-picomole level. We require a derivatization step which converts JHs to their 11-methoxy-d_3-10-hydroxy (MH) derivatives in good yield. Final analysis of the purified MHs uses a GC–quadrupole mass spectrometer in the SIM mode. We add a tritiated analogue of JH on tissue extraction for use as an internal standard for quantitative purposes, and for monitoring recoveries at any stage of the workup. Because of sensitivities attained, only gram-quantities of insect tissue are required for the assay.

This method has now been tested with hundreds of samples from several insect species, and has proven both remarkably specific for JH detection and general in its applicability. The versatility of this method is most vividly illustrated by our ability to detect, then subsequently identify by other means, the new, naturally occurring juvenile hormone, JH 0, the details of which are reported elsewhere[11].

MATERIALS AND METHODS

Reagents

Acetonitrile was "pesticide grade, distilled-in-glass" (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); pentane (technical, Phillips Petroleum, Bartlesville, OK, U.S.A.) and diethyl ether (analytical reagent, Mallinckrodt, St. Louis, MO, U.S.A.) were distilled through a glass helix-packed column before use. Dichloromethane (analytical reagent, Mallinckrodt) was purified as previously described[1]. Methanol-d_4 (99.9%) and trifluoroacetic acid (Aldrich, Milwaukee, WI, U.S.A.) were used as received. Neutral alumina (Woelm, Eschwege, G.F.R.) was heated to 600°C overnight, then supplied with 6% of its weight in water to bring the absorbent to activity III. Sodium chloride and Celite (Johns-Manville, Denver, CO, U.S.A.) were heated as above before use. All glassware was rinsed with deionized water, and heated at 500°C for 8–16 h before using for trace analysis.

Thin-layer chromatography

All thin-layer chromatographic (TLC) separations were performed on 5 × 20 cm, 0.5 mm thick, pre-coated plates of silica gel GF (Analtech, Newark, DE, U.S.A.). Plates were pre-washed by development in methanol and air-dried before use.

Liquid chromatography

Liquid chromatography (LC) was performed on a modular unit as previously described[1]. The column was prepacked silica gel (μPorasil, 30 × 0.4 cm I.D., Waters Assoc., Milford, MA, U.S.A.)*, and the eluting solvent was 3.4% diethyl ether in dichloromethane, 50% water-saturated; flow-rates were 1.5 ml/min at 24 atm, with peak detection by UV at 254 nm and 0.16–0.32 a.u.f.s.

Gas chromatography–mass spectroscopy

GC–MS was carried out on a Hewlett-Packard Model 5985 quadrupole mass spectrometer–computer system (electron impact, 70 eV, ion source temperature 200°C, emission current 300 μA, electron multiplier potential 2400–3000 V), using the appropriate SIM program. Other instrument operating parameters were optimized using perfluorotributylamine (PFTBA) standard. GC columns were 1.8 m × 2 mm I.D. packed with either 3% OV-225 Chromosorb W AW DMCS or Ultra-Bond II. (Supelco, Bellefonte, PA, U.S.A.). Separations were performed using a programmed temperature mode (190–230°C, Ultra-Bond II, 215–240°C, OV-225) at 4°C/min. Total time for elution was 5–6 min. Earlier a Hewlett-Packard Model 5984 GC–MS–data system unit was employed for titer determinations. Serious problems were encountered with peak tailing and inadequate precision and reproducibility, attributed to an inherently reactive interface between the chromatograph and the spectrometer, as well as inadequate ion source temperature control.

* We find that a LiChrosorb SI 100 column (25 × 0.46 cm, Brownlee Labs., Santa Clara, CA, U.S.A.) is directly interchangeable with μPorasil. No modification of mobile phase or marker compounds is required, and higher column efficiency is obtained.

Standards

JH 0, I, II, and III were obtained from the Zoecon Chemical Research group and purified by micro-preparative LC. Methyl (2*E*)-6,7-epoxy-3,7-dimethyl-oct-2-enoate (epoxy methyl geranoate, EMG) was prepared by epoxidation with *m*-chloroperbenzoic acid of methyl (2*E*,*Z*)-geranoate (gift of Dr. R. J. Anderson, Zoecon), followed by preparative LC to remove the (2*Z*)-isomer.

N,N-Diethyl-N′-phenylurea and N-*sec*.-butyl-N′-phenylurea, the LC marker compounds, were obtained by reaction of the appropriate amine with phenylisocyanate, and recrystallization of the crude product.

Internal standard

Both tritiated and non-labeled samples of the internal standard were prepared from a common intermediate using a scheme adopted from a stereoselective synthesis of JH I (ref. 12). 2,2-Dimethoxy-3-chloro-3-methylbutane, prepared as described[13], was reacted with 0.1 equivalent of methyl (2*E*)-6-hydroxy-3-methyl-7-methylene-2-nonenoate (gift of Dr. R. J. Anderson, intermediate 3 in ref. 12) with acidic catalysis, in refluxing toluene[12]. The product, methyl (2*E*,6*E*)-11-chloro-3,11-dimethyl-7-ethyl-10-oxo-2,6-tridecadienoate, was purified first on a silica open column eluted with 10% diethyl ether in hexane, and then rigorously purified by preparative LC (Zorbax SIL 22 × 0.79 cm, eluted with 3% diethyl ether in pentane at 9.8 ml/min). A portion of this pure chloroketone was reduced with excess $NaBH_4$ in methanol to a chlorohydrin, which was isolated and treated subsequently with dipotassium carbonate in methanol[12] to give the desired epoxide, methyl (2*E*,6*E*)-10,11-epoxy-3,11-dimethyl-7-ethyl-2,6-dodecadienoate, an isomer of JH II ("iso-JH II").

To prepare the labeled sample of iso-JH II, the above chloroketone was reduced with NaB^3H_4 (performed by New England Nuclear, Boston, MA, U.S.A.). The labeled chlorohydrin spontaneously cyclized during the reduction (apparently due to contamination of the NaB^3H_4 with base) to give the [10-^{3}H]epoxide directly. The crude labeled epoxide was purified by TLC (15% ethyl acetate in hexane, single development) to remove polar radio-labeled impurities.

Samples of nonlabeled and [10-^{3}H]iso-JH II were transesterified by treatment with dry ethanol in the presence of sodium cyanide according to Mori *et al.*[14] to afford the internal standard (IS, 5), iso-JH II ethyl ester. Each sample was first purified by TLC (as above), then by LC (Zorbax SIL, 22 × 0.46 cm, eluted with 7% diethyl ether in pentane). Final chemical and radiochemical purities were ⩾99%, as determined by LC using the above conditions.

The specific activity of [10-^{3}H]IS was determined by methane CI mass spectroscopy in the SIM mode, monitoring ions at *m/z* 323 $(M + C_2H_5)^+$, *m/z* 249 $(M + H - C_2H_5OH)^+$, and their corresponding isotopic ions at *m/z* 325 and 251. The ratio of ^3H:^1H was determined as 0.234, corresponding to a specific activity of 6.78 Ci/mmol (51,000 dpm/ng).

Preparation of methoxy-d_3-hydrin standards

Milligram amounts of JHs and IS were converted to the respective MH derivatives by stirring in 0.08 *M* d_4-methanolic trifluoroacetic acid (1.5 h, 20°C), quenching with 10 μl of 2 *M* aqueous potassium hydrogen carbonate, and purifying

by TLC. Standard solutions, in acetonitrile, were then obtained by serial dilution of stock preparations, and stored over sodium hydrogen carbonate at −20°C.

Insects

Colonies of the tobacco hornworm, *Manduca sexta*, and the tobacco budworm, *Heliothis virescens*, were reared on artificial diets for many generations. The diet for the former was prepared according to Yamamoto[15], while the diet for the latter was based on lima beans and split peas. Specimens from carefully synchronized stages were selected for workup and chemical assay.

Extraction, purification, derivatization and analysis of JH from insect tissue

Hemolymph processing. A volume of 3–6 ml of hemolymph is collected in increments in a chilled 13-ml centrifuge tube containing ≈5 ml acetonitrile and the requisite amount of [^{3}H]IS. The tube is agitated on a Vortex mixer between incremental additions of hemolymph, then briefly centrifuged to sediment precipitated solids. The supernatant is transferred to a separatory funnel containing 40 ml of pentane and 150 ml of 4% aqueous sodium chloride, and the tube contents then extracted three times with 5 ml of acetonitrile. The combined supernatants are added to the separatory funnel, shaken for a few minutes, the aqueous layer removed, and the organic phase washed with a 50-ml portion of brine. Upon solvent removal of the separated organic layer, an oily pentane-soluble residue (2–30 mg) is obtained, which is transferred in pentane to a 1-ml conical bottom vial (Microflex tube, Kontes, Vineland, NJ, U.S.A.). After solvent removal, the contents are vigorously agitated with 5 × 100 µl of acetonitrile, and each portion of acetonitrile is pipetted onto a pre-washed (5 ml acetonitrile) C_{18} Sep-Pak cartridge (Waters Assoc.). After the cartridge is loaded, 2.2 ml of acetonitrile is pumped through by means of a 2500-µl gas-tight syringe (Hamilton Reno, NV, U.S.A.). The eluent, containing the JH fraction (≤1 mg), is concentrated to dryness, then redissolved with a small (200 µl) volume of pentane.

The pentane solution is carefully transferred —with rinses— to a small (6 × 40 mm) glass column fashioned from a Pasteur pipet which is packed with 1.0 g alumina (Woelm, activity grade III), previously washed with ether. A 2.0-ml portion of diethyl ether is then percolated through the column, and the eluent, containing the JHs and IS is bulked and reserved for subsequent methanolysis.

The residue is supplied with ≈20 µg of EMG diluent, and the derivatization carried out in 150 µl of 0.08 *M* d_3-methanolic trifluoroacetic acid. Reaction conditions are as described for *Preparation of methoxy-d_3-hydrin standards*, exept after quenching all solvent is evaporated under vacuum. Ether is added and evaporated (to remove residual methanol), the residue is rinsed several times with ether, and the washings (filtered if necessary through a pre-washed plug of cotton) are evaporated with a stream of nitrogen, then supplied with 1–2 µg of the phenylurea markers, redissolved in dichloromethane and chromatographed on LC as described. The MH zone is collected, concentrated, and the MHs are transferred to 1-ml Microflex tubes by acetonitrile rinsing for GC–MS analysis. Usually 10–20% of a 40 µl solution is then injected on-column.

Whole-body processing. An amount of 5–15 g of insects, anesthetized with carbon dioxide if necessary, is placed in a 100-ml blending flask containing ≈40 ml acetonitrile and an amount of Celite equal to ≈1/3 of the sample weight of insects.

The IS is added, the glass homogenizer cup chilled in an ice bath, and the contents then homogenized using a high-speed blender (VirTis Model 23) for 3–5 min. The homogenate is filtered through a Buchner-type fritted glass funnel with 14/20 ground joint (Ace Glass 9439-08), and the cake is returned to the cup for a second homogenization. The combined filtrates are evaporated *in vacuo* (cautiously to avoid bumping or foaming) to $\approx$20 ml, then decanted into a separatory funnel for the pentane-brine partition. Further processing of the recovered lipoidal mass (usually 20–150 mg) is as described under *Hemolymph processing*.

Quantification. Titer data were derived from peak area values using an internal standard technique. A response factor (K), reflecting differences in ionization efficiencies among the various species of interest, was calculated for each JH from the following formula: $K = A_{JH}/A_{IS} \times W_{IS}/W_{JH}$, where A is the peak area and W a known mass of the MH derivative of the JH and the internal standard, respectively. Titer levels were subsequently calculated from the relationship $[C_{JH}] = A_{JH}/A_{IS} \times [C_{IS}]/K$, where $[C]$ is the concentration expressed in ng/g or ng/ml, derived from the known amount of IS added to the original mass (or volume) of insect tissue.

RESULTS

Isolation and purification

Because of the inherent specificity of the ion-monitoring detection method, we were able to develop a substantially shortened extraction and purification protocol essentially based on that utilized in our earlier GLC–ECD procedure[1]. Fig. 1 depicts the flow scheme for tissue processing that we ultimately have refined. The excellent

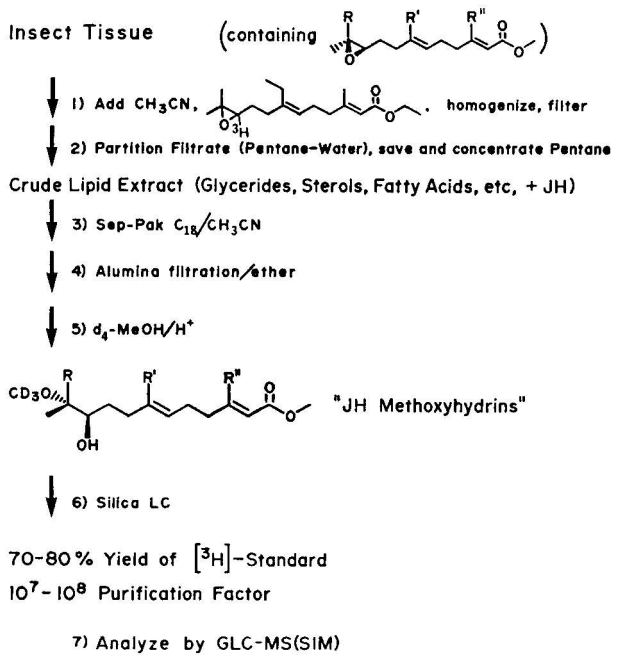


Fig. 1. Flow diagram for JH analysis by GC–MS (SIM) of insect tissue.

sensitivity of the method permits small sample sizes, minimizing the amounts of extractable material one must process.

We found that the combination of acetonitrile extraction of the crude lipoid fraction, followed by C_{18} non-aqueous reversed-phase chromatography quite efficiently separates JHs, fatty acids, and materials of similar molecular weight from the bulk of the sterols and di- and triglycerides which usually comprise the major portion of the pentane-soluble extract. It was necessary to include a final alumina filtration of the underivatized JHs in order to remove fatty acids. Certain insect samples were analyzed without the latter step; some gave apparently reliable results while others showed severe interferences. These interferences with one sample were found to be due to a 5- or 6-hydroxy fatty acid which was partly converted to a d_3-methyl ester during methan-d_3-olysis. The alumina step easily removed these impurities.

Finally, excellent selectivity of separation was achieved via LC using the dichloromethane-based system. Fig. 2a illustrates the LC profile of JH-MH standards and phenylurea markers, while Fig. 2b shows a typical chromatogram of a crude MH preparative cleanup from a hemolymph extract. Note the plethora of UV-absorbing substances eluting before the JH-MH zone. The fast-eluting region (forerun) contains even more extraneous material from whole-body workups, and the elution profile is indicative of the chromatographic selectivity obtained *vis-a-vis* the desired compounds of interest.

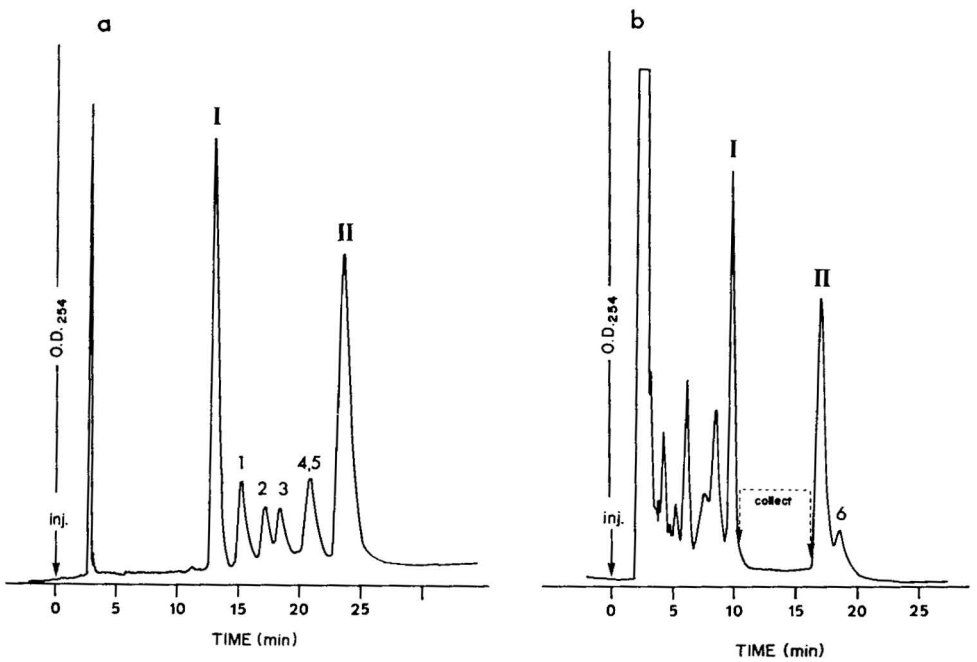


Fig. 2. LC chromatograms of (a) phenylurea markers and JH methoxyhydrin (MH) derivatives, and (b) derivatized JH extract from *Manduca sexta* Vth instar larval hemolymph. Peaks: I = N,N-diethyl-N′-phenylurea; II = N-*sec.*-butyl-N′-phenylurea; 1 = JH 0 MH; 2 = IS MH; 3 = JH I MH; 4, 5 = JH II and JH III MH; 6 = methyl geranoate MH. Conditions: column and solvent system as described in Materials and methods; flow-rate (a) = 1.3 ml/min; (b) = 1.9 ml/min; detector (254 nm) attenuation = 0.16 a.u.f.s.

Mass spectroscopy

Fig. 3 shows the electron impact mass spectrum of the IS methoxy-d_3-hydrin. The base peak, *m/z* 76, results from scission at C-10, producing the fragment $CD_3O^+C(CH_3)_2$. The latter carries 32% of the total ion current (TIC). An even higher value is obtained for the base peak of JH III MH (38%) while the analogous base peak of the MH derivatives of JH 0, I and II, *m/z* 90 [$CD_3O^+C(CH_3)(C_2H_5)$] carries 37, 47 and 47%, respectively, of the TIC. The substantial intensity of these fragments allows full exploitation of the inherent sensitivity of the SIM technique, in contrast to attempts to apply the SIM mode to electron impact analysis of underivatized JH[6].

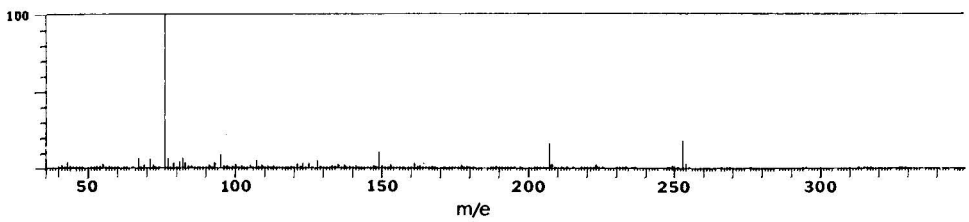


Fig. 3. Reference electron impact mass spectrum of internal standard (5) MH derivative.

A response factor *K* must be applied to reflect discrepancies between the base peak current for each of the JH–MH fragments. In turn, *K* is a direct function of instrumental operating parameters, and —for such reason— is calculated anew for each day's series of titer analyses. The basic instrument tuning parameters were obtained through the use of the H–P AUTOTUNE program; the sensitivity was further enhanced by appropriate adjustments of voltage parameters affecting the low mass end of the spectrum, namely, monitoring PFTBA fragments at *m/e* 69, 100 and 131 while optimizing their relative abundances. This technique biases the instrument's mass sensitivity toward the low mass end (within which the JH–MH peaks lie) to the detriment of higher masses, producing a gain in sensitivity of 3–4×.

Accuracy and precision of the method

Mass spectroscopic linearity studies were performed using standard solutions of the MHs, comprising a constant weight of IS (0.65 ng/ml) admixed with JHs whose weight ratios *vs.* the IS ranged from 1:50 to ≈ 40:1 (*i.e.*, a range of 2000-fold). Fig. 4 shows the excellent fit obtained for JH 0, as an example. Identical linearity characteristics were found for the other substrates, though not shown on the graph.

The accuracy of the method was determined by adding known amounts of JH standards to acetonitrile solutions containing 2.4 ng (≈0.48 ng/ml-equivalents) of IS, and working up as in Fig. 1. Table I illustrates the results obtained for levels equivalent to ≈ 6, 0.6 and 0.06 ng/ml. Acceptable correlations were found, though there was significant deviation from theoretical values in JH 0 at the 0.05 ng/ml level.

The precision of the method was checked further in the following manner: pools of insect hemolymph or whole bodies (*Manduca sexta*) were gathered from stages in which the JH titers were previously determined to be "high" (0.5–1.5 ng/ml or g)[16], and from stages having "low" titers (≤0.05 ng/ml)[16]. These were each divided into three equal portions, separately carried though the workup, and analyzed by

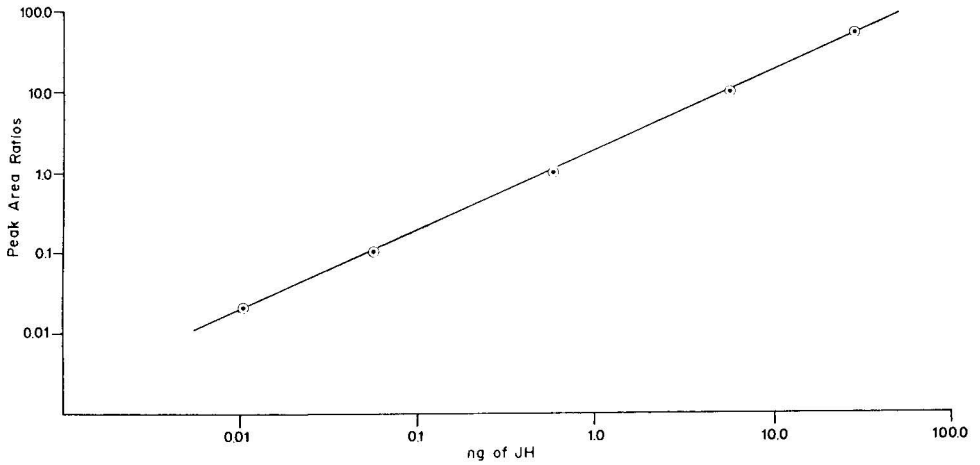


Fig. 4. Mass spectroscopic linearity-of-response curve for MH derivatives of JH 0 (0.011–28.5 ng) *vs.* IS (0.65 ng). Each point represents a minimum of three determinations.

TABLE I

ACCURACY OF METHOD

Data are expressed in ng/ml. Values are the mean of six determinations, except for JH 0 (values are the mean of three determinations).

	JH I			*JH II*		
Added	0.058	0.58	5.80	0.060	0.60	6.00
Found	0.067	0.64	5.84	0.065	0.62	5.90
Difference (%)	15.5	10.3	0.7	8.3	3.3	1.7
	JH III			*JH 0*		
Added	0.062	0.62	6.20	0.065	0.54	5.40
Found	0.066	0.61	5.95	0.086	0.64	5.67
Difference (%)	6.5	1.6	4.0	59.2	18.5	5.0

GC–MS (Table II). The data show reasonably good coefficient of variation (95% confidence limit) values for the "high level" replications (4.4–11.8%), while much higher values were obtained for the "low levels" (27–115%). However, it must be noted that the latter titer values are only 2–3× above our limits of detectability, and at such low levels (≈0.02 ng/ml) one would anticipate substantial inter-sample variation.

Recoveries

Through the aid of the tritiated IS, we were able to determine recoveries at any point in the workup scheme. Overall recoveries were virtually identical for "high level" *vs.* "low level" analyses: the recovery of IS added at 0.7 ng/ml (4.2 ng total) averaged 76%, while that at 0.043 ng/ml (0.26 ng total) averaged 78.5%. The latter data, in concert with standard recovery determinations, fully support our method of calculation of titers, which assumes identical losses in workup for endogenous JH and IS. For all samples, our recoveries were in the range of 75–80%.

TABLE II

PRECISION OF ANALYSIS AT VARIOUS TITER LEVELS, DETERMINED BY TRIPLICATE ANALYSIS OF EXTRACTS FROM THREE DIFFERENT PHYSIOLOGICAL STAGES (A, B, AND C) OF *MANDUCA SEXTA*

	Assay	*Mean (ng/g, ml)*	*Mean std. deviation ($\bar{S}_x$)*	*Coefficient of variation**
JH I	A	0.021	0.024	115
	B	0.17	0.020	11.8
	C	0.50	0.026	5.2
JH II	A	0.012	0.012	100
	C	0.71	0.031	4.4
	B	1.12	0.085	7.6
JH III	A	Undetectable		
	B	0.03	0.017	56.7
	C	0.043	0.012	27.2

* 95% confidence limit.

Biological applications

Hemolymph and/or whole animal samples from several insect species were processed to determine the general applicability of the method (Fig. 5a–c). Attention is drawn to Fig. 5b, wherein barely detectable levels of JH were found. Previous titer data reported for *M. sexta* mid-Vth instar larvae by bioassay[17] suggest JH levels at or below 0.05 ng/ml hemolymph; our data seem to confirm these observations. Fig. 5c shows SIM analysis of tissue extracts from an insect species (*Heliothis virescens* adult females) which contains "high" levels (1–2 ng/g) of JH I and II, and undetectable levels ($\leqslant$0.03 ng/g) of JH III. The *Heliothis* titer data confirms earlier *in vitro* studies by a Zoecon group[18], who found essentially the same qualitative ratio of JHs based on LC analysis of radiolabeled products from extracts of culture medium containing adult female *Heliothis* corpora allata incubated in the presence of [methyl-^{14}C]-methionine.

Procedural blanks are normally run concurrently with a series of 5–6 samples; background levels are determined at retention times corresponding to JH 0, I, II, and III derivatives, and these values are then factored into the computation program (see Discussion).

DISCUSSION

A particular shortcoming of all GLC–ECD assays[1–5] for JHs has been the need for two derivatization steps (to provide electron-capturing functionality), followed by extensive chromatographic purification in order to remove a host of both endogenous and introduced electron-capturing impurities. Specificity of detection, however, is still low even after rigorous cleanup. The specificity of detection inherent in MS, though, has enabled us to: (a) simplify and shorten heretofore cumbersome and time-consuming purification procedures; (b) use small sample sizes, thereby "miniaturizing" workup procedures, and sparing insect colonies of severe depletion due to assay requirements; (c) achieve desired sensitivities for low level analysis [at

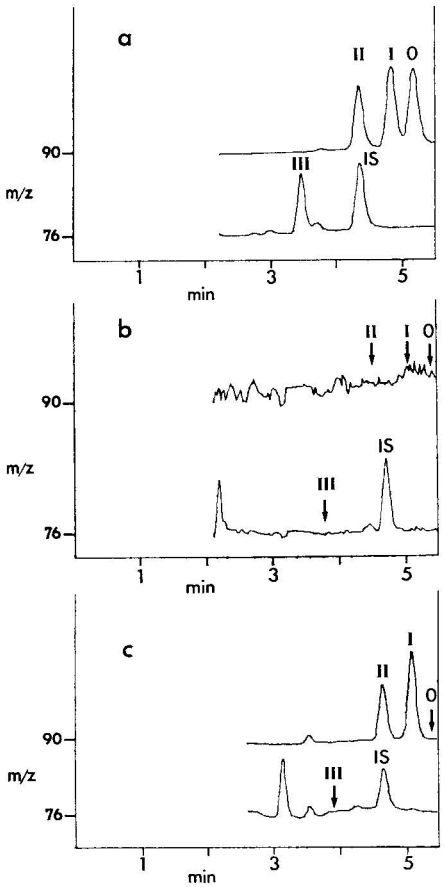


Fig. 5. Selected-ion chromatograms of (a) JH and IS MH standards (upper trace = m/z 90: JH II, I and 0; lower trace = m/z 76: JH III and IS), (b) analysis of *Manduca sexta* Vth instar larval hemolymph extract. The IS is present at 0.5 ng/ml of hemolymph, and no JH can be detected above $\approx$0.01 ng/ml, and (c) analysis of *Heliothis virescens* 0–24-h old adult female whole-body extract. IS = 0.76 ng/g; JH II = 1.4 ng/g; JH I = 2.1 ng/g; JH 0, JH III $\leq$0.02 ng/g. Analytical conditions: 2 m × 2 mm 3% OV-225 GC column programmed from (a) 215 to 250°C, or (b, c) 220 to 260°C, at 4°C/min, helium carrier at 40 ml/min, with a MS detector actuation delay of 2–2.4 min.

or below 0.01 ng/g (ml) (0.01 ppb, or $4 \cdot 10^{-11}$ M)] by "eliminating" interfering impurities through mass detection selectivity; and (d) obtain titer data of high confidence rapidly and accurately. Crucial to the success of this method is our development of a simple, streamlined cleanup procedure that does not require time-consuming low-temperature precipitation, molecular distillation, or preparative TLC purifications previously used for delipidation of JH-containing extracts. This has been achieved through a highly efficient and selective combination of acetonitrile extraction, nonaqueous reversed-phase filtration chromatography, and alumina mini-column chromatography. These rapid methods afford complete removal of glycerides, sterols, fatty acids, and hydrocarbons. The only additional purification after methan-d_3-olysis is an experimentally facile LC separation, wherein a broad zone of eluate is collected between the limits defined by two UV-active marker compounds.

In principle one could further extend the specificity of detection by monitoring a second or third ion fragment (*e.g.*, *m/z* 253 and 207 for IS–MH, Fig. 3); however, this significantly reduces overall sensitivity of itself, and one would at one point have to monitor more than four ions simultaneously, which is beyond the capability of our data system. Indeed, some methods[8] require monitoring several ions simultaneously for each JH, to compensate for a substantially lower degree of purification than that achieved in our assay.

We also evaluated CI mass spectroscopy in the SIM mode for detection, utilizing methane as reagent gas. However the base peak in the CI mass spectra of MH derivatives bears only about 20% of the TIC (about half the value found in the EI spectra). Also, a separate ion must be monitored for each JH (total of four); we encountered severe baseline difficulties due to column bleed in the 250–350 a.m.u. range, in which lie the ions of interest. These factors combine to reduce sensitivity and signal-to-noise ratio. Further it is generally found that protracted operation in the CI mode increases the rate of contamination of the ion source, thereby reducing sensitivity. Because of these considerations, we have not employed CI. In a different procedure, Mauchamp *et al.*[8] used CI with ammonia reactant gas for analysis of JH I as the underivatized epoxide.

We chose deuterated methanol as our derivatizing reagent, in preference to methanol, in order to raise the base peak of the analytes by 3 a.m.u. from *m/z* 73 and 87 to *m/z* 76 and 90. This avoids a need to monitor the troublesome *m/z* 73 ion, which is a high background ion from silicone stationary phases (trimethylsilyl ion). Monitoring an even mass ion is also advantageous due to their lower abundance in most spectra. We examined the suitability of perdeutero-ethanol to give a higher mass ion where background is lower still, and could obtain the analogous ethoxy-d_5-hydrin upon acid-catalyzed solvolysis of the juvenile hormones (ethoxy-d_5-hydrin base peaks at *m/z* 92 and 106). However, the ethanolysis was slower and very susceptible to "poisoning" by the presence of water, producing the diol competitively. Also, the selectivity of separation on LC was somewhat compromised by the decrease in polarity of the ethoxyhydrins *vs.* fast-eluting impurities.

The most appreciable loss in recovery occurs during methanolysis. The capricious nature of mineral acid-catalyzed solvolysis of the JH epoxide moiety on a micro scale is well documented[19,20]. With picomoles of substrate the acid is not a "catalyst", but actually an excess reagent. Difficulties with this hydration reaction provoked the development of enzymatic hydration[21] and weak acid-catalyzed hydration[20] procedures. We have examined the trifluoroacetic acid-catalyzed methanolysis carefully with the aid of the radiolabeled IS and in the presence of a carrier, EMG, and find consistently a 5–8% loss of material as polar by-products, and 2–6% as non-polar products. Without using EMG carrier, these losses vary greatly according to the particular sample. The amount of EMG added is typically at least 1000-fold the quantity of endogenous JH or IS. At such levels, impurities in the EMG are potential interferences, necessitating its rigorous purification before use. The MH derivative of EMG is selectively removed during the LC purification. Our use of a tritiated internal standard has been of key importance in ascertaining sample-to-sample reliability of the derivatization procedure. In our experience, methods for JH analysis[10] which require prior solvolysis of the epoxide group may not be reliable unless they include use of a JH-like carrier and a labeled internal standard.

The methanolysis is a highly regioselective reaction, affording a 97:3 ratio of the 11-methoxy-10-hydroxy derivative to the (analytically useless) 11-hydroxy-10-methoxy isomer. In contrast, solvolysis with nonafluorohexanol is reported to give about an 80:20 mixture of the corresponding alkoxy hydroxy isomers[10].

Excellent linearity of response for the MHs is observed over a >500:1 range; quite adequate accuracy and precision values (especially for levels of JH greater than 0.06 ng/g) allow confident data acquisition. As some insects have been reported to have two or more JHs present at a particular developmental state[9], one or more of which occurs at a substantially higher (or lower) level than the other(s), it is essential that the linearity encompass at least two orders of magnitude.

Having examined a host of biological samples, including a comprehensive study on *Manduca sexta* JH production over the complete life cycle[16], we routinely use only a single GC column per analysis. Our earlier work using GC–ECD[1] and that of Hagenguth and Rembold[3] and Peter *et al.*[2] employ a minimum of three columns of varying selectivity per analysis, in order to shift around interfering substances (unavoidably present by virtue of the methods) and to confirm retention time data for suspected JHs. Exceptions to our single-column analysis by GC–MS are sometimes made for first-time samples of an unusual hormone composition, or for mass spectral structure confirmation of JH present in unusually high quantities. This, however, is prudent analytical practice.

SIM analyses of "blanks" rarely show backgrounds exceeding 0.005–0.02 ng/ml for JH I and II, or 0.01–0.03 ng/ml for JH III. Currently, the only extraneous substance detected with regularity is a fast-eluting peak attributable to a phthalate ester (base peak *m/z* 149), that produces a minor fragment detectable at *m/z* 76. As this peak does not interfere with quantification, its presence is ignored. However, precautions are taken to minimize use of plastic or rubber apparatus throughout sample processing; in particular, nitrogen lines are of PTFE or metal to reduce possible phthalate contamination.

A dramatic illustration of the versatility of our method was recently furnished by the discovery of the fourth naturally-occurring juvenile hormone, JH 0 (ref. 11). We noticed the presence of this JH in extracts from eggs of *M. sexta* during routine SIM analysis, when a second component, monitored at *m/z* 90 and eluting slightly after JH I, was detected. Subsequent mass spectral analysis of the purified parent epoxide confirmed our findings and we now are examining other insect sources for presence of this hitherto unreported JH.

In conclusion, analysis to date of literally hundreds of hemolymph and whole-body extracts from diverse insect species for JH titers has convinced us that this assay procedure with SIM detection offers an excellent combination of specificity and sensitivity. The procedure as outlined allows an experienced technician to process 15–20 samples per week, substantially improving through-put when compared to many existing chemical assays. In most cases one will find that procurement and synchronization of specimen colonies is more time-consuming than the subsequent chemical processing and analysis.

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COMPORTEMENT ET SÉPARATION PAR CHROMATOGRAPHIE EN PHASE GAZEUSE SUR COLONNE CAPILLAIRE DES ACIDES DICARBOXYLIQUES À COURTE CHAÎNE

M. J. WAECHTER

Laboratoire de Chimie Analytique, Faculté de Pharmacie, Rue J.B. Clément, 92290 Chatenay-Malabry (France)

et

M. C. PFAFF-DESSALLES et G. MAHUZIER*

Laboratoire de Biologie, Centre Hospitalier Spécialisé de Villejuif, 54 Avenue de la République, 94800 Villejuif (France)

SUMMARY

Behaviour and separation of short-chain dicarboxylic acids by gas chromatography on glass capillary columns

The gas–liquid chromatographic behaviour of 26 dicarboxylic compounds usually found in biological fluids is described. After butylation by BF_3–butanol 14% (w/v) the esters are separated on a 11.5% Carbowax 20M, WCOT glass capillary column.

The compounds formed are identified by combined gas chromatography–mass spectrometry. Their capacity factors and methylene unit values can be applied to predict their separation.

INTRODUCTION

De nombreux dérivés dicarboxyliques à courte chaîne se rencontrent dans les milieux biologiques[1–3]. L'augmentation ou l'apparition de certains d'entre eux peuvent traduire des variations physiologiques[4] ou pathologiques[5,6], des désordres métaboliques ou génétiques[1,7–9] ou être consécutifs à certaines thérapeutiques tout particulièrement en milieu psychiatrique[10–12]. Les séparations les plus efficaces de dérivés dicarboxyliques sont actuellement effectuées par chromatographie en phase gazeuse (CPG) sur colonne capillaire[13–16] après, le plus souvent, transformation préalable de ces composés en dérivés silylés[5,7,17], méthylés[14,18], butylés[18–20] ou cyclohexylés[21].

La plupart de ces études ayant trait aux dérivés monocarboxyliques, le comportement chromatographique sur colonne capillaire des butylesters de 26 dérivés dicarboxyliques (C_2–C_{10}) les plus souvent rencontrés dans les milieux biologiques est ici envisagé.

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MATÉRIEL ET MÉTHODE

Produits de référence

Les dérivés dicarboxyliques étudiés sont les suivants: acide oxalique, succinique, glutarique, adipique, méthyl-3-maléique, maléique, fumarique, malique (Prolabo, Paris, France); acide pimélique, subérique, azélaïque, méthylmalonique, éthylmalonique, méthyl-3-glutarique, tartronique, hydroxy-2-méthyl malonique, hydroxy-2-succinique, hydroxy-3-méthyl-3-glutarique, oxomalonique, oxaloacétique, oxo-2-glutarique, oxo-3-glutarique (Fluka, Buchs, Suisse), acide malonique (J. T. Baker, Phillipsburg, N.J., U.S.A.), acide α-cétoadipique (Sigma, St Louis, MO, U.S.A.), hydroxy-2-glutarate disodique, citromalate dipotassique (Fluka). Le bibutyl adipate provient de Merck (Munich, Allemagne) et les carbures saturés de référence C_{16} à C_{18} d'Applied Sciences Labs. (State College, PA, U.S.A.).

Les solutions de ces acides sont préparées par dissolution directe dans le butanol (1 mg ml^{-1}).

Lorsque les dérivés dicarboxyliques sont commercialisés sous forme de sel alcalin, des solutions de ces sels à 2 mg ml^{-1} sont effectuées dans l'eau; les acides sont ensuite extraits par le butanol après acidification par l'acide chlorhydrique.

Réactifs et solvants

Le réactif de butylation BF_3–butanol 14% (w/v) provient d'Interchim (Montluçon, France), le chloroforme et le butanol de Prolabo normapur (Paris, France).

Appareillage

Chromatographie en phase gazeuse. L'appareil utilisé est un chromatographe Girdel 300 équipé d'un détecteur à ionisation de flamme et d'un injecteur-diviseur Girdel en verre pour colonne capillaire. La colonne capillaire (WCOT) de 50 m de long et 0.26 mm de diamètre interne est imprégnée d'un film de Carbowax 20M (11.5%) de 0.2 μm d'épaisseur. Le nombre de plateaux est de 30,000.

La température de l'injecteur est de 280°C, celle du détecteur 300°C. Les échantillons ont été chromatographiés soit en isotherme à 120 ou 150°C selon les cas, soit en programmation de température de 100 à 200°C (5°C min^{-1}). Le gaz vecteur utilisé est l'azote avec une pression d'entrée de 0.3 bar. La fuite est réglée pour obtenir un rapport de division d'environ 1/100ème.

L'appareil est relié à un enregistreur Servotrace 1 cm min^{-1}.

Chromatographie en phase gazeuse couplée à la spectrométrie de masse. Le chromatographe est un appareil Girdel couplé à un spectromètre de masse Nermag GC-MS R10-10B (Rueil-Malmaison, France). La colonne capillaire de 21 m de long et 0.21 mm de diamètre interne est imprégnée d'un film d'Ucon 75 H 90,000 de 0.147 μm d'épaisseur. Le gaz vecteur est de l'hélium dont la pression d'entrée dans la colonne est de 1.2 bar.

Technique de butylation

La butylation est effectuée selon la méthode proposée par Lambert et Moss[20] par action directe du réactif BF_3–butanol sur les solutions butanoliques des diacides.

Dans des tubes de 5 ml bouchés émeri, 100 μl de la solution butanolique de

diacide à 1 mg ml^{-1} sont mis en contact avec 100 μl de réactif BF_3–butanol et portés à 100°C pendant 10 min.

Après refroidissement l'ester formé est agité au Vortex pendant 1 min avec 100 μl de chloroforme. Après 10 min de repos à la température du laboratoire, l'excès de BF_3 est éliminé par lavage rapide à l'aide de 200 μl d'eau distillée. La phase aqueuse est ensuite éliminée et la phase chloroformique séchée sur sulfate de sodium.

0.25 à 1 μl de la solution chloroformique sont injectés directement.

RÉSULTATS ET DISCUSSIONS

Afin de déterminer les meilleures conditions de séparation, le comportement des dibutylesters a été étudié à différentes températures. Les facteurs de capacité, k', ont été calculés en température isotherme et les indices de méthylène en programmation de température. Ce calcul des indices utilisé avec profit pour l'étude du comportement des stéroides[22], des alcools à longues chaînes[23] et des sucres[24], à été effectué à l'aide d'une série de carbures saturés à nombre pair d'atomes de carbone compris entre l'hexadécane et l'octacosane.

Ces différents résultats sont regroupés dans les Tableaux I et II; le Tableau I concerne les diacides linéaires, ramifiés ou insaturés ne portant pas d'autres groupements fonctionnels, le Tableau II les diacides porteurs d'un groupement hydroxylé ou cétonique.

Dans chaque série un ou plusieurs dérivés ont été analysés en spectrométrie de masse.

Dérivés linéaires, ramifiés, saturés ou insaturés: Tableau I

Les dibutylesters formés ne donnent qu'un seul pic sur les chromatogrammes. Leur identité a été confirmée par spectrométrie de masse. A titre d'exemple le spectre

TABLEAU I

FACTEUR DE CAPACITÉ k' ET INDICE DE MÉTHYLÈNE DES BUTYLESTERS DE DIACIDES SATURÉS LINÉAIRÉS, RAMIFIÉS ET INSATURÉS

Nombre de C	*Acides*	k'	*Température (°C)*	*Indice de méthylène*
2	Oxalique	0.333	150	16.442
3	Malonique	0.611	150	17.594
4	Succinique	1.037	150	19.066
5	Glutarique	1.924	150	20.000
6	Adipique	1.877	160	21.166
7	Pimélique	1.821	170	22.187
8	Subérique	2.581	170	23.235
9	Azélaïque	1.757	190	24.125
10	Sébacique	3	190	25.133
4	Méthylmalonique	0.444	150	16.781
5	Éthylmalonique	0.611	150	17.594
6	Méthylglutarique	1.653	150	19.733
4	Maléique (*cis*)	1.423	150	19.366
	Fumarique (*trans*)	0.892	150	18.666
5	Méthylmaléique ou citraconique	0.620	150	19.500

TABLEAU II

FACTEUR DE CAPACITÉ DES BUTYLESTERS DE DIACIDES HYDROXYLÉS ET CÉTONIQUES

Nombre de C	*Acides*	*k'*	*Température (°C)*
3	Tartronique	*	
4	Malique	*	
5	Hydroxy-2-glutarique	1.076	150
4	Hydroxy-2 méthyl-2-malonique	0.551	150
5	Hydroxy-2 méthyl-2-succinique (citramalique)	0.616	150
6	Hydroxy-3-méthyl-3-glutarique	1.596	150
3	Oxomalonique	0.388	120
4	Oxaloacétique	0.666	120
5	Oxo-2-glutarique	1.70	120
—	Oxo-3-glutarique	0.750	120
6	Oxo-2-adipique	2.6	120

* Ininterprétable.

du bidutylester formé avec l'acide adipique a été comparé avec celui du dibutyladipate commercialisé.

Leurs facteurs de capacité ou leurs indices de méthylène montrent que ces différents composés peuvent être aisément séparés sur ce type de colonne et dans les conditions mentionnées (Fig. 1). Les temps de rétention augmentent avec le nombre d'atomes de carbone et sont influencés par la structure des dérivés: les composés ramifiés ont des temps de rétention nettement inférieurs à leurs isomères linéaires; l'ester de l'acide fumarique dérivé *trans*, insaturé est moins retenu que son homologue saturé le dibutyl succinate à la différence de son isomère *cis*, insaturé le dibutyl maléate. Contrairement à ce qui a été observé sur d'autres colonnes (OV-101, OV-17, EGS, NPGA[8], l'acide méthylmalonique se sépare des acides malonique ou éthyl malonique, ces deux derniers dérivés ayant par contre ici le même comportement.

Spectre de masse du dibutyladipate (Fig. 2a). Le spectre de masse du dibutyladipate formé par action du BF_3–butanol dans les conditions décrites et celui d'un dibutyladipate de référence, sont identiques.

On peut noter l'absence d'ion moléculaire ce qui est observé également dans tous les dérivés étudiés.

Le pic de base à *m/e* 129 est obtenu par rupture de la molécule en son milieu. Par élimination d'une molécule d'eau cet ion donne naissance à l'ion *m/e* 111. La rupture du radical ($C_4H_9O^+$) entraine la formation d'un autre ion abondant *m/e* 189, (M−73) comme dans le cas des esters butyliques d'acides monocarboxyliques linéaires et ramifiés étudiés par Choudhary et Moss[19], sans toutefois être le plus abondant. En accord également avec les résultats de Choudhary et Moss les ions *m/e* 56 ($C_4H_8^+$) et *m/e* 41 ($C_3H_5^+$) provenant de la rupture d'ions butyl sont observés.

La perte d'un groupement butoxycarbonyl ($C_4H_9OCO^+$) entraine la formation de l'ion *m/e* 101.

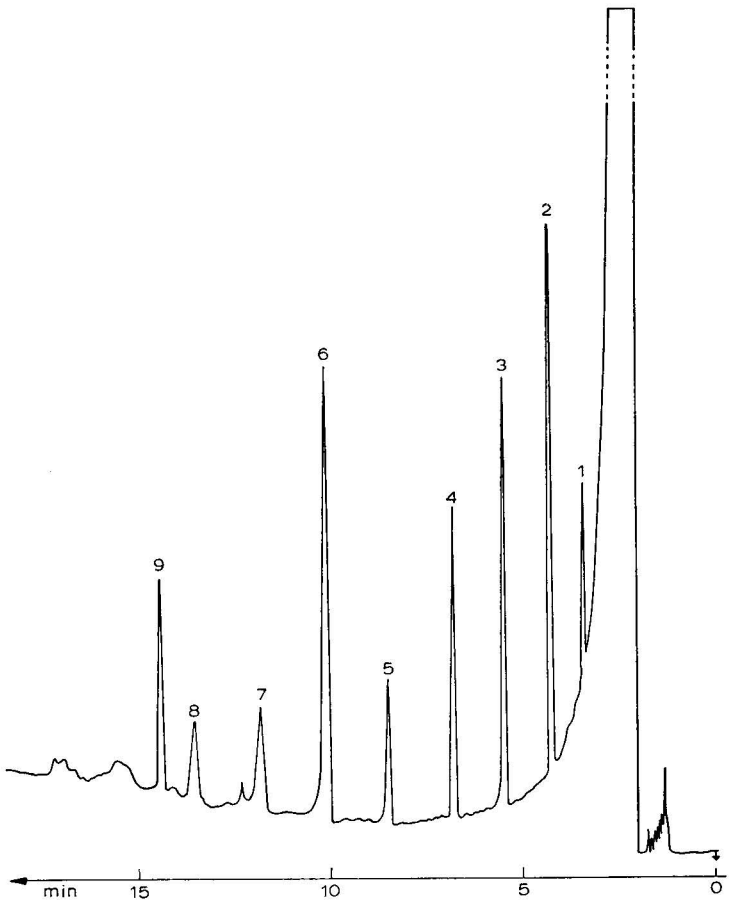


Fig. 1. Chromatogramme des dibutylesters des diacides saturés. 1 = Oxalique; 2 = malonique; 3 = succinique; 4 = glutarique; 5 = adipique; 6 = pimélique; 7 = subérique; 8 = azélaïque; 9 = sébacique.

Dérivés hydroxyles: Tableau II

L'acide hydroxy-2-glutarique et les acides en C_4, C_5 et C_6 hydroxylés et substitués par des groupements méthyles donnent chacun, sur les chromatogrammes, un pic bien isolé, identifié par spectrométrie comme étant un dibutylester, la butylation n'affectant pas les groupements hydroxylés.

Les dérivés méthylés et hydroxylés sortent au voisinage de leurs homologues non hydroxylés et par conséquent plus rapidement également que les dérivés linéaires correspondant.

Par contre, dans le cas des acides tartronique et malique plusieurs pics apparaissent et seules de très faibles quantités de butylester sont identifiées, les autres pics étant constitués par des produits de dégradation.

Spectres de masse des dérivés hydroxyles linéaires (Fig. 2b). Les spectres de masse des esters butyliques des acides tartronique, malique, hydroxy-2-glutarique ont été étudiés.

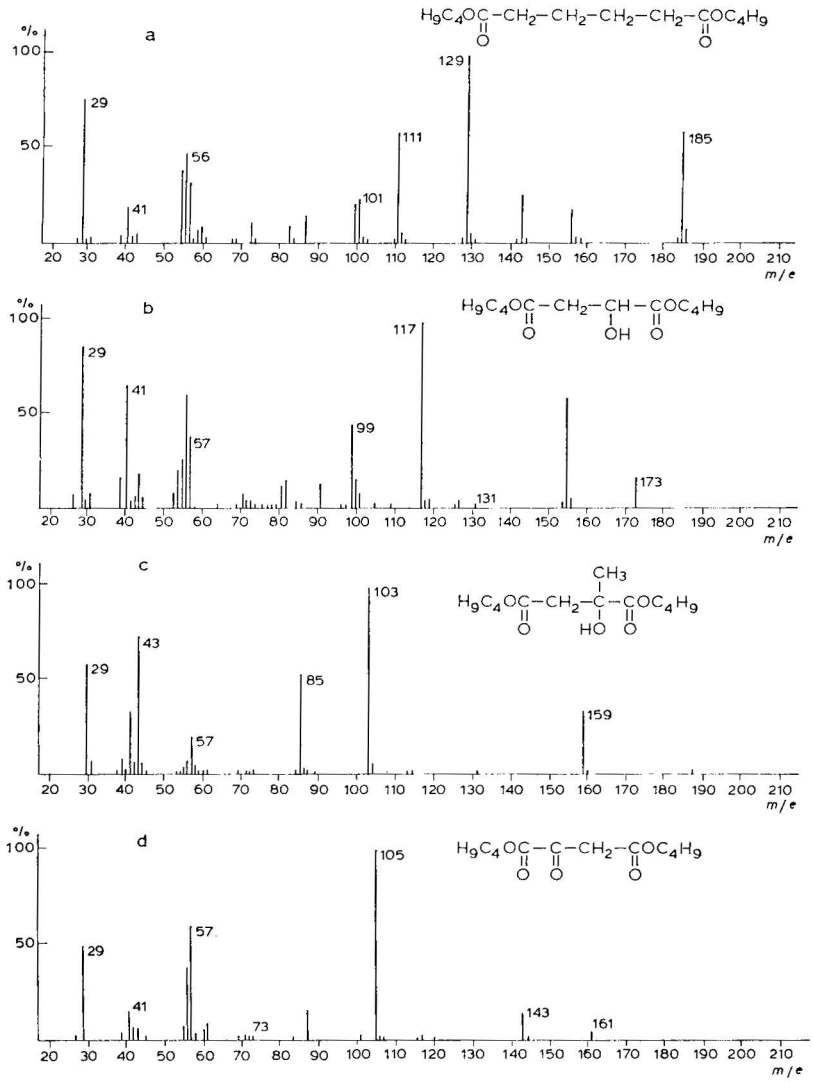


Fig. 2. Spectre de masse du dibutylester de l'acide adipique (a), malique (b), citramalique (c) et oxaloacétique (d).

L'ion *m/e* 131 bien que peu abondant est caractéristique de la série. Il provient de la rupture en α du groupement hydroxyle qui donne naissance à l'ion

OH
|
CH–COO $C_4H_9^+$.

Les autres ions caractéristiques M−73, M−129, M−147, s'expliquent par la perte d'un groupement butoxy suivi de celle d'un butyl puis de l'élimination d'une molécule d'eau.

Spectres de masse des dérivés hydroxyles ramifiés (Fig. 2c). Les spectres de

masse des esters butyliques des acides α-isomalique, citramalique, hydroxy-3méthyl-3-glutarique ont été étudiés.

La rupture en α du C tertiaire est typique de cette série, on obtient ainsi l'ion *m/e* 145 pour l'ester de l'α-isomalique et l'ion *m/e* 159 pour ceux des deux autres.

Cette rupture est suivie pour les trois dérivés de la perte d'un groupement butyl pour former les ions *m/e* 89 pour le dérivé de l'acide α-isomalique et *m/e* 103 pic de base des dérivés de l'acide citramalique et de l'hydroxy-3 méthyl-3-glutarique.

Enfin, pour ces derniers, l'élimination d'une molécule d'eau explique la présence de l'ion *m/e* 85.

On peut encore noter l'abondance des ions $C_4H_9^+$ ou $C_3H_5O^+$ (*m/e* 57), $C_3H_7^+$ (*m/e* 43) et $C_2H_5^+$ (*m/e* 29).

Dérivés cétoniques: Tableau II

Les dibutylesters sont très rapidement élués sur ce type de colonne et il faut descendre à 120°C pour obtenir leur séparation et calculer leur k'.

En programmation de température de 100 à 200°C, on constate la dégradation des acides oxaloacétique et oxomalonique, plusieurs pics précèdent alors les dibutylesters.

Spectres de masse des dérivés cétoniques (Fig. 2d). Les spectres de masse de quatre dérivés oxo-2 et d'un dérivé oxo-3 ont été étudiés: les dibutylesters des acides oxomalonique, oxaloacétique, α-cétoglutarique et α-cétoadipique d'une part, le dibutylester de l'acide oxo-3-glutarique d'autre part.

Dérivés oxo-2. L'ion à *m/e* M-101 observé dans tous les spectres de ces dérivés sauf dans celui de l'ester de l'acide oxomalonique provient de la rupture d'un groupement butoxycarbonyl en α du groupement cétonique. L'ion *m/e* 101 résultant de cette coupure constitue le pic de base de l'ester de l'acide α-cétoglutarique. Dans le cas du dérivé de l'acide α-acétoadipique le pic de base *m/e* 115 correspond à l'ion

$$C_4H_9O\overset{\overset{O}{\|}}{C}CH_2^+.$$

L'ion à *m/e* M−71 observé dans le spectre de l'ester de l'acide oxomalonique peut s'expliquer par le clivage d'un groupement butoxy suivi d'un réarrangement à double hydrogène.

Les ions à *m/e* M−83 caractéristiques des dérivés de l'acide oxaloacétique et de l'acide α-cétoglutarique semblent pouvoir être expliqués par analogie avec le butylester de l'acide α-cétopropanoïque[19]. Ces ions *m/e* 175 et *m/e* 161 dans les spectres de l'ester de l'acide α-cétoglutarique et l'acide oxaloacétique respectivement seraient dus au clivage du radical $CO\,C_4H_9^+$ suivi par un réarrangement à double hydrogène donnant d'une part l'ion $C_4H_9O\overset{\overset{O}{\|}}{C}-CH_2-CH_2-\overset{\overset{OH}{|}}{C}=OH^+$ et d'autre part l'ion $C_4H_9O\overset{\overset{O}{\|}}{C}-CH_2-\overset{\overset{OH}{|}}{C}=OH^+$.

Suite aux clivages donnant les ions M−71 ou M−83 la rupture d'un groupement butyl explique notamment le pic de base *m/e* 105 de l'ester de l'acide oxaloacétique.

A noter encore dans ce spectre l'importance des ions *m/e* 57 (pic de base de l'ester de l'acide oxomalonique) *m/e* 41 et *m/e* 29.

Dérivé oxo-3. Le pic à *m/e* M−99, de faible amplitude, est cependant caractéristique du dibutylester de l'acide oxo-3-glutarique. Il provient de la perte d'un groupement butoxycarbonyl suivi d'un réarrangement à double hydrogène. A partir de cet ion nous obtenons les autres ions caractéristiques par perte d'un butyl ou d'une molécule d'eau (*m/e* 103 et *m/e* 141) ou des deux à la fois (*m/e* 85).

Le pic de base est constitué par l'ion $C_4H_8^+$ *m/e* 56.

CONCLUSION

Les dégradations observées pour certains dérivés hydroxylés ou α-cétoniques à nombre d'atomes de carbones inférieur à C_5 ont été dans certains cas signalées par d'autres auteurs utilisant cependant une méthodologie différente[25]. Malgré cette limite, l'estérification par le BF_3–butanol étant trés aisée, cette étude montre qu'il est facile selon les conditions opératoires choisies de séparer la plupart des dérivés dicarboxyliques sous forme de dibutylesters.

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RÉSUMÉ

Le comportement chromatographique en phase gazeuse de 26 dérivés dicarboxyliques le plus fréquemment rencontrés dans les milieux biologiques, est exposé. Après butylation par le BF_3–butanol 14% (w/v), les esters sont séparés sur colonne capillaire WCOT Carbowax 20M (11.5%).

L'identification des composés formés est effectuée par couplage avec la spectrométrie de masse.

Leurs facteurs de capacité et leurs indices de méthylène peuvent être employés pour prévoir leur séparation.

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CHROM. 13,137

ACID-WASHED GRAPHITIZED CARBON BLACK AND ITS APPLICATION IN THE SEPARATION OF SOME ACIDS

ANTONIO DI CORCIA*, ARNALDO LIBERTI and CLAUDIO SEVERINI
Istituto di Chimica Analitica, Università di Roma, 00185 Rome (Italy)

SUMMARY

Acid-washed graphitized carbon black modified with a suitable liquid phase has been exploited for the analysis of mixtures of biological interest. Phenobarbital can be accurately determined at the 6 ppm level. 3-Hydroxybutyric acid can be determined at the physiological level by direct injection of undeproteinized serum.

INTRODUCTION

Although the first paper on gas chromatography (GC), by James and Martin[13] in 1951, dealt with the quantitative analysis of free fatty acies, and in spite of the large amount of subsequent work on the analysis of acids, there are still some unresolved or difficult problems: for example, trace analysis of acidic pollutants in water, fatty acids in fermentation broth, hydroxyacids in blood and barbiturates at the ppm level in biological materials.

In chromatography of solutions containing acidic compounds, the column is usually deactivated either with an acid (usually H_3PO_4) or with a liquid phase itself containing an acidic functional group. Generally, these columns have several limitations: thermal instability of H_3PO_4 at temperatures higher than 190°C; partial decomposition of the stationary phase by H_3PO_4 at temperatures higher than 190°C; anomalous interactions between H_3PO_4 or the acidic group of the liquid phase and non-acidic compounds, such as alcohols; strong interactions between the acidic liquid phase and acidic, polar eluates which results in long analysis times and poor selectivity for acidic compounds having similar molecular structure.

In recent years, graphitized carbon blacks (Carbopack) deactivated with H_3PO_4 (ref. 1), trimesic acid[2] or coated with FFAP[3,4] have been used for elution of acids. These chromatographic systems showed the same limitations as those mentioned above.

In our laboratory, we observed that if one part of Carbopack is immersed in 25 parts of water, the suspension continuously stirred for 24 h and the supernatant liquid then separated by filtration and placed in contact with the glass electrode of a pH electrometer, a reading is obtained corresponding to a pH of 10.5. It has also been noted[5] that washing the surface of graphitized carbon balcks (CGBs) with an acidic

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solution is effective in eliminating basic adsorption sites. Furthermore, after this chemical treatment, the pH of a suspension of Carbopack in water decreased to 6. Also, symmetrical peaks for acids were obtained after elution on even the uncoated surface of Carbopack.

In this work, the ability of acid-washed (AW) Carbopack to give symmetrical peaks for acids without the use of a deactivating acidic agent has been exploited in the analysis of some acid-containing solutions. In particular, by modifying the surface of Carbopack C with Apiezon and PEG 20M, a mixture of barbiturates was separated. Calibration curves for phenobarbital showed no chemisorption of this compound at the 5 ppm level. Moreover, by modifying the surface of Carbopack B with PEG 20M, we were able to detect about 30 ppm of 3-hydroxybutyric acid in blood by direct injection of undeproteinized plasma.

EXPERIMENTAL

Carbopack B and C, which are examples of graphitized carbon blacks, were kindly supplied by Supelco (Bellefonte, PA, U.S.A.) The procedure of washing Carbopacks with an acidic solution in order to obtain a carbon surface free from basic centres has been reported previously[5].

Coating of the Carbopack C surface was carried out in the usual way[6]. Both Apiezon and PEG 20M were deposited on Carbopack from methylene chloride: 0.2% Apiezon + 0.35% PEG 20M on Carbopack C AW was conditioned for 12 h at 250°C; 4% PEG 20M on Carbopack B AW was conditioned for the same time at 240°C.

A Model Gl gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame ionization detector was used. The nozzle of the detector was made of quartz. Tailed peaks for hydroxyacids were noted when the chromatographic apparatus was equipped with a stainless-steel nozzle.

RESULTS AND DISCUSSION

By using a 2-m column packed with Carbopack B AW + 5% PEG 20M, perfectly symmetrical peaks and baseline separations were obtained for elution of an aqueous mixture containing C_2–C_7 fatty acids, C_1–C_5 alcohols, ethyl acetate and acetaldehyde at 20 ppm. This kind of analysis is of biological interest as these substances may be produced by bacterial fermentation. It should also be noted that no chromatographic system is suitable for simultaneous elution of acids and alcohols. If the column is deactivated in order to obtain linear elution of acids, alcohols are eluted as tailed peaks due to anomalous interactions between eluates and H_3PO_4.

Because of the widespread use of barbiturates, therapeutic monitoring and drug overdose analysis is an important task in clinical laboratories. Several relevant gas–liquid chromatographic procedures have been described previously[7–11], although the plethora of methods only serves to illustrate the lack of general method to meet all needs. Many of the procedures reported give good results in the analysis of drug overdoses, where the blood barbiturate concentration is high, but fail in therapeutic monitoring, where the concentration levels in biological fluids are low. This is due to partial irreversible adsorption of barbiturates on the columns.

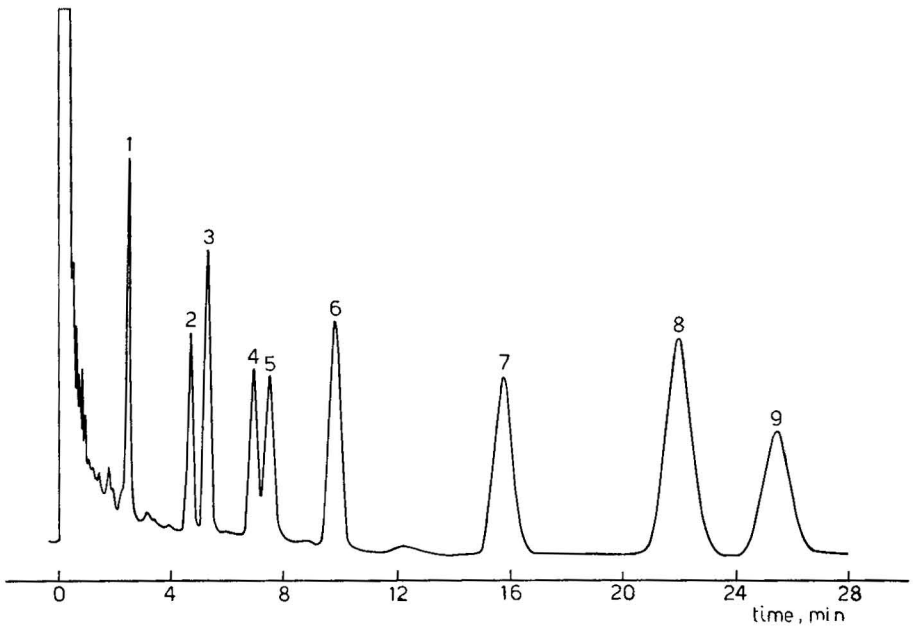


Fig. 1. Chromatogram of a barbiturate mixture. Packing: Carbopack C AW + 0.35% PEG 20M + 0.2% Apiezon. Column: 1 m × 2 mm. Carrier gas: hydrogen. Injected amount: 1 μl of a solution containing 80–100 ppm of each component. Dead time: 5.8 sec. Temperature: 235°C. Peaks: 1 = barbital; 2 = diallylbarbital; 3 = allylisobarbital; 4 = amobarbital; 5 = pentobarbital; 6 = secobarbital; 7 = cyclohexenylbarbital; 8 = phenobarbital; 9 = cycloheptylbarbital.

Fig. 1 shows the elution of a mixture of the most frequently encountered drugs on Carbopack C AW suitably modified with 0.35% PEG 20M and 0.2% Apiezon.

Chemisorption of barbiturates does not occur on the acid-washed surface of graphitized carbon blacks. To substantiate this and to determine the limit of detection of phenobarbital, quantitative measurements of this compound were made. A paraffin was chosen as internal standard since partial chemisorption of a given compound can be recognized by injecting it in variable amounts together with an inert eluate and measuring the response factor at any given concentration. Standard solutions at concentrations lower than 166 ppm were prepared by diluting the starting solution containing phenobarbital and the reference compound. Results are reported in Table I. It appears that the quantitative determination of phenobarbital at the ppm level can be made with an error not higher than 6%.

The value of gas chromatography in the diagnosis of, and studies on, human diseases is now well recognized. A number of metabolic disorders are characterized by increased concentrations of organic acids, in biological fluids and blood and urine samples are routinely examined by chromatographic methods in several hospital laboratories.

In many cases, the organic acids of biological interest are present in blood at the ppm level and are very polar in nature, such as the hydroxyacids. Consequently, the quantitative elution of these compounds presents serious problems due to chemisorption. Other sources of errors accrue from deproteinization, removal of charged

TABLE I

RESPONSE FACTOR FOR PHENOBARBITAL *VS.* HEXADECANE AT VARIOUS CONCENTRATIONS

Concentration (ppm, w/w)	*Response factor*	*S*
166	0.79	0.032
55	0.80	0.031
18	0.80	0.028
6	0.78	0.043

compounds and derivatization of organic acids. Serious difficulties are encountered in derivative preparation due to the inhibition caused by residual amounts of water.

It has been reported[12] that the concentration of 3-hydroxybutyric acid in blood can increase from about 20 ppm (physiological level) to about 60 ppm for patients affected by diabetes. The monitoring of this organic acid can be of great aid in the early diagnosis of this disease. Fig. 2 shows the elution of undeproteinized serum of a patient with a glycemia value equal to 2.2. A well-defined peak for 60 ng of 3-hydroxybutyric acid is observed. This elution was accomplished by the use of Carbopack B AW coated with 5% PEG 20M. The column was used over a long period of time, and accumulation in the injection port of proteins contained in serum did not affect the chromatographic profile.

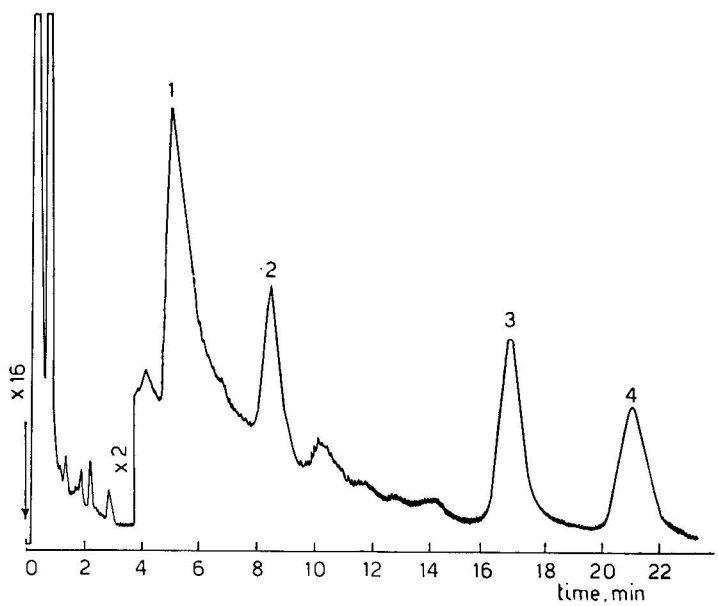


Fig. 2. Chromatogram of 3-hydroxybutyric acid from undeproteinized serum. Packing material: Carbopack B AW + 4% PEG 20M. Column: 1 m × 3 mm. Carrier gas: nitrogen. Injected amount: 2 μl of serum. Dead time: 12 sec. Temperature: 195°C. Peaks: 1 = lactic acid and artifact from acetoacetic acid; 2 = 3-hydroxybutyric acid; 3 = enanthic acid (internal standard), 10 ppm; 4 = unknown.

The same procedure was followed for 3-hydroxybutyric acid. Enanthic acid was chosen as internal standard. The results obtained showed no chemisorption of the hydroxyacid and a well-defined peak for 7 ppm of this acid in water. At this level of concentration, the error of quantitative measurement is $\leqslant 10\%$.

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APPLICATIONS IN DRUG ANALYSIS

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CHROM. 13,235

SYSTEMATIC USE OF TETRAHYDROFURAN IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AN EXAMPLE OF THE SELECTIVITY BENEFITS OF TERNARY MOBILE PHASES

ELISABETH ROGGENDORF* and REINHOLD SPATZ
Applications Laboratory, Spectra-Physics, Darmstadt (G.F.R.)

SUMMARY

Selectivity is described here as the ability of a solvent to exhibit specific solute interactions which another solvent of similar polarity does not exhibit. Selectivity differences are shown to provide an important and ready means of improving and controlling resolution. The selectivity effects of a water–methanol–tetrahydrofuran ternary system are discussed with respect to the separation of vanillin and syringaldehyde and that of eight cinnamic acid derivatives.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a powerful tool for solving difficult separations and subsequent quantitative analyses at trace levels and has therefore been extensively used, particularly in the biological and medical sciences. At present, reversed-phase HPLC using *n*-alkyl bonded phases is the most frequently selected separation mode. It is estimated that 60–80% of HPLC separations are accomplished using reversed-phase chromatography[4,5]. The advantage of reversed-phase HPLC compared with adsorption chromatography has been described by several workers[4,6,7] and indeed reversed-phase chromatography is used over a broad polarity range and in diverse applications.

The rôle of the mobile phase in reversed-phase chromatography and the expected solute interactions with the non-polar stationary phase have been described previously[3,8]. In particular, we have demonstrated[1,2,9] that mobile-phase control with three mixed solvents can be a potentially powerful tool for the optimization of selectivity. In those experiments several isocratic and gradient methods were used to study solvent selectivity of (1) binary mixtures of water–methanol, –acetonitrile and –tetrahydrofuran, and of (2) ternary mixtures of water, methanol and a third solvent chosen from acetonitrile, tetrahydrofuran, diethyl ether, methylene chloride, dimethylformamide, dimethyl sulphoxide and water 50% saturated with hexane. The results indicated that the retention of compounds containing certain functional groups can be systematically and precisely influenced by ternary solvent systems. Tetrahydro-

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furan, a solvent which is extensively used in reversed-phase HPLC[10–15], was found to be particularly useful as a ternary solvent in the separation of compounds which differ in their number of ether groups.

It should be mentioned at this point that compounds differing in the number of constituent ether groups can, as a rule, be easily separated by other chromatographic methods, *e.g.*, adsorption methods, including thin-layer chromatography (TLC). By contrast, separations of such compounds by reversed-phase HPLC are frequently poor, particularly for methoxy-containing compounds, on octyl- and octadecyl-bonded phases. However, reversed-phase HPLC has many methodological advantages which are of interest with respect to the solution of this separation problem.

The work of Tanaka *et al.*[3] illustrates vividly that the selectivity of some aromatic hydrocarbons seems to be greater in the tetrahydrofuran–methanol system than in acetonitrile–methanol and that a mixture of tetrahydrofuran, methanol and water would be an interesting ternary mobile phase for control of separation of substances with different functional groups. This work shows, among other things, that phenols are significantly retarded in tetrahydrofuran–water, relative to methanol–water, whereas in acetonitrile–water no special selectivity for phenols is observed relative to methanol–water.

The present work describes the reversed-phase separation of vanillin–syringaldehyde and eight cinnamic acid derivatives. The main difficulty was the complete resolution of all substances which differ only in the number of methoxy groups, because these compounds could not be separated by us on a reversed-phase system with a binary mobile phase of water–methanol or –acetonitrile. Based on experience gained from previous work[1,3], tetrahydrofuran in a ternary mobile phase with water–methanol was employed.

EXPERIMENTAL

Column, solvents and samples

Separations were performed on a 250 × 4.6 mm I.D. stainless-steel column packed with 10-μm LiChrosorb RP-8®, a totally porous granular material, with covalently bonded octyl groups. This column was obtained from Spectra-Physics, Santa Clara, CA, U.S.A.

The mobile phase consisted of different ternary mixtures of distilled water, methanol and tetrahydrofuran (THF) (E. Merck, Darmstadt, G.F.R.). No attempt was made to control the pH of the water in the analysis of vanillin–syringaldehyde. In the case of cinnamic acid metabolites, the pH was adjusted to 3 with acetic acid. All solvents were continuously degassed using helium. Samples were standards (commercial source), dissolved in methanol.

Control of flow, composition and temperature

A Spectra-Physics Model SP8000B liquid chromatograph was used. It employs a single pump, attached to a low pressure ternary proportioning valve. Up to three different solvents were mixed by the valve according to the operators instructions, in both isocratic and gradient elution. The temperature was controlled at 35°C in a forced-air oven.

Detection

The detector was a Spectra-Physics Model SP8310 multiple wavelength UV-photometer operated at 254 nm.

RESULTS AND DISCUSSION

Separation of vanillin and syringaldehyde

Vanillin and syringaldehyde differ only in the additional methoxy group in the *meta* position of syringaldehyde. As mentioned before, on a reversed-phase column with a binary mobile phase of water–methanol or water–acetonitrile, both substances are eluted with the same capacity factor, regardless of the relative composition of the mobile phase. Although simplified, the reasons for this behaviour lie in the retention increase due to the methyl group and the retention decrease due to the oxygen, the combined effect of which is no change in retention. With methanol–water (1:1) as mobile phase the capacity ratio (k') of vanillin and syringaldehyde was 1.3. As expected, water–methanol (3:2) as mobile phase increased the k' value to 1.5, but the separation was not improved. Fig. 1a shows the chromatogram with 70% water–30% methanol for which the k' value of both substances was 2.5.

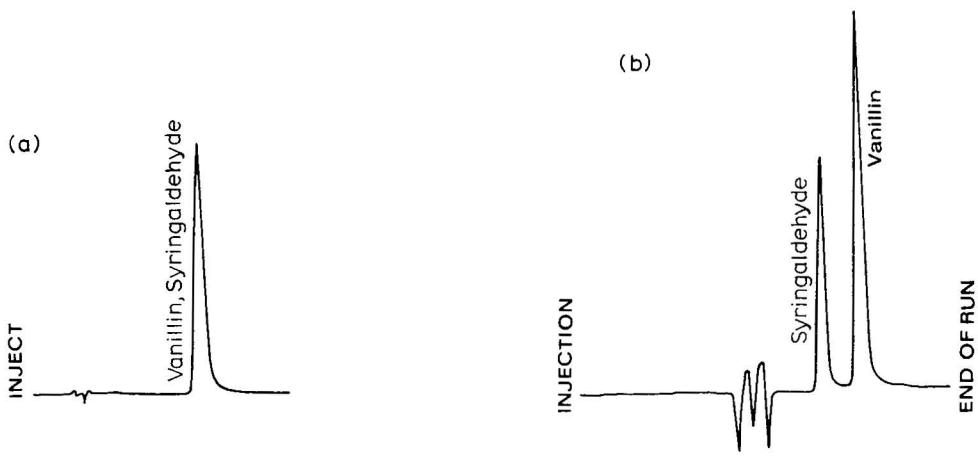


Fig. 1. Reversed-phase separation of vanillin and syringaldehyde. Conditions: SP8000B liquid chromatograph (Spectra-Physics); mobile phase, water–methanol (7:3) (a) or water–methanol–tetrahydrofuran (7:2:1) (b), flow-rate 1 ml/min; detector, SP8310 UV detector (Spectra-Physics), 254 nm; column, LiChrosorb RP-8, 10 μm.

The second step in improving the separation of vanillin and syringaldehyde was therefore the optimization of the selectivity of the mobile phase. Based on previous work, tetrahydrofuran was chosen because of its ability to accelerate compounds containing ether groups. Thus tetrahydrofuran causes syringaldehyde (two ether groups) to elute in front of vanillin (one ether group). Fig. 1b shows that a mixture of 10% tetrahydrofuran, 20% methanol and 70% water is the optimum mobile phase to achieve baseline separation. Another advantage of tetrahydrofuran is the shortening in retention time of both substances. The analysis is now completed in less than half the time of that required for the analysis with a binary mobile phase.

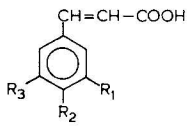
Separation of cinnamic acid metabolites

The metabolites of cinnamic acid (Table I) can be used as an indicator of the metabolism of drugs because the final amount of metabolites in urine gives an indication of body metabolism, and the activity of intestinal bacteria.

Preliminary work showed that about 75% water (pH 3) was required to achieve retention of the compounds on the RP-8 column; however, the binary water–methanol

TABLE I

CINNAMIC ACID METABOLITES



Cinnamic acid	R_1	R_2	R_3
m-Hydroxy	OH	H	H
p-Hydroxy	H	OH	H
3,4-Dihydroxy (caffeic acid)	OH	OH	H
m-Methoxy	OCH_3	H	H
p-Methoxy	H	OCH_3	H
3,4-Dimethoxy	OCH_3	OCH_3	H
3,4,5-Trimethoxy	OCH_3	OCH_3	OCH_3
3-Methoxy-4-hydroxy	OCH_3	OH	H

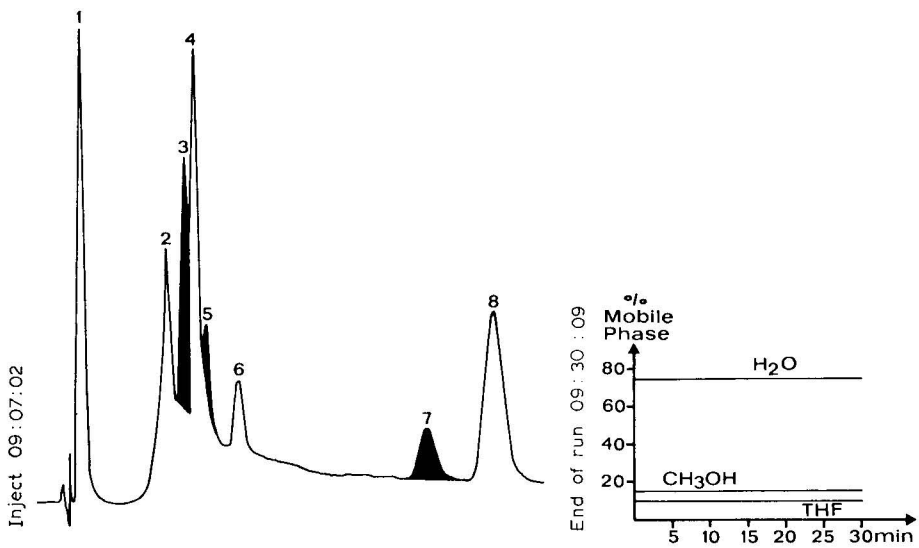


Fig. 2. Ternary isocratic separation of cinnamic acid metabolites. Mobile phase: water (pH 3)–methanol–THF. Other conditions as in Fig. 1. Cinnamic acids: 1 = 3,4-dihydroxy; 2 = 3-methoxy-4-hydroxy; 3 = 3,4-dimethoxy; 4 = *p*-hydroxy; 5 = 3,4,5-trimethoxy; 6 = *m*-hydroxy; 7 = *p*-methoxy; 8 = *m*-methoxy.

phase gave no separation of the mono-, di- and trimethoxy derivatives. From previous work involving methoxy-substituted compounds, as described above, this was to be expected. It followed that a selective separation of the metabolites could be achieved with a ternary mobile phase of water–methanol and THF. The separation was achieved in three steps.

Step 1. Fig. 2 shows the first analysis with THF in the ternary solvent. The water content was held constant at 75% to maintain retention and 10% THF was added as the modifier, the remainder being 15% methanol. The mono-, di- and trimethoxycinnamic acids were well separated but eluted together with hydroxy derivatives.

Step 2. Because of the complexity of the sample, a gradient was used. An initial composition consisting of 80% water, 10% methanol and 10% THF was held constant for 5 min to increase retention. The water content was then reduced to 70% and the methanol and the THF contents increased to 15%. Fig. 3 shows this ternary

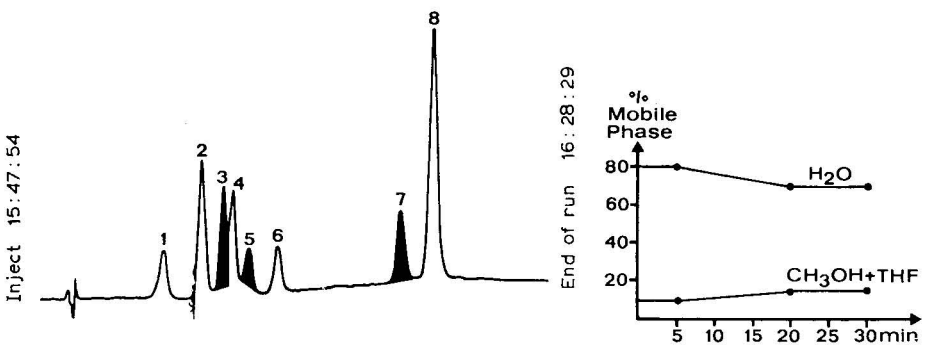


Fig. 3. Ternary gradient separation of cinnamic acid metabolites. For conditions see Fig. 2.

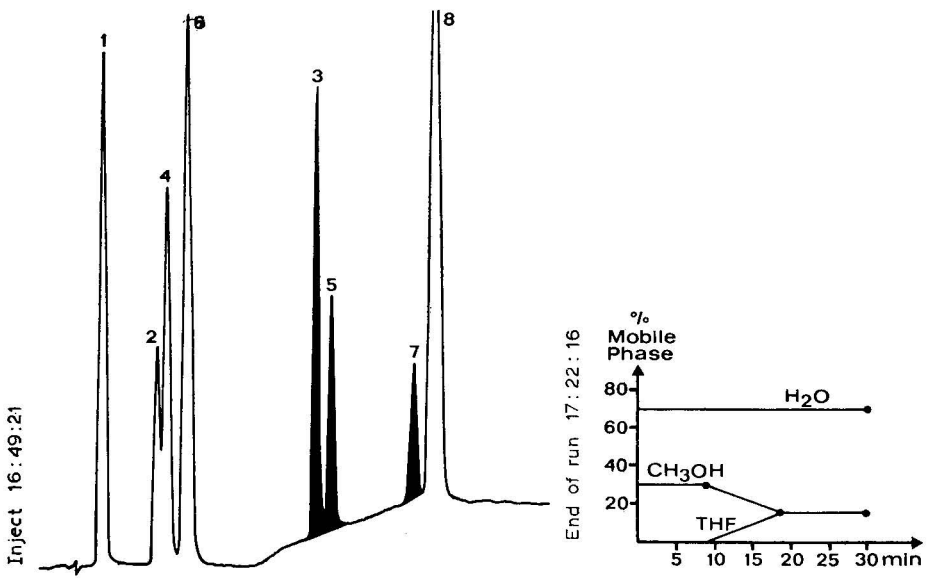


Fig. 4. Separation as in Fig. 3 but with a different gradient profile.

gradient separation where all three solvent concentrations were simultaneously changed. The three methoxycinnamic acids were preferentially accelerated, the monomethoxy more than either the tri- or dimethoxy. This may be explained by steric effects.

Step 3. The remaining difficulty was to improve the separation of *p*-hydroxycinnamic acid from the di- and trimethoxy derivatives. Here again, ternary solvent selectivity effects provided resolution.

Fig. 4 shows the HPLC separation of the metabolites, but in this case with a different gradient profile. Because of the absence of THF at the beginning of the analyses, the tri- and dimethoxy derivatives are selectively retained longer on the column while the first group of derivatives are eluted. THF is then rapidly added, causing the di- and trimethoxy derivatives to be eluted closely together but well separated from the mono-derivatives which of eluted last.

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CHROM. 13,261

SEPARATION OF 5-HYDROXYINDOLE-3-ACETIC ACID AND INDOLE-3-ACETIC ACID IN URINE BY DIRECT INJECTION ON A REVERSED-PHASE COLUMN CONTAINING A HYDROGEN-ACCEPTING STATIONARY PHASE

K.-G. WAHLUND* and B. EDLÉN

Department of Analytical Pharmaceutical Chemistry, Biomedical Center, Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

The separation of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid from urine is demonstrated. The urine is injected directly on a liquid chromatographic column which is coupled to a flow-through fluorescence detector or a UV detector. A hydrogen-accepting stationary phase, tributyl phosphate (TBP), which retains carboxylic acids selectively, was more selective than more common reversed-phase systems with alkyl-bonded stationary phases. The mobile phases were buffers, sometimes buffers containing methanol or acetonitrile.

A simple method of removing late peaks in the chromatogram, in order to reduce analysis time, is also presented.

INTRODUCTION

Determination of low-molecular-weight carboxylic acids in complicated matrices such as urine often requires time-consuming and laborious extraction, evaporation or pre-concentration steps. In addition, very hydrophilic acids may not be quantitatively extracted. Direct injection of urine on a liquid chromatograph with on-line separation and detection can greatly simplify the determination technique. However, due to the vast number of low-molecular-weight compounds in urine, the determination of an endogenous compound may place very high demands on the selectivity of both the separation and the detection systems. These demands will vary depending on the nature and concentration level of the compound in the urine.

Determination of carboxylic acids in urine, *e.g.*, the metabolites of serotonin and catecholamines, has often involved an initial extraction with solvents such as ethyl acetate or diethyl ether followed by various assay techniques such as the colorimetric measurement of 5-hydroxyindole-3-acetic acid[1] or gas–liquid chromatography for vanillylmandelic acid[2]. A notable simplification of procedures has been obtained by liquid chromatography with on-line UV absorbance detection after the extraction by ethyl acetate for vanillylmandelic acid[3,4]. The chromatograms are, however, often rather complex. The application of electrochemical detectors has also been discussed[4,5]. With an even more selective liquid chromatographic detection the urine can be directly

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injected as had been done for the determination of vanillylmandelic acid by an on-line post-column reaction detector[6].

This paper discusses the separation of 5-hydroxyindole-3-acetic acid (5-HIAA) and indole-3-acetic acid (IAA) from urine. The use of liquid chromatography in the determination of 5-HIAA has previously involved an initial extraction from the urine, either by liquid–liquid extraction[7], which was not quantitative, or by chromatography[8]. Chilcote[9] greatly simplified the determination of 5-HIAA and IAA by direct injection of urine on an anion-exchange column coupled on-line to a fluorimetric detector. However, 0.5 h was required for the separation of 5-HIAA and 1 h for IAA. Deproteinized urine has been injected on reversed-phase liquid chromatographic columns combined with a fluorometric detector, but 5-HIAA did not seem to be well resolved[10].

In the present work we have compared the selectivity of a few commonly used reversed-phase liquid chromatographic systems with a newly developed phase system which has a proton-accepting stationary phase, tributyl phosphate (TBP). On-line detection by UV and fluorescence was used. TBP has extensively been used as a stationary phase in separations of metal ions by extraction chromatography[11], but its use for the column liquid chromatographic separation of organic compounds has only recently been studied, namely for carboxylic acids[12], amino acids[12,13] and catecholamines[13]. The TBP column with a fluorimetric detector was the only system that gave resolution of 5-HIAA when urine was injected directly into the column. This clearly shows the need for phase systems with selectivities higher than that of the most commonly used systems in reversed-phase liquid chromatography.

Application of the TBP system to the determination of increased levels of 5-HIAA in urine, from patients suspected to be suffering from carcinoid tumors, will be discussed elsewhere[14]. The method is more rapid and simpler than existing methods for the determination of 5-HIAA in urine.

EXPERIMENTAL

Phosphate or citrate buffers with an ionic strength of 0.1 were used. Methanol and tributyl phosphate were of analytical grade from Merck (Darmstadt, G.F.R.). Acetonitrile was Baker (Phillipsburg, NJ, U.S.A.) analyzed HPLC solvent. 5-Hydroxyindole-3-acetic acid was from Sigma (St. Louis, MO, U.S.A.) and indole-3-acetic acid from Merck. Water was deionized and purified in a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

The separation columns (100 × 4.6 mm or 150 × 4.6 mm, 316 stainless steel; Handy & Harman) were packed with LiChrosorb RP-18, 5 μm (Merck), suspended in chloroform. Zero dead volume fittings with Altex 250-21 2-μm stainless-steel frits were used in both ends of the column.

The liquid chromatograph consisted of a Milton-Roy Minipump with pulse dampener (LDC Model 711-26), a Schoeffel FS 970 fluorescence detector with continuously variable excitation wavelength and a cut-off filter to isolate the emitted light and a SpectroMonitor III (LDC) variable-wavelength UV detector. Two sample valve injectors, one Rheodyne 7120 with a 1.1-ml loop connected in series before a Valco CV-6UHPa injector with 10-μl loop, were used. A HETO Model 02 PT 923 water-bath (HETO, Birkerød, Denmark) with external circulation was used to thermostat

the eluent reservoir and the chromatographic column, by means of a water-jacket, to 25.0 ± 0.1°C.

The eluent for the TBP phase system was prepared by mixing one volume of methanol with nine volumes of phosphate buffer. This mixture was saturated at 25.0 ± 0.1°C with TBP and nine volumes of the saturated solution were mixed with one volume of a corresponding unsaturated solution, which gave an eluent with 90% saturation of TBP.

The stationary TBP phase was applied by the injection technique[12]. After coating, the column was conditioned overnight.

Urine samples were filtered through 0.5–2-μm filters or centrifuged prior to injection.

RESULTS AND DISCUSSION

Choice of phase system in the separation column

The separation column consists of LiChrosorb RP-18 as the support coated with tributyl phosphate (TBP) as the stationary liquid phase. The eluent is a buffer mixed with 10% methanol and is partly saturated with TBP. The stationary phase is a strong hydrogen acceptor which can form hydrogen bonds to carboxylic acids such as 5-HIAA and IAA. A more detailed discussion of the properties of this phase system and of its ability to retain carboxylic acids is given elsewhere[12]. Due to its hydrogen-accepting properties this phase has a selectivity different from conventional reversed-phase systems where only the RP-18 phase is used as the stationary phase.

The advantage of using the TBP phase system is obvious from Fig. 1, which gives chromatograms of a urine pool obtained in different chromatographic phase systems and with fluorometric detection. Three of the phase systems are combinations of LiChrosorb RP-18 with eluents of methanol–buffer, acetonitrile–buffer or buffer only. In these systems a high noise level is obtained in the elution region of 5-HIAA due to the presence of fluorescent compounds in the urine, and 5-HIAA cannot be resolved at its normal concentration level in urine (about 10 μmol/l). Some experiments with different concentrations of methanol and acetonitrile and at different pH values of the buffer did not show any improvement of the resolution. In the fourth system, where LiChrosorb RP-18 is coated with TBP, most of the fluorescent compounds are eluted early whereas 5-HIAA and IAA are retained selectively, resulting in complete resolution of 5-HIAA at the normal concentration level.

Choice of detector

Detection by UV absorption and by fluorescence was tested. In the concentration range of interest for 5-HIAA, *i.e.*, above the normal level of about 10 μmol/l urine, it can be detected with the UV detector at the absorption maximum of 280 nm. However, as shown in Fig. 2 for a urine sample from a healthy individual, 5-HIAA was not completely resolved and the chromatogram was rather complicated. The advantage of using the more selective fluorescence detector, as in Fig. 1D, is obvious. It gave a much simpler chromatogram and 5-HIAA was completely resolved. Although this chromatogram was from a pooled urine sample, the conclusion is correct since the individual urine sample of Fig. 2 showed the same main peaks as in Fig. 1D when fluorescence detection was used.

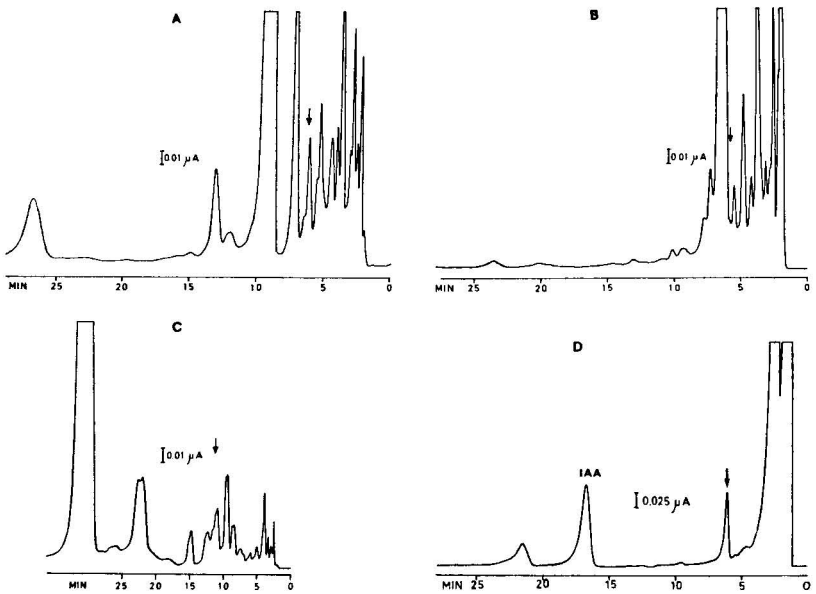


Fig. 1. Chromatograms of urine samples in different phase systems with fluorescence detection. Samples: 10 μl pooled urine from five healthy individuals. Stationary phases: RP-18 (A, B, C) and TBP (D). Eluents: A = buffer pH 5.0–methanol (85:15); B = buffer pH 3.0–acetonitrile (85:15); C = buffer pH 6.0; D = buffer pH 6.0–methanol (90:10) with 90% saturation of TBP. Excitation: 280 nm. Emission: 370 nm cut-off. Column length: 150 mm (A, B, C) and 100 mm (D). Flow-rate: 0.5 ml/min. Arrows indicate the retention time of 5-HIAA.

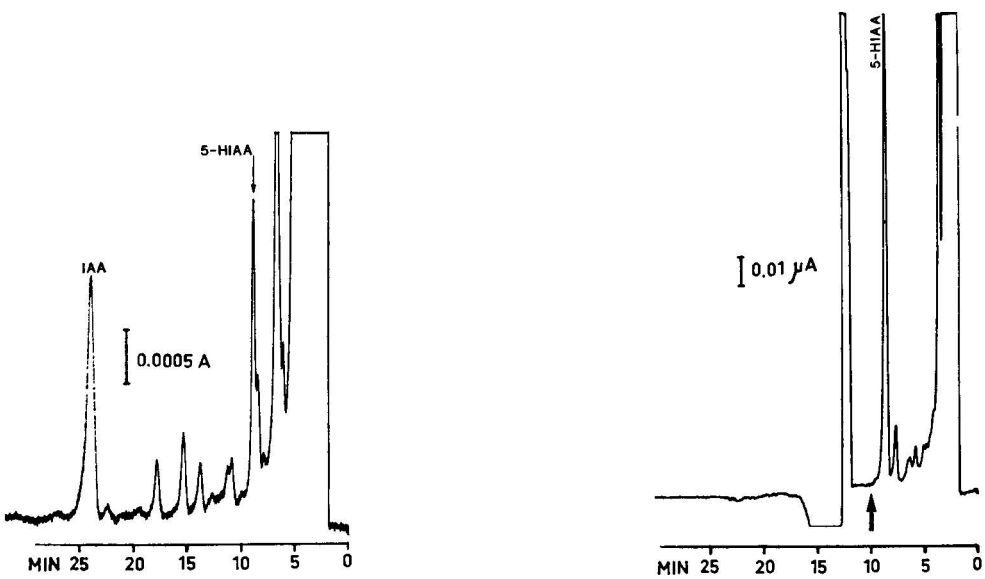


Fig. 2. Chromatogram of urine with UV-detection at 280 nm. Sample: 10 μl urine from a healthy individual. Stationary phase: TBP. Column length: 150 mm. Other conditions as in Fig. 1D.

Fig. 3. Removal of late peaks by a step change of pH of the eluent. Sample, phase system, column and flow-rate as in Fig. 2. Fluorescence detection conditions as in Fig. 1. The arrow indicates injection of 1 ml of buffer pH 8–methanol (9:1).

Stability of columns

The chromatographic phase system, based on TBP as the stationary phase, has a very high long-term stability, provided that the saturation degree of TBP in the eluent is carefully controlled, as discussed earlier[12]. No special precautions had to be taken when the urine samples were injected except that they were filtered or centrifuged before application. No deterioration in column performance due to the urine samples was observed on application of a total volume of urine of 1.0 ml corresponding to 100 samples.

Removal of late peaks by change of pH of the eluent

If only one of the components in the chromatogram has to be determined, those peaks that are eluted later increase the time intervals between sample injections. The retention time of the late peaks can be reduced by introducing a relatively small volume of a stripping eluent through the sample loop, provided that a fast equilibration is obtained on the column.

If only 5-HIAA is to be determined, IAA and some smaller peaks must be eluted before the next sample injection can be made. The retention of carboxylic acids can be decreased by increasing the pH of the mobile phase[12], *e.g.*, by injecting a 1-ml plug of buffer pH 8–methanol (9:1). At this pH IAA will have a capacity ratio 100 times smaller than at pH 6 and migrates with the front of the plug as in Fig. 3. A clean baseline is obtained within a much shorter time than in Fig. 1D, taken into account the difference in column size.

The minimum volume of stripping solvent that had to be applied to the columns was 1 ml. With smaller volumes the method was not effective. The technique was evaluated by injecting a series of 21 urine samples with intermittent injections of a pH 8 plug; 20 min were allowed between each urine injection. The retention time of 5-HIAA was unaffected by the pH 8 plugs provided that two column void volumes of eluent were passed down the column between the injection of the plug and that of the sample.

This principle can be used in many similar cases and can be automated easily.

ACKNOWLEDGEMENT

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CHROM. 13,101

ANALYTICAL ASPECTS OF BARBITURATE ABUSE

IDENTIFICATION OF DRUGS BY THE EFFECTIVE COMBINATION OF GAS–LIQUID, HIGH-PERFORMANCE LIQUID AND THIN-LAYER CHROMATOGRAPHIC TECHNIQUES

R. GILL*, A. H. STEAD and A. C. MOFFAT
Home Office Central Research Establishment, Aldermaston, Reading, Berks RG7 4PN (Great Britain)

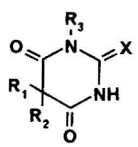
SUMMARY

Chromatographic retention data for a group of barbiturates have been measured on seven chromatographic systems (two gas–liquid chromatographic (GLC), three high-performance liquid chromatographic (HPLC) and two thin-layer chromatographic systems) and the value of these for barbiturate identification has been discussed. The overall correlations observed between pairs of systems are generally low; however, specific groups of barbiturates show very high correlations and this determines the approach to the selection of two or more systems to increase chromatographic discrimination of the barbiturate group. Column chromatographic techniques with lipophilic phases (GLC using SE-30, HPLC using ODS-silica) are most suitable for barbiturate identification. Changes of eluent pH in reversed-phase HPLC proved very effective for the separation of barbiturates with closely related structures.

INTRODUCTION

It has been estimated that of over 2500 barbiturates which have been synthesised more than 50 of these are presently marketed for clinical use throughout the world. Drug abuse involving barbiturates is widespread and the international nature of the illegal markets means that any forensic laboratory may encounter a vast range of these compounds. Furthermore, the abused barbiturates often occur in mixtures with other barbiturates, other drugs and/or excipients. The isolation and identification of a specific barbiturate thus poses a considerable analytical problem.

The barbiturates are all derivatives of barbituric acid ($R_1 = R_2 = R_3 = H$; $X = O$) and those with clinical use fall into three groups: 5,5-disubstituted oxybarbi-



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turic acids (R_1, R_2 = alkyl or aryl; R_3 = H; X = O); 1,5,5-trisubstituted oxybarbituric acids (R_1, R_2 = alkyl or aryl; R_3 = alkyl; X = O); 5,5-disubstituted thiobarbituric acids (R_1, R_2 = alkyl or aryl; R_3 = H; X = S).

All three groups of barbiturates can be distinguished by the nature of their ultraviolet (UV) spectra with changes in pH (ref. 1); however, the identification of individual compounds within any group is not possible using this method. Furthermore, UV spectroscopy may not be possible if the barbiturates are present in a mixture. The use of chromatographic techniques for the isolation and identification of barbiturates is clearly desirable. Although many procedures for the chromatographic separation of barbiturates have been published, the principles of selecting the most efficient systems (or combinations of systems) have not been discussed.

Of the clinically useful barbiturates the thiobarbituric acids have a rapid onset of action and are used almost exclusively as anaesthetics. The oxybarbituric acids are generally slower acting drugs and are used as sedative-hypnotics or anticonvulsants; these compounds are more likely to be abused. In the following study chromatographic data are presented for 28 oxybarbiturates which are likely to be encountered in forensic and clinical laboratories. A comparison of the separation of these barbiturates by gas–liquid chromatography (GLC), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) systems has been made and the combination of these systems to give maximum discrimination has been investigated.

EXPERIMENTAL

GLC was performed on a Pye 104 gas chromatograph fitted with a flame ionization detector and a glass column (2 m × 4 mm I.D.) packed with 3% SE-30 on Chromosorb G HP (80–100 mesh). Nitrogen flow-rates of 45–50 ml/min with oven temperatures of 190–200°C were used. Barbiturates were injected as solutions in ethanol. On-column methylation was carried out with Methelute (Pierce and Warriner, Chester, Great Britain).

HPLC was performed with a constant-flow pump (Waters M6000), a variable-wavelength UV detector (Pye-Unicam LC-UV) and a valve injector (Rheodyne 7120) fitted with a 20-μl loop. The normal-phase column (250 × 4.6 mm I.D.) and the reversed-phase column (150 × 4.6 mm I.D.) were packed with Hypersil and ODS-Hypersil (Shandon Southern Products, Runcorn, Great Britain) respectively. The reversed-phase eluents were prepared by mixing aqueous sodium dihydrogen phosphate (0.1 *M*) and methanol then adjusting the final pH with sodium hydroxide or phosphoric acid. Flow-rates of 2 ml/min were used throughout. The detector was operated at 216 nm for reversed-phase and 250 nm for normal-phase chromatography. Barbiturates were injected in ethanolic solution.

TLC was conducted using glass-coated silica gel 60 F254 plates, 20 × 20 cm, 0.25 mm thickness from E. Merck (Darmstadt, G.F.R.). The spots were detected by spraying with ethanolic diphenylcarbazone and mercuric chloride[2].

The seven chromatographic systems examined in this study were as follows:

(1) GLC using SE-30 stationary phase;

(2) GLC using SE-30 stationary phase with co-injection of Methelute;

(3) HPLC using ODS-silica with an eluent of 40% methanol at pH 3.5;

(4) HPLC using ODS-silica with an eluent of 40% methanol at pH 8.5;

(5) HPLC using silica with an eluent of isooctane–acetic acid–isopropanol (200:3:2, v/v/v);

(6) TLC using silica plates with a developing solvent of chloroform–acetone (4:1, v/v);

(7) TLC using silica plates with a developing solvent of isopropanol–chloroform–ammonia (9:9:2, v/v/v).

RESULTS AND DISCUSSION

Choice of chromatographic systems

Barbiturates have been chromatographed using a wide range of GLC stationary phases and Berry[3], considering the needs of the clinical toxicologist, has recommended the CDMS phase. The choice of SE-30 in the present work reflects the wide application of this phase to forensic problems and the vast amount of data on drugs which is already available[4,5]. Other GLC stationary phases are certainly capable of providing satisfactory separations of the barbiturates.

Hydrocarbonaceous bonded phases have been widely used in HPLC for the separation of pharmaceutical compounds including small groups of barbiturates. Separations using ODS-silica[6–12], SAS-silica[13] and methyl-silica[14] have been reported and the limited data available for aqueous methanolic eluents show a common order of elution and suggest that similar mechanisms are operating on these phases. Initial experiments showed that differences in selectivity could be achieved by changing the pH of the eluent. Two of the systems examined involve the chromatography of barbiturates on ODS-silica. Both eluents contain 40% methanol and the two differ only in the pH. Only a few publications give data concerning the separation of barbiturates by HPLC using microparticulate silica[15–19]. Our initial experiments showed that the barbiturates give poor peak shapes with silica when neutral or basic eluents were used. A system was subsequently developed using an acidic eluent which gave good peak shapes and efficiencies.

Many TLC systems for the separation of barbiturates have been recorded in the literature[20,21]. The two systems included in this study were chosen as examples of systems frequently used in forensic casework[2]. One involves a neutral developing solvent (chloroform–acetone) and the other, a basic solvent (isopropanol–chloroform–ammonia).

The chromatographic retention data for the 28 barbiturates on the seven system are given in Table I.

Comparison of the chromatographic systems

Any chromatographic system which is proposed as a routine analytical procedure must fulfill several requirements: the analysis time must be as short as possible; the system should be simple to set up (*e.g.*, isothermal GLC, isocratic HPLC); the system must achieve the necessary level of reproducibility both within-laboratory and between-laboratory; the detection method for the system must be sufficiently sensitive for the application. Any chromatographic system which meets these general criteria must then be examined with reference to the separation of the group of compounds of interest.

TABLE I

CHROMATOGRAPHIC RETENTION DATA FOR 28 BARBITURATES

System 1, GLC using SE-30; system 2, GLC using SE-30 with methylation; system 3, HPLC using ODS-silica (pH 3.5); system 4, HPLC using ODS-silica (pH 8.5); system 5, HPLC using silica; system 6, TLC using silica (chloroform–acetone); system 7, TLC using silica (isopropanol–chloroform–ammonia).

Barbiturate	*Structure*			*GLC (RI)*		*HPLC (k')*			*TLC ($R_F \times 100$)*	
	R_1	R_2	R_3	*System 1*	*System 2*	*System 3*	*System 4*	*System 5*	*System 6*	*System 7*
Allobarbitone	Allyl	Ally	H	1586	1491	2.46	1.33	9.03	59	53
Amylobarbitone	Ethyl	Isopentyl	H	1700	1600	10.91	7.05	7.17	66	74
Aprobarbitone	Isopropyl	Allyl	H	1600	1513	3.42	2.22	8.10	59	66
Barbitone	Ethyl	Ethyl	H	1482	1415	1.11	0.63	11.95	51	51
Brallobarbitone	Allyl	2-Bromoallyl	H	1842	1741	3.09	1.72	10.88	60	47
Butalbital	Isobutyl	Allyl	H	1658	1553	6.17	3.48	6.68	63	67
Butobarbitone	Ethyl	*n*-Butyl	H	1645	1557	5.43	3.42	7.60	64	68
Cyclobarbitone	Ethyl	Cyclohex-1-enyl	H	1945	1850	5.25	2.61	10.04	59	59
Cyclopentobarbitone	Allyl	Cyclopent-2-enyl	H	1858	1751	6.00	3.84	8.13	64	62
Enallylpropymal	Isopropyl	Allyl	Methyl	1559	1522	8.65	6.96	1.90	88	87
Heptabarbitone	Ethyl	Cyclohept-1-enyl	H	2035	1932	9.90	4.93	9.42	61	62
Hexethal	Ethyl	*n*-Hexyl	H	1835	1748	34.28	20.39	6.31	71	74
Hexobarbitone	Methyl	Cyclohex-1-enyl	Methyl	1850	1800	7.37	5.67	4.87	77	85
Ibomal	Isopropyl	2-Bromoallyl	H	1866	1759	4.01	2.58	10.27	63	61
Idobutal	*n*-Butyl	Allyl	H	1698	1608	8.12	4.77	6.33	68	71
Metharbitone	Ethyl	Ethyl	Methyl	1450	1410	2.69	1.99	2.75	79	86
Methohexitone	Allyl	1-Methylpent-2-ynyl	Methyl	1768	1721	27.61	20.48	1.48	88	93
Methylphenobarbitone	Ethyl	Phenyl	Methyl	1882	1832	7.27	3.84	2.91	80	72
Nealbarbitone	2,2-Dimethylpropyl	Allyl	H	1720	1609	10.22	6.19	5.79	69	78
Pentobarbitone	Ethyl	1-Methylbutyl	H	1733	1632	10.96	8.07	6.78	65	76
Phenobarbitone	Ethyl	Phenyl	H	1934	1831	3.09	1.23	12.57	56	38
Phenylmethylbarbituric acid	Methyl	Phenyl	H	1875	1789	1.48	0.94	24.20	41	27
Probarbitone	Ethyl	Isopropyl	H	1550	1480	2.15	1.57	8.96	57	65
Quinalbarbitone	1-Methylbutyl	Allyl	H	1770	1670	16.28	11.47	5.63	72	78
Secbutobarbitone	Ethyl	1-Methylpropyl	H	1650	1564	4.89	3.32	7.59	60	69
Sigmodal	1-Methylbutyl	2-Bromoallyl	H	2031	1901	19.58	12.37	5.87	73	73
Talbutal	1-Methylpropyl	Allyl	H	1704	1592	7.25	4.67	6.50	65	71
Vinbarbitone	Ethyl	1-Methylbut-1-enyl	H	1755	1650	4.83	2.32	9.88	62	56

The effective separation of a given group of compounds with a chromatographic system is controlled by two factors. Firstly, the band spreading which occurs as the compounds migrate through the stationary phase determines the number of compounds which can be resolved across the chromatographic range (*e.g.*, the number of spots which can be separated across a TLC plate). Minimum band spreading is clearly desirable and for column chromatography (GLC, HPLC) this is expressed as a high plate count. No attempt has been made in this study to optimise this factor for the seven chromatographic systems. In general, the GLC and HPLC systems are capable of resolving more compounds than the TLC systems. Longer GLC or HPLC columns would give greater plate counts but only at the cost of longer analysis times. The second factor which determines the effectiveness of a system concerns the frequency distribution of the compounds across the chromatographic range. Maximum discrimination occurs when the retention parameters for the group show an even distribution over the entire range.

The frequency distributions for the retention parameters of the 28 barbiturates on the GLC, HPLC and TLC systems are shown in Fig. 1. The retention index (RI) values for the barbiturates with SE-30 stationary phase show a good distribution over the chromatographic range (Fig. 1a); no more than four compounds lie within any range of 50 RI units. Comparison of these results with those for GLC with methylation (Fig. 1b) shows an overall move towards smaller RI values with a slight contraction of the distribution. This is expected as the addition of methyl groups increases the volatility of the barbiturates. Opposed to this, methylation gave an improvement in peak symmetry for most compounds. Two peaks were observed when one of the barbiturates containing a 2-bromoallyl substitutent (brallobarbitone, ibomal and sigmodal) was co-injected with Methelute. The peak with the longer retention time represented the dimethyl derivative while the other peak resulted from the elimination of hydrogen bromide from this derivative. Peaks were identified by mass spectrometry.

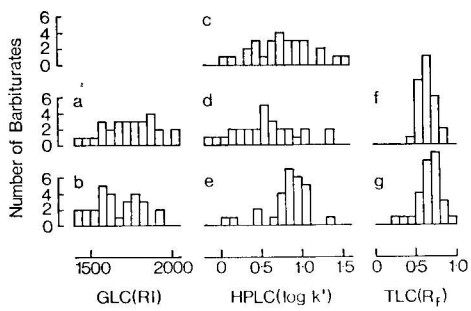


Fig. 1. Frequency distributions of retention parameters for 28 barbiturates on 7 chromatographic systems: (a) GLC using SE-30; (b) GLC using SE-30 with methylation; (c) HPLC using ODS-silica at pH 3.5; (d) HPLC using ODS-silica at pH 8.5; (e) HPLC using silica; (f) TLC using silica plates with chloroform–acetone; (g) TLC using silica plates with isopropanol–chloroform–ammonia.

Figs. 1c and 1d show the frequency distributions for the reversed-phase HPLC systems which differ only by the pH of the eluent. The data in Fig. 1c (pH 3.5) show a shift towards longer retention times relative to those in Fig. 1d (pH 8.5) and reflect the increase in lipophilicity of the barbiturates as they change from the ionised to the

TABLE II

LINEAR CORRELATION COEFFICIENTS FOR THE COMBINATION OF RETENTION DATA FROM PAIRS OF CHROMATOGRAPHIC SYSTEMS

Calculations involving GLC and TLC data use RI and R_F values respectively. Combinations of HPLC data with other HPLC data use k', combinations with GLC data use $\log k'$ and combinations with TLC data use R_F values; $R_F = 1/(1 + k')$ (ref. 24).

Chromatographic system combinations	*Linear correlation coefficients (r)*		
	All barbiturates (28)	*5,5-Dialkyl barbituric acids (7)*	*5-Alkyl-5-allyl barbituric acids (6)*
a GLC (SE-30) *vs.* HPLC (ODS-silica; pH 3.5)	0.379	0.995	0.986
b GLC (SE-30) *vs.* HPLC (silica)	0.216	0.944	0.959
c GLC (SE-30) *vs.* TLC (chloroform–acetone)	0.050	0.967	0.919
d HPLC (ODS-silica; pH 3.5) *vs.* HPLC (silica)	0.557	0.935	0.969
e HPLC (ODS-silica; pH 3.5) *vs.* TLC (chloroform–acetone)	0.673	0.968	0.965
f HPLC (silica) *vs.* TLC (chloroform–acetone)	0.901	0.965	0.967
g GLC (SE-30) *vs.* GLC (SE-30 with methylation)	0.989	0.997	0.989
h HPLC (ODS-silica; pH 3.5) *vs.* HPLC (ODS-silica; pH 8.5)	0.986	0.997	0.992
i TLC (chloroform–acetone) *vs.* TLC (isopropanol–chloroform–ammonia)	0.876	0.898	0.912

neutral form. Nevertheless, both reversed-phase systems do show a wide distribution of data. Fig. 1e shows that the distribution across the normal-phase HPLC system is not so even with a concentration of data around $k' = 7$.

The frequency distributions of the R_F values for the two TLC systems are shown in Figs. 1f and 1g; considerable clustering can be seen in both systems. The chloroform–acetone system (Fig. 1f) has 25 barbiturates with R_F values between 0.5 and 0.8 while the isopropanol–chloroform–ammonia system has only a slightly better distribution with 21 drugs within this range.

It is clear that the two GLC systems and the two reversed-phase HPLC systems have better distributions of chromatographic retention parameters than any of the systems based on silica as the stationary phase (*i.e.*, the normal-phase HPLC system and the two TLC systems). The lipophilic nature of the SE-30 and ODS-silica stationary phases thus provides a better separation of the group of barbiturates. This reflects the fact that the barbiturates differ from each other by the nature of their lipophilic substituents. Of the three chromatographic systems based on silica (two TLC systems and one HPLC system) the normal-phase HPLC system shows the best frequency distribution; furthermore it is capable of resolving a greater number of compounds across its chromatographic range.

Combination of chromatographic systems

The number of compounds which can be resolved on a single chromatographic system is limited and hence the combination of data from different systems to give extra discrimination is desirable. It is generally regarded that the combination of chromatographic systems to give maximum discrimination requires a low correlation between the systems[22]. An examination of the relationships between the present experimental systems has therefore been performed (see Table II). Although the overall correlations between pairs of systems for the 28 barbiturates may be low, specific groups of barbiturates show very high correlations.

This is well illustrated by the combination of the GLC data obtained for the barbiturates using SE-30 and the reversed-phase HPLC data at pH 3.5. The 28 barbiturates show a low overall correlation between these two systems ($r = 0.379$); however, the dialkylbarbituric acids and alkyl, allylbarbituric acids show high linear correlations ($r = 0.995$ and $r = 0.986$ respectively). The regression lines for these groups are parallel and very close together (Fig. 2; lines C and D). The dialkylbarbituric acids and the alkyl, allylbarbituric acids can be combined with allobarbitone (5,5-diallylbarbituric acid) with no significant change in the overall linear correlation coefficient ($r = 0.992$). It thus appears that an allyl group contributes to the chromatographic retention properties of the barbituric acids on these two systems in a similar way to an alkyl group.

Fig. 2 shows four other lines drawn parallel to lines C and D; these represent further specific groups of barbiturates. Line A passes through enallylpropymal (1-methyl-5-isopropyl-5-allylbarbituric acid); line B passes through metharbitone (1-methyl-5,5-diethylbarbituric acid); line E passes through the two alkyl, bromoallylbarbituric acids (ibomal and sigmodal); line F passes through the two alkyl, phenylbarbituric acids (methylphenobarbitone and phenobarbitone). The proximity of line A (1-alkyl-5-alkyl-5-allyl barbiturates) to line B (1-alkyl-5,5-dialkyl barbiturates) and the observation that brallobarbitone (an allyl, bromoallylbarbituric

acid) falls close to line E (the alkyl, bromoallylbarbituric acids) give further evidence that an allyl group contributes to the GLC and HPLC retention properties in a very similar way to an alkyl group.

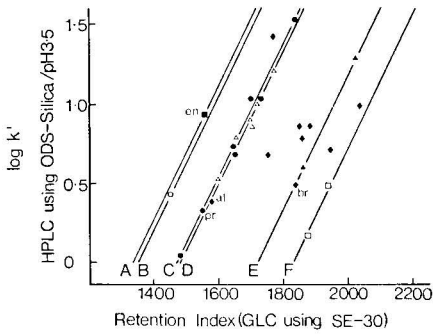


Fig. 2. Correlation of GLC retention data (SE-30 stationary phase) for 28 barbiturates with HPLC retention data (ODS-silica; eluent pH 3.5). ■ = 1-methyl-5-alkyl-5-allylbarbituric acids (line A); ○ = 1-methyl-5,5-dialkylbarbituric acids (line B); ● = 5,5-dialkylbarbituric acids (line C); △ = 5-alkyl-5-allyl-barbituric acids (line D); ▲ = 5-alkyl-5-bromoallylbarbituric acids (line E); □ = 5-alkyl-5-phenylbarbituric acids (line F); ◆ = all other barbiturates. al = allobarbitone, br = brallobarbitone, en = enallylpropymal and pr = probarbitone.

Table II gives correlation data for combinations of the experimental chromatographic systems. Table II (a–f) represents the possible combinations of the GLC system without methylation, the reversed-phase HPLC system at pH 3.5, the normal-phase HPLC system and the TLC system with chloroform–acetone developing solvent. Linear correlation coefficients have been calculated for the total group of 28 compounds as well as the dialkylbarbituric acids and the alkyl, allylbarbituric acids for each pair of systems. The linear correlation coefficients for the total group of barbiturates are generally low, the highest value being observed for the combination of the normal-phase HPLC and TLC systems ($r = 0.901$). The sub-division of the 28 barbiturates into structurally related groups leads to a large increase in correlation coefficient in all cases. The lowest linear correlation for the group of dialkylbarbituric acids is observed for the combination of the normal-phase and reversed-phase HPLC systems ($r = 0.935$); however, these data points lie on a smooth curve and hence calculation of a linear correlation coefficient cannot totally reflect the inter-relationship between the two sets of data. Similar non-linear correlations are observed with other combinations of systems.

The combinations of systems already considered represent different modes of chromatography (*e.g.*, HPLC using silica *vs.* TLC using silica) or different stationary phases (*e.g.*, HPLC using silica *vs.* HPLC using ODS-silica). Table II (g–i) gives combinations of systems involving the same mode of chromatography with identical stationary phases. The data show that the overall correlations for these pairs are high, however sub-division of the barbiturates into structurally related groups still leads to an increase in correlation in most cases. A plot showing the combination of the two GLC systems shows that the data points for the group of disubstituted barbiturates fall around one line ($r = 0.997$) while the trisubstituted barbiturates fall on a separate but parallel line ($r = 1.000$).

The identification of an unknown compound using chromatographic techniques involves the unique matching of the chromatographic properties of the compound with a single member of a defined group of compounds. Such a unique match differentiates the unknown from other members of the defined group but does not exclude compounds from outside the group. If an unknown compound does not show a unique match with the first chromatographic system (*i.e.*, the unknown compound shows identical chromatographic properties with a group of unresolved compounds within the group) a second system must be chosen which can separate these unresolved compounds. The high correlations for structurally related groups of barbiturates between different chromatographic systems which have been observed in this study must influence the choice of the second chromatographic system. It can be concluded that the second system must be chosen with reference to the nature of the compounds which need to be separated and not on the overall correlation of the two systems. If the unresolved barbiturates belong to different structural groups the second system should be selected such that the combination gives maximum separation of the regression lines for the structurally related groups. Enallylpropymal (a trisubstituted barbituric acid) and probarbitone (a disubstituted barbituric acid) provide an example of this type. The free barbiturates are not resolved by GLC (SE-30) but are separated from the remaining 26 barbiturates in the group. The correlation of the GLC data with the data from reversed-phase HPLC at pH 3.5 shows that the regression lines on which the data points for the two compounds fall are well separated (Fig. 2). The reversed-phase HPLC system is therefore a good choice for the second chromatographic system and enables these compounds to be separated. Similarly, the two compounds lie on well separated regression lines when the two GLC systems are correlated and hence GLC using SE-30 with methylation would also be a satisfactory second system.

Alternatively, when the barbiturates not resolved on the first chromatographic system are of the same structural type (*e.g.*, both dialkylbarbituric acids) the second system must be chosen such that the correlation of this structural group between the two systems is as low as possible. The separation of such compounds is often difficult, *e.g.*, butobarbitone and secbutobarbitone can only be separated by reversed-phase HPLC at pH 3.5. The use of the two reversed-phase HPLC systems (one at pH 3.5, the other at pH 8.5) in combination proved very useful for the separation of such difficult pairs. The pK_a values of the barbiturates fall within the range 7–8.5 (ref. 23) and therefore the compounds are fully protonated at pH 3.5 but only partially so at pH 8.5. Although the overall correlation between these two systems is high (Table II, h) many changes in selectivity occur between them, *e.g.* amylobarbitone–pentobarbitone and cyclobarbitone–butobarbitone pairs can be resolved at pH 8.5 but not at pH 3.5 while amylobarbitone–enallylpropymal, butobarbitone–secbutobarbitone and cyclobarbitone–vinbarbitone pairs can be resolved at pH 3.5 but not at pH 8.5.

In conclusion, the present study has shown that TLC retention data have limited value for the identification of barbiturates although it should be remembered that the application of specific visualisation reagents (*e.g.* potassium permanganate for barbiturates with unsaturated substituents) can give some increase in discrimination. The column chromatographic systems with lipophilic stationary phases (GLC using SE-30, HPLC using ODS-silica) are to be preferred as they show a wide distribution of retention parameters. The effective combination of different chromato-

graphic systems to increase discrimination must consider the high correlations observed for groups of structurally related barbiturates and should not be based on the overall correlation of the retention data between the systems. The separation of barbiturates with very similar structures is best approached by using the effect of eluent pH on reversed-phase HPLC.

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RETENTION DATA METHODS FOR THE DETERMINATION OF DRUG–PROTEIN BINDING PARAMETERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BERNARD SEBILLE* and NICOLE THUAUD

Laboratoire de Physico-Chimie des Biopolymères, Université de Paris Val de Marne, Avenue du Général de Gaulle, 94010 Creteil Cedex (France)

and

JEAN PAUL TILLEMENT

Département de Pharmacologie, Université de Paris Val de Marne, Avenue du Général Sarrail, 94010 Creteil Cedex (France)

SUMMARY

The binding to human serum albumin of some drugs (warfarin, furosemide and phenylbutazone) has been studied by high-performance liquid chromatography. Two methods have been used and compared, based on the measurement of the ligand retention volume under different conditions. The obtained total affinities of the drugs for the protein are in accordance with our previous results. The equilibrium saturation method leads easily to n_i and K_i parameters from the retention volume of the ligand.

INTRODUCTION

Gel filtration chromatography has been widely applied for the determination of binding parameters of macromolecules–low-molecular-weight ligands[1]. Except in the case of very stable associations, where zonal chromatography is suitable, it is necessary to use specific processes to prevent dissociation of the complex during its passage through the column.

In a previous paper[2], we have described the application of the Hummel and Dreyer method[3] with a high-performance liquid chromatograph for the determination of binding parameters of warfarin–human serum albumin. It is also possible to obtain these parameters by an equilibrium saturation method with the same apparatus[4]. In the present work, we have used another method which relies upon the determination of the retention volume of a drug eluted on an appropriate column with a protein solution. We have previously used this method in the case of L-tryptophan–human serum albumin (HSA)[5]. This method has some features similar to the work of Uekama *et al.*[6] on steroid–cyclodextrin associations and of Horváth *et al.*[7] on ion–nucleotide complexes.

We have compared the results of the equilibrium saturation method with those obtained by retention volume determination.

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THEORETICAL

The reversible binding of a ligand to a macromolecule is governed by the multiple equilibria theory[8–10] which yields the equation

$$\bar{r} = \sum_{i=1}^{i=m} n_i K_i [A]_f / (1 + K_i [A]_f)$$

where $\bar{r}$ is the mean number of ligands per mole of macromolecule, m is the number of classes of independent sites where each class, i, has n_i sites with binding affinity K_i and $[A]_f$ is the concentration of free (unbound) ligand. The retention volume of the ligand, V_R, is given by the usual chromatography equation

$$V_R = V_0 + K v_f$$

where V_0 is the retention volume of an unretained compound, v_f is the stationary phase volume and K is the distribution coefficient of the ligand between the stationary and mobile phases. If the mobile phase contains a polymer of concentration $[P]_0$ giving with the ligand A complexes PA, PA_2 ..... PA_n a new distribution coefficient, K', needs to be considered because of the different equilibria presented in Table I. The ligand retention volume becomes:

$$V'_R = V_0 + K' v_f$$

If we define α as the ratio of the concentration of bound ligand, $[A]_b$, to the concentration of total ligand, $[A]_0$, we obtain the following equation:

$$\alpha = [A]_b / [A]_0 = \bar{r} [P]_0 / [A]_0 \tag{1}$$

Since by definition $K' = [A]_{st}/[A]_0$ and $K = [A]_{st}/[A]_f$, where $[A]_{st}$ is the ligand concentration in the stationary phase, it follows that $K' = (1 - \alpha)K$ and:

$$\frac{V'_R - V_0}{V_R - V_0} = \frac{K'}{K} = 1 - \alpha = 1 - \frac{\bar{r}[P]_0}{[A]_0}$$

Then, since

$$[A]_0 = [A]_f + [A]_b = [A]_f \left(1 + r \frac{[P]_0}{[A]_f}\right)$$

We obtain:

$$\frac{V'_R - V_0}{V_R - V_0} = \frac{1}{1 + ([P]_0/[A]_f)} = \frac{1}{1 + \sum_{i=1}^{i=m} n_i K_i [P]_0 / (1 + K_i [A]_f)} = \frac{K'}{K} \tag{2}$$

This equation shows the dependence of the retention volume, V'_R, on the association parameters, n_i and K_i, polymer concentration, $[P]_0$, and free ligand concentration, $[A]_f$ (dependent upon the amount of injected ligand).

TABLE I

EQUILIBRIUM BETWEEN STATIONARY AND MOBILE PHASES IN THE PRESENCE OF PROTEIN

Stationary phase		*Mobile phase*
A_{st} ligand in stationary phase	$\rightleftharpoons$	A_f free ligand in mobile phase
		+ P ⇅ $PA + PA_2 + \ldots PA_n$ bound ligand in mobile phase

If the amount of injected ligand is small enough to render $K_i[A]_f$ negligible compared with 1, eqn. 2 simplifies to

$$\frac{V'_{R1} - V_0}{V_R - V_0} = \frac{1}{1 + \sum_{i=1}^{i=m} n_i K_i [P]_0} = 1 - \alpha_1 = \frac{K'}{K} \tag{3}$$

where V'_{R1} and α_1 are the limit values of V'_R and α when the injected amount of ligand tends to zero.

$$\alpha_1 = \sum_{i=1}^{i=m} n_i K_i [P]_0 / 1 + \sum_{i=1}^{i=m} n_i K_i [P]_0 \tag{4}$$

Eqn. 3 leads to the "total affinity" $\sum_{i=1}^{i=m} n_i K_i$ since:

$$\sum_{i=1}^{i=m} n_i K_i = \frac{1}{[P]_0} \cdot \frac{1}{1 - \alpha_1} \tag{5}$$

For a specific value of $[P]_0$, it is possible to determine $V'_{R1} - V_0$ which is just half of $V_R - V_0$ when $\alpha_1 = 0.5$. Under these conditions

$$\sum_{i=1}^{i=m} n_i K_i = 1/[P]_0 \tag{6}$$

and we can readily obtain the total affinity.

EXPERIMENTAL

Apparatus

A 6000 A pump, U6K injector and a 440 UV detector, all from Waters Assoc. (Milford, MA, U.S.A.), were used for all the experiments. The monitored wavelength was 280 nm for furosemide and phenylbutazone and 313 nm for warfarin. The stainless-steel column (15 cm × 4.7 mm I.D.) was filled by a slurry packing technique with 10-μm LiChrosorb Diol support (E. Merck, Darmstadt, G.F.R.).

Materials

Warfarin (Merell-Toraude Laboratory, Paris, France), furosemide (Hoechst, Frankfurt/M,G.F.R.) and phenylbutazone (Ciba-Geigy, Basle, Switzerland) were gifts. HSA (A 1887, essentially fatty acid free albumin) was from Sigma (St. Louis, MO, U.S.A.). All products were dissolved in a 0.067 *M* phosphate buffer, pH 7.4. In all the experiments, the column temperature was maintained at 37°C.

RESULTS AND DISCUSSION

The retention volumes of some drugs injected onto a size exclusion chromatographic support eluted by different solutions of human serum albumin were measured. Fig. 1 shows the variation of retention volume with the injected amount of different drugs. As predicted by eqn. 2, the retention volume reaches a limit V'_{R1} when the injected quantity tends to zero. This limiting retention volume allows the determination of $\Sigma n_i K_i$ by using eqn. 3.

We observed that peak dissymetry grows with increasing warfarin injection (Fig. 2). This can be explained by eqn. 2 in which K' depends on the injected amount of ligand. If $K_i[A]_f$ is small enough for eqn. 3 to be valid, K' is a constant and the ligand peak is symmetrical.

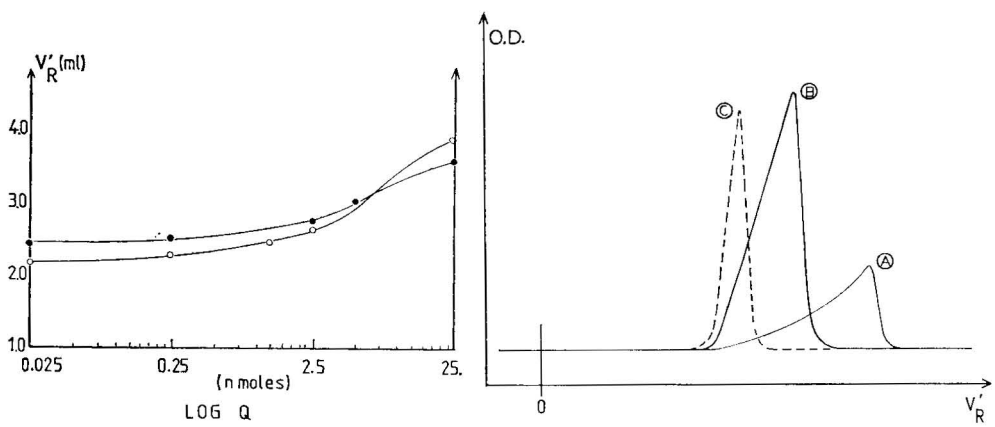
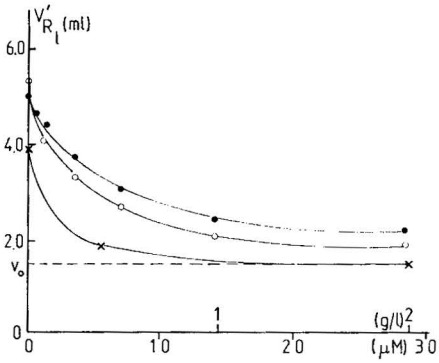


Fig. 1. Influence of injected ligand amount, Q, on retention volume. Eluent: 1 g/l HSA (buffered at pH = 7.4); flow-rate 0.4 ml/min. ○, Warfarin; ●, furosemide.

Fig. 2. Shape of chromatograms as a function of injected ligand amount. Eluent: 2 g/l HSA (buffered at pH = 7.4); Flow-rate 0.4 ml/min. Injection (25 μl warfarin): A, 10^{-3} *M*, 0.5 O.D. units full scale; B, $2 \cdot 10^{-2}$ *M*, 0.2 O.D. units full scale; C, 10^{-5} *M*, 0.02 O.D. units full scale.

The variation of limiting retention volume of the ligand as a function of protein concentration is shown in Fig. 3, and it is seen that the curve has a hyperbolic profile as predicted by eqn. 3. With the V'_{R1} values and using eqn. 3 it is possible to calculate α_1 and to plot α_1 as a function of log $[P]_0$. The curve of Fig. 4 has the shape predicted by eqn. 5.

It is possible to calculate the total affinity $\Sigma n_i K_i$ either from the specific value of $1/[P]_0$ when $\alpha_1 = 0.5$, or by using eqn. 4 for each value of α_1 and different $[P]_0$.



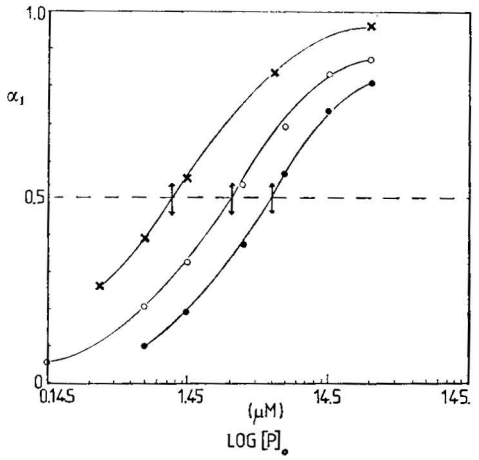


Fig. 3. Limiting retention volume, V'_{R1}, of different drugs as a function of HSA concentration in the eluent. ○, warfarin; ●, furosemide, ×, phenylbutazone.

Fig. 4. Variation of α_1 as a function of HSA concentration, $[P]_0$, in the eluent. For symbols, see Fig. 3.

In Table II, we have compared the results obtained by these different procedures for three drugs: warfarin, furosemide and phenylbutazone. The results previously reported[2,11] using Hummel and Dreyer's method are in good agreement, as were those of the equilibrium saturation method applied to warfarin–HSA binding[3].

TABLE II

TOTAL AFFINITY $\sum_{i=1}^{i=m} n_i K_i$ (M^{-1}) OF VARIOUS DRUGS FOR HSA

	Phenyl butazone	*Warfarin*	*Furosemide*
$n_1K_1 + n_2K_2$ (Hummel and Dreyer's method)	820.000 (ref. 11)	301.000 (ref. 2)	191.000 (ref. 2)
$\sum_{i=1}^{i=m} n_i K_i = 1/[P_0]$ (for $\alpha_1 = 0.5$)	860.000	320.000	180.000
$\sum_{i=1}^{i=m} n_i K_i$, mean value of	845.000	325.000	186.000
$\frac{1}{[P_0]} \cdot \frac{\alpha_1}{(1-\alpha_1)}$ (from eqn. 5)	845.000	325.000	186.000

We now discuss in detail a point mentioned in our previous work on the equilibrium saturation method[3]. In this method, the eluent is a buffered solution of drug and protein. The injection of 25 μl of buffer alone leads to the appearance of two negative peaks: the first corresponds to the liganded protein and the second to the free ligand. We noticed that in this case the retention volume of the ligand, V'_R,

was dependent upon the extent of association with protein[3]. From the theoretical results mentioned above, we are now able to explain this phenomenon. Indeed, from eqn. 1, V'_R is directly related to α:

$$V'_R - V_0 = (V_R - V_0)(1 - \alpha) \tag{7}$$

In this formula, there is a linear relationship between V'_R and $(1 - \alpha)$. We have verified this by plotting V'_R as a function of $(1 - \alpha)$. The parameter α is obtained, using eqn. 1, from the measurement of ligand peak area.

The results obtained from our previous experiments[3,12] are described in Fig. 5. There is satisfactory agreement between the experimental values and those provided by eqn. 7, especially for the protein solutions at low concentrations.

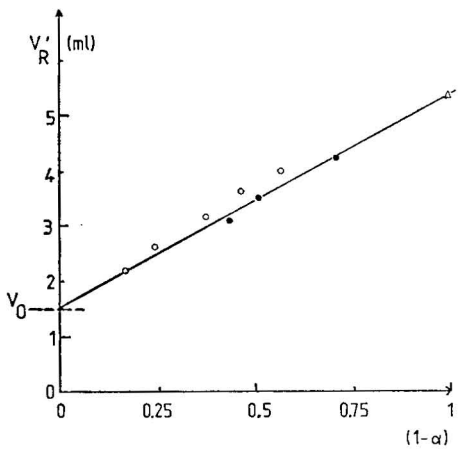


Fig. 5. Warfarin retention volume as a function of the free drug fraction, $(1 - \alpha)$, in the equilibrium saturation method. ○, 2 g/l HSA; ●, 0.4 g/l HSA; △, warfarin retention volume in the buffer alone as eluent ($\alpha = 0$).

Owing to the linear relationship between α and V'_R, it is possible to determine α from experimental V'_R values and to calculate $\bar{r}$ from eqn. 1. Then, a Scatchard plot leading to n_i and K_i parameters can be drawn[3].

It may be concluded that, in the saturation method, the mean number of bound ligands, $\bar{r}$,can be obtained either from measurement of the ligand peak area or from the ligand retention volume.

CONCLUSION

The retention volume of a ligand eluted by a protein solution depends on the association of the ligand with the macromolecules. It is possible to work out very easily the total affinity $\Sigma n_i K_i$ from the limiting retention volume, *i.e.*, the retention volume extrapolated to a zero amount of injected ligand. The results obtained for warfarin, furosemide and phenylbutazone association with HSA are in a good agreement with previous results[2,11] obtained by using Hummel and Dreyer's method.

The observed change of the ligand retention volume with extent of association has allowed us to explain some features of the equilibrium saturation method that we have previously described[3]. We have demonstrated that, in this method, the binding parameter, $\bar{r}$, and more generally the parameters n_i and K_i can be obtained from the determination of the retention volume of the ligand. This very easy procedure offers new possibilities for studying ligand–macromolecule interactions.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARDIAC GLYCOSIDES

V. Ya. DAVYDOV, M. ELIZALDE GONZALEZ and A. V. KISELEV*
Chemistry Department, State University of Moscow, Moscow 117234 (U.S.S.R.)

SUMMARY

Highly efficient, short columns have been prepared for the separation of mixtures of cardiac glycosides at relatively small pressure drops. The selectivities of silica adsorbents with different chemically modified surface layers for cardiac glycosides were determined. The influence of the structure of cardiac glycoside molecules on their retention characteristics in liquid chromatography was investigated. The relative number of hydrophobic and hydrophilic groups in the glycoside molecules as well as the configurations are very important for the separation.

INTRODUCTION

The chromatographic investigation of cardiac glycosides is of great interest, first because these natural biologically active compounds are used increasingly in medicine and secondly because the large number of compounds in this class with different structures enables the investigation of the relation between their structures and retention volumes. Moreover, in the course of optimizing the chromatographic separation of glycosides it is also possible to investigate some physico-chemical problems of adsorption from dilute solution and of the surface chemistry of the adsorbents.

Although a number of studies[1–9] have been devoted to the chromatographic analysis of cardiac glycosides, many problems remain to be solved, one of which is the correlation between their structures and retention volumes. The determination of such correlations in liquid chromatography requires the use of adsorbents with homogeneous surfaces, as in gas chromatography[10].

Hydroxylated silica and silicas with chemically modified surfaces are generally used as adsorbents in liquid chromatography[11–13]. Most often, long hydrocarbon chains are attached to the silica surface and it is assumed that these form a "brush" on the surface. The use of diphenyldichlorosilane for the chemical modification of silica surfaces makes it possible to obtain a hydrophobic surface with a large carbon content that is very important for increasing the capacity factor. At the same time the phenyl groups are rigid and they can only vibrate around the carbon–silicon bond.

The chemical modification of silica surfaces by diphenyldichlorosilane is carried out in toluene at 80–100°C. The surface is first covered by the electron donor

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triethylamine which accelerates the reaction. A convenient method of qualitative and quantitative control of the reaction is infrared (IR) spectroscopy which enables the evaluation of the surface concentration of phenylsilyl groups, α, and observation of the attached phenyl groups. Since α for LiChrosorb Si 100 (particle size 30 μm) with attached phenylsilyl groups was determined earlier independently of the carbon content on the surface, and since the extinction coefficient, K, is the same for all samples, it is possible to evaluate the concentration of attached diphenylsilyl groups by the spectroscopic method.

Fig. 1 shows the IR spectra of silica with different specific surface areas after chemical modification by diphenyldichlorosilane. The band at $\nu = 3083\ \mathrm{cm}^{-1}$ may be used for the evaluation of the surface concentration of phenylsilyl groups. The optical density, D, is related to the surface concentration, α, by

$$D = \ln I_0/I = K\alpha\varrho s \tag{1}$$

where K is the extinction coefficient, α is the surface concentration (μmol/m^2), ϱ is related to the thickness of the tablet (g/cm^2) and s is the specific surface area (m^2/g) of the adsorbent under investigation. If D, ϱ and s are known it is possible to determine the products $K\alpha$ and to compare them for different chemically modified adsorbents. Such a comparison makes it possible to evaluate the relative degree of chemical modification of surfaces.

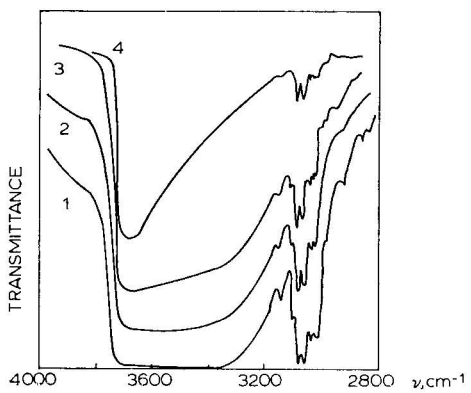


Fig. 1. Infrared spectra of silica gels modified by diphenyldichlorosilane evacuated at 200°C and in carbon tetrachloride. Adsorbents: 1 = LiChrosorb Si 60; 2 = LiChrosorb Si 100; 3 = LiChrospher Si 500; 4 = LiChrospher Si 1000.

For the spectroscopic investigation a Zeiss UR-20 infrared spectrometer was used. The spectra were taken in the region 4000–2700 cm^{-1} with a LiF prism. The tablet of adsorbent under investigation was evacuated at 200°C to 10^{-5} Torr. It was then placed in carbon tetrachloride to reduce the scattering and the spectrum of the sample was taken.

In Table I the values of $K\alpha$ and α are presented for different modified silicas. It is seen that the surface coverage of attached groups is practically the same for the different silicas. Thus for the preparation of modified adsorbents with reproducible

TABLE I

SURFACE CONCENTRATIONS OF DIPHENYLSILYL GROUPS ATTACHED TO SILICA
Spectral data from Fig. 1.

Adsorbent	d_p (μm)	s (m^2/g)	D	ϱ (g/cm^2)	$10^4 K\alpha$	α ($\mu mol/m^2$)
LiChrosorb Si 60	5	500	3.16	0.0418	0.15	1.56
LiChrosorb Si 100	30	285	1.21	0.0215	0.20	2.04
LiChrosorb Si 100*	7	285	0.74	0.0136	0.19	1.98
LiChrospher Si 500	10	50	0.68	0.0651	0.21	2.16
LiChrospher Si 1000	10	20	0.14	0.0185	0.38	3.91

* Spectral data from Fig. 2.

surface properties it is possible to use the reaction of silica with diphenyldichlorosilane in the presence of triethylamine as electron donor in a liquid phase.

It is interesting to compare the separation of cardiac glycosides on adsorbents with attached phenyl and octadecyl groups. The adsorbent was LiChrosorb Si 100 (particle size 7 μm) modified by diphenyldichlorosilane and octadecyltrichlorosilane in the presence of triethylamine under the same conditions. Fig. 2 shows the IR spectra of the initial adsorbent and after the modification with phenyl and octadecyl groups. The spectra exhibit the bands of phenyl and octadecyl groups and the bands of hydrogen-bonded hydroxyl groups which do not take part in the reaction of chemical modification.

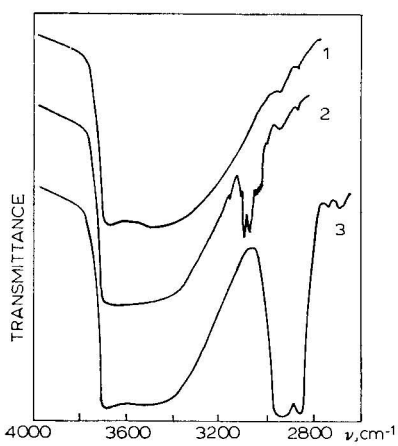
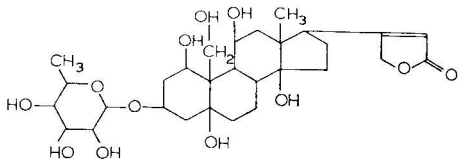
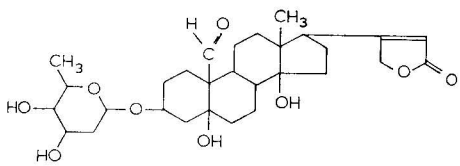
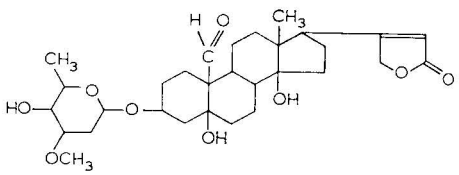
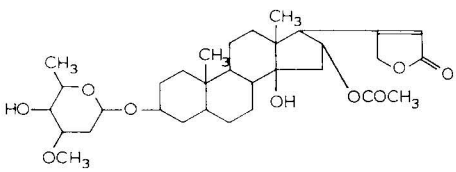
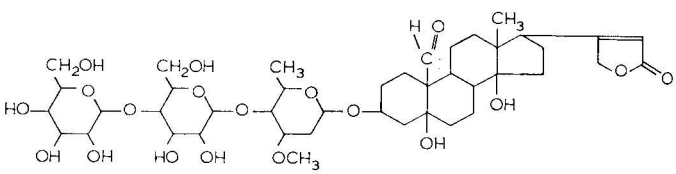
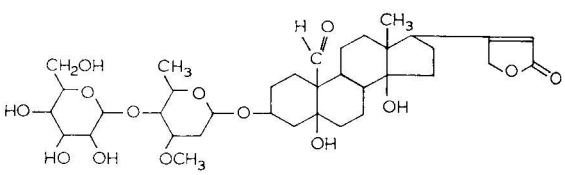
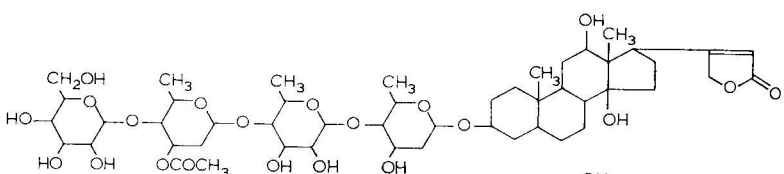
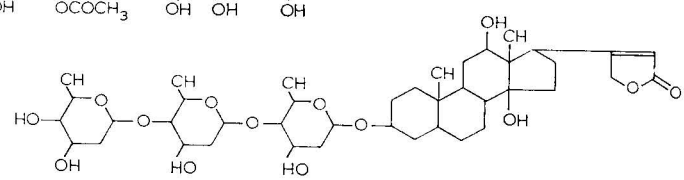


Fig. 2. Infrared spectra of LiChrosorb Si 100 (1) and of the same silica modified by diphenyldichlorosilane (2) and octadecyltrichlorosilane (3) evacuated at 200°C and placed in carbon tetrachloride.

To determine the differences in the adsorption properties of silica surfaces having different functional groups we investigated the effect of temperature and eluent composition on the retention volumes of some cardiac glycosides in columns of these adsorbents. A mixture of glycosides having a monosaccharide in the glycone part of the molecules was chosen. To determine the main correlation between the retention volumes and the structure of the glycoside molecules the eight-component

TABLE II

INVESTIGATED CARDIAC GLYCOSIDES

Name	*Structural formula*
G-Strophanthin	
Erysimin	
Cymarin	
Neriolin	
K-Strophanthoside	
K-Strophanthin	
Isolanid	
Digoxin	

mixture of cardiac glycosides was separated on modified silica adsorbents. In Table II the structural formulae of the cardiac glycosides are presented.

A Spectra-Physics liquid chromatograph was used. The separation was effected with water–ethanol eluents at temperatures from 20 to 60°C. The temperature of the columns was maintained within ± 0.1°C by a thermostated water-jacket. Fig. 3 shows the separation of G-strophanthin, erysimin, cymarin and neriolin on LiChrosorb Si 100 (7 μm) with columns of 125 mm length and 4.8 mm I.D. The first glycoside to be eluted from the columns is G-strophanthin, *i.e.*, the most hydrophilic molecule[6], then erysimin and cymarin which have the same steroid part but different glycone part of the molecules. Cymarose in cymarin is more hydrophobic than digitoxose in erysimin. Thus cymarin is eluted later than erysimin. Neriolin, which contains a more hydrophobic aglycon oleandrigenin than aglycon strophanthidin in cymarin and erysimin, is eluted last.

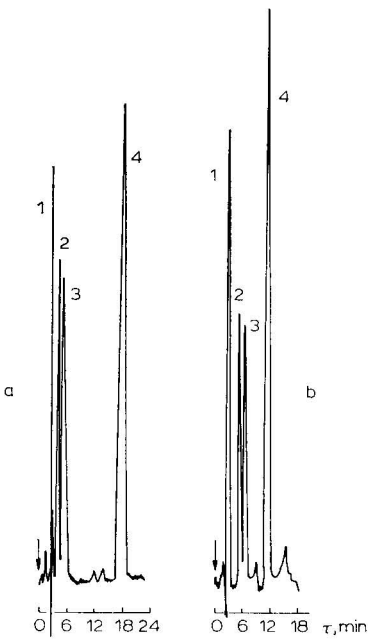


Fig. 3. Separation of cardiac glycoside mixture on LiChrosorb Si 100 (7 μm) modified by octadecyltrichlorosilane (a) and diphenyldichlorosilane (b). Column: 125 × 4.8 mm; temperature 40°C. Eluent: ethanol–water (40:60); flow-rate 0.7 cm^3/min. Peaks: 1 = G-strophanthin; 2 = erysimin; 3 = cymarin; 4 neriolin.

Figs. 4 and 5 show the dependences of the logarithm of retention volume of these cardiac glycosides on inverse temperature and on the composition of the eluent for columns of silica modified by octadecyltrichlorosilane (Fig. 4) and diphenyldichlorosilane (Fig. 5). It is seen that increasing the ethanol content in the eluent decreases the retention volume of glycosides on the adsorbent with octadecyl groups faster than on the adsorbent containing phenyl groups. But increasing the temperature decreases the retention volumes on the adsorbent with phenyl groups faster than on

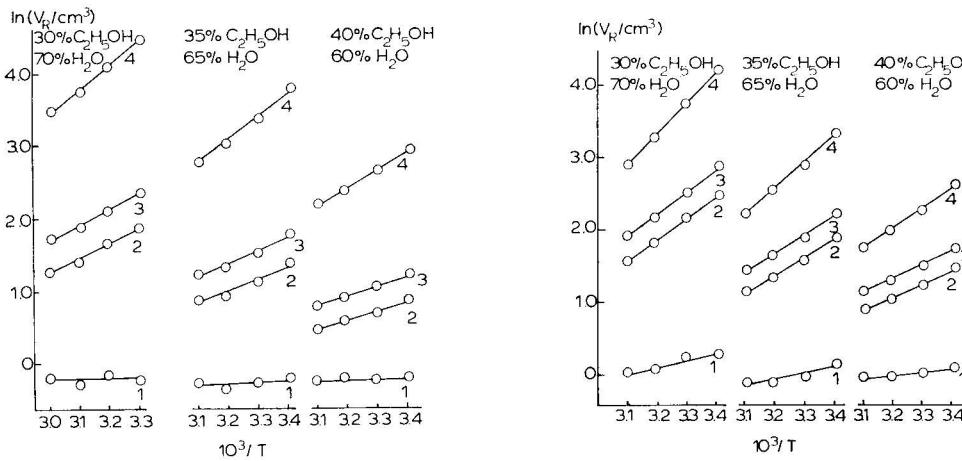


Fig. 4. Dependences of retention volume on temperature and eluent composition for cardiac glycosides on a column of LiChrosorb Si 100 (7 μm) modified by octadecyltrichlorosilane. Glycosides as in Fig. 3.

Fig. 5. Dependences of retention volume on temperature and eluent composition for cardiac glycosides on a column of LiChrosorb Si 100 (7 μm) modified by diphenyldichlorosilane. Glycosides as in Fig. 3.

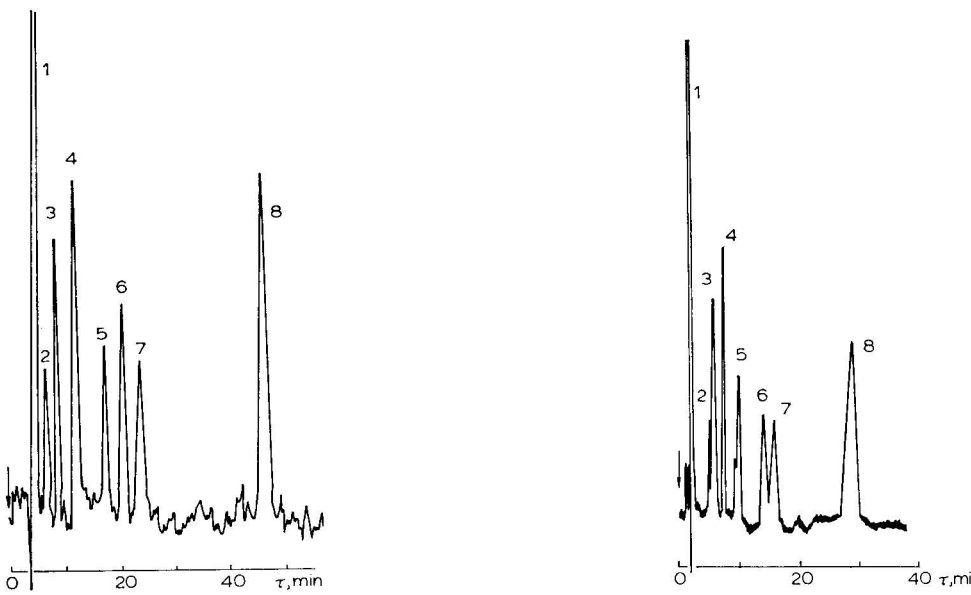


Fig. 6. Separation of cardiac glycoside mixture on LiChrosorb Si 60 (5 μm) modified by diphenyldichlorosilane. Column: 125 × 4.8 mm; temperature 50°C. Eluent: ethanol–water (40:60); flow-rate 0.34 cm³/min. Pressure drop 38 atm. Peaks: 1 = G-strophanthin; 2 = K-strophanthoside; 3 = K-strophanthin; 4 = erysimin; 5 = cymarin; 6 = isoland; 7 = digoxin; 8 = neriolin.

Fig. 7. Separation as in Fig. 6 but on LiChrosorb Si 100 (7 μm) modified by diphenyldichlorosilane. Column: 125 × 4.8 mm; temperature 40°C. Eluent: ethanol–water (30:70); flow-rate 0.7 cm³/min. Pressure drop 35 atm.

the adsorbent with octadecyl groups. Thus for the separation of cardiac glycosides on silica with octadecyl groups it is better to change the composition of eluent, but on silica with phenyl groups it is better to change the temperature.

Figs. 6 and 7 show the separations of eight components of cardiac glycosides on LiChrosorb Si 60 ($d_p = 5\ \mu m$, $s = 500\ m^2/g$) and on LiChrosorb Si 100 ($d_p = 7\ \mu m$, $s = 285\ m^2/g$) with attached phenyl groups. To reduce the analysis time on the LiChrosorb Si 60 column the eluent with a higher ethanol content was used in comparison with LiChrosorb Si 100, the capacity factor of which is smaller. For comparison, the same mixture was separated (Fig. 8) on the HiBar pre-packed column of LiChrosorb RP-8 (Merck).

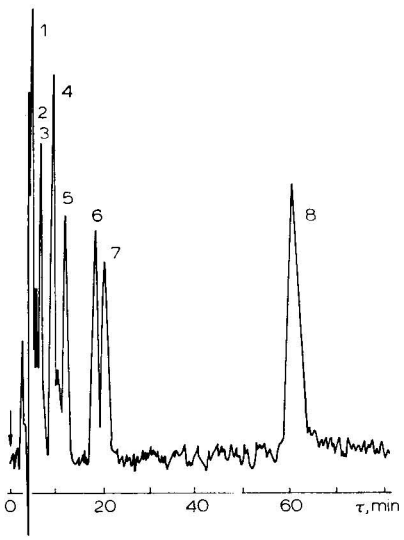


Fig. 8. Separation as in Fig. 6 but on HiBar pre-packed column of LiChrosorb RP-8 (7 μm). Column: 250 × 3 mm; temperature 50°C. Eluent: ethanol–water (30:70); flow-rate 0.34 cm^3/min. Pressure drop 135 atm.

It has been shown previously[14–17] that a particle size of 7 μm and a column of 100–150 mm length and ≈ 5 mm I.D. are optimal as regards the pressure drop in the column, the efficiency and the time of analysis. In the case of the cardiac glycosides, the column packed with LiChrosorb Si 100 ($d_p = 7\ \mu m$) with attached phenyl groups gave better separations.

The elution order of glycosides from the columns under investigation is determined by the relative content of hydroxyl and hydrophobic groups in the molecules, but not by molecular weight.

Fig. 9 shows the selectivity, α, of the columns packed with silica modified by octadecyltrichlorosilane and diphenyldichlorosilane. It is seen that α for erysimin, cymarin and neriolin relative to G-strophanthin decreases with increasing ethanol concentration and with increasing temperature on both adsorbents. The dependence on ethanol concentration was stronger for the silica modified by octadecyltrichlorosilane.

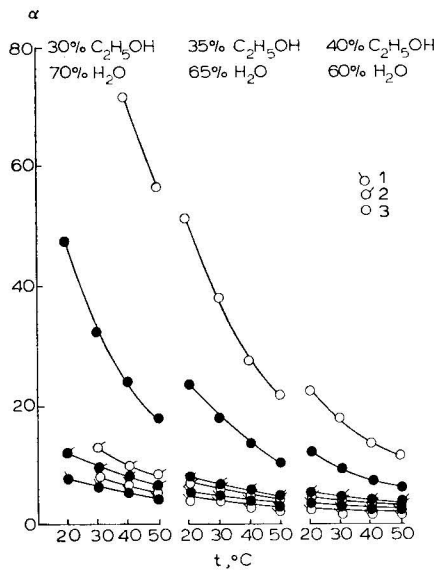


Fig. 9. Dependence of selectivity, α, on temperature and eluent composition for columns packed by LiChrosorb Si 100 (7 μm) modified by octadecyltrichlorosilane (open symbols) and diphenyldichlorosilane (closed symbols). Curves: 1 – erysimin; 2 – cymarin; 3 – neriolin, all relative to G-strophantin.

Such a large difference in the dependence of α on the ethanol content in the eluent is probably connected with the nature of the modifying layer. The layer formed by long hydrocarbon chains changes thickness with the ethanol concentration in water, while phenyl groups attached to the surface form a stable layer the structure of which is not dependent on the eluent composition. In the latter case the adsorption mechanism of separation operates, but in the former case the separation mechanism is quasi-partition and will depend on the eluent composition, although one probably cannot regard the long hydrocarbon chains attached to the surface as stationary liquid phase on the surface because the density of the hydrocarbon "brush" is less than that of the hydrocarbon liquid and because the chains are attached at one end. In a non-polar solvent, for example some hydrocarbons, these attached chains may

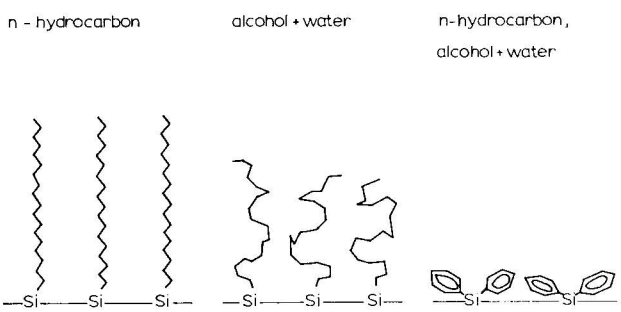


Fig. 10. Schematic picture of the dependence of the possible conformations of the functional groups attached to the silica surface on the eluent composition.

indeed form a "brush", but in the presence of water the chains may roll up and interact with each other and the thickness of the modifying layer will change.

Fig. 10 shows possible structures of modifying layers consisting of octadecyl and phenyl groups. Owing to conformational mobility, the hydrocarbon chains may be arranged in different ways near the surface, but in the case of attached phenyl groups the modifying layer will be independent of eluent composition.

The high carbon content of the phenyl rings attached to the surface results in a high capacity factor and a stable modifying layer in any eluent.

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DETERMINATION OF CLOMIPRAMINE OR IMIPRAMINE AND THEIR MONO-DEMETHYLATED METABOLITES IN HUMAN BLOOD OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. GODBILLON* and S. GAURON

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

SUMMARY

A high-performance liquid chromatographic method for the quantitative assay of clomipramine or imipramine and their mono-demethylated metabolites in human blood or plasma is described. After addition of the internal standards, imipramine for clomipramine, desipramine for desmethylclomipramine and conversely, the compounds are extracted from blood or plasma at pH 10 into heptane containing 1% isoamyl alcohol; they are then back-extracted into an acidic aqueous phase and re-extracted at a basic pH into heptane. After evaporation, the residue is dissolved in 300 μl of mobile phase and 150 μl is injected.

Both drugs and their mono-demethylated metabolites are well separated from the blood or plasma components and the other metabolites on a silica gel column using ethanol–hexane–dichloromethane–diethylamine ($30:62:8:5 \cdot 10^{-3}$) as the mobile phase at a flow-rate of 1.5 ml/min. The limit of sensitivity is 5 ng/ml for clomipramine and imipramine and 10 ng/ml for the corresponding mono-demethylated metabolites.

This method has been used to analyse plasma from subjects given therapeutic doses of clomipramine or imipramine.

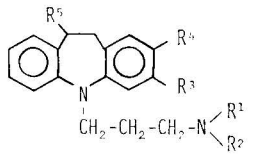
INTRODUCTION

Several liquid chromatographic procedures have been described for the analysis of the tricyclic antidepressant drugs clomipramine (CMI) or imipramine (IMI) and their respective demethylated metabolites (Fig. 1), desmethylclomipramine (DCMI) and desipramine (DMI)[1–27].

A few of these methods have been applied only to pure solutions[1–5]. Those described for the assay in biological materials involved either ion-pair partition chromatography[6–11], adsorption[12–22] or reversed-phase chromatography[23–27]. The sensitivity ranged from 1 ng/ml with fluorimetric detection[16,25] to 5–20 ng/ml with UV detection[6–10,12,13,17,18,23,24].

We tried to reproduce the method described by Westenberg and co-workers[12,13] for CMI and DCMI in plasma because it was reported as being "selective, rapid and simple, and especially suitable for routine analysis"[12]. It requires a single-step extrac-

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Compound	R^1	R^2	R^3	R^4	R^5
Clomipramine[1] (CMI)	CH_3	CH_3	Cl	H	H
Desmethyl-clomipramine (DCMI)	CH_3	H	Cl	H	H
Bis-desmethyl-clomipramine	H	H	Cl	H	H
Imipramine[2] (IMI)	CH_3	CH_3	H	H	H
Desipramine[3] (DMI)	CH_3	H	H	H	H
2-hydroxy-imipramine (2-OH-IMI)	CH_3	CH_3	H	OH	H
2-hydroxy-desipramine (2-OH-DMI)	CH_3	H	H	OH	H
10-hydroxy-imipramine (10-OH-IMI)	CH_3	CH_3	H	H	OH
10-hydroxy-desipramine (10-OH-DMI)	CH_3	H	H	H	OH
Bis-desmethyl-imipramine (DDMI)	H	H	H	H	H

1 Anafranil® Ciba-Geigy.
2 Tofranil® Ciba-Geigy.
3 Pertofran® Ciba-Geigy.

Fig. 1. Clomipramine, imipramine and their metabolites.

tion before injection, and IMI is used as internal standard for *both* CMI and DCMI. We applied this method using chromatographic conditions very similar to those described and the reported extraction procedure, but were not able to obtain acceptable and reproducible blank plasma extracts. An interfering peak having the same retention time as CMI was observed with eight different samples of blank human plasma; its height was subject-dependent. This interfering compound could be removed only by using a three-step extraction procedure.

This paper describes an high-performance liquid chromatographic (HPLC) method for the simultaneous determination of CMI and DCMI or IMI and DMI in blood and plasma with new extraction conditions. As already suggested by Gupta and Molnar[28], two internal standards were used to improve the reproducibility: IMI for CMI, DMI for DCMI and conversely. This method was applied to plasma samples from patients given repeated doses of clomipramine and from healthy subjects given a single oral dose of imipramine.

EXPERIMENTAL

Chemicals

CMI, IMI and their metabolites (Fig. 1) were supplied by Ciba-Geigy (Basle, Switzerland). Dichloromethane, *n*-heptane, isoamyl alcohol, pH 10 buffer (Titrisol) and 5-μm LiChrosorb Si 60 were purchased from E. Merck (Darmstadt, G.F.R.), ethanol from Prolabo (Paris, France), diethylamine from Fluka (Buchs, Switzerland)

and hexane (Spectrosol quality) from S.D.S. (Valdonne, France). Internal standards and calibration solutions were prepared in 0.01 *N* hydrochloric acid from the hydrochlorides.

Chromatography

The chromatography was performed on a Hewlett-Packard Model 1084 B instrument, equipped with a fixed-wavelength detector (254 nm) and a variable-volume injector. The column was a stainless-steel tube (12.5 cm × 4.6 mm) filled with 5-μm LiChrosorb Si 60 (Merck 9388) using the balanced-density slurry packing technique. The slurry, made of 1.8 g 5-μm LiChrosorb Si 60 dispersed in 7.8 ml carbon tetrachloride, was forced into the column with methanol. The degassed mobile phase, ethanol–hexane–dichloromethane–diethylamine ($30:62:8:5\cdot10^{-3}$), was used at a flow-rate of 1.5 ml/min.

With a new column, the retention times were 3.3, 4.1, 11.3 and 15.3 min for CMI, IMI, DCMI and DMI, respectively. The maximum pressure was about 25 bar. After about 800 injections, the pressure increased to 40 bar and the retention times decreased to 2.3, 2.8, 7.5 and 10.1 min, respectively. The mobile phase and the column were at room temperature.

Sample preparation

To 1 ml plasma or blood in a 10-ml glass tube were added 100 μl internal standard solution (added amount: CMI, 60 ng; DCMI, 150 ng; IMI, 35 ng; DMI, 100 ng), 1 ml buffer (0.2 *M* boric acid, 0.2 *M* potassium chloride, 0.176 *M* sodium hydroxide), pH 10 and 5 ml *n*-heptane–isoamyl alcohol (99:1). The stoppered tube was shaken mechanically for 5 min at 3000 rpm (Infors shaker) and centrifuged at 1400 *g* for 10 min. The *n*-heptane–isoamyl alcohol phase was transferred with a Pasteur pipette into another tube and 1 ml 0.1 *N* H_2SO_4 was added. The tube was shaken for 5 min and centrifuged for 3 min. It was then dipped into a mixture of methanol and solid CO_2 to freeze the aqueous phase, and the organic phase was discarded.

The acidic aqueous phase was left to thaw at room temperature. Then 0.5 ml 1 *N* NaOH and 5 ml *n*-heptane–isoamyl alcohol were added. The tube was shaken for 5 min and centrifuged for 3 min. The *n*-heptane–isoamyl alcohol phase was transferred into a conical glass tube and evaporated to dryness under nitrogen at 60°C. The residue was dissolved in 300 μl of mobile phase.

This solution must be prepared just before injection. After mixing for 20 sec on a Vortex mixer, 150 μl were injected.

Calibration curves

Samples were prepared by adding 100 μl of reference solutions of both compounds, CMI and DCMI or IMI and DMI, to 1 ml of blood or plasma. These samples were then worked up according to the procedure described above. The calibration curves were obtained by plotting either the peak area ratio for CMI and IMI or the peak height ratio for DCMI and DMI against concentration.

Acidic solutions of both the internal standards and the reference compounds have to be used to minimize adsorption on glass or plastic surfaces, which occurs with solutions in distilled water[29].

RESULTS AND DISCUSSION

Plasma interference

When a single-step extraction (heptane + buffer pH 10) as indicated by Westenberg *et al.*[12] was used, an interfering peak having the same retention time as CMI was observed with eight different samples of blank human plasma; its height was subject-dependent. This interfering compound could be removed only by a three-step extraction procedure: extraction into heptane at a basic pH, back-extraction into an acidic aqueous phase and re-extraction into heptane at a basic pH.

The chromatograms corresponding to the extract of a 1-ml sample of blank human plasma or of plasma spiked with CMI, IMI, DCMI and DMI are shown in Fig. 2. Similar chromatograms are obtained with blank human blood.

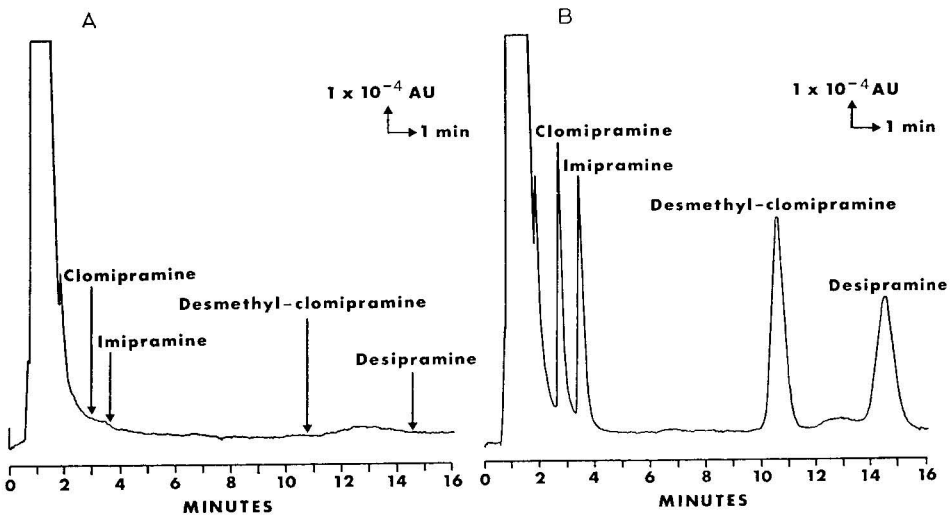


Fig. 2. Chromatograms corresponding to the extract of 1 ml of blank plasma (A), and 1 ml of plasma spiked with 30 ng of IMI, 60 ng of DMI, 35 ng of CMI and 100 ng of DCMI (B).

Choice of internal standard

In most of the published methods, a single internal standard, either a tertiary or a secondary amine, was used for the estimation of both tertiary (CMI and IMI) and secondary (DCMI and DMI) bases: protriptyline[14,18,21], DCMI[6,11,16], DMI[6], CMI[6,15], IMI[12,13], trimipramine[7,8,25], maprotiline[24], promazine[15,22], β-naphthylamine[10] and mesopridazine[26].

Using IMI as internal standard for DCMI, we were unable to obtain a good linear relationship between the peak height ratio, DCMI/IMI, and the concentration of DCMI, and the results were not reproducible. The use of a secondary amine (DMI) as internal standard for DCMI gave more reliable results. The losses of CMI and DCMI during the analytical procedure were well compensated by IMI and DMI, respectively.

Linearity

The calibration curves were established as described above. Their equations were calculated by least-squares linear regression. A good linear relationship was obtained in the range of 10–400 ng/ml for DCMI and DMI, and in the ranges of 5–50 and 50–400 ng/ml for CMI and IMI.

For routine analysis, a calibration curve is established every week, and test samples are analysed during the week to validate the method.

Accuracy, precision and sensitivity

The results summarized in Tables I and II show that concentrations down to 5 ng of CMI or IMI can be accurately and precisely determined in 1 ml of plasma by the present method. This corresponds to a peak height ranging from 0.4 to 1 cm. The sensitivity limit in blood is 10 ng/ml for CMI and 5 ng/ml for IMI. For DCMI and DMI, the sensitivity limit in blood or plasma is 10 ng/ml (Tables I and II): this corresponds to a peak height ranging from 0.4 to 1.5 cm.

Lower concentrations can be estimated, but the accuracy is poorer (Table I).

TABLE I

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR CMI AND DCMI (SPIKED HUMAN PLASMA SAMPLES)

Compound	*CMI*				*DCMI*			
Amount added (ng/ml)	2.5	5	50	100	10	15	100	300
Amount found (ng/ml)	3.9	5.4	50.5	98.5	9.9	14.0	106	299
(*n* replicates)	(6)	(6)	(10)	(6)	(6)	(6)	(6)	(6)
Coefficient of variation (%)	9.5	9.5	6.1	3.0	16.0	9.5	4.2	5.4
Mean recovery (%)	158	108	101	98.5	99	93	106	99.8

TABLE II

ACCURACY, PRECISION AND WITHIN-DAY REPRODUCIBILITY FOR IMI AND DMI (SPIKED HUMAN PLASMA SAMPLES)

Compound	*IMI*				*DMI*			
Amount added (ng/ml)	5	10	30	200	5	10	150	400
Amount found (ng/ml)	5.1	9.7	29.4	203	4.2	9.6	157	392
(*n* replicates)	(10)	(9)	(7)	(7)	(12)	(12)	(12)	(12)
Coefficient of variation (%)	6.6	2.7	2.1	3.3	11.0	7.3	2.8	2.9
Mean recovery (%)	102	97	98	102	84	96	104	98

Specificity

Clomipramine. N-Demethylation is a main pathway of biotransformation of clomipramine in man[30,31]. Hydroxylation occurs at various positions of the molecule, but the hydroxylated metabolites, eliminated as glucuronides or sulphates in urine, are only present in small amounts in the plasma.

As bis-desmethylclomipramine does not interfere in the assay of CMI and DCMI, the described method can be considered specific.

Imipramine. N-Demethylation and hydroxylation at two positions of the molecule are the main pathways of biotransformation of imipramine[32]. Desipramine is a therapeutically active metabolite, but recently the 2-hydroxy metabolites of both IMI and DMI have been shown to be biologically active[16,33–35].

As all the hydroxylated and demethylated metabolites are well separated from the parent drug and the internal standards under the chromatographic conditions described in the present method (see Fig. 3), their determination together with the parent drug was investigated with CMI as internal standard for IMI, 2-OH-IMI and 10-OH-IMI, and DCMI as internal standard for DMI and DDMI; the hydroxylated metabolites of desipramine, 2-OH-DMI and 10-OH-DMI, which were eluted as broadened peaks, were not considered.

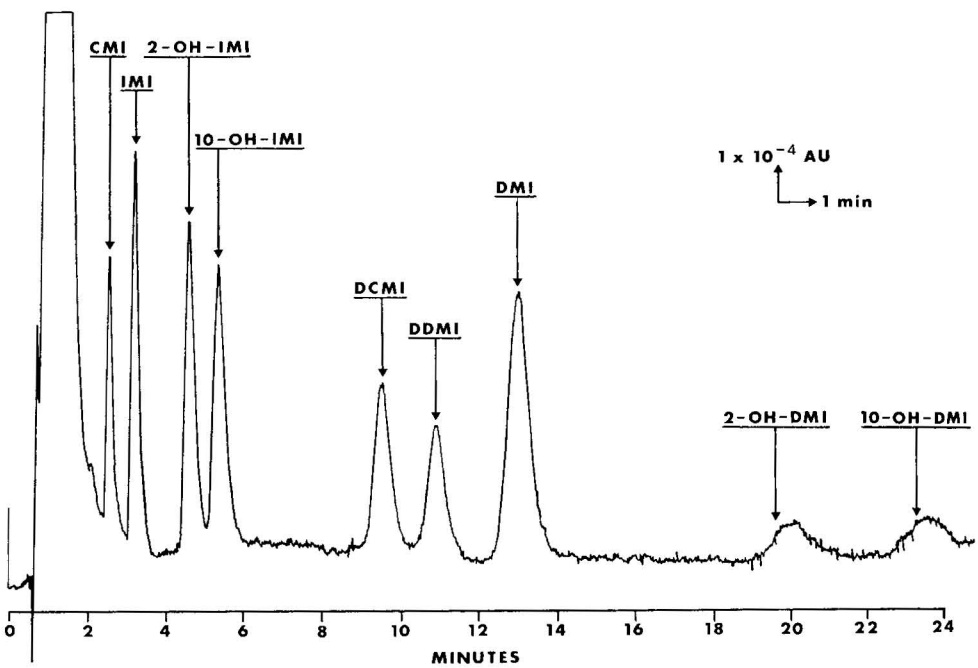


Fig. 3. Separation of CMI, IMI and their metabolites. Spiked calf plasma sample extracted with dichloromethane–diethyl ether (1:2).

When heptane with isoamyl alcohol was used as extraction solvent, 2-OH-IMI was not extractable from plasma. The solvent mixtures *n*-butanol–heptane (20:80) or dichloromethane–diethyl ether (40:60) increased the extraction yield, but resulted in an impure extract of blank human plasma: an interfering peak having the same retention time as that of CMI was observed. All attempts to eliminate this interfering compound were unsuccessful.

It is likely that 10-OH-IMI could be assayed with the present extraction and chromatographic conditions, but this remains to be verified. DDMI was extracted into heptane with good yield, but could not be accurately assayed because the reproducibility of the DDMI/DCMI ratio was poor. Only imipramine and desipramine can be precisely and specifically assayed by the present HPLC method.

Stability

As mentioned previously, the dry residue must be dissolved in 300 μl of the mobile phase just before injection to ensure good reproducibility. However, the dry evaporation residue remains stable for 5 days when stored in the refrigerator.

Application

Steady-state plasma levels of CMI and DCMI. The present method was used to determine the steady-state plasma levels of CMI and DCMI reached in patients given daily 75–150-mg doses of clomipramine. Blood samples were taken in the morning on days 26 and 27 of treatment, just before the first daily administration. Plasma was separated and stored at −20°C until analysis. The results summarized in Fig. 4 show that on days 26 and 27 the pre-dose mean plasma levels of CMI and its metabolite DCMI were not linearly related to the daily dose, and the concentrations of the metabolite DCMI were larger than those of the unchanged drug during chronic treatment with clomipramine.

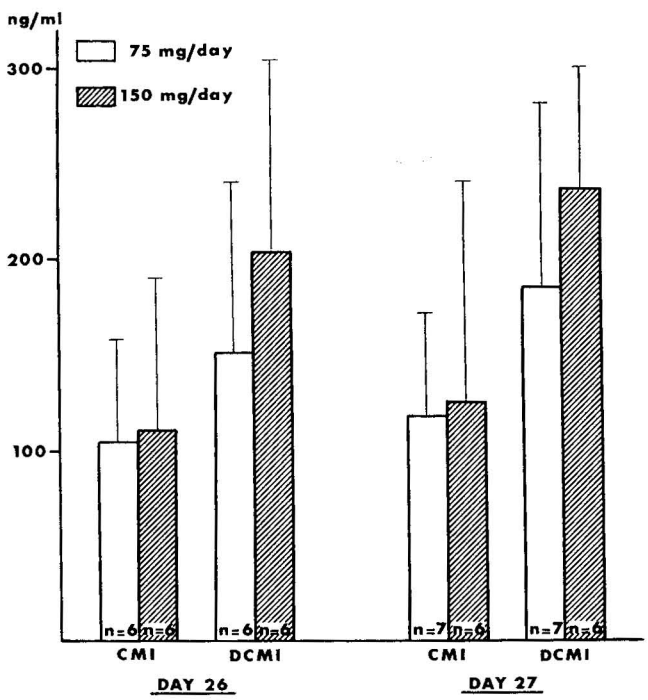


Fig. 4. Mean (± S.D.) steady-state plasma levels of CMI and DCMI achieved in patients given daily 75- or 150-mg doses of clomipramine (25-mg Anafranil tablets).

Imipramine plasma levels after oral administration of a single 50-mg dose. The time course of the plasma levels of imipramine following the administration of a single 50-mg oral dose of imipramine hydrochloride was determined in six healthy volunteers. The average curve is shown in Fig. 5. IMI levels peaked at 18 ng/ml 4 h after dosing and declined to 5 ng/ml at 24 h. DMI levels were lower than 10 ng/ml.

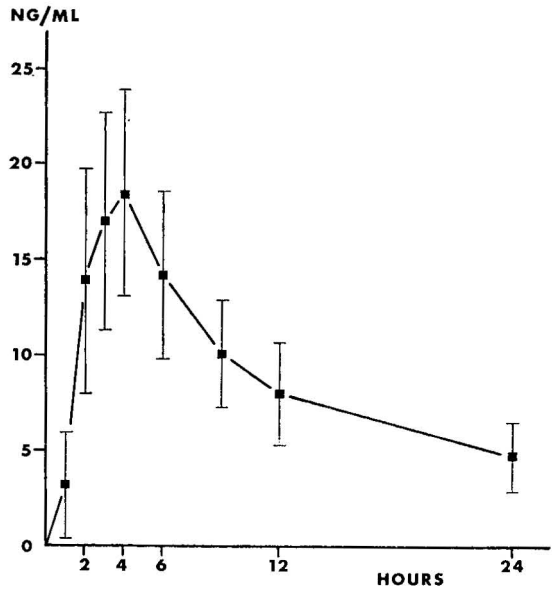


Fig. 5. Time course of plasma concentrations of imipramine following administration of a single 50-mg oral dose of imipramine hydrochloride (25-mg Tofranil tablets). Average curve ± S.D. from six healthy subjects.

CONCLUSION

The described method is sensitive enough for clinical pharmacology studies and for drug monitoring: concentrations down to 5–10 ng/ml can be determined using 1-ml blood or plasma samples.

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QUANTITATION OF NIKKOMYCINS IN BIOLOGICAL FLUIDS BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H.-P. FIEDLER
Institut für Biologie II, Lehrstuhl Mikrobiologie I, Auf der Morgenstelle 28, D-7400 Tübingen 1 (G.F.R.)

SUMMARY

In contrast to biological evaluation methods the use of high-performance liquid chromatography has allowed the quantitation of five nikkomycin components from the filtrate of the fermentation broth in which they are cultured.

INTRODUCTION

Nikkomycins (Fig. 1) are nucleoside–peptide antibiotics, similar in structure to polyoxins, and have been isolated from the culture broth of *Streptomyces tendae*[1–4]. These antibiotics are interesting from an agricultural point of view because they possess a high and selective antifungal and insecticidal activity. The site of action is associated with the biosynthesis of chitin in the cell wall[1,5]. An analytical method was then sought for the quantitation of the whole spectrum of these substances during the fermentation process. Biological testing using agar plate diffusion had disadvantages in that no quantitation of single components was possible, for although the antibiotically active substances could be determined the inactive substances could not and 2 days were necessary to evaluate the test plates using *Mucor hiemalis* as the test organism.

High-performance liquid chromatography (HPLC) is a powerful technique in quantitation of antibiotics in pharmaceutical analysis, but there have been few investigations of the use of this method for the control of antibiotic production. The quantitation of antibiotics from the fermentation broth by HPLC was reported in the cases of cephalosporin C[6,7], erythromycin and tetracyclin[8] and penicillins[9]. This paper demonstrates the use of HPLC for quantitation of five nikkomycin compounds from the culture filtrate of the fermentation broth.

EXPERIMENTAL

Chromatographic apparatus

The system consisted of a Spectra-Physics Model SP 8000-10 liquid chromatograph, a Spectra-Physics Model 8010 autosampler and a Schoeffel Model SF 770 variable-wavelength detector. A reversed-phase column packed with LiChrosorb

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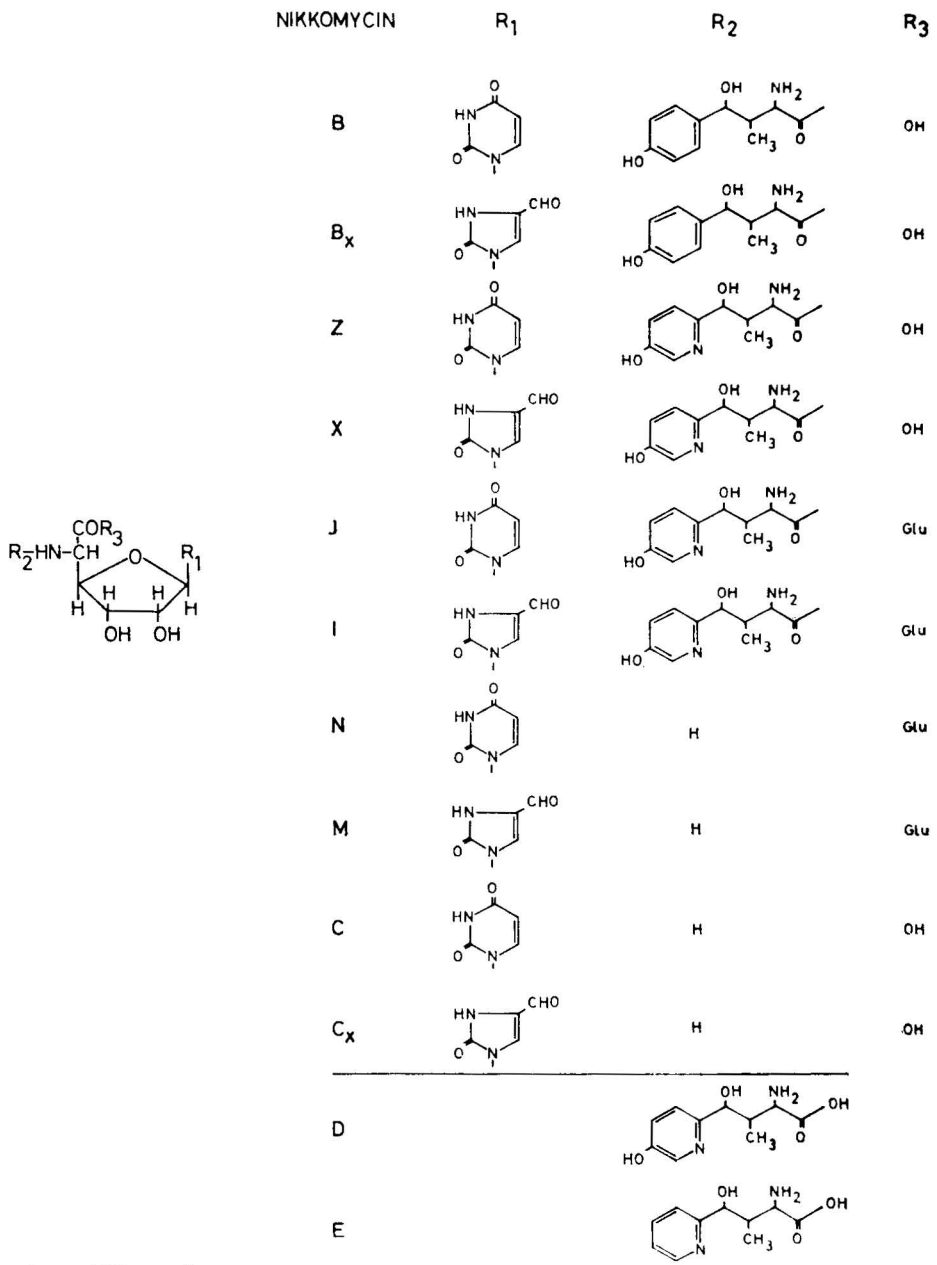


Fig. 1. Nikkomycin structures.

RP-8 (5 μm) of dimensions 120 $\times$ 4.6 mm I.D., supplied with a pre-column of dimensions 40 $\times$ 4.6 mm I.D., was obtained from Knauer (Berlin, G.F.R.).

For isocratic elution the mobile phase was 30 m*M* ammonium formate buffer, pH 4.7, containing 1 m*M* heptanesulphonic acid. The flow-rate was set to 1 ml/min and the pressure was approximately 100 bar. For gradient elution, solvent A was

30 m*M* ammonium formate buffer, pH 3.75, containing 1 m*M* heptanesulphonic acid, and solvent B was methanol containing 30 m*M* ammonium formate (corresponding to pH 3.75) and 1 m*M* heptanesulphonic acid. The flow-rate was set to 1 ml/min.

The detector wavelength was set at 290 nm and the sensitivity kept at 0.1 absorbance units full scale (a.u.f.s.).

Sample preparation

The fermentation broth was centrifuged and the supernatant containing the nikkomycins was filtered with a membrane filter (pore diameter 0.6 μm). A 10-μl sample was injected onto the column.

RESULTS AND DISCUSSION

Ion-pair reversed-phase chromatography seemed to be a promising method for separation of the nikkomycins because of the polar character of these substances and because quantitation has to be performed in an aqueous medium. Use of a mobile phase without a lipophilic counter-ion did not result in the separation of all nikkomycins. Ammonium formate buffer or ammonium acetate buffer, pH 4–6, separated nikkomycins C, D and I, but not Z and X, the last two components being eluted as one peak. The addition of a lipophilic basic counter-ion, such as tetramethyl-, tetraethyl- or tetrabutylammonium chloride, to the mobile phase did not result in the separation of all nikkomycins. When a lipophilic acidic counter-ion, such as dodecyl sulphate or heptanesulphonic acid, is included in the ammonium buffer, only with heptanesulphonic acid was the separation achieved of nikkomycins C, D, B, Z, X and I. A strong dependence was found between the pH of the mobile phase and the k' values, as shown in Table I.

Fig. 2 shows the separation of five nikkomycin standards and the HPLC analysis of a fermentation sample after a fermentation period of 145 h, under isocratic elution conditions. Fig. 3 demonstrates the separation of the standards using gradient elution.

The retention times and amounts recovered were reproducible whether isocratic or gradient elution was employed, as shown in Table II. Only the recovery of nikkomycin Z is unsatisfactory, because there is no baseline separation between Z and X, and therefore, Z was integrated as a rider peak.

When using gradient elution, only a short equilibration time under the initial conditions is necessary to achieve reproducible results. In isocratic elution the column must be equilibrated for 3 days after the regeneration with methanol in order to obtain reproducible results. On the other hand, when using isocratic elution conditions, the analysis time of approximately 18 min is shorter because of a 10 min initial delay in gradient elution, giving a total analysis time of 25 min.

Both methods are designed for the analysis of a great number of samples. Using isocratic elution, about 300 injections could be performed before regeneration of the column. When using a pre-column, about 3000 injections could be effected. However, it was necessary to change the pre-column after approximately 500 injections because of irreversible adsorption of substances from the culture filtrate on the stationary phase which resulted in poor separations.

TABLE I

CORRELATION BETWEEN pH OF THE AMMONIUM FORMATE BUFFER AND k' VALUES OF THE NIKKOMYCINS

Nikkomycin	*k' value*					
	pH 5.0	*4.9*	*4.8*	*4.7*	*4.6*	*4.5*
C	0.35	0.35	0.35	0.35	0.35	0.40
D	1.9	1.9	1.95	2.0	2.2	3.1
Z	3.2	3.35	3.5	3.65	4.8	6.5
X	3.65	3.8	4.0	4.2	5.6	7.5
I	5.8	6.8	9.3	10.7	19.7	30

Fig. 4 shows the quantitation of nikkomycins in the course of a fermentation period of 280 h, as well as biological testing, by HPLC analysis using isocratic elution conditions.

The advantages of HPLC are obvious. Several components could be detected in one run with a short analysis time. Not only the antibiotically active nikkomycins

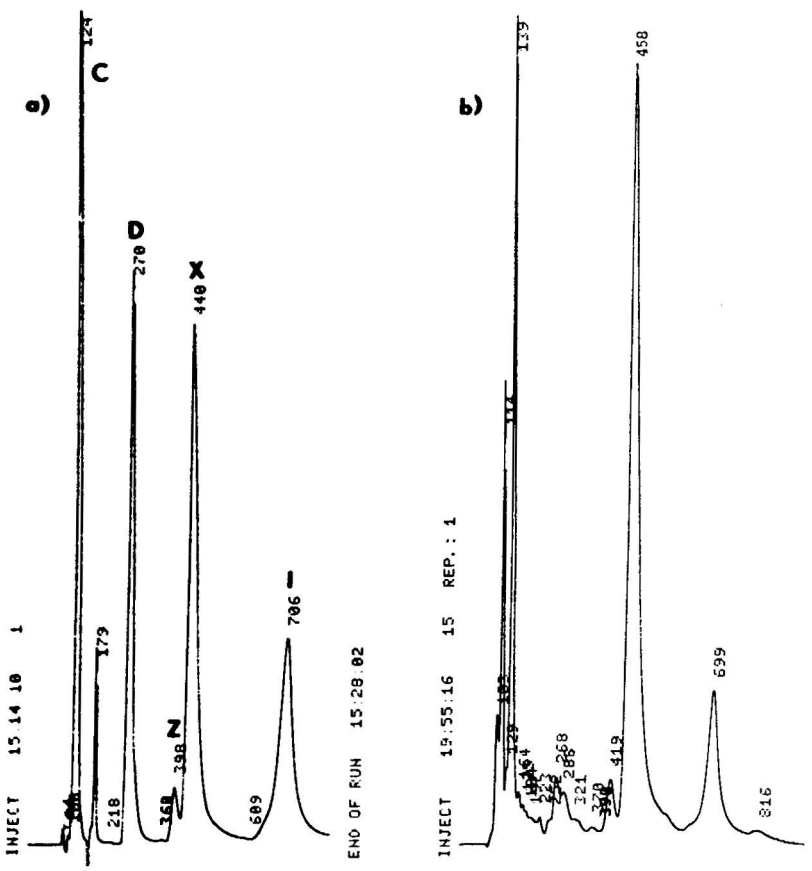
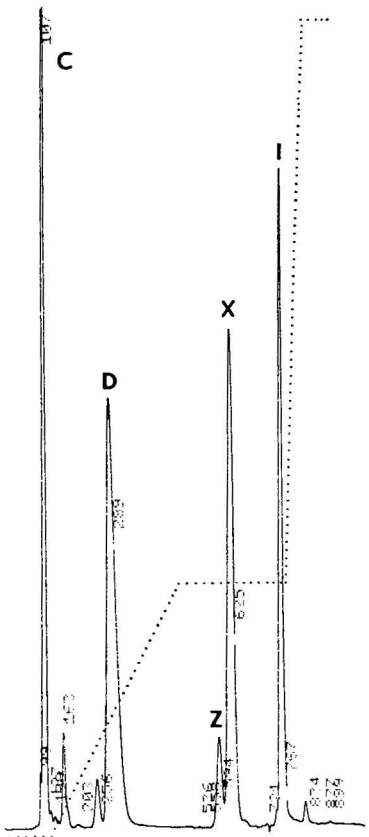


Fig. 2. HPLC analysis of nikkomycins using isocratic elution conditions. Retention times in seconds. a, Nikkomycin standard (1 mg/ml of each component); b, culture filtrate (fermentation period 145 h).



Gradientprofile

time (min)	% A	% B
0	100	0
2	100	0
8	70	30
13	70	30
14	0	100
15	0	100

Fig. 3. HPLC analysis of nikkomycin standards using gradient elution conditions. Retention times in seconds.

Z, X and I, but also the inactive components C and D could be quantitated. The amount of nikkomycin as determined by agar plate diffusion using *Mucor hiemalis* as the test organism corresponded to the sum of nikkomycins Z, X and I as determined by HPLC. Nikkomycins B/B_x were not produced under these fermentation conditions. Nikkomycins M and N are not biological products and therefore could not be

TABLE II

RELATIVE STANDARD DEVIATIONS (%)

Nikkomycin	*Isocratic elution*		*Gradient elution*	
	Retention time	*Concentration*	*Retention time*	*Concentration*
C	0.45	1.25	1.5	1.25
D	0.55	1.39	1.0	1.24
Z	0.85	8.52	0.66	6.3
X	0.88	1.50	0.3	2.36
I	1.14	2.52	1.0	2.51

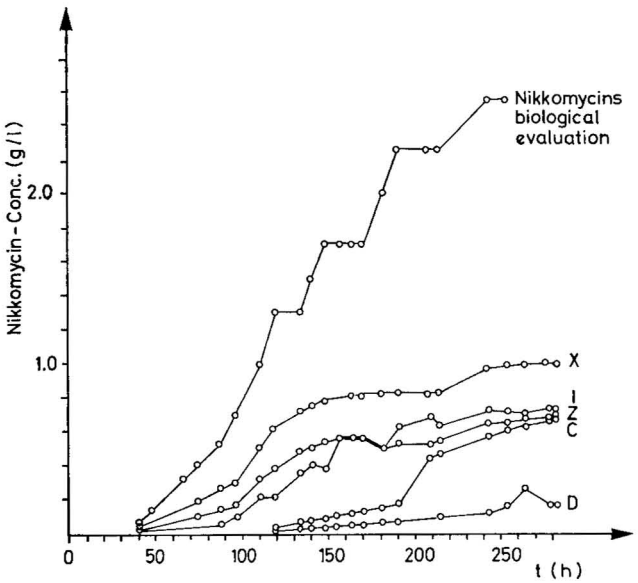


Fig. 4. Production control of nikkomycins in the course of a fermentation cycle.

detected in the fermentation broth. Both substances result from chemical hydrolysis of nikkomycins I and J.

The limit of detection using absorption spectrophotometry at 290 nm was approximately 0.05 μg of each component.

ACKNOWLEDGEMENTS

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REVERSED-PHASE LIQUID CHROMATOGRAPHY FOR THE SEPARATION OF CHLORPROMAZINE, IMIPRAMINE AND SOME OF THEIR METABOLITES

J. FEKETE*, P. DEL CASTILHO and J. C. KRAAK*

Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, Amsterdam (The Netherlands)

SUMMARY

Reversed-phase liquid chromatography on a C_{18} bonded silica with water–alcohol mixtures containing decylamine as the mobile phase was investigated for its applicability to the separation of chlorpromazine, imipramine and their metabolites. The addition of decylamine to the mobile phase was found to be necessary in order to obtain symmetrical peaks. The influence of the decylamine and ethanol concentrations and of the pH of the mobile phase was investigated and these parameters were found to be useful for the adjustment of the retention. The phase system developed was found to be suitable for the required separation. The phase system was applied to the analysis of imipramine and its metabolites, desmethylimipramine and didesmethylimipramine, and desmethylchlorpromazine in human plasma.

INTRODUCTION

The tricyclic antidepressants are by far the most commonly prescribed drugs for the treatment of psychiatric patients suffering depressions. The monitoring of the level of the antidepressant drugs and their metabolites in plasma is important for various reasons: (i) it provides more insight into the relationship between the level of the drug and its clinical effect; (ii) it is useful for discriminating poor biological availability from fast metabolic degradation or unreliable drug administration to patients; and (iii) it provides valuable information for pharmocokinetic studies.

Of the variety of analytical techniques applied for this purpose, high-performance liquid chromatography (HPLC) has been shown to be pre-eminently suitable[1–4] and has been applied succesfully to the analysis of imipramine and desmethylimipramine and also its two hydroxy metabolites[5]. However, the didesmethyl metabolite of imipramine was not included in these studies. Chlorpromazine has only been determined by HPLC in drug formulations[6]. A main drawback of the liquid chromatography of basic substances such as tricyclic antidepressent, is frequently the poor peak shape, probably caused by interactions of these bases with the support.

* On leave of absence from the Technical University, Budapest, Hungary.

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In this paper we report the results of an investigation to apply reversed-phase (RP) HPLC with alkyl-modified silica as the stationary phase and water–alcohol mixtures containing a long-chain alkylamine as the mobile phase for the separation of imipramine, chlorpromazine and their metabolites. The phase system was tested for its suitability for the analysis of plasma extracts from patients receiving these tricyclic drugs.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from commercially available parts and consisted of a Varian 8500 syringe pump, a Rheodyne 7105 high-pressure sampling valve, a Pye Unicam variable-wavelength UV detector and a linear potentiometric recorder (Servogor, Goerz, Austria). In all experiments stainless-steel columns of I.D. 4.6 mm and length 125 mm were used.

Materials

In all experiments doubly distilled water was used. All solvents and chemicals were of analytical-reagent grade and used without any further pre-treatment. The alkyl-modified silica was Hypersil ODS, mean particle size 5 μm (Shandon, London, Great Britain). The drugs and their metabolites were kindly donated by Dr. R. G. Muusze, St. Joris Gasthuis, Delft, The Netherlands. Their structures and abbreviations are given in Table I.

Procedures

The columns were packed by a balanced slurry technique and were washed successively with 100 ml of methanol, 50 ml of the appropriate water–organic solvent mixture and finally with the eluent until constant retention of the solutes was achieved. Blank pooled plasma and plasma samples containing chlorpromazine, imipramine or desmethylimipramine were stored at −20°C. After thawing, the plasma samples were treated according to the following extraction scheme[6]: a mixture of 2 ml of plasma, 2 ml of 1 *M* sodium hydroxide solution and 5 ml of *n*-heptane–isoamyl alcohol (99:1) was shaken for 10 min and then centrifuged for 3 min at 300 *g*. To 4 ml of the organic layer was added 0.2 ml 0.05% orthophosphoric acid and the mixture was shaken for 2 min and then centrifuged for 3 min at 300 *g*. A 100-μl volume of the aqueous layer was injected into the column. The amount of adsorbed alkylamine was determined by means of the breakthrough method[7].

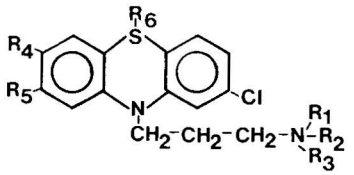
RESULTS AND DISCUSSION

As the metabolites of the antidepressants under investigation are more polar and usually present in plasma at considerable lower concentrations than the parent drugs, RP-HPLC was chosen as the separation method. In this type of liquid chromatography the metabolites will usually be eluted with a smaller capacity ratio than the parent drugs, which is advantageous with respect to the detection limit of these metabolites[3].

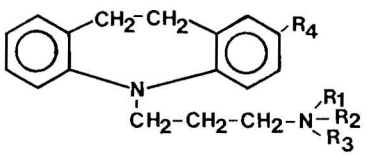
A general problem in the liquid chromatography of basic substances is severe

TABLE I

STRUCTURES OF THE ANTIDEPRESSANTS AND THEIR METABOLITES



Compound	*Code*	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Nor II CPZS → O	1	H	H	—	—	—	O	—
CPZS → O	2	CH_3	CH_3	—	—	—	O	—
Nor I CPZS → O	3	CH_3	—	—	—	—	O	
7-OH-CPZ	4	CH_3	CH_3	—	OH	—	—	—
6-OH-CPZ	5	CH_3	CH_3	—	—	OH	—	—
Nor II-CPZ	6	H	H	—	—	—	—	—
Nor I-CPZ	7	CH_3	H	—	—	—	—	—
Chlorpromazine (CPZ)	8	CH_3	CH_3	—	—	—	—	—
Dichlorpromazine	9	CH_3	CH_3	—	—	—	—	Cl



Compound	*Code letters*	R_1	R_2	R_3	R_4
Imipramine	a	CH_3	CH_3	—	—
Desmethylinipromino	b	CH_3	H	—	—
Didesdemethylimipiramine	c	H	H	—	—
2-Hydroxyimipramine	d	CH_3	CH_3	—	OH
Imipramine-N-oxide	e	CH_3	CH_3	O	—

peak tailing, probably due to the specific interactions of the bases with the support. These interactions can be supressed in three ways: (i) by post-silanization of the RP support[8]; (ii) by applying basic eluents[3,8,9]; or (iii) by using ion-pair chromatography[7,10,11].

All of these possibilities were initially investigated using a C_{18} modified silica (Hypersil ODS) as the column support. Post-silanization of the Hypersil ODS material by refluxing with a 10% (v/v) solution of dichloromethylsilane in toluene for 24 h did not result in any significant improvement in the peak shapes of the solutes. On applying ion-pair chromatography[7,11], by adding 0.1% (w/v) of sodium lauryl sulphate to the mobile phase, it was found that, apart from a considerable difference in retention behaviour, the peak shape improved significantly for a number of solutes. However, for some metabolites (in particular the hydroxylated ones) a poor peak shape was still observed.

By far the best results were obtained when basic eluents were applied. For this purpose 0.2% (v/v) of decylamine was added to the mobile phase. Under these con-

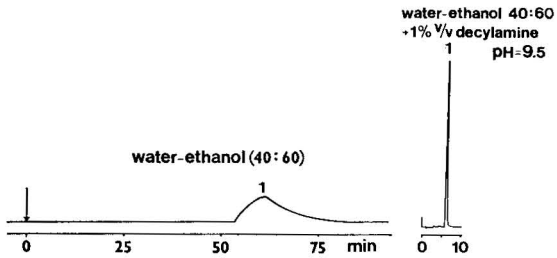


Fig. 1. Effect of the presence of decylamine in the mobile phase on the peak shape and retention of chlorpromazine (1).

ditions the peak shape of all solutes improved greatly (see Fig. 1). Therefore, in all further experiments basic eluents containing decylamine were used.

In order to determine the optimal conditions for the separation of the solutes, the capacity ratio (k_i') was measured as a function of the organic modifier content, the amount of decylamine and of the pH of the mobile phase. Fig. 2 shows the effect of the methanol content of the mobile phase on the k_i' of chlorpromazine and its metabolites. It can be seen that k_i' decreases with increasing methanol content. For some solutes a linear dependence of log k_i' on the percentage of methanol was found, whereas, particularly for the hydroxylated metabolites, a deviation from linearity

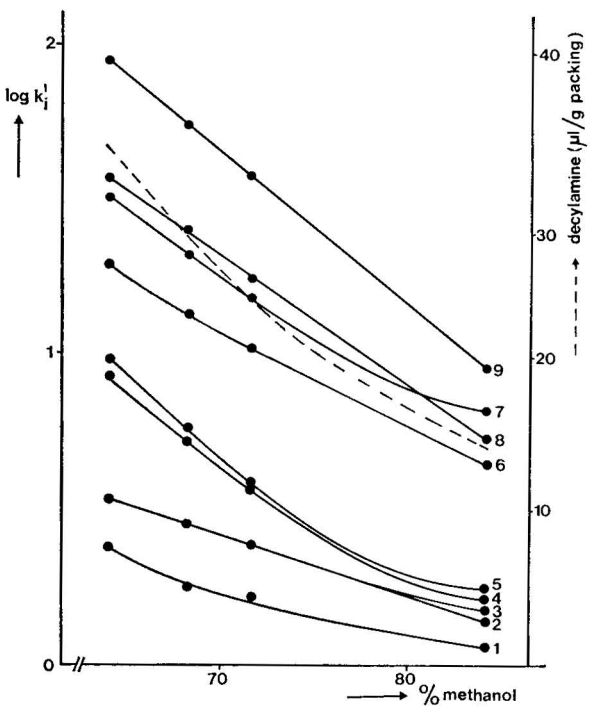


Fig. 2. Influence of the methanol content on the capacity ratio (k_i') of chlorpromazine and its metabolites. Mobile phase: water–methanol + 0.28% (v/v) of decylamine. For identification of compounds, see Table I.

occurred at higher methanol contents. As the amount of adsorbed decylamine is influenced by the methanol content (see the broken line in Fig. 2), this deviation from linearity might be partly attributed to a change in the specific nature of the dynamically modified stationary phase.

The influence of the amount of decylamine in the mobile phase on the k_i' of chlorpromazine and its metabolites is shown in Fig. 3. It can be seen that, the k_i' values decrease sharply from 0.02% (v/v) to about 0.1% (v/v) of decylamine and then decrease gradually with further increase in the amount of decylamine. The change in k_i' coincides well with the shape of the adsorption isotherm of decylamine, as indicated by the broken line in Fig. 3. For imipramine and its metabolites a similar dependence of k_i' on the methanol and decylamine concentrations was found.

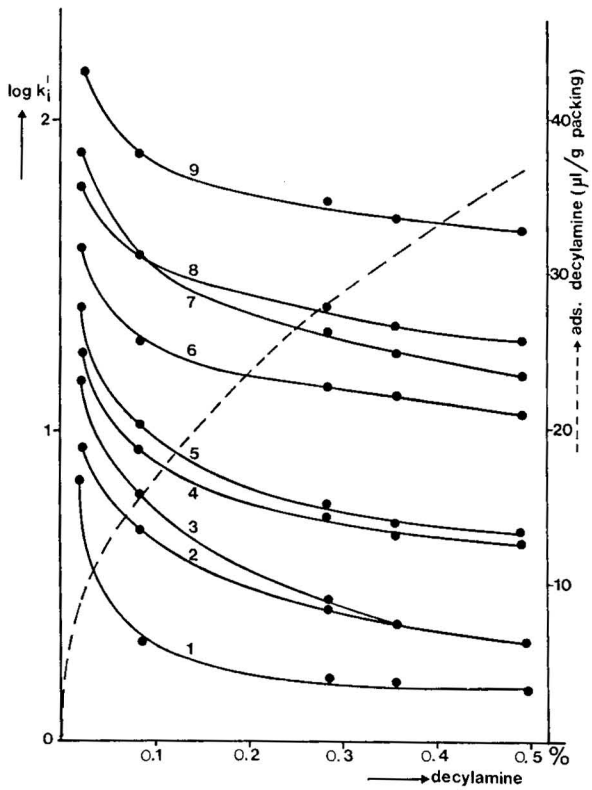


Fig. 3. Influence of the amount of decylamine on the k_i' of chlorpromazine and its metabolites. Mobile phase: water–methanol (3:7) + decylamine. For identification of compounds, see Table I.

To obtain symmetrical peaks it was necessary to add at least 0.1% (v/v) of decylamine with chlorpromazine and its metabolites and about 0.05% (v/v) with imipramine and its metabolites.

When the decylamine content of the mobile phase is changed the pH will also be changed. In order to investigate the effect of the pH of the mobile phase on the retention, k_i' values were measured at pH 9.5 and 11.0 (adjusted with perchloric acid) and the results are shown in Fig. 4. It can be seen that the k_i' values of chlorpromazine

and its metabolites are much larger at pH 9.5 than at pH 11.0. Moreover, the elution order changes for some pairs of solutes. For imipramine and its metabolites the changes are smaller.

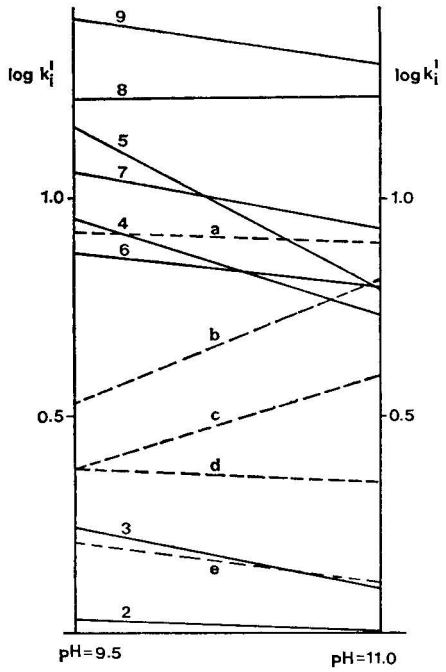


Fig. 4. Influence of the pH of the mobile phase on the k'_i of chlorpromazine (solid line) and imipramine (broken line) and their metabolites. Mobile phase: for chlorpromazine and its metabolites, water–ethanol (1:1) + 2% of decylamine; for imipramine and its metabolites, water–ethanol (4:6) + 0.2% of decylamine. For identification of compounds, see Table I.

With respect to the amount of adsorbed decylamine, it was found that at pH 9.5 about 80 μl of decylamine per gram of silica were adsorbed and about 200 μl/g at pH 11.0. This difference certainly has an influence on the nature of the stationary phase and hence on the retention. With respect to the type of organic modifier, ethanol was also tested. Although ethanol acts as a strong modifier, no significant differences were found in comparison with methanol. However, it was found that decylamine dissolves better in water–ethanol than in water–methanol mixtures, and therefore the final separations were performed with ethanol instead of methanol as organic modifier.

The results in Figs. 2–4 show that the organic modifier and decylamine concentrations and the pH are useful parameters for adjusting the retention of the antidepressants and their metabolites.

Fig. 5 shows chromatograms of the separation of test mixtures of (a) chlorpromazine and (b) imipramine and their metabolites. Dichlorpromazine, a less common antidepressant, was included in the study as a possible internal standard.

Quantitative aspects

The precision and linearity of the HPLC method were determined by injecting

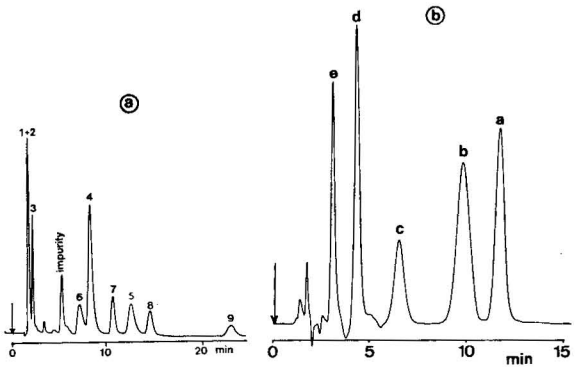


Fig. 5. Separation of test mixtures of (a) chlorpromazine and (b) imipramine and their metabolites. Mobile phase: (a) water–ethanol (1:1) + 2% of decylamine, pH = 9.5 adjusted with $HClO_4$; (b) water–ethanol (4:6) + 0.2% of decylamine, pH = 11.0. For identification of compounds, see Table I.

100 μl of solutions of the various solutes at different concentrations and measurement of the peak heights. The calibration graphs were linear up to 1 μg/ml for the parent drugs and main metabolites with correlation coefficients ranging from 0.998 to 0.999. The precision of the method was determined from repeated injections ($n = 5$) of 0.1 μg/ml solutions of imipramine and chlorpromazine and was found to be $\pm$ 4% for both solutes. The peak-to-peak value of the baseline noise was determined to be 10^{-5} absorbance unit. This leads to a calculated limit of detection, for a signal-to-noise ratio of 3, for imipramine and its metabolites of about 1 ng for the most UV-active compound (2-hydroxyimipramine) and about 3 ng for the least UV-active compound (didesmethylimipramine). For chlorpromazine and its desmethylated metabolites the limit of detection was calculated to be 2 ng.

The recovery and reproducibility of the extraction were determined by spiking blank plasma samples with known amounts of the parent drugs and their main metabolites (40 ng/ml) and extracting as described under *Procedures*. The recoveries were 85 $\pm$ 3% for imipramine, 81 $\pm$ 2% for desmethylimipramine and 74 $\pm$ 4% for didesmethylimipramine ($n = 4$).

Chlorpromazine was not extracted at all under the chosen extraction conditions. This is in contradiction with reports in the literature[12,13] in which excellent recoveries of this drug were obtained with extraction procedures similar to that used in this study. Also with more polar extraction solvents such as diethyl ether or dichloromethane only a small percentage of chlorpromazine was extracted. Adsorption on to the surface of the glassware used for the extraction or decomposition of chlorpromazine might be the reason for the very low recovery. Both possibilities were investigated in more detail.

Stability tests with solutions of chlorpromazine showed that this compound decomposes rapidly in basic solutions. However, with respect to the extraction time this cannot fully explain the very low recovery of this drug. The main losses of chlorpromazine were due to adsorption onto the surface of the glassware and on to the synthetic material in the screw-stopper of the extraction tubes. Replacement of these tubes with tubes fitted with glass stoppers decreased the adsorption losses, but the recoveries were still poor (5–20%) and varied significantly from tube to tube.

So far we have not been able to overcome the losses due to adsorption. An alternative might be to add an excess of a compound that is preferentially adsorbed on the glass surface, as proposed by Fenimore *et al.*[12]. However, this has not yet been investigated.

Desmethylchlorpromazine could be extracted with a recovery of about 60%. However, also for this compound large variations in recovery were found (± 20%), which seriously hampers its quantitative measurement.

Analysis of plasma extracts of patients

The developed RP system was applied to the analysis of the plasma from patients receiving daily doses of imipramine or desmethylimipramine. Although chlorpromazine could not be extracted for the reasons outlined above, plasma from a patient receiving chlorpromazine daily was also investigated for the presence of desmethylchlorpromazine. Fig. 6a and b show the chromatograms of extracts of blank plasma and of plasma spiked with 100 n*M* of imipramine and some of its metabolites.

Fig. 6c shows the chromatogram of the plasma from a patient receiving 100 mg of imipramine daily. It can be seen that this patient shows an unusually high metabolic conversion into desmethyl- and didesmethylimipramine. The plasma levels in this patient were determined to be 29 nmol/l of imipramine, 86 nmol/l of desmethylimipramine and 10 nmol/l didesmethylimipramine. This example shows the need to determine the main metabolites of drugs in cases of rapid metabolization.

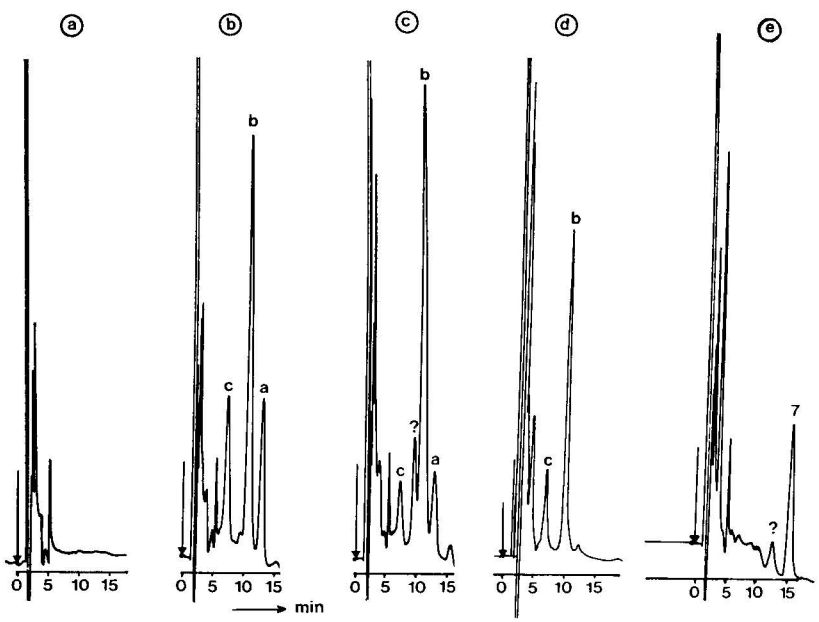


Fig. 6. Chromatograms of extracts of (a) blank and (b) spiked plasma and of plasma of patients receiving daily (c) 100 mg of imipramine, (d) 100 mg of desmethylimipramine and (e) 75 mg of chlorpromazine. Conditions as in Fig. 5. For identification of compounds, see Table I.

Fig. 6d shows the chromatogram of the plasma from a patient receiving 100 mg of desmethylimipramine daily. It can be seen that the parent drug and the didesmethyl metabolite can easily be determined by the proposed method. The plasma levels in this patient were determined to be 75 nmol/l of desmethylimipramine and about 40 nmol/l of didesmethylimipramine.

Fig. 6e shows the chromatogram of the plasma from a patient receiving 75 mg of chlorpromazine daily. No chlorpromazine could be detected in this sample because of the failure of the extraction procedure. However, the main metabolite of this drug, desmethylchlorpromazine, was found in the plasma at a concentration of about 40 nmol/l (estimated value).

CONCLUSION

RP-HPLC using mobile phases containing decylamine was found to be useful for the separation of chlorpromazine, imipramine and their metabolites. The presence of decylamine in the mobile phase improves considerably the peak shapes of these basic substances.

With the column material used (Hypersil ODS), no damage of the column packing due to the basic eluent was observed during a period of 6 months. The RP system is applicable to the analysis of imipramine and its two desmethyl metabolites in human plasma extracts. The same would apply to chlorpromazine and its desmethyl metabolite, provided that the extraction of these substances, which in this study was seriously affected by adsorption on to the surface of the glassware, can be improved.

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CHROM. 13,315

DETERMINATION OF NIFUROXAZIDE IN BIOLOGICAL FLUIDS BY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LARGE-VOLUME INJECTION

P. R. GUINEBAULT*, M. BROQUAIRE and R. A. BRAITHWAITE

Clinical Research Department, Synthélabo-Lers, 31 Av. Paul Vaillant Couturier, 92220 Bagneux (France)

SUMMARY

A high-performance liquid chromatographic method for the measurement of nifuroxazide in plasma is described. The technique is based on the single extraction of the drug from buffered plasma with chloroform, using nifuratel as internal standard. The chromatographic system consisted of a 15 cm × 4.6 mm I.D. stainless-steel column packed with Spherisorb ODS, 5 μm, and the mobile phase was acetonitrile–orthophosphoric acid (pH 2.5) (30:70). The method was able to measure accurately plasma nifuroxazide concentrations down to 2 ng·ml^{-1} using 2 ml of sample with no interference from endogenous compounds. The coefficients of variation of the method at 200 and 2 ng·ml^{-1} were 3% and 15%, respectively, and the calibration graph was linear in this range. The use of automatic injection makes the method suitable for the routine analysis of large numbers of samples.

INTRODUCTION

Nifuroxazide {4-hydroxybenzoic acid [(5-nitro-2-furanyl)methylene]hydrazide} is the active principle of Ercefuryl, which is used for the treatment of acute bacterial diarrhoea. Although this drug has been available for 15 years, no analytical method has yet been developed to determine "therapeutic" nifuroxazide concentrations in biological fluids.

In this study a method was developed involving a single extraction followed by high-performance liquid chromatography (HPLC). The different steps of the extraction and the stability of nifuroxazide were investigated.

EXPERIMENTAL

Solvents

Reagent grade chloroform used for the extraction, analytical-reagent grade orthophosphoric acid and LiChrosolv acetonitrile used for the mobile phase were purchased from Merck (Darmstadt, G.F.R.).

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Standards

Nifuroxazide was synthesized in the laboratories of Synthélabo (Bagneux, France). The internal standard, methylmercadone or nifuratel, 5-[(methylthio)-methyl]3-{[(5-nitro-2-furanyl)methylene]amino}-2-oxazolidone, was kindly supplied by Fumouse (Ile Saint-Denis, France). Standard solutions were prepared by dissolving nifuroxazide in 0.01 *M* sodium hydroxide solution and the internal standard in acetonitrile.

Equipment

Routine analyses were carried out on a liquid chromatographic system consisting of a Constametric II G pump (LDC, Riviera Beach, FL, U.S.A.), a Micromeritics 725 automatic injector and a Micromeritics Chromonitor UV detector.

Chromatographic analysis

The mobile phase (acetonitrile–orthophosphoric acid (pH 2.5), 3:7) was adjusted to a flow-rate of 1.0 ± 0.01 ml·min^{-1} through a stainless-steel column (15 cm × 4.6 mm I.D.), packed in the laboratory[1] with Spherisorb ODS, 5 μm (batch 17/49) (Phase Separations, Queensferry, Great Britain). The detector wavelength was set a 362 nm, which corresponded to the highest optical absorption of nifuroxazide dissolved in the mobile phase.

Extraction procedure

A 2-ml volume of plasma together with 1 ml of phosphate buffer, pH 5.0, was introduced into a conical tube containing 150 ng of the internal standard (30 μl of a 5 ng·μl^{-1} solution in acetonitrile). This mixture was shaken on a Vortex mixer and then extracted with chloroform (7 ml) on a "rock and roll" shaker for 30 min. The two phases were then separated by centrifugation (1000 *g*, 5 min at 4°C) and the aqueous phase was discarded; 6.5 ml of the chloroform extract were transferred into a second tube and evaporated to dryness at 60°C under a gentle stream of nitrogen. The drug extract was then dissolved in 800 μl of acetonitrile–water (10:90) by agitation on a Vortex mixer. This solution was transferred into an injection vial and injected onto the column by means of an automatic injector with a 500-μl loop.

RESULTS

Two chromatograms, one obtained from a blank plasma extract and the other from a plasma extract spiked with nifuroxazide and internal standard, are presented in Fig. 1b and a, respectively. The corresponding peaks for the two compounds were well resolved and no endogenous material extracted at the same time interfered in the analysis.

The retention times of nifuroxazide and methylmercadone were 5.2 and 8.8 min, respectively, with reduced plate heights of 3.4 and 3.5, respectively.

The procedure was quantified by the internal standard technique using the peak-height ratio method. The coefficient of variation of the slope of the calibration graph was 5.5%. The response was linear between 2 and 200 ng·ml^{-1} under the conditions described above. The accuracy and reproducibility of the method were determined by analysing spiked plasma samples of nifuroxazide at various concentrations. The mean values, the standard deviation and the coefficient of variation are given in Table I.

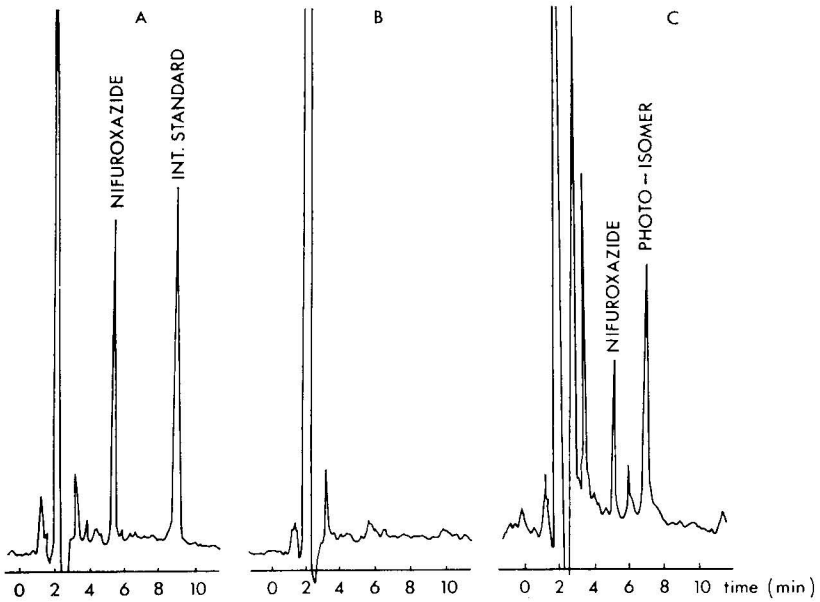


Fig. 1. Typical chromatograms of human plasma extracts for nifuroxazide determinations. A, Spiked plasma (100 ng·ml^{-1} of nifuroxazide and the internal standard); B, blank plasma; C, spiked plasma (nifuroxazide and its photo-isomer).

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR THE MEASUREMENT OF NIFUROXAZIDE AT DIFFERENT CONCENTRATIONS IN PLASMA

Concentration prepared (ng·ml^{-1})	*No. of measurements*	*Mean concentration obtained (ng·ml^{-1})*	*Standard deviation (ng·ml^{-1})*	*Coefficient of variation (%)*
2	6	1.8	0.3	15
5	6	5.6	0.6	10
10	11	9.9	0.8	8
20	18	20.5	1.0	5
100	10	100.5	5.3	5
200	4	199.5	6.7	3

All of the parameters involved in the extraction of nifuroxazide from plasma were investigated because there was no literature available on this subject. The stability of standard solutions was checked for nifuroxazide and the internal standard. Methylmercadone was stable in organic solvents whereas nifuroxazide exhibited rapid photodecomposition in methanol and acetonitrile. One of the main products of the light transformation was identified by IR and NMR spectroscopy as the imine double-bond isomer of nifuroxazide. This compound was chromatographed using the same conditions as nifuroxazide and had a retention time of 7.0 min (Fig. 1c). To prevent this isomerization, the nifuroxazide standard solutions were prepared in 0.01 *M* sodium hydroxide solution, which seemed to be stable if stored at 4°C.

In order to determine the optimal extraction pH, a series of plasma standards (1 ml) spiked with 200 ng of nifuroxazide and 300 ng of internal standard were extracted at different pHs (1 ml of buffer) with chloroform. The extract was dissolved in 100 μl of injection solvent and 50 μl were injected, the peak heights being measured at different pH values. The maximal extraction pH was found to be between 5.8 and 6.7 (Fig. 2).

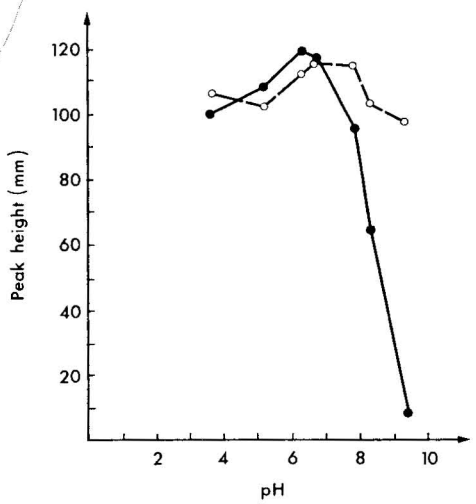


Fig. 2. Relative extraction (recovery) for nifuroxazide at different pH values. ●—●, Nifuroxazide; ○- - -○, internal standard.

Different extraction solvents were tried, using the conditions described above for chloroform. The recovery for nifuroxazide increased in the order *n*-hexane < chloroform < diethyl ether = ethyl acetate. Diethyl ether and ethyl acetate extracted a large amount of endogenous material which was trapped on the top of the column and not eluted. This created an increase in pressure drop and band broadening. Chromatograms of plasma extracted by diethyl ether or ethyl acetate exhibited interferences with nifuroxazide and the internal standard. These interferences did not appear with chloroform even after extraction for 4 h. The recovery of nifuroxazide extracted with chloroform was about 25% less than the recovery with diethyl ether; this 25% improvement in recovery with diethyl ether decreased the sensitivity from 2 to 1.5 $ng \cdot ml^{-1}$, which was relatively unimportant in comparison with the possibility of interference.

DISCUSSION

In other studies[2] it was demonstrated that reproducibility, sensitivity and efficiency of the analysis of a number of drugs by HPLC was greatly improved when the sample was injected dissolved in a large volume of a "non-eluting solvent" (*i.e.* a solvent acting as a relatively weak eluent for the compounds under analysis), compared with the more traditional method of injecting the sample dissolved in a

small volume of mobile phase or some other eluting solvent. The chromatographic processes appear not to be modified by the repeated injection of a large volume of a solvent that is not an eluent, providing this is made by using one of the components of the mobile phase[2]. This finding was also observed in the present method, where the mobile phase consisted of 30% acetonitrile in acidified water and the non-eluting solvent was 10% acetonitrile in pure water. Using automated injection it was possible to analyse at least 40–50 samples daily without any problem due to the repeated injection of relatively large volumes of the non-eluting solvent.

In conclusion, the HPLC method for nifuroxazide that has been developed is simple, sensitive, accurate and suitable for routine studies.

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CHROM. 13,155

DETERMINATION OF VINCAMINE IN HUMAN PLASMA USING AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

C. DUBRUC*, H. CAQUERET and G. BIANCHETTI
Synthélabo-Lers Clinical Research Department, 58 Rue de la Glacière, 75013 Paris (France)

SUMMARY

A specific and sensitive high-performance liquid chromatographic method for the quantitative determination of vincamine at therapeutic concentrations in plasma is described. The column was packed with Spherisorb ODS 5 μm, and the mobile phase was acetonitrile–potassium phosphate (0.02 *M*, pH 2.3) (50:50) with a flow-rate of 10 ml/min. Detection was at 230 nm. Using automated large-volume injection of a non-eluting solvent (0.02 *M* potassium phosphate) the method was capable of the analysis of a large number of samples daily. The coefficient of variation of the procedure was 4.8% at a plasma vincamine concentration of 10 ng/ml.

INTRODUCTION

Vincamine is one of the major indole alkaloids derived from *Vinca minor*[1]. Despite its widespread use in the treatment of cerebrovascular disorders[2] there have been no simple and reliable analytical methods described for the measurement of vincamine at therapeutic concentrations in biological fluids. Those methods which have been described either lack specificity[3] and sensitivity[4] or require very elaborate and expensive instrumentation[5]. The present described method is based on reversed-phase high-performance liquid chromatography (HPLC) with UV detection and automatic injection. Because of its simplicity and speed of operation the technique offers many advantages over existing methods.

EXPERIMENTAL

Standards and reagents

Vincamine, (3α,14β,16α)-14,15-dihydro-14-hydroxyeburnamenine-14-carboxylic acid methyl ester, and its internal standard desoxyvincaminamide, (3α,14β,16α)-14,15-dihydroeburnamenine-14-carboxamide, were synthesized at the laboratories of Synthélabo-Lers at Bagneux.

The following reagents were used: glass-distilled diethyl ether, acetonitrile, potassium and sodium phosphate, phosphoric acid (Merck, Darmstadt, G.F.R.).

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Stock solutions

Standard solutions of vincamine (1 μg/ml) and desoxyvincaminamide (10 μg/ml) in acetonitrile were prepared and kept at 4°C. Under these conditions the solutions were stable for at least one month.

Calibration curve and quantification

Vincamine and desoxyvincaminamide standard curves were prepared by adding 10, 50, 100 and 200 ng of vincamine and 200 ng of desoxyvincaminamide to 1 ml of drug-free plasma. The plasma standards were processed exactly the same way as the samples according to the method described below.

The ratio of the peak areas for vincamine/desoxyvincaminamide were used to calculate a calibration curve, the slope of which was used in the quantification of vincamine in plasma.

Extraction procedure

For each analysis 1 ml of plasma sample was used, and 200 ng of a solution of desoxyvincaminamide internal standard (10 ng/μl in acetonitrile) were added to each sample in a glass centrifuge tube. 1 ml of phosphate buffer (pH 8) and 8 ml of glass-distilled diethyl ether were added and the tubes stoppered and shaken vigorously for 10 sec at room temperature on a Vortex mixer. The tubes were then centrifuged for 5 min at 1000 *g*. The ether phase was transferred to another series of clean glass tubes, and evaporated under a gentle stream of nitrogen at 37°C. The dry residue was dissolved into 880 μl of 0.02 *M* potassium phosphate (pH 2.3, adjusted with phosphoric acid) and transferred into vials for automatic injection.

Apparatus

An LDC Constametric II G HPLC pump equipped with an LDC Spectro-Monitor III UV spectrophotometer was used with a stainless-steel column (15 cm × 4.6 mm I.D.) packed with ODS Spherisorb 5 μm (Phase Separations, Queensferry, Great Britain).

The automatic injector, Micromeritics model 725, holding 64 samples, was coupled to a Perkin-Elmer Σ10 integrator–calculator.

Operating conditions

The eluent was acetonitrile–potassium phosphate (0.02 *M*, pH 2.3, adjusted with phosphoric acid) (50:50) at a flow-rate of 1 ml/min. The detector was set at $\lambda = 230$ nm.

RESULTS AND DISCUSSION

The chromatogram obtained from blank plasma to which known amounts of vincamine and desoxyvincaminamide were added is shown in Fig. 1 together with those obtained from drug-free plasma and from a patient receiving chronic treatment with vincamine. The vincamine and desoxyvincaminamide peaks were well separated, and no interfering peaks from endogenous substances or concomitant therapy were observed.

To determine the best pH for the extraction of vincamine, the peak heights

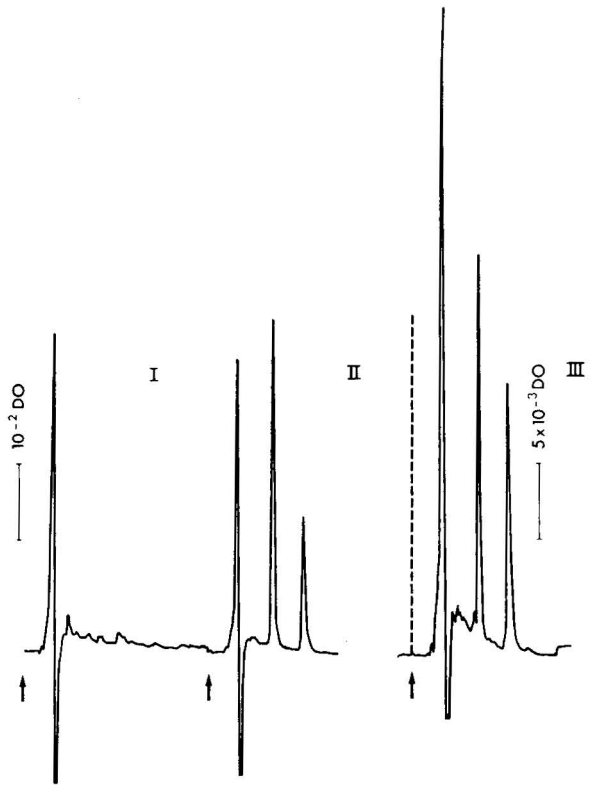


Fig. 1. Typical chromatogram obtained after extraction of 1 ml of plasma. I, Blank. II, Spiked with vincamine and its internal standard (200 ng). III, From a patient treated with vincamine.

obtained after extraction of drug from plasma at different pH values were measured and the optimum pH was found to be 8.0, giving a recovery of 99% for vincamine and 92% for desoxyvincaminamide. The chromatographic characteristics of vincamine and desoxyvincaminamide are listed in Table I.

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF VINCAMINE AND DESOXYVINCAMINAMIDE

	Vincamine	*Desoxyvincaminamide*
pK_a	8.39	8.03
pH of extraction	8	8
Solvent	Ether	Ether
Recovery after extraction (%)	99	92
Limit of sensitivity (ng/ml)	10	
Retention time (min)	6.49	4.37
Height equivalent to one theoretical plate (μm)	36.2	35.0

A standard calibration curve obtained after the extraction of vincamine from plasma is shown in Fig. 2, and the coefficient of variation of the method at different concentrations is reported in Table II.

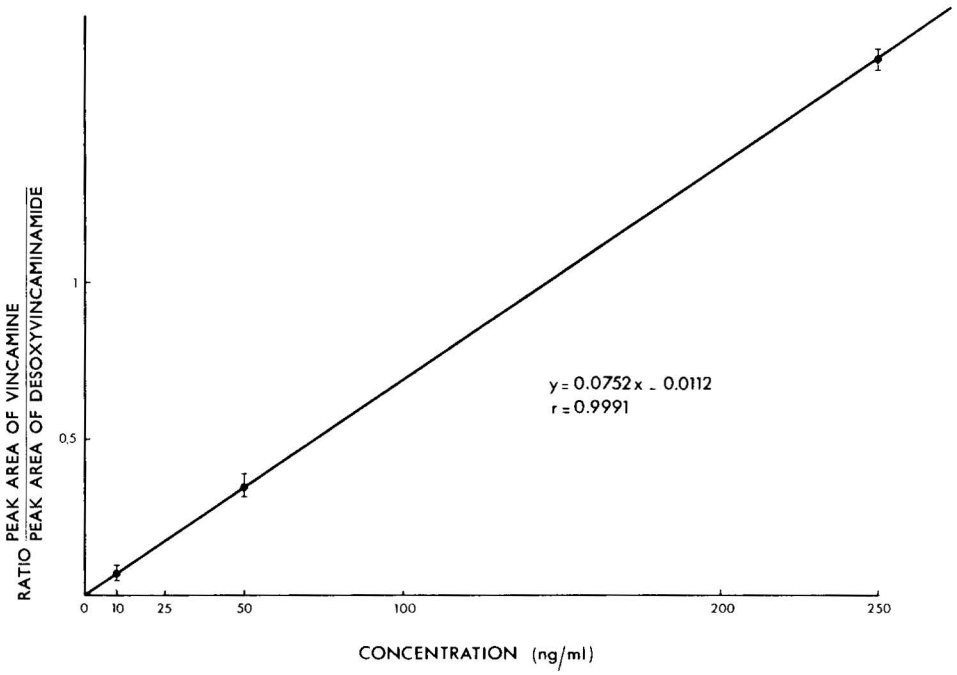


Fig. 2. Standard calibration curve obtained after extraction of vincamine from plasma.

The injection loop had a volume of 500 μl, but the drug extract was redissolved in 880 μl of solvent in order to fill the vials and rinse the loop between each injection. This was possible without any problem of band-broadening because of the choice of a non-eluting solvent (the aqueous phase of the eluent, potassium phosphate 0.02 *M*). Under these conditions the dry extract containing vincamine was perfectly redissolved and concentrated on the top of the HPLC column, without any distortion in its equilibrium. Moreover, the sensitivity of the method was as good as that obtained when injecting the total volume of a sample redissolved in only 30 μl of acetonitrile in order to prevent band-broadening. In this latter situation, it was obviously impossible to use automatic injection.

The method was found suitable for pharmacokinetic studies and the monitoring of plasma levels in patients receiving chronic treatment with vincamine. This was clearly demonstrated in a bioavailability study comparing chronic treatment with two different formulations of vincamine in ten elderly patients.

TABLE II

COEFFICIENT OF VARIATION OF THE METHOD AT DIFFERENT CONCENTRATIONS

Number of determinations: 10.

Vincamine concentration added to plasma (ng/ml)	*Mean value of concentration found*	*S.D.*	*Coefficient of variation*
10	11.2	0.52	4.8
50	47.2	1.93	4.1
250	248.9	11.20	4.5

The results indicate that the method described is not only specific and sensitive, but also very simple and rapid. The coupling of an automatic injector holding 64 samples with an integrator–calculator allows a large number of analysis to be carried out in one day.

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CHROM. 13,156

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF EBURNANE ALKALOIDS

I. SEPARATION ON REVERSED PHASES

G. SZEPESI* and M. GAZDAG
Chemical Works of Gedeon Richter, Ltd., Budapest (Hungary)

SUMMARY

Separation of eburnane alkaloids using reversed-phase high-performance liquid chromatography was investigated on μBondapak C_{18} and LiChrosorb RP-8 columns with acetonitrile–aqueous 0.01 *M* ammonium carbonate as eluent. The method can be successfully applied for the group separation of eburnane alkaloids as well as for the separation of stereoisomers and of ester homologues.

INTRODUCTION

In the last few years the importance of eburnane alkaloids in pharmacy has considerably increased. Besides vincamine, new representatives of eburnane alkaloids such as apovincaminic acid ethyl ester have been introduced in medical practice.

Only a few methods can be found in the literature for the separation of vincamine and apovincaminic acid ethyl ester. Recently, paper and thin-layer chromatography[1–7] as well as gas chromatography[8,9] have been used for solving special analytical problems. Considering the characteristics and structures of eburnane alkaloids, high-performance liquid chromatography (HPLC) seems to be the most appropriate method for their separation owing to the low selectivity and efficiency of thin-layer chromatography and the thermal instability and low volatility of many eburnane alkaloids.

In the present paper, we report studies using reversed-phase HPLC.

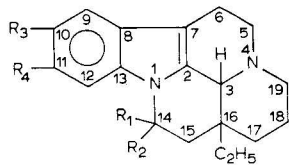
EXPERIMENTAL

The liquid chromatograph consisted of a Model OE-312 Liquopump reciprocating pump (Labor-MIM, Esztergom, Hungary), a Model OE-308 variable-wavelength UV detector (Labor MIM) and a Rheodyne 7120 loop injector (Rheodyne, Berkeley, CA, U.S.A.). The separations were performed on columns of μBondapak C_{18} (300 × 3.9 mm I.D.) (Waters Assoc., Frankfurt/M, G.F.R.) and of 10 μm

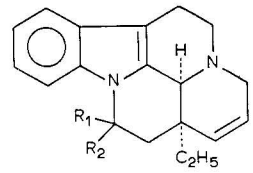
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TABLE I

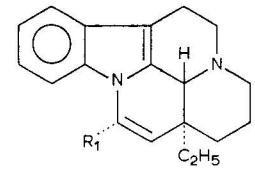
STRUCTURES OF EBURNANE ALKALOIDS INVESTIGATED



Type A
14,15-Dihydroeburnamenine



Type B
Eburnamenine



Type C
14,15-Dihydro-17,18-dehydro-eburnamenine

No.	*Alkaloid*	*Type*	*Substituents*				*Configuration of*			
			R_1	R_2	R_3	R_4	R_1	R_2	*3-H*	*16-*C_2H_5
I	(+)-*cis*-Vincaminic acid	A	OH	COOH	H	H	β	α	α	α
II	(+)-*cis*-Vincamine	A	OH	$COOCH_3$	H	H	β	α	α	α
III	(−)-*cis*-Vincamine	A	OH	$COOCH_3$	H	H	β	α	β	β
IV	(+)-*cis*-Epivincamine	A	OH	$COOCH_3$	H	H	α	β	α	α
V	(−)-*cis*-Epivincamine	A	OH	$COOCH_3$	H	H	α	β	β	β
VI	(+)-*cis*-Vincaminic acid ethyl ester	A	OH	$COOC_2H_5$	H	H	β	α	α	α
VII	(+)-*trans*-Vincaminic acid ethyl ester	A	OH	$COOC_2H_5$	H	H	β	α	β	α
VIII	(+)-*cis*-Epivincaminic acid ethyl ester	A	OH	$COOC_2H_5$	H	H	α	β	α	α
IX	(+)-*trans*-Epivincaminic acid ethyl ester	A	OH	$COOC_2H_5$	H	H	α	β	β	α
X	(+)-*cis*-Vincamone	A	O		H	H	—	—	α	α
XI	(+)-*cis*-Vincanole	A	OH	H	H	H	β	α	α	α
XII	(+)-*cis*-Isovincanole	A	OH	H	H	H	α	β	α	α
XIII	(+)-*cis*-10-Bromovincamine	A	OH	$COOCH_3$	Br	H	β	α	α	α
XIV	(+)-*cis*-11-Bromovincamine	A	OH	$COOCH_3$	H	Br	β	α	α	α
XV	(+)-*cis*-Apovincaminic acid	B	—	COOH	H	H	—	—	α	α
XVI	(+)-*cis*-Apovincamine	B	—	$COOCH_3$	H	H	—	—	α	α
XVII	(+)-*cis*-Apovincaminic acid ethyl ester	B	—	$COOC_2H_5$	H	H	—	—	α	α
XVIII	(+)-*trans*-Apovincaminic acid ethyl ester	B	—	$COOC_2H_5$	H	H	—	—	β	α
XIX	(+)-*cis*-Apovincaminic acid phenyl ester	B	—	$COOC_6H_5$	H	H	—	—	α	α
XX	(+)-*cis*-Vincamenine	B	—	H	H	H	—	—	α	α
XXI	(+)-*cis*-Dehydrovincamine	C	OH	$COOCH_3$	H	H	β	α	β	β
XXII	(+)-*cis*-Dehydroepivincamine	C	OH	$COOCH_3$	H	H	α	β	β	β

LiChrosorb RP-8 (250 × 4.6 mm I.D.) (Pierce Eurochemie, Rotterdam, The Netherlands).

The chemicals and solvents used were of analytical grade (Reanal, Budapest, Hungary). All solvents were freshly distilled and degassed before use. The compounds investigated were prepared in our laboratories and in the Institute for Organic Chemistry, Budapest Technical University, and were considered to be of the highest available quality.

RESULTS AND DISCUSSION

The structures of eburnane alkaloids investigated are summarized in Table I. According to their structures the eburnane alkaloids can be divided into three groups:

(a) vincamine type consisting of a 14,15-dihydroeburnamenine skeleton

(b) apovincamine type consisting of an eburnamenine skeleton

(c) dehydrovincamine type consisting of a 14,15-dihydro-17,18-dehydroeburnamenine skeleton.

Considering the structures and physico-chemical properties of the compounds (Table I), the analytical tasks can be summarized as follows: group separation of the three different types of eburnane alkaloids; separation of stereo- and structural isomers as well as of ester homologues; comparison of the separation possibilities both on octadecyl and octyl silica, and optimization of the separation system. Table II shows the capacity ratios, k', measured for the compounds both on octadecyl and octyl silica stationary phases using different mixtures of acetonitrile and aqueous ammonium carbonate as eluent.

It can be seen from Table II that the group separation of eburnane alkaloids can be achieved on both phases. The elution order of the compounds having similar structure is as follows: *cis*-dehydroepivincamine; *cis*-dehydrovincamine; *cis*-epivincamine; *cis*-vincamine; and *cis*-apovincamine.

As regards the separation of stereoisomers, some interesting conclusions can be drawn. For example, in case of vincaminic acid ethyl ester isomers (their structures are in Fig. 1) eight different stereoisomers should be found depending on the relative positions of the hydrogen atom and ethyl group in positions 3 and 16, and of the ester and hydroxy groups in position 14. However, the (+)-*cis* and (−)-*cis* isomers cannot be separated from each other (see Table II) and we assume the same situation applies for (+)-*trans* and (−)-*trans* isomers [we did have not the (−)-*trans* isomer]; thus only the separation of four isomers can be achieved. In Fig. 1 the dependence of the capacity ratios on the eluent composition using octadecyl and octyl silica stationary phases is shown.

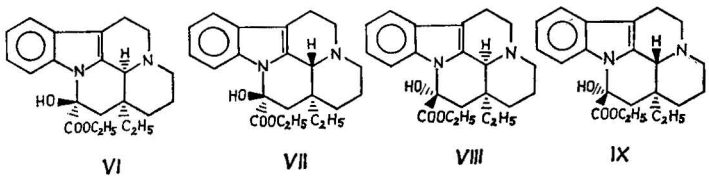
It can be seen from Fig. 1 that a linear relationship between log k' and eluent composition was obtained on both phases. In other respects, however, the results obtained significantly differ from each other. Thus the elution orders are different, and while on octyl silica the elution order is independent of the eluent composition, on octadecyl silica the elution order of *cis*-epivincaminic acid ethyl ester (VIII) and *trans*-epivincaminic acid ethyl ester (IX) can be reversed by changing the eluent composition (Fig. 2).

As has already been mentioned, a limitation of the systems investigated is that the optical isomers of eburnane alkaloids cannot be separated from each other.

TABLE II

CAPACITY RATIOS, k', FOR EBURNANE ALKALOIDS ON OCTADECYL AND OCTYL SILICA PACKINGS WITH DIFFERENT ELUENTS

No.	*Alkaloid*	*Ratio of acetonitrile and 0.01 M $(NH_4)_2CO_3$*								
		4:6	*5:5*	*6:4*	*7:3*	*8:2*	*4:6*	*5:5*	*6:4*	*7:3*
		μBondapak C_{18}					*10 μm LiChrosorb RP-8*			
XI	(+)-*cis*-Apovincaminic acid	0.05	0.05	0.0	0.0	0.0	0.0	0.0	0.0	0.0
I	(+)-*cis*-Vincaminic acid	0.10	0.10	0.07	0.05	0.0	0.0	0.0	0.0	0.0
XXII	(+)-*cis*-Dehydroepivincamine	4.65	2.57	1.57	1.04	0.57	4.61	2.14	1.30	0.66
XXI	(+)-*cis*-Dehydrovincamine	6.03	3.21	1.86	1.31	0.57	5.65	2.60	1.55	0.98
VII	(+)-*trans*-Vincaminic acid ethyl ester	8.93	4.29	2.29	1.32	0.57	10.81	4.43	2.19	1.30
IV	(+)-*cis*-Epivincamine	9.23	4.71	2.71	1.79	0.75	6.02	2.60	1.55	1.04
V	(−)-*cis*-Epivincamine	9.23	4.71	2.71	1.79	0.75	6.02	2.60	1.55	1.04
VIII	(+)-*cis*-Epivincaminic acid ethyl ester	12.4	6.36	3.43	1.97	0.95	8.45	3.60	1.92	1.30
IX	(+)-*trans*-Epivincaminic acid ethyl ester	14.4	6.36	3.43	1.79	0.75	19.8	7.04	3.26	1.83
II	(+)-*cis*-Vincamine	12.4	6.36	3.43	2.14	0.90	10.9	3.53	1.87	1.30
III	(−)-*cis*-Vincamine	12.4	6.36	3.43	2.14	0.90	10.9	3.53	1.87	1.30
X	(+)-*cis*-Vincamone	13.7	7.21	4.04	2.57	1.43	—	5.51	3.19	1.87
XI	(+)-*cis*-Vincanole	15.6	8.07	4.29	3.14	1.71	—	3.60	2.26	1.55
XII	(+)-*cis*-Isovincanole	18.8	9.43	5.43	3.71	1.71	—	4.30	2.83	1.87
VI	(+)-*cis*-Vincaminic acid ethyl ester	16.9	8.47	4.38	2.57	1.43	12.4	5.03	2.57	1.55
XVI	(+)-*cis*-Apovincamine	23.9	14.1	6.78	4.11	2.64	—	9.42	4.79	2.66
XVIII	(+)-*trans*-Apovincaminic acid ethyl ester	30.3	18.5	7.76	3.97	2.03	—	21.8	8.64	4.23
XVII	(+)-*cis*-Apovincaminic acid ethyl ester	32.5	20.2	9.00	6.20	3.27	—	13.6	6.36	3.47
XX	(+)-*cis*-Vincamenine	46.5	31.2	14.0	8.71	5.57	—	19.2	10.4	5.26
XIX	(+)-*cis*-Apovincaminic acid phenyl ester	56.1	36.8	14.0	7.07	4.00	—	26.4	9.80	4.81
XIII	(+)-*cis*-10-Bromovincamine	13.4	9.11	4.86	3.54	1.70	—	7.32	3.57	2.11
XIV	(+)-*cis*-11-Bromovincamine	13.4	9.11	4.86	3.54	1.70	—	7.32	3.57	2.11



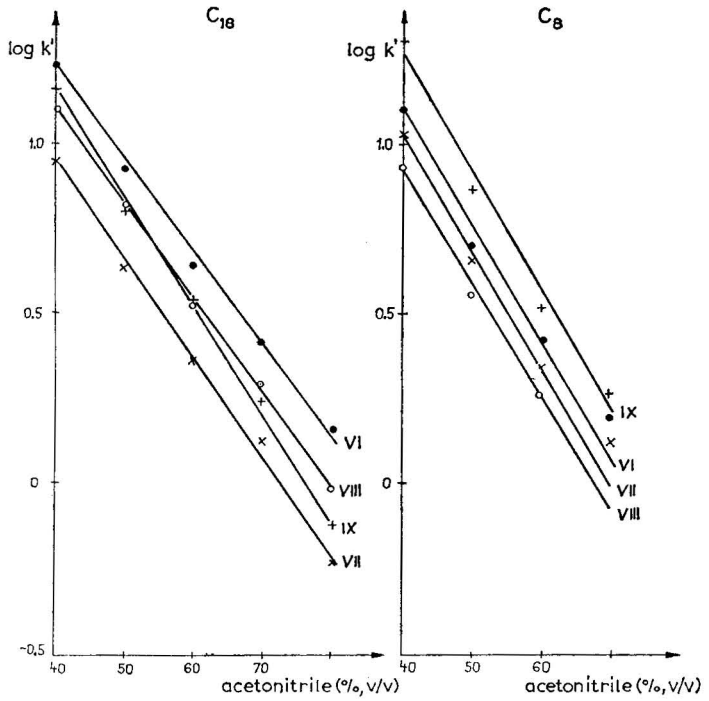


Fig. 1. Dependence of the capacity ratio, k', for vincaminic acid ethyl ester isomers on the eluent composition. Columns: μBondapak C_{18}, 300 × 3.9 mm I.D.; 10 μm LiChrosorb RP-8, 250 × 4.6 mm I.D. Detector: UV, 280 nm. Flow-rate: 1 cm^3/min.

Another limitation can be seen in Table II, namely the structural isomers of eburnane alkaloids substituted in the aromatic ring also cannot be separated, being eluted with virtually identical retention in the systems investigated. As shown in Table II and Figs. 3 and 4, the ester homologues of apovincaminic acid and vincaminic acid are well separated both on octadecyl and octyl silica.

To optimize the separation system, a mixture of eburnane alkaloids (Table I) was analyzed. On both octyl silica and octadecyl silica stationary phases, acetonitrile–aqueous 0.01 *M* ammonium carbonate (6:4) was found to be optimal. The separation of these components is shown in Figs. 3 and 4. It thus seems that the best resolution was achieved on octadecyl silica, and by increasing the chain length of the bonded alkyl group on the silica surface, the capacity ratios and to some extent also the selectivity increase for the compounds.

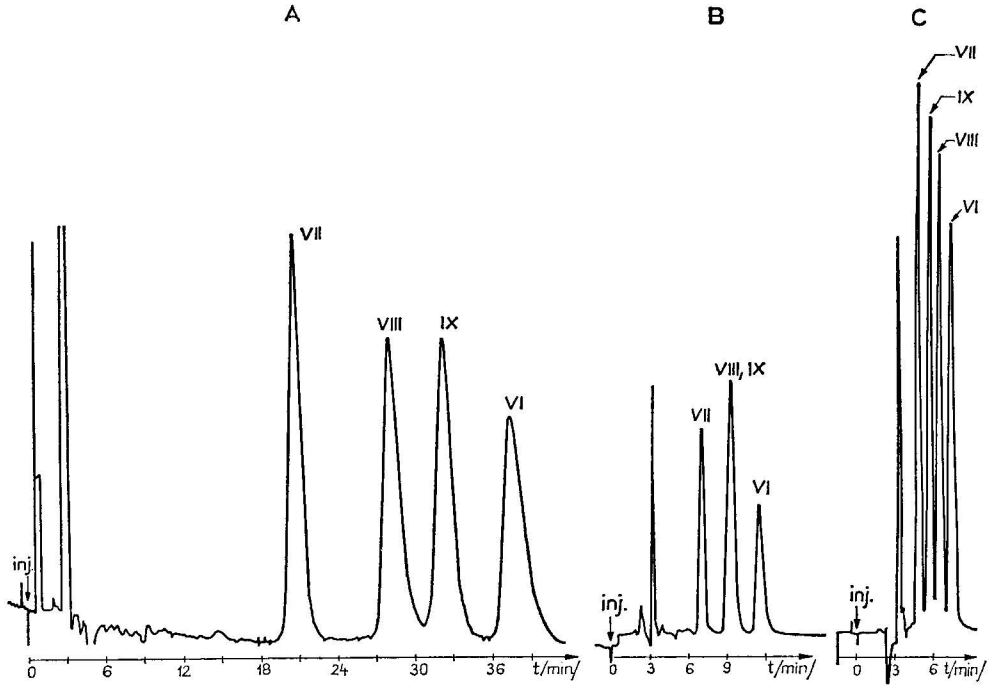


Fig. 2. Separation of vincaminic acid ethyl ester isomers. Column: μBondapak C_{18}, 300 × 3.9 mm I.D. Eluents: (A) acetonitrile–0.01 *M* ammonium carbonate (4:6); (B) 6:4; (C) 7:3. Other conditions as in Fig. 1. For compounds see Table I.

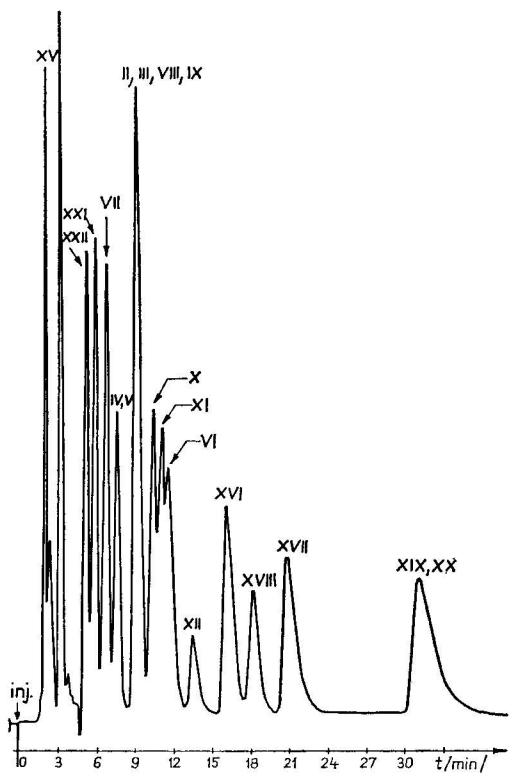


Fig. 3. Separation of eburnane alkaloids on octadecyl silica. Eluent: acetonitrile–0.01 *M* ammonium carbonate (6:4). Other conditions as in Fig. 1. For compounds see Table I.

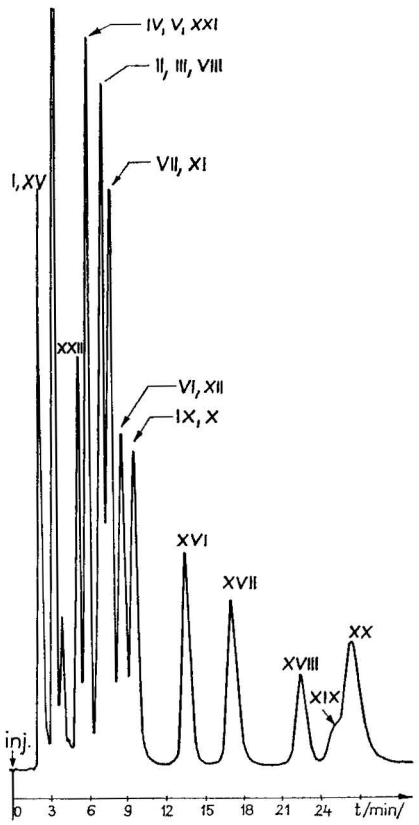


Fig. 4. Separation on octyl silica stationary phase. Conditions as in Fig. 3.

CONCLUSIONS

The separation of eburnane alkaloids by reversed-phase HPLC was studied. It was found that only some of the problems can be solved by the systems investigated. The method is suitable for the separation of closely related eburnane alkaloids as stereoisomers and ester homologues; group separation can also be carried out. However, optical isomers and structural isomers cannot be separated. The investigation of the effect of the chain length on the separation characteristics has revealed a significant difference in selectivity between the octyl and octadecyl silica stationary phases.

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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF MAPROTILINE AND OXAPROTILINE IN HUMAN PLASMA

H. J. KUSS* and E. FEISTENAUER

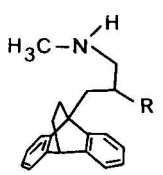
Department of Neurochemistry, Psychiatric Hospital, University of Munich, Nussbaumstr. 7, D-8000 Munich 2 (G.F.R.)

SUMMARY

A procedure is described which permits the determination of maprotiline and oxaprotiline in human blood or plasma at therapeutic concentrations. The biological fluids are extracted with base and the extract is injected into a high-performance liquid chromatograph. The substances are detected by a UV detector operating at 214 nm, amitriptyline being used as internal standard. Concentrations as low as 2 ng per sample can be measured.

INTRODUCTION

Maprotiline (Ludiomilz, Ciba-Geigy, Basel, Switzerland) is an antidepressant drug, the main action of which is thought to be the strong inhibition of the noradrenaline uptake into neurons[1]. In pharmacological experiments[2] the side-chain hydroxy derivative of maprotiline (oxaprotiline, Fig. 1) inhibited the neuronal uptake of noradrenaline by a factor of 10.



R = H maprotiline
R = OH oxaprotiline

Fig. 1. Structural formulae of maprotiline and oxaprotiline.

Some methods concerning the quantitative determination of maprotiline have been published[3–10], most of which involve gas chromatographic separations. As detectors, the electron-capture detector[3], the nitrogen-selective flame-ionization detector and the mass spectrometer[8,9] can be used. In 1974 a double radioisotope derivatization technique, which made use of a thin-layer separation, was described[7]. Recently, the first high-performance liquid chromatographic (HPLC) assay was published[10], in which a derivatization step is also necessary. However, the most frequently used

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HPLC detector with a wavelength of 254 nm is not suitable for the determination of maprotiline and its derivatives because of their very low absorption in this region. Only below 220 nm is the extinction of the UV-radiation high enough to allow the estimation of these drugs in the therapeutic range.

In this paper, an HPLC method for the determination of maprotiline and oxaprotiline without derivatization is described. Hundreds of quantitative determinations of both these compounds have been carried out in our laboratory, thus establishing the method as a routine determination.

EXPERIMENTAL

The simple high-performance liquid chromatograph consisted of an Altex A 110 pump, a Rheodyne 7120 injection valve, a commercially available Nucleosil C_{18} column and an LDC UV-monitor III with a zinc source operating at 214 nm. The mobile phase was a mixture of 35 % acetonitrile and 65 % of a 0.05 *M* phosphate buffer, pH 2.7. It was vacuum-filtered and then degassed ultrasonically. All water used was double-distilled. The acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) had an UV cut-off of 190 nm. Reagents for the extraction were of analytical grade.

In the morning before the first drug dose, blood from patients was drawn into collection tubes containing lithium heparinate. The plasma was separated and stored frozen in polypropylene tubes until analysis. Each tube contained exactly 1 ml and the contents were used directly for extraction.

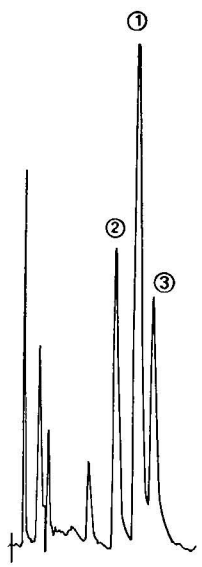
The extraction procedure is summarized in Table I. After thawing the plasma, 1 ml 2 *M* $NaHCO_3$ solution and 100 ng amitriptyline were added. The alkaline solution was mixed with 6 ml hexane and centrifuged for 10 min. After separating the

TABLE I

EXTRACTION PROCEDURE

1 ml plasma
+ 100 ng amitriptyline
+ 1 ml 2 *M* $NaHCO_3$ solution
↓
+ 6 ml hexane ← shake and centrifuge
↓
+ 1 ml 0.1 *N* H_2SO_4 ← shake and centrifuge
↓
+ 0.1 ml 5 *N* NaOH solution
↓
+ 2 ml hexane ← shake and centrifuge
↓
evaporate to dryness
solve in 0.02 ml eluent
inject all
↓
HPLC
↓
integrator → result

hexane layer with a pasteur pipette it was mixed with 1 ml 0.1 N H_2SO_4 for 10 min and centrifuged for another 10 min. Then the hexane layer was aspirated. A 0.1-ml volume of 5 N NaOH solution was added to the aqueous phase followed by 2 ml hexane. The solution was mixed for 10 min and then centrifuged. The hexane was evaporated in a vacuum and the residue dissolved in 20 μl of the eluent. As much as possible of this solution was then transferred to the injection valve of the high-performance liquid chromatograph. For automatic estimation of the results an integrator was used. Fig. 2 shows the chromatogram of a plasma extract from a patient receiving 150 mg maprotiline for therapy. It can be seen that both maprotiline and desmethylmaprotiline can be determined. Fig. 3 shows a similar chromatogram for oxaprotiline. For these substances the lower limit of detection is 2 ng/ml using 1 ml plasma for the extraction.



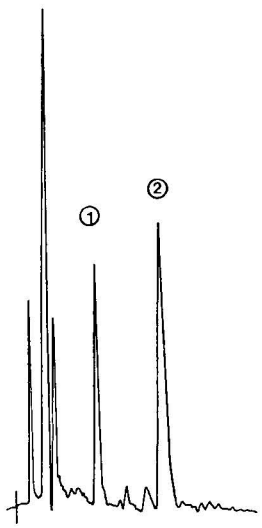


Fig. 2. HPLC of plasma extract from a patient receiving 150 mg maprotiline. Mobile phase, 35% acetonitrile and 65% 0.05 M phosphate buffer, pH 2.7. Detection at 214 nm at 0.08 a.u.f.s. Peaks: 1 = maprotiline, 2 = desmethylmaprotiline, 3 = amitriptyline.

Fig. 3. HPLC of plasma extract from a patient receiving 150 mg oxaprotiline. Conditions as in Fig. 2. Peaks: 1 = oxaprotiline, 2 = amitriptyline.

RESULTS

The range of concentrations found is shown in Table II. The values are in accord with other studies of maprotiline in plasma. So far, no measurements of oxaprotiline concentrations in plasma have been published.

One possible explanation for the lower concentration range of oxaprotiline as compared with maprotiline is the direct glucuronidation of the hydroxy group. In our determination the desmethyl derivatives are also included. The pharmacological importance of these metabolites is unknown, but the measured plasma concentration is so high that an action on the nervous system cannot be excluded. We established a

TABLE II

PLASMA CONCENTRATIONS (ng/ml) FOUND

Twenty patients were sampled in each case.

Dosage	*Maprotiline*		*Desmethylmaprotiline*		*Oxaprotiline*	
	Found	*Mean*	*Found*	*Mean*	*Found*	*Mean*
150 mg maprotiline per day	37–208	107	13–111	40		
150 mg oxaprotiline per day					5–48	25

negative correlation at the 2% level of the therapeutic effect and the plasma level of maprotiline, and a negative correlation at the 0.1% level with the concentration of desmethylmaprotiline. No correlation could be found for the plasma level of oxaprotiline and the patients' improvement[11].

In Fig. 4 the pharmacokinetics of oxaprotiline in human blood is shown. It can be seen that the method can also be used to analyze the low concentrations obtained after a single dose.

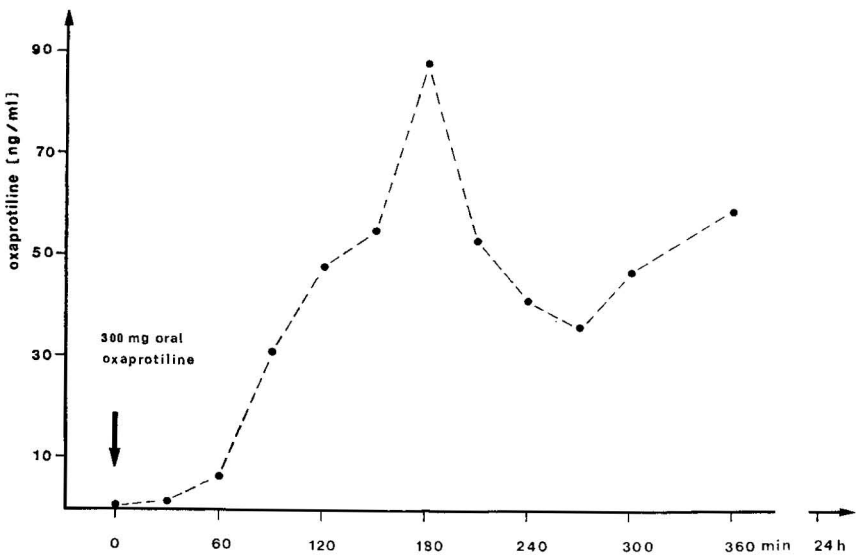


Fig. 4. Pharmacokinetics of oxaprotiline in human blood.

DISCUSSION

Gas chromatography–mass spectrometry is the best tool for analyzing the metabolic fate of drugs in the body, but is very expansive. For routine analysis in a clinical laboratory, HPLC is the most valuable method for determining the concentration of drugs in human blood during therapy. Some manufactures are also developing immuno-assays, but these are not as specific as chromatographic analyses. The quantitative separation of the parent compound and its active metabolites is the principal requirement (*cf.* ref. 12) for progress in clinical psychopharmacology.

Using a 214-nm detector with a zinc source we could achieve a 20-fold increase

in sensitivity, as compared to the usual 254-nm detector, for the detection of maprotiline and oxaprotiline. This high sensitivity allowed us to analyze plasma concentrations of these two substances in the therapeutic range. For the extraction we used a very common method including two back-extraction steps. The mean recovery was 50% or lower. Each day a recovery curve was constructed from spiked plasma samples. All the plasma concentrations of maprotiline, desmethylmaprotiline and oxaprotiline were corrected for recovery. The concentrations of desmethyloxaprotiline were in the range of the detection limit.

An important problem in plasma level monitoring is the presence of more than one drug. In our assay some of the tricyclic antidespressants and of the benzodiazepines can result in interferences. Monitoring the effluent of the column at 214 and 254 nm allows detection of these interferences. If there is no interference the 254-nm detector will not show any signal at the retention time of the drugs determined When there is any doubt regarding the identification of the peaks, the effluent can be injected into a gas chromatograph for further identification. Our values are in good agreement with the measurements of Gupta *et al.*[6] using the only assay procedure, which included the concentration of desmethylmaprotiline. These workers found a mean concentration of 117 ng/ml for maprotiline (range 40–237) and 52 ng/ml for desmethylmaprotiline (range 20–132); for comparison see Table II.

Some authors prefer the extraction of whole blood. We use blood concentration measurements only for pharmacokinetic purposes (Fig. 4). For therapeutic drug monitoring we prefer plasma, because most studies dealing with concentrations and the therapeutic effect make use of plasma extractions.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. W. Riess, Ciba-Geigy, Basle, for supplying the substances maprotiline, desmethylmaprotiline, oxaprotiline and desmethyloxaprotiline. They also thank Professor N. Matussek for his generous advice.

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SPECIFIC AND SENSITIVE METHOD FOR THE DETERMINATION OF TOBANUM® USING GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE AND MASS FRAGMENTOGRAPHIC DETECTION

EVA TOMORI* and ISTVAN ELEKES
Institute for Drug Research, P.O. Box 82, 1325 Budapest (Hungary)

SUMMARY

Quantitative gas–liquid chromatographic (GLC) and GLC–mass spectrometric (MS) methods for the determination of Tobanum®, a new β-blocking agent, in human plasma have been developed. After solvent extraction, a bis-trifluoroacetyl derivative is formed which is measured by an electron-capture detector. The quantitation is controlled by using an internal standard, propranolol hydrochloride, which is added to all samples. The electron-capture detector response is linear in the concentration range used, 5–150 ng/ml. The identity and quantity of the GLC peaks is confirmed by GLC–MS. The minimum detectable concentration of Tobanum® is 1 ng using 3-ml plasma samples.

INTRODUCTION

The determination of Tobanum® in plasma is important for the understanding of the mechanism of action of this reagent. Tobanum hydrochloride [1-(*tert.*-butylamino)-3-(2,5-dichlorophenoxy)-propanol-2 hydrochloride] is a new β-adrenergic receptor antagonist having antihypertensive, antiarrhythmic and anticonvulsant activity[1,2].

Several methods have been reported for quantitative determinations of β-blocking drugs. Propranolol and sotalol have been determined fluorimetrically[3,4], practolol colorimetrically[5] and alprenolol by gas–liquid chromatography (GLC)[6]. An improved method based on GLC of a fluorinated derivative has been developed for determination of propranolol and its metabolite[7]. A gas chromatography–mass spectrometry (MS)–computer technique[8] has been employed for assay of urinary propranolol and its metabolites.

Our aim was to combine specificity with high sensitivity; therefore two methods have been developed based on GLC and GLC–MS–computer techniques. This study presents, for the first time, methods for the quantitative determination of Tobanum in plasma. Conditions for extraction, derivatization and detection are described.

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EXPERIMENTAL

Standard and reagents

Propranolol hydrochloride was kindly supplied by ICI (Macclesfield, Great Britain). Tobanum hydrochloride was prepared in our laboratories. The following reagents were used: freshly distilled *n*-hexane and nanograde benzene (Mallinckrodt, St. Louis, MO, U.S.A.); trifluoroacetic anhydride as the derivatization reagent (Pierce, Rockford, IL, U.S.A.); 6 *N* sodium hydroxide, 1.25 *N* sulphuric acid, 0.5 *M* phosphate buffer, pH 6.0, prepared with glass-distilled water and stored in glass bottles.

After cleaning with chromic acid, all glassware was silanized with dimethyldichlorosilane (Pierce) and rinsed with methanol and toluene.

Apparatus

For GLC, a Hewlett-Packard 5710A gas chromatograph with a frequency-modulated ^{63}Ni electron-capture detector was used. The glass chromatographic columns (3 ft. × 4 mm I.D.) were packed with 3% OV-17 and 3% OV-1 stationary phases on Gas-Chrom Q (100–120 mesh) (Applied Science Labs., State College, PA, U.S.A.). The carrier gas was argon–methane (95:5) at a flow-rate of 50 ml/min. Temperatures: injection port, 250°C, column, 180°C and 170°C and detector, 250°C.

For GLC–MS, a Hewlett-Packard 5992A instrument was used. The mass spectrometric conditions were: ionization beam energy, 70 eV; electron multiplier, 2800 V. The glass column (3 ft. × 2 mm I.D.) was packed with 2% OV-101 on Gas-Chrom Q (100–120 mesh) and operated at 170°C with a helium gas flow-rate of 25 ml/min.

Extraction procedure and preparation of derivatives

To samples of 3 ml of plasma were added 300 ng of internal standard, propranolol hydrochloride, and 0.25 ml of 6 *N* NaOH in a glass-stoppered 10-ml centrifuge-tube. The samples were shaken with 3 ml of benzene for 5 min and centrifuged at 3000 *g* for 5 min. The benzene phase was transferred to a tube containing 2 ml of 1.25 *N* H_2SO_4. The tube was shaken for 5 min and centrifuged. The acidic aqueous phase was transferred to another tube, made alkaline (pH 12) with 0.75 ml of 6 *N* NaOH and shaken with 3 ml of benzene for 5 min. The benzene phase was transferred to another tube with a PTFE screw cap and evaporated to a small volume (50 μl) with a gentle stream of nitrogen at 60°C. A 70-μl volume of 1 *M* trimethylamine in benzene and 25 μl of trifluoroacetic anhydride were added. The tubes, after being tightly stoppered, were heated for 10 min at 60°C in a sand-bath. After cooling the reaction mixtures were shaken vigorously for 30 sec with 1 ml of 0.5 *M* phosphate buffer, pH 6.0, and centrifuged. Part of the benzene phase was taken for electron-capture GLC and GLC–MS assay.

Construction of standard calibration curves

Aliquots (3 ml) of control plasma samples were spiked with Tobanum hydrochloride in the range 5–150 ng and extracted according to the back-extraction procedure using 300 ng of propranolol hydrochloride as internal standard. In the case of GLC, peak area ratios of Tobanum to propranolol were measured and plotted as a

function of Tobanum concentration. In the case of GLC–MS the calibration curve was obtained from intensity ratios for the ion at m/z 266 in the spectra of Tobanum and propranolol.

RESULTS AND DISCUSSION

Fig. 1 shows a typical gas chromatogram obtained from plasma extract (pH 12) on the apolar silicon phase OV-1; a good separation is achieved ($\alpha = 1.57$).

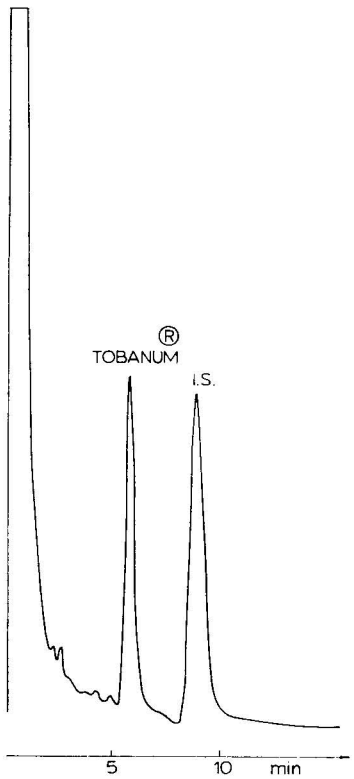


Fig. 1. Gas chromatogram of a plasma extract obtained from a patient 4 h after administration of 3 × 5 mg Tobanum. The column was OV-1 and the temperature was 170°C. Conditions as in Experimental.

The acylation of Tobanum was studied between 5 and 60 min at 30°C and 60°C. The relative peak size appeared to be independent of the acylation conditions investigated; hence, both Tobanum and I.S. are converted into their derivatives at the same rate and to the same extent. The use of propranolol as an internal marker was found to be satisfactory. The physico-chemical properties of this compound are closely related to those of Tobanum. Standard curves obtained from plasma B in Table I do not significantly differ from that obtained from water (A), indicating no significant interference from plasma binding.

TABLE I

STANDARD CURVES FOR TOBANUM

Curves: A = extraction from distilled water; B and C = extractions from plasma. A and B were measured by electron-capture GLC and C by GLC–MS. Conditions as in Experimental.

Curve	$y = a + bx$		
	a	*b*	*Correlation coefficient*
A	+0.02	0.009	0.997
B	+0.04	0.0073	0.994
	+0.03	0.0068	0.996
	+0.18	0.009	0.997
C	+0.006	0.0066	0.996

According to Table I, there are daily variations in the slope of the standard curves. Over a 2-month period, during which more standard curves were run, the slopes for Tobanum ranged from 0.006 to 0.009. All curves, however, passed through the origin and correlation coefficients were all high. The relative recovery from human plasma was constant in the range examined with a mean of 47 $\pm$ 0.05% (n = 5), and from water was 76 $\pm$ 0.02% (n = 5). The minimum detectable amount is about 1 ng using 3 ml of plasma, by both methods.

The GLC–MS assay confirmed the identity and the quantity of the GLC peaks. GLC peaks of Tobanum bis-trifluoroacetate should give a molecular ion at m/z 483 (0%). The mass spectrum (Fig. 2) exhibited an ion at m/z 468 (2%) corresponding to the loss of a methyl group from the molecular ion. The ion at m/z 162 (14%) is formed by loss of the side-chain after H-rearrangement, and the fragment ions at m/z 266 (55%) and m/z 152 (5%) arise from cleavage of the side-chain at various positions.

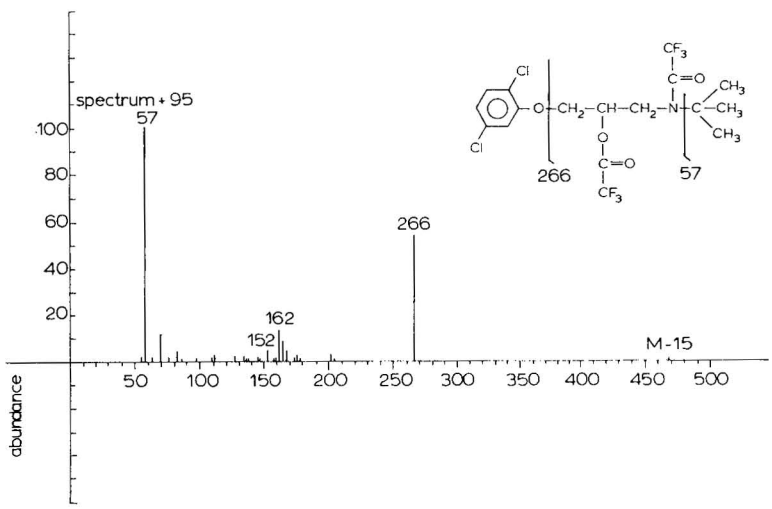


Fig. 2. Low-resolution mass spectrum of Tobanum bis-trifluoroacetate. Conditions as in Experimental.

The base peak at m/z 57 (100%) corresponds to the *tert.*-butyl group. The intense fragment ion at m/z 266 is characteristic of aryloxy β-blocking drugs[9].

For the quantitative determination of Tobanum the ion at m/z 266 was monitored. Typical single-ion recordings of plasma extracts are shown in Fig. 3.

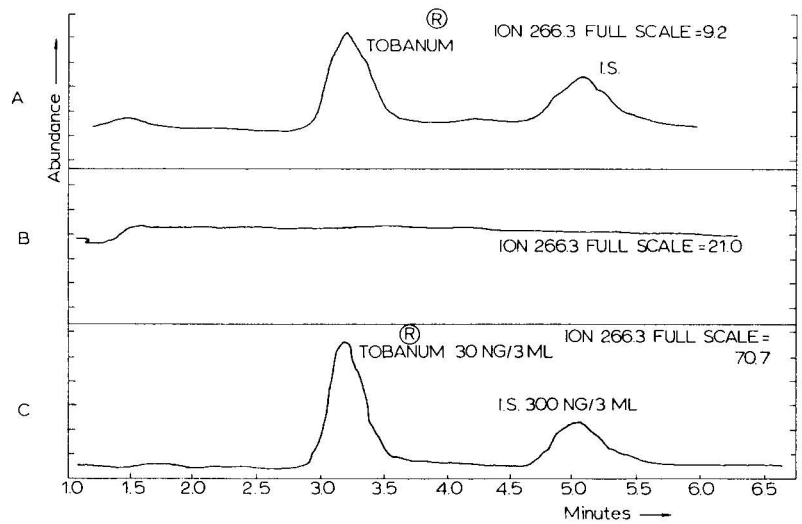


Fig. 3. Single-ion (m/z 266) recordings of plasma extracts at pH 12. Conditions as in Experimental. Curves: A = plasma from a patient treated with Tobanum; B = blank plasma; C = blank plasma spiked with known quantities of Tobanum and propranolol.

The methods described above have been applied to the determination of Tobanum levels in human plasma. Fig. 4 shows the plasma Tobanum levels in patients administered orally with 3 × 5 mg of Tobanum in tablets. The plasma levels were determined over a period of 24 h. Tobanum was still detectable (5 ng/ml) 24 h following administration. The calculated plasma half-life, $t_{\frac{1}{2}}$, was 6 h. The kinetic parameters calculated from the concentration in plasma were: $k_1 = 0.5577\ h^{-1}$

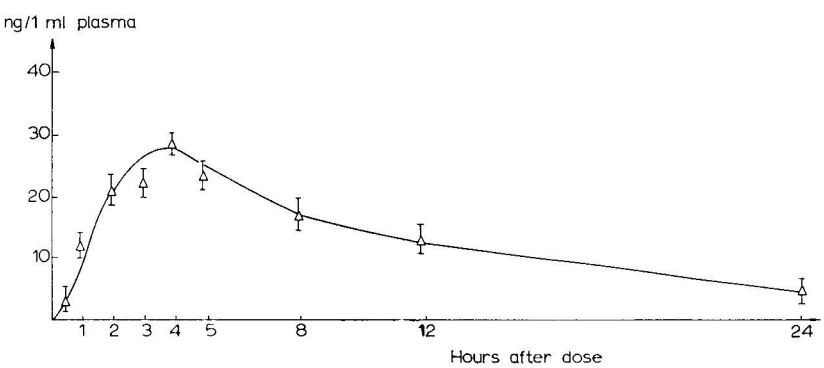


Fig. 4. Curve of Tobanum blood level *vs.* time in ten patients receiving 3 × 5 mg orally. Conditions as in Experimental.

(invasion constant); $k_2 = 0.0845\ h^{-1}$ (elimination constant). The value of k_2 shows that the elimination of the agent from the plasma is very slow. A concentration maximum of 28 ng/ml was observed in the fourth hour following administration. The absorption from the gastrointestinal tract was very rapid, resulting in high and lasting serum levels. The plasma levels obtained by GLC (Fig. 4) showed good agreement with results obtained by GLC–MS. This means that interfering compounds were not likely to be present in the samples.

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RAPID ISOTHERMAL GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF TRICYCLIC ANTIDEPRESSANTS IN SERUM WITH USE OF A NITROGEN-SELECTIVE DETECTOR

JAN ERIK BREDESEN* and ODD FINN ELLINGSEN

Division of Clinical Pharmacology and Toxicology, The Central Laboratory, Ullevål Hospital, Oslo (Norway)

and

JAN KARLSEN

Institute of Pharmacy, University of Oslo, Oslo (Norway)

SUMMARY

A rapid and simple gas–liquid chromatographic method suitable for routine use is described for quantitation of tricyclic antidepressants. The method can be used for quantitation of amitriptyline, nortriptyline, imipramine, desmethylimipramine, doxepine, desmethyldoxepine, clomipramine, desmethylclomipramine, trimipramine, desmethyltrimipramine and dibenzepine. The method involves one extraction with hexane–isopropyl alcohol, no derivative formation of the metabolites and chromatography using an OV-25 liquid phase which permits isothermal quantitation of the different drugs within 5–10 min. Standard curves for the different tricyclic antidepressants were linear over the concentration range tested (10–1500 μg/l) and the maximum coefficient of variation was 7.7%.

INTRODUCTION

Tricyclic antidepressants are widely used in treatment of mental disorders and routine monitoring of plasma concentration should provide useful clinical information. Plasma concentrations in patients treated with tricyclic antidepressants may be as low as 10–20 ng/ml while the concentration after an overdose with tricyclic antidepressants can reach several milligrams per litre[1]. During the past decade numerous methods haven been described for routine determination of plasma levels of tricyclic antidepressants[2]. Previously reported methods include spectrophotometry[3], fluorimetry[4], radioimmunoassay[5], thin-layer chromatography (TLC)[6], mass spectrometric procedure[7], isotope-derivative dilution technique[8], high-performance liquid chromatography (HPLC)[9,10] and gas–liquid chromatography (GLC) using electron-capture detection[11], flame ionization detection[12] or nitrogen-selective detection[13,14]. Nevertheless, the routine determination of tricyclic antidepressants still remains difficult. The spectrophotometric, fluorimetric, radioimmunological and TLC procedures have too low sensitivity, are non-specific or time-consuming. The major disadvantage of the

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mass spectrometric technique is the high cost of the equipment, and probably this technique will be available to only a few in the near future. Previously reported HPLC methods are often time-consuming and lack adequate sensitivity. However, the methods described by Vandermark *et al.*[9] and Reece *et al.*[10] seem to offer appreciable sensitivity. The sample pretreatment is fairly rapid, and these procedures may be an alternative to GLC with a nitrogen-selective detector. The GLC methods reported previously are more or less suitable for routine purposes. Procedures using an electron-capture detector are sensitive, and have acceptable specificity, precision and accuracy, but give no information on the *tert.*-amine concentration[2]. In procedures with flame ionization detectors a time-consuming purifaction step is necessary and the sensitivity of *ca.* 20 μg/l may be insufficient[1]. Recently, GLC with nitrogen-selective detectors has been recommended. These detectors combine sensitivity, specificity and flexibility. Nevertheless, previously reported GLC procedures employing a nitrogen-selective detector still lack routine reliability. Some use derivatization of the secondary amines, and most include extensive cleaning of the glassware, as well as time-consuming purification of the plasma[2,13,14]. The tendency of the drugs to be adsorbed on to surfaces complicates extraction procedures at low concentrations, and contamination occurs easily[12].

In our laboratory the work-load and the high turnover rate of the technicians made it necessary to develop methodological techniques that were as simple and rapid as possible, within the limits set by quality and reliability. These criteria are met in the present method, which includes a simple cleaning and deactivation procedure of the glassware, one extraction step, no derivative formation of the demethylated metabolites, and simultaneous isothermal quantitation of the different drugs and their respective demethylated metabolites within 5–10 min.

MATERIAL AND METHODS

Reagents

All reagents were analytical-reagent grade, and aqueous solutions were prepared using glass-distilled water. The extracting solvents, hexane and isopropyl alcohol, were obtained from Merck (Darmstadt, G.F.R.). The tricyclic antidepressants were supplied by the following companies: clomipramine, desmethylclomipramine, imipramine and desimipramine by Ciba-Geigy (Basel, Switzerland); amitriptyline and nortriptyline by Lundbeck (Copenhagen, Denmark); doxepine and desmethyldoxepine by Pfizer (New York, NY, U.S.A.); trimipramine and desmethyltrimipramine by Rhone-Poulenc (Birkeröd, Denmark); and dibenzepine by NOVO (Copenhagen, Denmark).

Preparation of standard solutions

An amount of each drug equivalent to 100 mg of base was dissolved in HCl (0.01 mol/l) to give 100 ml of stock solution. Known amounts of each drug and metabolite were added in increasing concentrations to plasma. Standard curves from 10 to 1500 μg/l were prepared. Promazine was used as internal standard: 50 μg/l in HCl (0.01 mol/l).

Glassware

All glassware used in the procedure was rinsed in acetone in an ultrasonic bath for 20 min, then soaked in triethylamine–methanol (5:95) and dried in air.

Apparatus

A gas chromatograph equipped with a nitrogen–phosphorus-selective detector was used (Sigma 4, Perkin-Elmer, Norwalk, CT, U.S.A.). The glass column (1800 × 2 mm I.D.) was packed with 3% OV-25 on Gas-Chrom Q (100–120 mesh) (Applied Science Labs., State College, PA, U.S.A.). The operating conditions were: nitrogen flow-rate, 15 ml/min; hydrogen flow-rate, 2.0 ml/min; air flow-rate, 70 ml/min; column temperature, 245°C; detection temperature, 275°C; attenuation, 128–1024. Extractions were carried out with the aid of a horizontal mixer, Desaga type EM 4.

Extraction procedure

Blood samples were collected in Venoject, evacuated blood-collection tubes, red stopper (no additive) or green stopper (containing 142 U.S.P. units of sodium heparin) (Terumo, Tokyo, Japan). A 0.1-ml volume of the internal standard solution, 0.1 ml of NaOH (5 mol/l), and 10 ml of hexane–isopropyl alcohol (98.3:1.7) were added to 2 ml of serum in a centrifuge tube. The sample was extracted for 20 min on a horizontal shaker and centrifuged for 10 min at 400 *g*. The organic layer was transferred to a clean centrifuge tube containing 1 ml of methanol and evaporated to dryness at 60°C under a stream of nitrogen. The residue was dissolved in 20 μl of ethanol and 3 μl were injected for analysis. The peak-height ratio of drug to the internal standard was calculated for each sample, and the concentration of the drug was read from a standard curve constructed by plotting the peak-height ratios of drug and internal standard for known standards against the concentrations.

RESULTS

A typical chromatogram obtained from a sample containing amitriptyline, nortriptyline and internal standard is shown in Fig. 1. Sera containing other tricyclic antidepressants gave corresponding chromatograms. Blank sera assayed by this procedure gave no peaks that might interfere with the analysis. The retention times, in minutes, relative to amitriptyline for the tricyclic antidepressants studied and for caffeine on four different packing materials, are given in Table I.

The linearity of the method was tested on the basis of extracted samples in the concentration range 10–1500 μg/l for each drug. A linear relationship ($r = 0.99$) was observed for each drug over the range studied. The results of the analytical recoveries and reproducibility of the method are summarized in Table II. To test the analytical recoveries of the extraction procedure, known amounts of the drugs were added to blank serum samples, and the extraction procedure was performed without the internal standard, which was added immediately before the injection into the gas chromatograph. The analytical recoveries were constant over the concentration range tested. The sensitivity of the method, calculated from the minimum detectable amount of twice the noise level under normal operating conditions, was 1 μg/l for each drug.

The extractability for lipid components from plasma was influenced by isopropyl alcohol in the extracting solvent. The influence is shown in Table III. The cholesterol and triglyceride contents in the plasma extracted were 8.2 and 5.0 mmol/l.

We screened 33 commonly used basic drugs for interference. The drugs tested, but showing no interference, were amfetamine, antipyrine, caffeine, chlorpromazine, clemazine, codeine, chlorprotixen, dexchlorpheniramine, dextropropoxyphene, diaze-

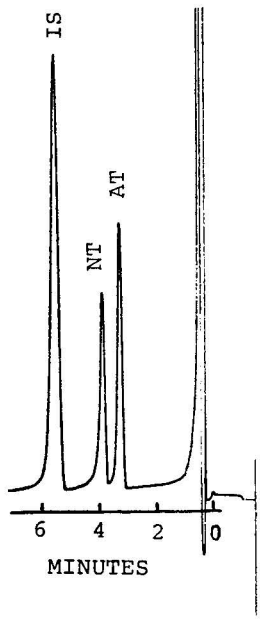


Fig. 1. Gas chromatogram of a serum sample containing 110 μg/l of amitriptyline (AT), 125 μg/l of nortriptyline (NT) and promazine as internal standard (IS).

TABLE I

RETENTION TIMES (min), RELATIVE TO AMITRIPTYLINE, FOR TRICYCLIC ANTIDEPRESSANTS AND CAFFEINE ON VARIOUS LIQUID PHASES

Drug	*3% OV-17*	*3% OV-25*	*3% OV-225*	*1.5% Carbowax 20M + 1.5% KOH*
Caffeine	1.9	2.0	3.5	4.6
Amitriptyline	3.4	3.4	3.4	3.4
Nortriptyline	3.9	4.0	4.6	4.6
Imipramine	3.7	3.7	4.0	3.8
Desmethylimipramine	4.3	4.4	5.4	5.3
Trimipramine	3.5	3.4	3.4	3.2
Desmethyltrimipramine	4.2	4.2	5.0	4.6
Doxepine	3.9	4.0	4.3	4.6
Desmethyldoxepine	4.6	4.8	5.9	7.0
Clomipramine	6.5	6.0	7.4	7.4
Desmethylclomipramine	7.8	7.7	10.5	11.4
Dibenzepine	10.2	10.3	18.9	18.7

pam, diphenhydramine, disopyramide, ephedrine, etenzamid, flunitrazepam, hydralazine, levomepromazine, lidocaine, medazepam, metaqualone, methadone, methylphenidate, morphine, neostigmine, orphenadrine, oxazepam, pethidine, physiostigmine, procainamide, prochlorperazine, promazine, pyridostigmine and quinidine. Nor was any interference observed from more acidic drugs such as barbiturates, carbamazepine, glutethimide, meprobamate, paracetamol, primidone, salicylate and theophylline.

TABLE II

ANALYTICAL RECOVERY AND REPRODUCIBILITY (n = 20) OF ANALYSES OF ELEVEN DRUGS

Drug	*Recovery 300 μg/l (%)*	*Reproducibility: CV (%)*	
		100 μg/l	*300 μg/l*
Amitriptyline	98	2.1	4.7
Nortriptyline	96	4.9	4.1
Imipramine	99	4.0	2.1
Desmethylimipramine	98	5.0	3.5
Trimipramine	93	4.7	2.2
Desmethyltrimipramine	88	4.2	1.7
Doxepine	100	3.3	3.7
Desmethyldoxepine	90	6.3	7.7
Clomipramine	91	5.0	7.2
Desmethylclomipramine	91	3.3	7.2
Dibenzepine	90	3.0	3.8

TABLE III

EFFECT OF ISOPROPYL ALCOHOL ON LIPID CONTENT IN THE INJECTION SAMPLE

Isopropyl alcohol in hexane (%)	*Cholesterol (mmol/l)*	*Triglycerides (mmol/l)*
1	3.0	0.5
1.5	3.8	1.3
1.7	3.9	1.5
2	4.0	2.8
3	45.0	11.3

DISCUSSION

Most of previously described GLC procedures for quantitation of tricyclic antidepressants need a time-consuming purification step of the samples as well as a time-consuming cleaning and deactivation procedure of glassware[2].

Incomplete deactivation of the glass walls will reduce recovery of the drugs to a great extent. The cleaning and deactivation procedure described is simple and rapid and sufficient to avoid adsorption of the drugs on to the glass walls.

The most characteristic feature of the method described is the single extraction step, which still avoids extraction of impurities and other drugs from plasma. To achieve this it is of vital importance to use a nitrogen-selective detector. A flame ionization detector necessitates a time-consuming purification of the extract. On the hand, when working with a nitrogen-selective detector and a single extraction, it becomes crucial to avoid interfering substances such as plasticizers and other contaminants originating from rubber stoppers, tubes and additional equipment used for sampling and analytical procedures. The sampling tubes used gave no interfering peaks.

Most of the previously described procedures for quantitation of tricyclic antidepressants use n-hexane or n-heptane as extracting solvents[2]. During solvent

evaporation, the temperature should not exceed 60°C because that will increase adsorption on to the glass walls. For this reason *n*-hexane may be preferable, because it is more easily evaporated at 60°C. Another problem with the use of *n*-hexane or *n*-heptane as extracting solvent, is that samples high in lipid content easily become emulsified or gelatinated during extraction. Samples high in lipid content are common, and an extraction procedure where such samples become emulsified or gelatinated is impracticable. The problem can be overcome by modifying the polarity of *n*-hexane with isopropyl alcohol. However, isopropyl alcohol in the extracting solvent will increase the lipid content in the extract. As shown in Table III, an increase in the alcohol content from 1.7% to 3% will increase the lipid content in the extract *ca.* ten-fold and this again will interfere with the chromatographic determination. A content of 1.7% of isopropyl alcohol in the extracting solvent is sufficient to avoid emulsification or gel formation in all samples extracted.

Different packing materials can be used for chromatography[2]. As seen in Table I, a more polar liquid phase will improve the separation between the tertiary amines and their demethylated metabolites as well as the separation between the different drugs. However, a higher polarity of the packing materials will, at the same time, give a longer retention time of caffeine compared with the retention time of the different tricyclic antidepressant drugs. Table I shows that caffeine will interfere with the analysis of amitriptyline on OV-225 and with imipramine on Carbowax 20M. Caffeine in blood samples is common and should be considered as a normal component of the blood. Of the packing materials tried, OV-25 gave the best separation of the different drugs without giving interference from caffeine. Unfortunately, neither OV-25 nor any other packing material we have tried can separate all different tricyclic antidepressants and the demethylated metabolites. On OV-25 doxepine and nortriptyline have the same retention time. This interference causes minimal problems for therapeutic drug monitoring, because different tricyclic drugs should rarely be administered together. For toxicological diagnosis this may become a problem. However, if the retention times of both the parent drug and the demethylated metabolite are used, the drug taken should, as a rule, be identified.

The extractability, sensitivity and reproducibility are as good as or better than methods described earlier[2], and the present method is quicker. The method is convenient for therapy control and toxicological diagnosis as well as for pharmacokinetic studies. Scoggins *et al.*[2] state that enzyme immunoassay will be the method of choice for routine analysis of tricyclic antidepressants in the future. For therapy control and toxicological diagnosis, there is a need both to identify different drugs and to monitor the concentrations. A complete analytical system based on immuno-assay has to include may be as many as twenty different antisera. Immunoassay can make a valuable contribution for specific purposes, but a complete analytical system still has to be based on GLC or HPLC. Accordingly, a comprehensive comparative study of the recent method with an appropriate liquid chromatographic procedure would be of the greatest relevance for the future choice of methodology.

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APPLICATIONS IN THE ANALYSIS OF FOODS, FLAVORS AND FRAGRANCES

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QUANTITATIVE ANALYSIS OF RESIDUAL SOLVENTS IN FOOD PACKAGING PRINTED FILMS BY CAPILLARY GAS CHROMATOGRAPHY WITH MULTIPLE HEADSPACE EXTRACTION

B. KOLB*, P. POSPISIL and M. AUER
Bodenseewerk Perkin-Elmer & Co. GmbH, D-7770 Überlingen (G.F.R.)

SUMMARY

A procedure for stepwise gas extraction at equal time intervals has been developed, which is called "multiple headspace extraction" (MHE), because it uses the same equipment as for a standard headspace analysis. The method is suitable as a calibration method for the headspace analysis of volatile trace compounds in solid samples which otherwise cannot be analysed quantitatively. A BASIC program has been used to perform the necessary calculations for quantitative determinations.

The application of the MHE procedure is demonstrated on the example of the analysis of residual solvents in printed films which are used as food packaging material, and for the determination of vinyl chloride monomer in a PVC resin. Particularly for the solvent analysis a good glass capillary column is required to separate the complex mixtures which are used for printing the films.

INTRODUCTION

Volatile compounds in solid samples can easily be determined by headspace gas chromatography (GC), but any attempt to quantitate the results meets with the problem of how to prepare calibration standards. It is difficult, if not impossible, to add trace amounts of volatile compounds to a solid material. Such problems are known where residual solvents have to be determined quantitatively in printed plastic or aluminium films which are used as food packaging materials. In any case, headspace analysis needs an equilibrium based on the partition of the volatiles between the sample and the surrounding gas phase in a closed sample vial. The prevailing partition coefficient of each compound is included in the calibration factor, together with the detector response, the sample volume and other instrumental parameters[1]. This calibration factor is always determined experimentally under the conditions of analysis, and it is obvious that the calibration sample must therefore reproduce exactly the prevailing equilibrium system. If, for practical reasons, such a calibration standard cannot be prepared, the method of headspace analysis can be used for quantitative determinations only if the sample is analysed independently by a different method and is used further as a calibration standard for headspace analysis.

A stepwise gas extraction procedure, as has been used by Suzuki *et al.*[2] for the

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determination of occluded solvents in adhesive tape seems to be attractive for this purpose, as a few extractions are sufficient to be able to calculate, from the exponential decrease in the concentration of each solvent in the resulting gas samples, its original concentration in the solid sample. Kolb and Pospisil[3,4] determined volatiles such as monomers, solvents, ethylene oxide and water in solid plastic materials by a similar approach and even aqueous samples have been analysed by McAuliffe[5,6] and by Ioffe and Vitenberg[7] by such a stepwise gas extraction method.

Such a stepwise extraction procedure is usually too time consuming, even if only a few extractions are carried out, to be applied for routine analysis, where a high sample throughput is required, particularly if the printing process of such films must be controlled. Automated headspace analysis is apparently the method of choice, but a stepwise gas extraction procedure, if used for calibration purposes only, can take more time and is not necessarily carried out automatically. Such a combinations eems to be ideal, therefore, provided that both methods can be performed with the same equipment, particularly using the same vials. However, this meets with the following problem. The various applications of a stepwise gas extraction as cited above use a heated tube or chamber as a sample container, from which, after equilibration, the gas phase is completely separated from the sample and transferred on to the column for subsequent GC analysis. Such a complete separation of both phases is not possible with the sampling device for headspace analysis as used here, because only an aliquot is taken from the gas phase and this aliquot is usually not large enough to cause a significant extraction effect. An additional problem arises if capillary columns must be used owing to their small sample capacity, particularly for gas samples. The gas sample which is taken from the sample vial should be as large as possible in order to achieve an effective extraction, but the capillary column requires a small gas sample only for capacity reasons and in order to avoid peak broadening during a long sampling time. We first used a high splitting ratio to combine both requirements, but this was often difficult to achieve with columns of low back-pressure, such as glass capillary columns. High-efficienty glass capillary columns, however, are indispensable for separating solvent mixtures, which are mostly of very complex composition; a typical example is given in Fig. 1. Capillary columns work well with the pneumatic sampling system used here, as has first been shown by Kuck[8] and discussed later more in detail[9,10].

For all of these reasons it has been found necessary to modify the stepwise gas extraction in such a way that the extraction is carried out independently of the analysis. This becomes possible if not the gas extract is used for the analytical determination, but rather the remaining sample, which can be used equally well if the extraction yield is to be determined. It is necessary only to perform the gas extraction between two sample analyses, which in this instance are carried out by the simple headspace technique. The extraction of the sample by a gas also becomes very simple, because with the pneumatic sampling system used here the sample vials are under pressure after each headspace analysis and it is necessary only to vent the pressure to the atmosphere in order to achieve an effective extraction.

Based on these considerations, a procedure of stepwise gas extraction has been developed which we shall call "multiple headspace extraction" (MHE) in order to indicate that it is in fact a repeated headspace analysis with a gas extraction in between, and that it uses the standard headspace sampling system.

This MHE procedure is demonstrated on the determination of residual solvents in printed films which are used as food packaging material. The need for and the possibilities of analysing such samples by headspace GC for their content of solvents and monomers have been discussed by Jeffs[11] and Lüthi[12].

MULTIPLE HEADSPACE EXTRACTION PROCEDURE

The procedure of MHE needs an electropneumatic sampling system as used in a special headspace instrument, the F45 Automatic Headspace Analyzer, as well as in the HS6 Semiautomatic Headspace Injector, an accessory for gas chromatographs of the Sigma series (Perkin-Elmer, Norwalk, CT, U.S.A.). This sampling system has already been described in detail[9,13], but will be discussed briefly again here.

The sample to be analysed is placed in a glass vial which is closed by a septum and secured against pressure by crimping an aluminium cap on it. The vials are thermostated in a heated aluminium block, which is mounted as a turntable in front of the headspace sampling system, which comprises a dosing needle and a solenoid valve in the carrier gas supply line and which is operated from a control unit.

When the dosing needle pierces the septum, the sample vial is first pressurized up to the head pressure of the column. After a short pressurization period, the sample transfer on to the column is performed by stopping the carrier gas supply for a few seconds by means of the solenoid valve, thus causing the pressurized gas in the vial to expand together with all of the volatiles along the needle on to the column. The injection is stopped when the solenoid valve opens the carrier gas supply again and it is important to note that the vials thus remain under pressure after each headspace analysis.

It is possible now to achieve an effective extraction of the sample if the pressure in the vial is released to the atmosphere simply by puncturing the septum manually with a dosing needle from a syringe. If, for example, the internal pressure is 1 bar, half of the gas content in the vial is vented and with higher pressures even more. This extraction is followed by the necessary equilibration time, during which the chromatographic separation is performed, after which the next headspace analysis can be carried out. This procedure can be continued several times and a series of chromatograms are obtained with decreasing peak heights for each solvent peak, as shown in Fig. 1, in which the first two chromatograms from a series of five extraction steps are presented. If this procedure were to be continued experimentally until exhaustive extraction, it would be necessary only to add the peak areas from each component in all of the chromatograms to obtain the total peak area, which corresponds to the total amount or the original concentration of this compound in the sample. This value must, of course, be calibrated, and it is necessary for this purpose to repeat the whole procedure with a calibration sample, which is easily prepared by injecting a few microlitres of the respective compounds into a closed and empty glass vial through the septum. After complete vaporization at the prevailing temperature, the whole procedure is repeated, and again a series of chromatograms are obtained, in which the peak heights usually decrease at a faster rate compared with the decrease in the chromatograms from the analysis. It has been found, as with all other such stepwise extraction techniques, that a straight line is obtained if the logarithm of the peak areas is plotted against the number of extractions[2,3,6,7], and such a plot is shown in

Fig. 2 for three extractions of ethyl acetate from a printed aluminium film by the MHE procedure. This relationship enables the total peak areas from each solvent to be determined from only a few extractions as the sum of a geometric progression. At least two extractions are necessary and have often been found sufficient for practical purposes. Applying a statistical evaluation by linear regression calculation improves the precision of the measurement, but at the expense of an increased number of experimental determinations.

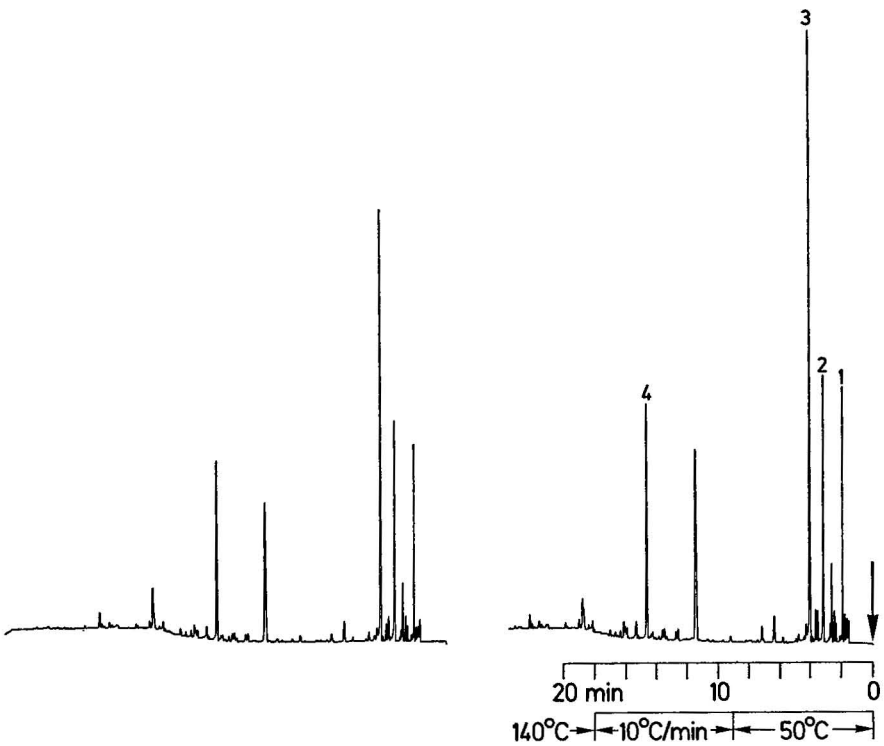


Fig. 1. Analysis of residual solvents in a "sandwich-structured" printed aluminium film by multiple headspace extraction. Instrument, Sigma 2 and HS6 semiautomatic headspace injector (Perkin-Elmer); 35 m × 0.3 mm I.D. glass capillary with Marlophen M87 liquid stationary phase; temperatures, column programmed as indicated, sample 140°C for 45 min; detector, FID, 10 × 16; sample, 50 cm^2. Compounds: 1 = *n*-hexane, 1.8 mg/m^2; 2 = methanol, 9 mg/m^2; 3 = ethanol, 12 mg/m^2; 4 = ethyl glycol, 6 mg/m^2.

RESULTS AND DISCUSSION

A printed aluminum film with ethyl acetate as the main volatile constituent was extracted three times by the MHE procedure. Three peak area values were obtained both from the foil sample (shown in the computer printout in Fig. 2) and from the calibration sample, from which the area total was calculated and is given in Fig. 2. The calibration sample was prepared by injecting 1 μl of ethyl acetate into an empty vial with a 10-μl syringe. The analysis was carried out using the F45/FID automatic headspace analyzer with the Sigma 10 Chromatographic Data System (Perkin-Elmer)

and with a 50 m × 0.25 mm I.D. glass capillary with Carbowax 1000 as the stationary phase. A 150-cm² sample was used and thermostated at 110°C for 50 min.

Fig. 2 gives the result of a linear regression calculation using a BASIC program with the Sigma 10 Chromatographic Data System. A concentration of 1.018 μg/cm² of ethyl acetate in the aluminium film sample was determined.

```
/BR GASEX.1
PROGRAM   57
OK
57 : 1
                    MULTIPLE HEADSPACE EXTRACTION
                    -----------------------------

      CALIBRATION RUNS  Y/N  ?N
      NO. OF EXTRACTION STEPS?3
      COMPOUND NAME           ?ETHYLACETATE

                     PEAK AREA
      1 .EXTRACTION   ?12.8441
      2 .EXTRACTION   ?6.3510
      3 .EXTRACTION   ?3.2955
      CONCENTRATION OF CALIBRATION SAMPLE MG/ML?900.5
      VOLUME OF CALIBRATION SAMPLE  UL          ?1
      SAMPLE WEIGHT(MG) OR SAMPLE AREA(CM2)     ?150,CM2
      PEAK AREA OF CALIBRATION COMPOUND         ?152.247
      PEAK AREA OF BLANK                        ?0
      PEAK AREA FOR ADDITION                    ?0

                -------->LOG PEAK AREA  ETHYLACETATE
      1 -I-----------------------------------------+-----
         I                                              12.8441
         I
         I
         I
      2 -I                          +   6.351
         I
         I
         I
      3 -I      +   3.2955

                      EXTRAPOLATED FINAL AREA    S= 25.82
         CONCENTRATION MG/M2 OF ETHYLACETATE     C= 10.1812

57 : 1  1000 H 100
```

Fig. 2. Print-out from the Sigma 10 chromatographic data station for the analysis of ethyl acetate in a printed aluminium film by multiple headspace extraction with three extraction steps. Concentration of ethyl acetate in this 150-cm² sample = 1.018 μg/cm².

From this result, it is now possible to derive a correction factor that can be applied to further headspace analyses of similar samples. This correction factor is obtained from the first peak area with 12.8441 units and the corresponding ethyl acetate concentration of 1.018 μg/cm²; the resulting value of 0.7926 can be handled

as a response factor (RF) for further data processing by using external standard calibration.

This method was then applied to the analysis of three more samples, which were cut out in equal sizes from the same roll of printed film. These three samples were analysed by the standard headspace procedure but using the correction factor, and a mean value for the ethyl acetate concentration of 0.92 $\mu g/cm^2$ was determined with a relative standard deviation of 11%. Despite the fact that this precision is an order of magnitude worse than the precision of the measurement, it is sufficiently good for the practical purpose of this analysis, and is caused mainly by the inhomogeneous distribution of the ethyl acetate across the film areas of these three samples. Sample homogeneity is the main problem with solid samples, particularly if the reproducibility has to be determined, and this situation is different from that with liquid samples.

The accuracy which is obtainable by the MHE procedure cannot be determined with samples such as printed films, because there is no alternative method available for comparing the results. The MHE procedure was therefore applied to the determination of vinyl chloride monomer (VCM) in a PVC resin, because this sample offered the possibility of analysis by a different approach with no calibration problems according to the method of Puschmann[14] by headspace analysis of a solution, and a concentration of 1.28 ppm (w/w) of VCM was found. Four further samples from the same batch of PVC resin were analysed by the MHE procedure and a concentration of 1.35 ppm (w/w) of VCM was determined, with a relative standard deviation of 3.4%, thus showing much better sample homogeneity in this instance and fairly good agreement between the results of the two methods.

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ÉTUDE PAR CHROMATOGRAPHIE GAZEUSE DE LA VARIABILITÉ DES ACIDES GRAS DES GRAINES DE MIL

M. PANSU*, S. TOSTAIN et M. PINTA
Laboratoire de Spectrographie, Orstom, 70–74 Route d'Aulnay, 93140 Bondy (France)

SUMMARY

Gas chromatographic study of variations in the amounts of fatty acids in millet seeds

To investigate new millet hybrids more accurate methods for the analysis of seed components are needed. For example, the study of small variations in amounts of fatty acids requires a better knowledge of sources of errors in their quantitative chromatographic determination. The results of the extraction–transmethylation method used have been verified by saponification and methylation. The resulting variability is not higher than that due to chromatographic determination (0.5%). Precautions to avoid some unexpected errors have been taken. The multivariate analysis of the results, seed by seed, makes it possible to collect those from the same ear. Sterols determined in these seeds cannot be used as biochemical tracers, since their quantities are too low and not sufficiently variable.

INTRODUCTION

La teneur en huile des graines de mil a été estimée à 4–5%. Elle est similaire et même légèrement supérieure (surtout pour l'acide palmitique) à celles du maïs, sorgho et autres céréales[3]. Si la composition en acides gras de cette huile a été plusieurs fois déterminée, Jellum et Powell[1] ont été les premiers à étudier les variations de composition pour 65 lignées de mil cultivées dans les mêmes conditions. Ils observent des variations importantes entre ces lignées. Les corrélations entre acides sont, cependant, moins fortes que pour le maïs. L'acide stéarique varie le plus. Entre les acides oléïques et linoléïques, les coefficients de corrélation (négatifs) sont les plus importants. Pour une variété donnée, la taille des graines semble introduire des différences significatives dans la composition, notamment pour les acides oléïques et linoléniques. La teneur de ce dernier croit en raison inverse de cette taille.

L'état de mûrissement des graines joue un rôle important dans la composition. Les changements les plus rapides et les plus importants se produisent dans les seize jours après l'anthèse. Ces changements sont plus faibles après 24 jours. Les fractions d'acides palmitiques et linoléniques décroissent avec le mûrissement tandis que les acides stéariques et linoléïques augmentent. La composition varie peu dans les graines mûres, quelle que soit la date de la récolte; les températures durant la maturation n'ont pas d'influence sur la composition de l'huile de ces graines.

Les résultats d'analyses sont affectés par le choix du solvent d'extraction. Les

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solvants apolaires extraient surtout les triglycérides (65 à 80% chez l'avoine), alors que les solvants polaires extraient les lipides polaires liés à l'amidon de l'endosperme.

Notre but est de préciser la fiabilité d'une méthode d'analyse totale des acides gras sur des graines mûres. En effet, l'interprétation des liaisons stochastiques pouvant exister entre les compositions de différentes souches, ne peut avoir de valeur que si l'on est assuré de la totalité de l'extraction et si la variabilité de cette dernière, ainsi que du dosage, sont faibles devant la variabilité des concentrations des divers échantillons.

Cette étude peut donc se diviser en quatre parties.

(1) Choix au laboratoire de la méthode d'extraction et transestérification avec contrôle de la totalité de l'extraction par une saponification suivie d'une méthylation des produits saponifiés.

(2) Étude sur une farine homogène de la variabilité des opérations d'extraction et transestérification, et de la variabilité propre à l'analyse chromatographique; comparaison des variabilités des concentrations relatives et des teneurs absolues lorsque l'on utilise un étalon interne.

(3) Étude de la variabilité entre farines de quelques souches différentes.

(4) Étude de la variabilité entre graines d'une même chandelle, entre graines de plusieurs chandelles d'une même souche, et comparaison avec les graines d'une autre souche.

PARTIE EXPÉRIMENTALE

Extraction et transméthylation

Les graines sont pesées et broyées dans un mortier en pyrex à piston rodé, type VSC, puis transférées dans des fioles Sovirel de 25 ml, à bouchage à vis. Lors des analyses sur une seule graine pesant 10 à 25 mg, il a été vérifié que les pertes, en poids, n'excèdent pas 5% dans ces opérations. Pour une bonne extraction des lipides polaires et apolaires, nous avons choisi le mélange 2 ml méthanol–2 ml éther de pétrole. L'acide heptadécanoïque utilisé comme étalon interne est dissous dans l'éther de pétrole d'extraction à raison de 1 mg/ml pour les extractions sur 200 mg de farine, ou 0.1 mg/ml pour les extractions sur une seule graine. Après extraction une nuit, la transméthylation est réalisée selon une technique analogue à celle de Jellum et Powell[1], 70°C pendant 5 h, avec du méthanol anhydre à 3% d'acide sulfurique. Après refroidissement et adjonction d'eau, les esters en solution dans l'éther de pétrole sont séchés et recueillis dans des tubes gradués cylindroconiques à bouchage à vis. Ces tubes, fabriqués selon nos plans, permettent, selon les prises d'essai, de concentrer les solutions sous courant d'azote au volume désiré pour ne pas modifier les conditions chromatographiques. Les extraits peuvent être conservés à -10°C ou injectés directement dans le chromatographe.

Contrôle de la totalité de l'extraction

On effectue une saponification à reflux sur les farines avec de la potasse méthanolique 2 *N* pendant 2 h. Le mélange réactionnel est amené ensuite en milieu potasse 0.1 *N* dans le méthanol à 50% et agité avec de l'éther de pétrole. La phase éther de pétrole contient les insaponifiables. Elle est concentrée pour un dosage des stérols aprés formation des dérivés triméthylsylés. La phase hydro-alcoolique est

acidifiée et les acides gras sont extraits à l'éther éthylique. Après évaporation de l'éther, la méthylation est effectuée comme dans la première méthode.

Conditions chromatographiques

Le dosage des stérols est effectué avec une colonne pyrex de 2.10 m à 300°C, remplie de OV-1 à 3% sur Chromosorb W AW HMDS.

Pour les acides gras, diverses colonnes ont été expérimentées (DEGS sur Chromosorb W, Silar 10C sur Volaspher A2 100–120 mesh) et nous avons obtenu, finalement, les meilleurs résultats avec une colonne inox de 3 m × 2 mm, remplie de 20% BDS + 2% H_3PO_4 sur Chromosorb W (80–100 mesh). Cette colonne donne une bonne séparation de tous les acides gras ainsi qu'une plage de séparation entre l'acide palmitoléïque et l'acide stéarique permettant d'utiliser l'étalon interne (acide heptadécanoïque), sans interférence avec les autres acides. En outre, le logarithme des distances de rétention des acides gras saturés est rigoureusement proportionnel à leur nombre d'atomes de carbone.

Le chromatographe utilisé est un Girdel 300 avec détection par ionisation de flamme air–hydrogène. Le volume injecté est 1 μl, la sensibilité 64 × 1. Les températures sont respectivement 200°C pour la colonne, 270°C pour l'injecteur et 250°C pour le détecteur, et la pression d'entrée de l'azote gaz vecteur 2 bars.

RÉSULTATS

Intervalles de confiance des resultats d'analyse

L'analyse des stérols sur six variétés fixées montre des teneurs faibles et peu variables pour ces composés. On observe deux pics de faible amplitude. Celui de temps de rétention le plus élevé correspond au β-sitostérol à une teneur d'environ 0.06%. L'autre pic peut être du stigmastérol ou du campostérol à une teneur d'environ 0.02%.

L'analyse des acides gras, après saponification, fournit des chromatogrammes analogues à ceux obtenus par l'extraction au mélange méthanol–éther de pétrole et transméthylation. Cette dernière méthode est donc satisfaisante pour l'analyse totale dans les graines et nous avons étudié la variabilité due à l'échantillonnage et au dosage.

Nous avons attaqué, pour celà, un nombre de 12 échantillons (p) de 200 mg d'une farine homogène et effectué sur le produit méthylé 4 injections (n) par échantillon dans le chromatographe, soit 48 injections (pn). Toutes les injections ont été faites au hasard pour éliminer les effets de dérive éventuelle de l'appareil.

Soit pour un acide gras donné: x_{np}, le résultat de chaque injection; $\bar{x}_p$, les moyennes de chaque échantillon; $\bar{x}$, la moyenne globale; σ_2^2, la variance due aux injections; σ_1^2, la variance due à l'échantillonnage.

La variance de l'échantillonnage est estimée par $S_1^2 = n\sum_p (\bar{x}_p - \bar{x})^2/(p-1)$ qui est une estimation de $\sigma_2^2 + n\,\sigma_1^2$. La variance due au dosage par $S_2^2 = \sum_p \sum_n (x_{np} - \bar{x}_p)^2/(np-p)$ qui est une estimation de σ_2^2. La variance globale par $S_t^2 = \sum_{np} (x_{np} - \bar{x})^2/(np-1)$.

Le rapport $F = S_1^2/S_2^2$ permet de définir l'influence de l'échantillonnage. Les résultats sont donnés dans le Tableau I. Chaque ligne de ce tableau comprend deux séries de résultats. Les résultats du haut sont calculés pour les douze échantillons et

TABLEAU I

ETUDE DE LA VARIANCE DES RÉSULTATS

C.R. = Concentration rélative; C.T. = concentration totale.

	Acide											
	$C_{16:0}$		$C_{16:1}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$	
	C.R. (%)	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*
$\bar{x}$	19.70	0.977	0.56	0.028	5.48	0.272	25.60	1.270	44.72	2.222	2.57	0.128
	19.68	0.981			5.46	0.272	25.56	1.274	44.84	2.235	2.58	0.129
F	4.1	42.7	0.6	1.2	4.1	7.4	10	56.6	4.1	73.3	1.8	6.5
	3.9	20.6			2.0	7.3	3.3	40.7	4.8	35.2	1.0	2.6
σ_1^2	0.010				0.003		0.020	0.001	0.16	0.004	0.001	
	0.008				0.001		0.005		0.023	0.002		
σ_2^2	0.013		0.001		0.004		0.009		0.20		0.005	0.005
	0.012				0.004		0.009		0.024			
σ_t^2	0.022		0.001		0.007		0.027	0.001	0.35	0.004	0.006	
	0.019				0.005		0.014		0.045	0.002	0.005	
$\frac{\sigma_1}{\bar{x}}$ %	0.51	1.8			1.1	1.5	0.55	1.8	0.9	2.7	1.2	3.1
	0.46	1.3			0.5	1.5	0.27	1.4	0.33	1.8		1.15
$\frac{\sigma_2}{\bar{x}}$ %	0.56	0.61	4.3		1.1	1.1	0.35	0.47	1.0	0.6	2.7	3.1
	0.56	0.61			1.1	1.1	0.35	0.47	0.33	0.6		1.5
$\frac{\sigma_t}{\bar{x}}$ %	0.76	1.9	4.3	3.6	1.5	1.8	0.66	1.8	1.3	2.7	3.1	4.7
	0.71	1.4			1.3	1.8	0.47	1.4	0.47	1.8	2.7	3.1

les résultats du bas, après élimination d'un échantillon défectueux (fuite d'un bouchon lors des transméthylations). En ce qui concerne les concentrations relatives, les rapports F trouvés sont peu différents de ceux donnés par les tables de Snédécor au niveau de confiance 99% ($F_{11.36} = 2.8$). La variation due à l'échantillonnage est donc du même ordre que celle due aux injections et la variance globale est approximativement la somme des variances. La variance de l'échantillonnage devient toujours la plus faible après élimination de l'échantillon défectueux dont la cause d'erreur est toujours détectable. Par contre, pour les concentrations totales, cette variance est toujours forte devant celle des injections. L'élimination de l'échantillon défectueux dans ce cas provoque une diminution de l'erreur d'échantillonnage moins importante que précédemment. Celle-ci reste toujours analogue à l'erreur globale tandis que les variations entre injections sont beaucoup plus faibles et analogues aux variations précédentes. Ces considérations montrent que l'erreur d'échantillonnage, dans l'analyse des teneurs totales, semble provenir exclusivement des variations, entre échantillons, du pic étalon interne. Ce dernier a été prélevé dans la même solution-mère, avec une même pipette de 2 ml au 1/20ème pour chaque échantillon. Cependant, l'erreur due au pipetage semble prépondérante par rapport à l'ensemble des autres erreurs.

A partir des résultats précédents, les coefficients de variation, de part et d'autre des moyennes, sont calculés au niveau de confiance 95% par $\sigma t_{0.975}/\bar{x}\sqrt{m}$ afin de choisir le nombre d'attaques p et de dosages n nécessaires pour la précision désirée

ultérieurement. On indique dans le Tableau II la valeur la plus grande de la variation: échantillonnage (1), injection (2) ou globale (3). Les valeurs de t des tables de Student sont respectivement 2.228 pour l'échantillonnage, 2.036 pour les injections, 2.021 pour la variation globale, et les valeurs de m respectivement: le nombre d'attaques, le nombre d'injections par attaque, le nombre total d'analyse.

TABLEAU II

ERREUR DE PRÉCISION EN VALEUR RELATIVE

La source de variation la plus grande est indiquée: (1), échantillonage; (2), injection; (3), globale.

p	*n*	*Acide*	$C_{16:0}$		$C_{16:1}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$		$C_{18:3}$		$C_{20:0}$	
			C.R. (%)	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*	*C.R. (%)*	*C.T.*
1	1	1.4	2.9	8.7	7.2	2.5	3.7	0.9	3.1	0.9	4.0	6	6.3	11	12
		(3)	(1)	(3)	(3)	(3)	(3)	(3)	(1)	(3)	(1)	(3)	(2)	(3)	(3)
1	2	1.0	2.9	6.1	5.1	1.8	3.3	0.6	3.1	0.8	4.0	4.3	4.5	8	8.8
		(1)	(1)	(3)	(3)	(3)	(1)	(1)	(1)	(1)		(3)	(2)	(3)	(3)
1	3	1.0	2.9	5.0	4.2	1.4	3.3	0.6	3.1	0.8	4.0	3.5	3.6	6.5	7.2
		(1)	(1)	(3)	(3)	(3)	(1)	(1)	(1)	(1)		(3)	(3)	(3)	(3)
2	2	0.8	2.0	4.2	3.6	1.6	2.3	0.5	2.2	0.5	2.8	3.0	3.6	5.5	6.2
		(2)	(1)	(3)	(3)	(2)	(1)	(2)	(1)	(1)		(3)	(2)	(3)	(3)
2	3	0.8	2.0	3.5	2.9	1.3	2.3	0.4	2.2	0.5	2.8	2.3	3.6	4.6	5.1
		(1)	(1)			(2)	(1)	(1)	(1)	(1)		(3)	(2)		(3)

Avec une attaque et deux injections, la précision sur les teneurs relatives des acides gras les plus concentrés est inférieure à 1 %. Elle est suffisante pour observer des variations entre échantillons qui sont égales ou supérieures à 10 %, avec quelques réserves pour les deux pics les plus faibles: palmitoléïque et arachidique. Les échantillons où se produira une fuite durant l'attaque devront être éliminés.

La mesure des teneurs totales pourra être rendue plus fiable en améliorant la précision du prélèvement de l'étalon interne (seringue, pipette automatique, etc.). La farine de référence, les extraits et les chromatogrammes de l'étude statistique sont conservés pour des contrôles de fidélité. Lors des analyses ultérieures, il pourra être nécessaire de modifier légèrement la pression d'entrée du gaz vecteur pour obtenir des temps de rétention identiques et assurer une bonne reproductibilité des mesures. Pour la même raison, on évitera de changer le volume injecté et l'atténuation. Il est préférable de diluer ou de concentrer les extraits sous courant d'azote.

Variabilité observée entre farines de plusieurs origines fixées génétiquement

Huit variétés fixées cultivées en serre mais dans des conditions différentes ont été analysées. Les teneurs totales varient de 4.8 à 7.6 % du poids de farine. Les écarts extrêmes entre les teneurs relatives sont résumés dans le Tableau III, en comparaison avec Jellum et Powell[1] pour 65 lignées de mil et De la Roche *et al.*[2] pour neuf lignées d'avoine.

TABLEAU III

ÉCARTS EXTRÊMES ENTRE LES TENEURS RELATIVES EN COMPARAISON AVEC CEUX DE JELLUM ET POWELL[1] ET DE LA ROCHE *et al.*[2]

Acides	*Huit lignées de mil (%)*	*Soixante-cinq lignées de mil (%)*	*Neuf lignées d'avoine (%)*
Acide palmitique	19.6–23.4	16.7–25.0	17.2–23.6
Acide palmitoléïque	0.4– 0.8	0.3– 0.8	–
Acide stéarique	2.6– 5.5	1.8– 8.0	0.8– 1.8
Acide oléïque	22.1–32.1	20.2–30.6	26.5–47.5
Acide linoléïque	38.9–48.4	40.3–51.7	33.2–42.2
Acide linolénique	2.2– 4.0	2.3– 5.8	0.9– 2.4

Comme Jellum et Powell, nous observons une forte corrélation négative entre les teneurs relatives des acides oléïque et linoléïque. Pour les autres acides, les corrélations entre ces teneurs ne semblent pas significatives. Une augmentation de la teneur totale en acides gras se traduit par une augmentation de celle des acides palmitique, oléïque, linoléïque et linolénique. Une augmentation de la quantité de chacun de ces trois derniers acides entraine une augmentation de celle de l'acide palmitique. Ces résultats restent à confirmer sur un plus grand nombre de lignées, dans des conditions de culture identiques.

Variabilité observée entre graines d'un même pied mère entre différents pieds mères

Nous avons dosé des graines de quatre faux épis d'origine Malienne. Sur chaque chandelle, seize graines ont été analysées[4]. Trois chandelles (A, B, C) provenaient d'une même souche B 237 et une chandelle d'une autre souche B 257. L'analyse en composantes principales des sept caractères (six acides gras et poids de chaque graine) montre qu'il est possible de regrouper les graines d'un même épis et de séparer des épis d'origine différente (Fig. 1). L'axe 1, de plus grande variabilité, représente la somme des acides gras; les autres axes de variabilités importante sont des combi-

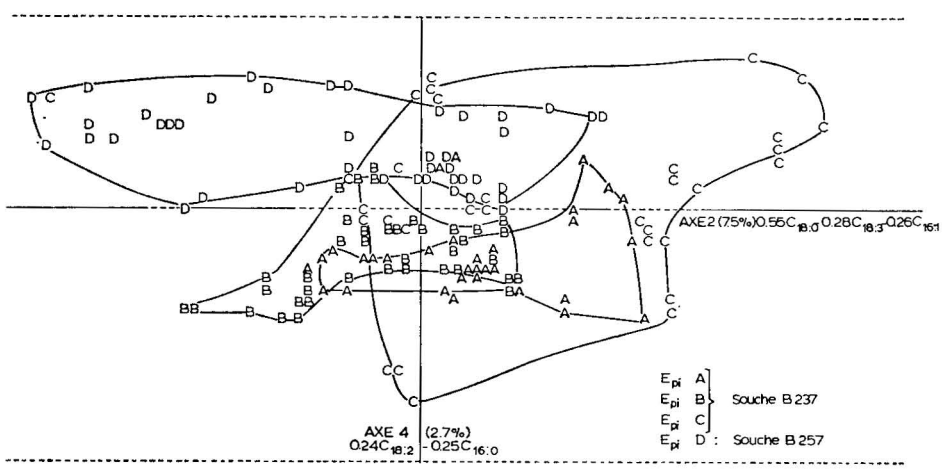


Fig. 1. L'analyse en composantes principales des sept caractères.

naisons des acides gras les moins corrélés. Une augmentation du poids de graine correspond à une diminution de teneurs. Ces résultats intéressants restent à confirmer sur des variétés cultivées dans les mêmes conditions. Des comparaisons entre croisements et parents seraient souhaitables.

CONCLUSION

La chromatographie en phase gazeuse est une méthode précise et reproductible, très utile pour l'observation de variations de faible amplitude comme les teneurs en acides gras des graines de mil. La méthode d'extraction et transméthylation utilisée permet le dosage de la totalité de ces acides. En tenant compte des précautions indiquées, au niveau de confiance 95% avec une seule extraction et deux injections, la précision sur les teneurs relatives des acides gras les plus concentrés est inférieure à 1%; la précision sur les teneurs absolues qui peut être améliorée est inférieure à 4%.

Les extraits et chromatogrammes témoins de l'étude de variabilité permettent d'assurer une bonne fidélité des analyses.

Les premiers résultats de comparaison entre souches, graines et épis sont encourageants, l'analyse multivariable semblant permettre de regrouper les graines d'un épis déterminé.

Les analyses doivent être poursuivies sur des souches fixées, cette fois obtenues en serre, dans les mêmes conditions de culture, et utilisées, par ailleurs, dans d'autres études génétiques. Enfin, l'analyse séparée des acides gras des triglycérides et phospholipides peut permettre d'aller plus loin dans l'étude du caractère, bien qu'il soit difficile d'obtenir une séparation de ces lipides du même ordre de précision que la chromatographie gazeuse.

RÉSUMÉ

La recherche de nouveaux hybrides de mil nécessite une précision accrue des méthodes d'analyse des constituants des graines.

Ainsi, lors de la détermination chromatographique des teneurs en acides gras, l'observation des faibles variations entre les graines nécessite une meilleure connaissance des sources d'erreurs. Les résultats de la méthode d'extraction et transméthylation choisie ont été contrôlés par saponification et méthylation. La variabilité obtenue n'excède pas celle due à la méthode chromatographique (0.5%). Les précautions à prendre pour éviter certaines erreurs accidentelles ont été déterminées.

Avec cette méthode bien calibrée, l'analyse multivariable des résultats, graine par graine, permet de rassembler celles appartenant à un même épi. Les stérols dosés dans ces graines sont en quantités trop faibles et trop peu variables pour servir de marqueurs biochimiques.

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CHROM. 13,229

DETERMINATION OF PHENOLIC PLASTIC ADDITIVES IN EDIBLE OILS BY THIN-LAYER CHROMATOGRAPHY

G. HAESEN* and B. LE GOFF
Joint Research Centre, Petten Establishment, P.O. Box 2, 1755 ZG Petten (The Netherlands)

SUMMARY

An analytical method has been developed for the determination of five sterically hindered phenolic plastic additives in different edible oils. The procedure is based on thin-layer chromatography (TLC), after extraction of the additives from the matrix. The co-extracted oil components are eliminated by the use of pre-treated-silica plates. The additives are revealed according to the procedure developed by Boute and determined quantitatively by a monochromator TLC scanner. The detectable concentration of the individual antioxidants, in 5 g of the oil, ranges from 50 ppb* to 2 ppm, depending on the nature of the additive and the oil. The validity of the proposed method is based on investigations using sesame oil, safflower oil, sunflower oil, corn-germ oil and olive oil.

INTRODUCTION

The plastic packaging of foodstuffs has proven to be most suitable in conservation processes. However, the quality of the packed product may change as a result of the migration of plastic components into the food. In order to understand and control such migration mechanisms, it is necessary to develop accurate and simple methods for the detection of these contaminants in the complex food matrix. Most of the earlier work has been concentrated on the identification of food preservatives[1–6].

The aim of this paper is the analyses of traces of some plastic additives in edible oils. The choice of both the phenolic antioxidants and the oils has to be related to important parameters of the migration mechanism, namely the size of the migrant and the composition of the foodstuff[7,8]. Earlier attempts to apply luminescence spectroscopy to achieve the identification of commercial plastic additives[9] in food simulants, including work in our laboratory[10], met with little success when applied to edible oils.

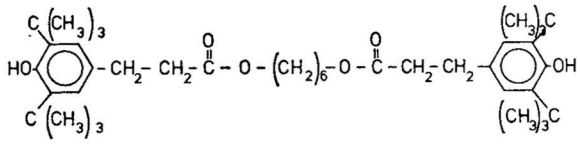
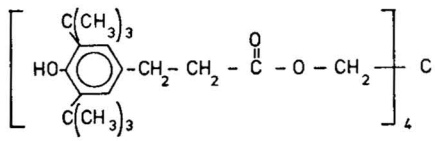
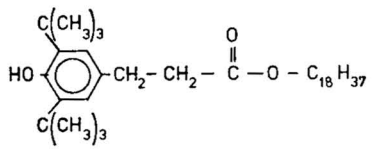
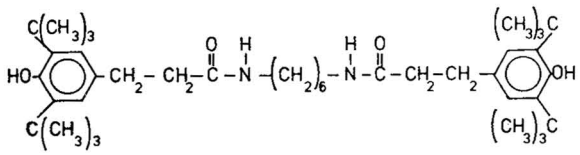
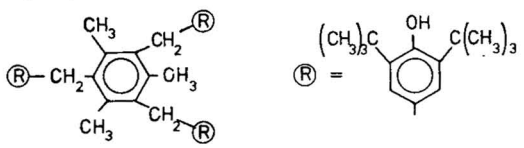
The selected plastic additives are: 1,6-hexanediol bis-[3-(3,5-di-*tert.*-butyl-4-hydroxyphenyl)propionate]; pentaerythrityl tetrakis-[3-(3,5-di-*tert.*-butyl-4-hydroxyphenyl)propionate]; octadecyl 3-(3,5-di-*tert.*-butyl-4-hydroxyphenyl)propionate; N, N′-hexamethylenebis-(3,5-di-*tert.*-butyl-4-hydroxyhydrocinnamamide); 1,3,5-trimethyl-2,4,6-tris-(3,5-di-*tert.*-butyl-4-hydroxybenzyl) benzene, subsequently referred to as antioxidant I, II, III, IV and V, respectively (see Table I).

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

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TABLE I

CHEMICAL STRUCTURES OF THE FIVE ANTIOXIDANTS AND THE SOLVENTS USED FOR EXTRACTION

Antioxidant	*Chemical structure*	*Extractant*
I		Acetonitrile
II		Acetonitrile
III		Dimethylformamide
IV		Methanol
V		Dimethylformamide

EXPERIMENTAL

The thin-layer chromatography (TLC) apparatus comprised an automatic injector and a TLC scanner with monochromator (Camag, Muttenz, Switzerland). The TLC plates used were precoated with SiO_2 HR 25-UV 254 (Machery, Nagel & Co., Düren, G.F.R.). Antioxidants I, II, III, IV and V were obtained from Ciba–Geigy (Basle, Switzerland) and Shell (Amsterdam, The Netherlands), thioglycol acid 2-ethylhexyl ester ("RSH") and the unrefined edible oils were respectively purchased from Fluka (Buchs, Switzerland) and general commercial sources.

Stock solutions of the five antioxidants in chloroform were added to the edible oils. The chloroform was evaporated, and the oils (spiked with the additive) were used for extraction and recovery measurements. Aliquots of the stock solutions and oil extracts were spotted on a TLC plate and treated as described under *Procedures*. The peak area of the reference spot was integrated with a computing integrator (Techmation 306), and the calibration graph was constructed by plotting the peak area (arbitrary counts) of each antioxidant against the concentration. A linear

detector response for each substance was obtained by establishing an absolute calibration curve for sample volumes ranging from 1–25 μl.

Procedures

(A). For the extraction of antioxidants I and II from the different oils, 5 g of the oil was diluted with 20 ml of 2-methylbutane [containing 100 ppm of butylated hydroxytoluene (BHT) and 250 μl of RSH] and extracted with a total volume of 20 ml of acetonitrile (5 ml per extraction). The acetonitrile extract was washed with 5 ml of 2-methylbutane and evaporated to dryness on a rotary evaporator; the residue was dissolved in 1 ml of chloroform. Aliquots of this solution were spotted on a TLC plate that had been sprayed (8 cm) with aqueous 10% $AgNO_3$ solution and dried at 120°C for 5 min. The plate was developed in benzene–chloroform (95:5). After evaporation of the residual solvent and revelation of the antioxidants by the Boute reaction[11], the yellow spot of the nitroso-derivative was scanned at 405 nm for antioxidant II and 436 nm for antioxidant I.

(B). Antioxidants III and V were extracted from the oil by four successive 5-ml portions of dimethylformamide. The extract was diluted with 200 ml of 5% (v/v) aqueous phosphoric acid and then extracted with a total of 60 ml of benzene in four extractions. After evaporation of the benzene, the residue was dissolved in chloroform, and aliquots were spotted on a 3-cm $AgNO_3$-impregnated TLC plate; the plate was developed successively with benzene–chloroform (90:10) and with trichloroethylene over a distance of 5 and 15 cm, respectively. The spot was revealed as described in (A) above.

(C). The extraction of antioxidant IV from 5 g of oil, diluted with 20 ml of *n*-heptane (8% of RSH), was incomplete with four successive extractions with 5 ml of methanol. After evaporation of the solvent, the residue was dissolved in chloroform, and aliquots were spotted on a TLC plate (with a concentration zone), which was developed with dichloromethane–diethyl ether (60:40); spots were visualised by using the Boute reaction and measured at 436 nm.

RESULTS AND DISCUSSION

Extraction

The direct identification of small quantities of antioxidants in edible oils was not feasible, owing to interference by other materials in the oil. The antioxidants were therefore extracted from the matrix prior to identification procedures. Good recoveries of antioxidants I, II and IV were achieved if the initial oil sample was diluted with a non-polar solvent that was not miscible with the extractant. Also, the addition of RSH increased the recovery by a factor of almost 2. The extraction of antioxidant III from safflower and sesame oil was very poor.

Thin-layer chromatography

In order to reveal the antioxidants on the TLC plate, a very sensitive spray reagent was used. Phenolic substances form nitroso derivatives according to a reaction described by Boute[11]. The high sensitivity of this colour reaction (related to the number of phenolic groups on each molecule) makes it possible to detect trace amounts of the five antioxidants. Table II summarises the detection limit of each

TABLE II

DETECTION LIMITS AND RECOVERY OF FIVE ANTIOXIDANTS IN DIFFERENT EDIBLE OILS BY THIN-LAYER CHROMATOGRAPHY

Antioxidant	*Oil*	Q_{max}* (μl)	*DL*** (ng)	*DA**** (ppb)	*Recovery*§ (%)	
					Without RSH	*With RSH*
I	Olive oil	80	30	125	45	79.9
	Safflower oil	80	50	125	55	86.1
	Sesame oil	25	50	400	45	65.5
	Sunflower oil	80	50	125	—	77.7
	Corn-germ oil	25	50	400	—	75.2
II	Olive oil	80	20	50	36	68.4
	Safflower oil	80	20	50	35.5	74.1
	Sesame oil	25	20	150	32.2	77.7
	Sunflower oil	80	20	50	—	85.9
	Corn-germ oil	25	20	160	—	78.1
III	Olive oil	25	50	800	97	
	Safflower oil	15	50	1.400	25	
	Sesame oil	15	50	1.400	33	
	Sunflower oil	15	50	1.600	61	
	Corn-germ oil	10	50	2.500	70	
IV	Olive oil	5	50	2.000		40
	Safflower oil	5	50	2.300		30
	Sesame oil	5	80	2.500		35
	Sunflower oil	5	50	2.000		30
	Corn-germ oil	5	50	2.000		40
V	Olive oil	25	5	80	83.6	
	Safflower oil	15	5	140	82.6	
	Sesame oil	15	5	140	67.8	
	Sunflower oil	15	5	140	68.3	
	Corn-germ oil	10	5	250	93.4	

* Volume applied to the plate.
** Detection limit on the plate.
*** Detectable amount of antioxidant in the oil.
§ Recovery percentage after extraction.

antioxidant in different oils, the recovery data of the oil extraction and the amount of each substance detectable in the oil.

CONCLUSION

A procedure has been devised for the routine analysis of some plastic migrants in edible oils. The selected sterically hindered phenols, which can migrate from the packaging plastic into the contacting oils, will only be present in trace amounts. Before quantification of the different migrants, pre-concentration of the additives has to be carried out (*e.g.*, extraction). The phenolic antioxidants are successfully separated from the co-extracted oil, and from interfering oil components, by TLC on $AgNO_3$-impregnated SiO_2 layers. After development, the antioxidants are identified by the formation of a nitroso derivative[11]. The detection limit for each antioxidant on the

TLC plate is between 5 and 80 ng, depending on the chemical structure of the substances. The combination of extraction and TLC makes it possible to detect 200 ppb of antioxidants I, II, and V in some edible oils.

The scope of the method could be extended to a wide variety of fatty food products, provided that the extraction step was sufficiently selective. For analysis purposes, TLC has a great potential in the identification of plastic additives, particularly phenolic antioxidants, which migrate into fatty foods.

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ANALYSIS OF NUTMEG OIL USING CHROMATOGRAPHIC METHODS

H. P. SCHENK and D. LAMPARSKY*
Givaudan Research Company Ltd., CH-8600 Dübendorf (Switzerland)

SUMMARY

Various chromatographic methods were used for the analysis of nutmeg oil. In addition to the fourteen known monoterpene hydrocarbons, α,*p*-dimethylstyrene has been identified together with sixteen monoterpene alcohols, seven esters and eight sesquiterpene hydrocarbons. The occurrence of the missing *trans*-isomer of *p*-menth-2-en-1-ol and a bifunctional derivative of the same type (*trans-p*-menth-2-ene-1,4-diol) are of special interest. Besides the terpenoid constituents, two unsaturated aliphatic compounds, namely, (*E*)-3-methyl-4-decen-1-ol and its acetate, were detected for the first time in a natural substrate.

INTRODUCTION

The characteristic odours of natural substrates (spices, essential oils, foods in general) are mainly caused by volatile components. Gas–liquid chromatography (GLC) is the most important tool for the analysis of these compounds. Older analytical methods worked well only for the main constituents, but were unsuitable for the trace components which so often are responsible for the overall flavour or odour. The new methods developed for all kinds of chromatographic techniques allow much easier qualitative and quantitative monitoring of compositions. The rapid development of capillary columns in gas chromatography has facilitated the investigation of complex mixtures such as essential oils.

In the field of spicy essential oils, nutmeg oil and mace oil play an important rôle in the flavour and fragrance industry. Owing to their known content of noxious substances such as safrol and myristicin[1], it was desirable to obtain more information about the qualitative and quantitative compositions in relation to the odour and flavour characteristics of these oils. Based on the compilation of Van Straaten[2], we have now confirmed all of the components already known and added to the list of nutmeg constituents the following compounds: one monoterpene hydrocarbon; six sesquiterpene hydrocarbons; six monoterpene alcohols; three esters; vanillin and two aliphatic compounds detected for the first time in nature, (*E*)-3-methyl-4-decen-1-ol and its acetate (see Table I).

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EXPERIMENTAL

Nutmeg oil of commercial grade (Roure-Bertrand, Grasse, France) was first distilled *in vacuo*. Certain fractions were combined and then chromatographed on silica gel 60 (E. Merck, Darmstadt, G.F.R.) using hexane–diethyl ether in various ratios (100:0 to 0:100) in order to isolate specific components of the oil. Thus, 30 g of a distillation fraction (b.p. 34–39°C/0.04 mmHg; 1.8% of the total oil) were first chromatographed on 150 g of silica gel 60. After elimination of the hydrocarbons, hexane–diethyl ether (10:1) was used to elute 4.3 g of a complex mixture which was rechromatographed on 130 g of silica gel 60. Elution with hexane–diethyl ether (10:1) then afforded a mixture (0.6 g) consisting mainly of the known *trans*-sabinene hydrate, *cis*- and *trans-p*-menth-2-en-1-ol. The previously unknown *trans*-isomer was isolated in pure form (64 mg) by means of preparative GLC.

Another distillation fraction (b.p. 49–65°C/0.03 mmHg; 34 g, 1.9% of the total oil) was treated in the same way to yield 200 mg of (*E*)-3-methyl-4-decen-1-ol (purity 80%) using hexane–diethyl ether (5:1) as eluent. Final elution with diethyl ether afforded *trans-p*-menth-2-ene-1,4-diol in 70% purity. Both constituents were further purified by preparative GLC.

All GLC measurements were conducted on a Carlo Erba GI apparatus. A glass column (3.8 m × 11 mm I.D.) packed with 10–5% Carbowax 20M (loading decreasing stepwise with increasing distance from column entrance) on Supelcoport, treated with terephthalic acid, was used for preparative work. Temperature: isothermal, 180 or 200°C. Carrier gas: nitrogen, 200 ml/min. A glass column (3 m × 3 mm I.D.) packed with 2% Carbowax 20M on Chromosorb G HP (80–100 mesh), treated with terephthalic acid, was used for the quantitative determination of myristicin. Temperature: programmed from 60 to 230°C at 6°C/min. Carrier gas: helium, 55 ml/min. Capillary columns (50 m × 0.31 mm I.D.) were coated with UCON HB 5100 or Carbowax 1000, respectively. Temperature: programmed from 50 to 185°C at 3°C/min. Carrier gas: helium, 2.5 ml/min.

Spectroscopic data of isolated compounds were recorded on Perkin-Elmer Model 157 (infrared), Bruker WH-360 (^{1}H nuclear magnetic resonance, NMR) and Varian XL-100A (^{13}C NMR) spectrophotometers. A Varian-MAT CH-5 or 212 in combination with Varian SS 100 or Finnigan Incos 2400 computer systems was used for mass spectrometry (MS) and GLC–MS coupling experiments.

RESULTS AND DISCUSSION

Monoterpene hydrocarbons represent about 70% of nutmeg oil. In GLC experiments, only Carbowax 1000 resolved all fifteen monoterpene hydrocarbons. With this stationary phase even 1,8-cineol is well separated from the adjacent hydrocarbons (limonene and β-phellandrene). α,*p*-Dimethylstyrene, already known to occur in other natural sources, was newly detected as a trace component in nutmeg oil by GLC–MS coupling experiments.

The capillary columns used showed efficient separation of sesquiterpene hydrocarbons from monoterpene alcohols, *i.e.*, α-copaene from *trans*-sabinene hydrate or caryophyllene from *trans-p*-menth-2-en-1-ol. They also enabled the detection of a sesquiterpene hydrocarbon which seems to decompose during the pretreatment via

silica gel column chromatography. We assume that this is a germacrene-type sesquiterpene hydrocarbon in view of the other identified compounds which are related to a common intermediate (Fig. 1). Germacrenes are known to be very sensitive and prone to skeletal rearrangements under acidic conditions[3–5].

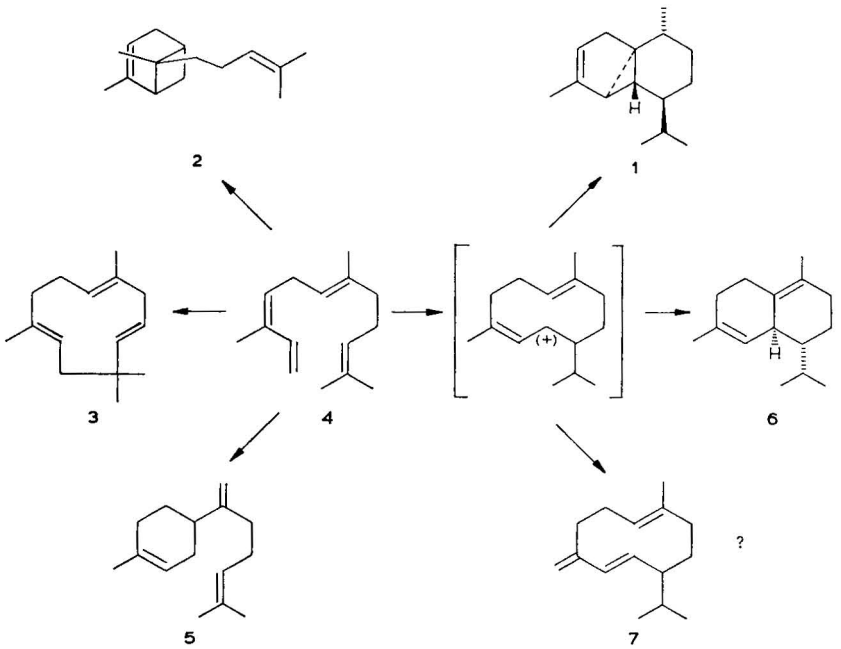


Fig. 1. New sesquiterpene hydrocarbons in nutmeg.

Two pairs of *cis/trans*-isomeric monoterpene alcohols usually occur together in essential oils of several spices (especially in Laminaceae-derived oils, for instance rosemary oil[6]), namely, *cis/trans*-sabinene hydrate and *cis/trans-p*-menth-2-en-1-ol. Three of the compounds are known to occur in nutmeg[7]. The missing fourth compound (*trans-p*-menth-2-en-1-ol) has now also been identified via its isolation and comparison of spectra with an authentic specimen[8].

Another interesting finding was the identification of *trans-p*-menth-2-ene-1,4-diol in nutmeg. This diol, already known as a constituent of Mitcham peppermint oil[9], is the first bifunctional monoterpene derivative detected in nutmeg oil. Its structure was assigned by comparison with synthetic specimens of known stereochemistry: ascaridol was reduced with triphenylphosphine[10] and gave after hydrolysis the *trans*-isomer identical in all respects with the isolated material. In contrast, reduction of ascaridol with lithium aluminium hydride[11] leads to the *cis*-isomer, as yet undetected in our natural substrate.

The occurrence of (*E*)-3-methyl-4-decen-1-ol ($[\alpha]^{22} - 32.05°$, $c = 1.045$, $CHCl_3$) in a natural material is here reported for the first time. The structure of the isolated alcohol follows from spectroscopic data and comparison with an authentic, synthetic sample[12]. This represents a good example of the successful isolation and structure

elucidation by combination of a tedious chromatographic step with modern spectroscopic means. The natural alcohol seems to be optically pure, because no additional lines were observed when an optically active Eu(III)-shift reagent was added. In contrast, the synthetic specimen shows doubling of the characteristic signals due to the presence of both possible isomers.

Additionally, 3-methyl-4-decen-1-yl acetate was detected as a trace component by GLC–MS where the mass spectrometer was operated in the chemical ionization (CI) mode: the CI spectrum using isobutane as reaction gas exhibits intense signals at

TABLE I

COMPOSITION OF NUTMEG OIL

The newly identified compounds are marked with an asterisk (*).

Name	*Content (%)*	*Name*	*Content (%)*
Monoterpene hydrocarbons		*Aromatics*	
α-Pinene	17.2	*trans*-Methylisoeugenol	
α-Thujene	0.9	5-Methoxyeugenol	
Camphene	0.4	Myristicin	14.0
β-Pinene	14.8	Elemicin	
Sabinene	21.0	*cis*-Isoelemicin	
Δ^3-Carene	1.4	*trans*-Isoelemicin	
Myrcene	1.6		
α-Phellandrene	1.6	*Monoterpene alcohols*	
α-Terpinene	1.4	Linalool	1.0
Limonene	4.1	Fenchyl alcohol* (α-fenchol)	
β-Phellandrene	2.0	Borneol	
γ-Terpinene	2.1	Terpinen-4-ol	6.3
p-Cymene	0.5	α-Terpineol	1.4
Terpinolene	1.0	Citronellol	
α,*p*-Dimethylstyrene*		Nerol*	
		Geraniol	
Oxides		*cis*-Piperitol	
1,8-Cineol		*trans*-Piperitol*	
		cis-Sabinene hydrate	
Sesquiterpene hydrocarbons		*trans*-Sabinene hydrate	
Total	1.0	*cis*-*p*-Menth-2-en-1-ol	
α-Copaene (0.3)		*trans*-*p*-Menth-2-en-1-ol*	
α-Cubebene* 1		*trans*-*p*-Menth-2-ene-1,4-diol*	
α-Bergamotene* 2		*p*-Cymen-8-ol*	
Carophyllene			
α-Humulene* 3		*Esters*	
α-Farnesene* 4		Bornyl acetate	
β-Bisabolene* 5		Linalyl acetate	
δ-Cadinene* 6		Citronellyl acetate*	
Germacrene D* 7		Neryl acetate*	
		Geranyl acetate	
Aromatics		Terpinen-4-yl acetate	
Safrol	3.3	α-Terpenyl acetate*	
Eugenol	0.3		
cis-Isoeugenol		*Aliphatics*	
trans-Isoeugenol		3-Methyl-4-decen-1-ol*	
Methyleugenol	0.3	3-Methyl-4-decenyl acetate*	
Vanillin*			

$m/e = 153$ and 213, whereas the normally recorded electron impact spectrum completely lacks the molecular ion at $m/e = 212$.

The analysis of the so-called mace oil, the oil of the dried arillode of nutmeg reveals only quantitative but no significant qualitative differences when compared with the real nutmeg oil. The higher percentage of caryophyllene and the slightly lower amounts of safrol and myristicin are to be noted. For the latter, a noxious constituent with psycgoactive, toxic properties, a quantitative GLC method was developed using diethyl phthalate as internal standard. Our interest in developing such a method was aroused by the fact that the literature[7] values obtained by GLC measurements show some discrepancy. The results from our method correspond well to the values obtained by conventional work-up (13–14%): nutmeg 13.9 $\pm$ 0.5%; mace 10.1 $\pm$ 0.5%.

In Table I the approximate composition of nutmeg oil is given for the main components, thus underlining the predominance (70%) of monoterpene hydrocarbons.

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CHROM. 13,125

ANALYTICAL AND STRUCTURAL STUDY OF SOME FOOD EMULSIFIERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND OFF-LINE MASS SPECTROMETRY

G. SUDRAUD*, J. M. COUSTARD and C. RETHO

Laboratoire Central de Recherches et d'Analyse, 21 Avenue de la République, 91300 Massy (France)

M. CAUDE and R. ROSSET

Laboratoire de Chimie Analytique des Processus Industriels, ESPCI, 10 Rue Vauquelin, 75231 Paris Cédex 05 (France)

and

R. HAGEMANN, D. GAUDIN and H. VIRELIZIER

Service de Spectrométrie de Masse, Centre d'Études Nucléaires de Saclay, B.P. No. 2, 91190 Gif-sur-Yvette (France)

SUMMARY

Components of various food emulsifiers distilled monoglycerides (E 471), sodium stearoyllactylate (E 481), calcium stearoyllactylate (E 482), diacetyltartaric acid esters of mono- and diglycerides (E 472 e) and mixed acetic and tartaric acid esters of mono- and diglycerides (E 472 f), have been separated analytically and on a semi-preparative basis by high-performance liquid chromatography. The various products were identified by off-line high-resolution mass spectrometry.

INTRODUCTION

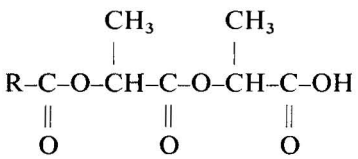
Emulsifiers are widely used in the food industry because they facilitate the processing of food and improve the quality of the resulting products. They are derived from edible fats, which consist of molecules with both hydrophilic and lipophilic characters, thus conferring tensioactive properties upon the emulsifiery. Emulsifiers may also complex with proteins (*e.g.*, anionic and polyethoxylated emulsifiers) or starch (*e.g.*, amylose forms a helical complex around the hydrocarbon chain of saturated monoglycerides), resulting in the dough strenghening or bread softening properties well known in baking[1]. When these compounds possess both an anionic part and a long saturated hydrocarbon chain, *e.g.*, diacetyltartaric acid esters of mono- and diglycerides, they behave both as dough strengheners and bread softeners[2].

The different emulsifiers are generally described by a generic name which refers to the major components. In fact they are mixtures of well defined chemical compounds the proportions of which vary according to the starting materials and processing conditions: temperature and reaction time. Their contents in different basic constituents[3,4] may be specified, *e.g.*, total lactic acid in sodium stearoyllactylate.

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Much work has been done on the separation and identification of emulsifiers in food by thin-layer chromatography (TLC)[5] and gas–liquid chromatography (GLC)[6]. High-performance liquid chromatography (HPLC) has been used for the separation of glycerides[7–10] and for fingerprinting of some food emulsifiers[11]. In contrast, the separation and identification of the components of complex emulsifiers has been little investigated.

The present study was undertaken mainly on two emulsifiers widely used respectively in the U.S.A. and Europe in the baking industry, namely: sodium (and calcium) stearoyllactylate (E 481*, E 482*); diacetyltartaric acid esters of mono- and diglycerides (E 472 e*) and mixed acetic and tartaric acid esters of mono- and diglycerides (E 472 f*). The first are manufactured by reacting fatty acids with lactic acid and a suitable base[12,13]. The main product is thought to be



because the fatty acid:lactic acid molar ratio is rougly 1:2 in the emulsifier. TLC[6,12] or GLC[6] has been used for their identification. The latter are very complex mixtures manufactured either in two steps[13,14] or in one step[13] using acetic acid (or anhydride), tartaric acid and mono- and diglycerides as starting materials. They are identified by fingerprinting TLC or after saponification[6,15].

In this study, HPLC and off-line mass spectrometry were used for separation and identification of various components of these two types of emulsifiers.

EXPERIMENTAL

Chromatography

Varian 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a loop injector (Valco) and a Pye Unicam LC–UV spectrophotometer detector (Pye Unicam, Cambridge, Great Britain) at 213 nm was used. Mass spectrometry was performed on an AEI MS-9 high-resolution mass spectrometer, calibrated with perfluorokerosine (electron energy 80 eV). The columns (15 × 0.47 cm) were filled with 5-μm Partisil 5 silica gel (Whatman, Clifton, NJ, U.S.A.) or 10-μm LiChrosorb RP-18, octadecyl silica (Merck, Darmstadt, G.F.R.). The solvents hexane and acetonitrile, LC grade, were supplied by S.D.S. (Peypin, France); 2-propanol, spectroscopic grade, and perchloric acid (70%) were obtained from Prolabo (Paris, France).

Samples

1-Monostearine and 1-monopalmitine were purchased from Sigma (St. Louis, MO, U.S.A.); 1,2-dipalmitine, 1,3-dipalmitine, 1,2-distearine, tristearine and tripalmitine were from Fluka (Buchs, Switzerland). Monoglycerides and tartaric acid esters were dissolved in chloroform. Sodium and calcium stearoyllactylate (1 g) were

* European Economic Community (EEC) code number.

added to aqueous 1 *M* HCl (20 ml) and extracted with diethyl ether (3 × 7 ml). The organic layers were recombined and washed with water (7 ml), then with saturated sodium chloride solution (4 ml) (yield ≈ 75%).

Fatty acids analysis

Samples were saponified and esterified with boron trifluoride–methanol before injection in a Fractovap 2160 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a glass capillary column (48 m × 0.2 mm I.D.) coated with Carbowax 20M. Carrier gas: hydrogen, flow-rate 2 ml/min. Temperature gradient: 150°C to 190°C at 2°C/min.

RESULTS

Distilled monoglycerides (emulsifier type E 471)

Analysis of these compounds by GLC[16,17] and HPLC on a polar stationary phase[10] has been described previously. Monoglycerides distilled under high vacuum contain more than 90% monoesters. Fig. 1 shows a separation obtained on a LiChrosorb RP-18 column and Table I the identified compounds from a sample of saturated distilled monoglycerides. As expected this emulsifier is mainly a mixture of monopalmitine and monostearine, and was probably manufactured from hydrogenated lard.

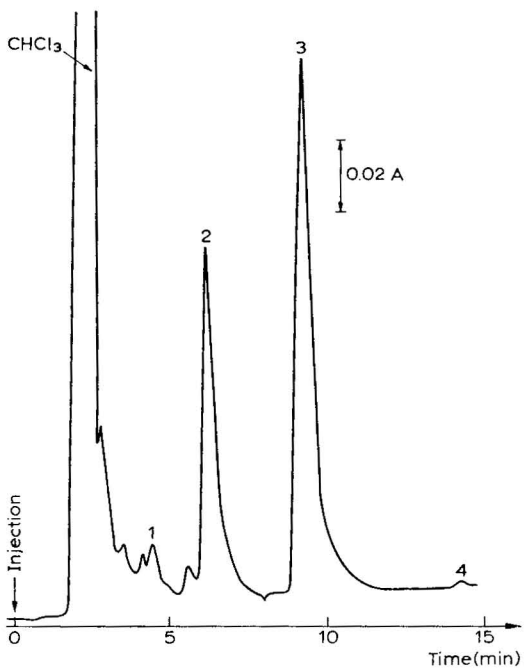


Fig. 1. Separation of saturated distilled monoglycerides by reversed-phase chromatography. Column: 15 × 0.47 cm I.D. Stationary phase: LiChrosorb RP-18, 10 μm. Mobile phase: Acetonitrile; flow-rate 0.9 ml/min. UV detection at 204 nm. Peaks: 1 = monomyristine; 2 = monopalmitine; 3 = monostearine; 4 = monoarachidine.

TABLE I

IDENTIFIED COMPOUNDS IN A SAMPLE OF DISTILLED MONOGLYCERIDES (E 471)
Second column gives formula number in Appendix.

Identified compound	*Formula number in Appendix*
Monomyristine	1a
Monopalmitine	1b
Monostearine	1c

Calcium and sodium stearoyllactylates (emulsifier types E 481, E 482)

A chromatographic study was made of four samples from two manufacturers. We observed a good resolution according to the lactoyl group number by adsorption chromatography (Fig. 2a, b) and according to the carbon number of the fatty acid radicals by reversed-phase chromatography (Fig. 3a, b). The compounds identified by off-line mass spectrometry are shown in Table II. The most typical fragmentations were at $m/e = M - \{p[OCH(CH_3)CO] + OH\}$ where $0 \leqslant p \leqslant n$ and n is the total lactoyl group number in the molecule and M the molar weight.

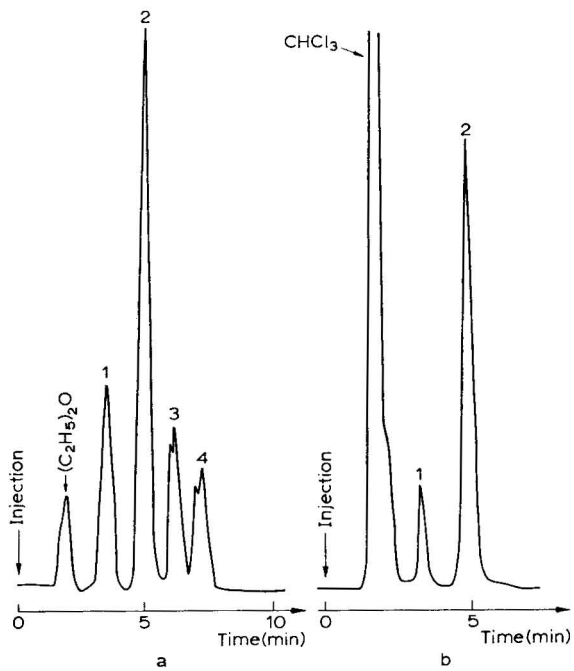


Fig. 2. Separation of calcium (a) and sodium (b) stearoyllactylate by adsorption chromatography. Column: 15 × 0.47 cm I.D. Stationary phase: Partisil 5, 5 μm. Mobile phase: hexane–2-propanol (with 2% water and 0.5% perchloric acid) (99.1:0.9 v/v). flow-rate 1.3 ml/min. UV detection at 204 nm. Peaks: 1 = fatty acids; 2 = fatty acids ester of lactic acid; 3 = fatty acid esters of lactoyl-lactic acid; 4 = fatty acid esters of di(lactoyl)lactic acid.

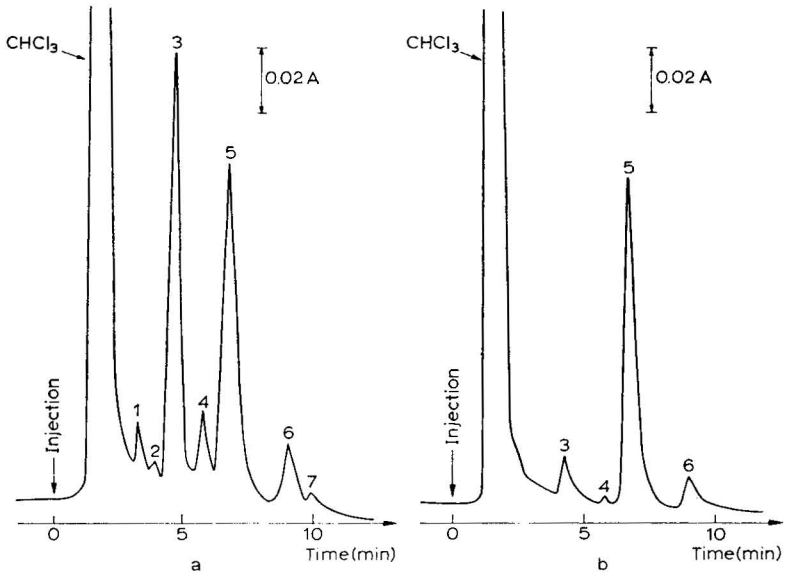


Fig. 3. Separation as in Fig. 2 but by reversed-phase chromatography. Column: 15 × 0.47 cm I.D. Stationary phase: LiChrosorb RP-18, 10 μm. Mobile phase: acetonitrile + 10^{-4} *M* $HClO_4$; flow-rate 1.3 ml/min. UV detection at 204 nm. Peaks: 1 = myristoyllactic acid and myristoylpolylactic acid; 2 = myristic acid; 3 = palmitoyllactic acid and palmitoylpolylactic acids; 4 = palmitic acid; 5 = stearoyllactic acid and stearoylpolylactic acids; 6 = stearic acid; 7 = arachidoyllactic acid and arachidoylpolylactic acids.

TABLE II

IDENTIFIED COMPOUNDS IN SAMPLES OF CALCIUM AND SODIUM STEAROYL-LACTYLATES (E 481, E 482)

Identified compound	*Formula number in Appendix*
Fatty acids	2a, b, c
Fatty acid esters of lactic acid	3a, b, c, d
Fatty acid esters of lactoyllactic acid	4a, b, c, d
Fatty acid esters of di(lactoyl)lactic acid	5a, b, c

In each sample, the main components are fatty acid esters of lactic acid, and not, as generally thought (except in Regula's TLC work[12]) fatty acid esters of lactoyllactic acid. In three samples, fatty acid esters of polymeric lactic acid were found.

A semi-quantitative analysis shows that about half of the total lactic acid content of the emulsifier is esterified with fatty acids.

Acetylated tartaric acid esters of mono- and diglycerides (emulsifier types E 472 e, E 472 f)

The study was done by adsorption liquid chromatography on ten samples

from four manufacturers; although identification by mass spectrometry was carried out only on four samples, the results obtained may be extended to all ten samples.

Most of the components were separated in a semi-preparative way with four separate isocratic runs using mobile phases of decreasing elution strength. In the first run the more polar products were isolated and the less retained ones were recovered as a mixture. In the second run, with a weaker mobile phase, the mixture previously recovered was chromatographed; the more retained products were isolated and the less retained ones were again recovered as a mixture. In this way (and with two other runs), most of the components were isolated.

The chromatographic separation of the more polar compounds from two

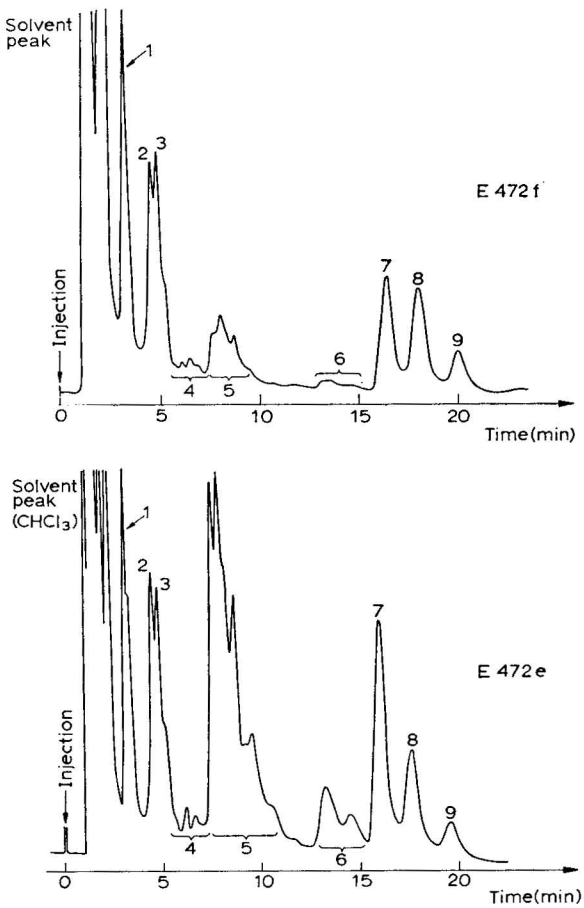


Fig. 4. Separation of tartaric acid esters by adsorption chromatography. Column: 15 × 0.47 cm I.D. Stationary phase: Partisil 5, 5 μm. Mobile phase: hexane–2-propanol [2% water and 0.2% perchloric acid (70%)] (92:8, v/v); flow-rate 1.5 ml/min. UV detection at 213 nm. Peaks: 1 = acetic acid and acetylated monoglycerides; 2 = diacetyltartaric acid ester of diglycerides; 3 = acetic and diacetyltartaric acid esters of monoglycerides; 4 = monoglycerides; 5 = diacetyltartaric acid esters; 6 = acetyltartaric acid esters; 7 = tartaric acid esters; 8 = di(diacetyltartaric acid) esters; 9 =acetyl- and diacetyltartaric acid esters of monoglycerides.

saturated* emulsifiers is shown Fig. 4a, b and the compounds identified are listed in Table III. The different products were identified by off-line mass spectrometry of their trimethylsilyl derivatives** and direct admission into the ionizing chamber. The most typical fragmentations were at $m/e = M' - CH_3$, $M' - RCOO$ and $M' - RCOOCH_2$ where R = fatty acid radical or $(CH_3)_3SiOCOCH(OR_1)CH(OR_2)$ with R_1, $R_2 = Si(CH_3)_3$, CH_3CO. Ions $[RCOOC_3H_6O]^+$ were observed in every spectrum. These kinds of fragmentations are well-known with silanized derivatives of diglycerides[18], we also observed them for trimethylsilyl derivatives of the different tartaric acid esters of monoglycerides.

TABLE III

IDENTIFIED COMPOUNDS IN SAMPLES OF ACETYLATED TARTARIC ACID ESTERS OF MONO- AND DIGLYCERIDES (E 472 e, f)

Identified compound	*Formula number in Appendix*
Acetyl- and diacetyltartaric acid esters of monoglycerides	6
Di(diacetyltartaric acid) esters of monoglycerides	7
Tartaric acid esters of monoglycerides	8
Acetyltartaric acid esters of monoglycerides	9
Diacetyltartaric acid esters of monoglycerides	10
Acetic and diacetyltartaric acid esters of monoglycerides	11
Diacetyltartaric acid esters of diglycerides	12

Separation of medium polar and apolar compounds was achieved with a saturated emulsifier (containing stearic acid as more than 80% of the total fatty acid content). Chromatographic separations are shown Fig. 5, and the results are in Table IV. The different products were identified by their mass spectra[18] as determined by gas chromatography–mass spectrometry.

TABLE IV

MEDIUM POLAR AND APOLAR COMPOUNDS IDENTIFIED IN A SAMPLE OF DIACETYLTARTARIC ACID ESTERS OF MONO- AND DIGLYCERIDES (E 472, e)

Mobile phase composition (% v/v)		*Identified compound*	*Formula number in Appendix*
Hexane	*2-Propanol (0.8% water)*		
		Acetic acid	—
96.3	3.7	Acetylated 1,3-monoglycerides	13
		Acetylated 1,2-monoglycerides	14
99.1	0.9	1,3-Diglycerides	15
		Fatty acids	2
99.75	0.25	Triglycerides	16

* With nearly the same fatty acid composition, as determined after saponification, methylation and GC analysis. Stearic and palmitic acids are the major fatty acids.

** Obtained with N,O-bis(trimethylsilyl)acetamide (BSA) in dichloromethane.

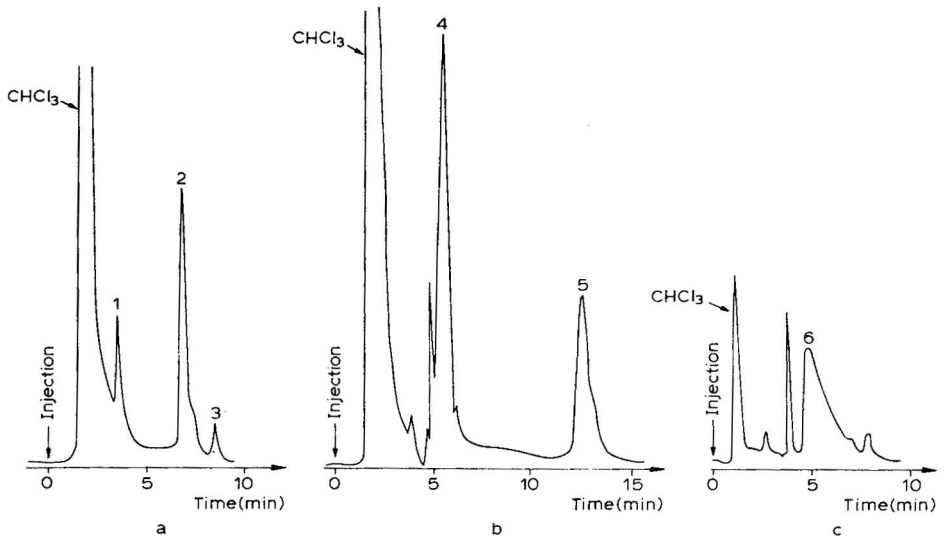


Fig. 5. Separation polar and apolar compounds in a sample of diacetyltartaric acid esters of mono- and diglycerides. Column: 15 × 0.47 cm I.D. Stationary phase: Partisil 5, 5 μm. Flow-rate 1.3 ml/min. UV detection at 204 nm. Mobile phase: hexane–2-propanol (0.8% water) (96.3:3.7) (A), (99.1:0.9) (B) and (99.75:0.25) (C). Peaks: 1 = acetic acid, 2 = acetylated 1,3-monoglycerides; 3 = acetylated 1,2-monoglycerides; 4 = fatty acids; 5 = 1,3-diglycerides; 6 = triglycerides.

As expected from the starting materials, these emulsifiers are very complex mixtures which show only quantitative differences in liquid chromatographic analysis. These differences may be due to the processing conditions (temperature, reaction time) and the ratio and source of the starting materials.

Aging of an emulsifier sample was also studied. A sample which was previously a powder became a pasty block having a strong odour of acetic acid on storage at

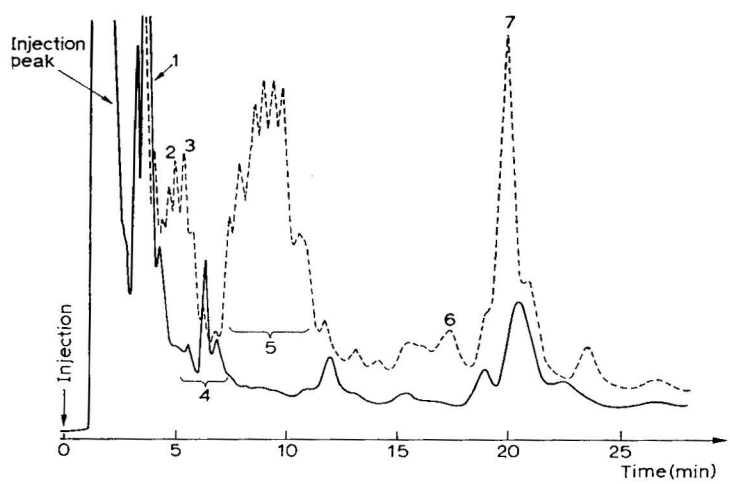
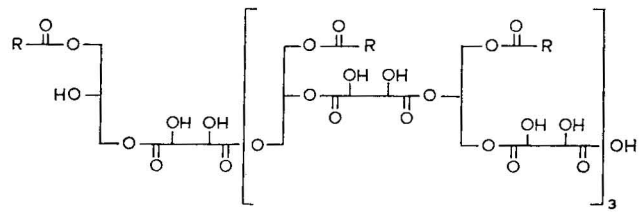


Fig. 6. Aging of an emulsifier sample. Operating conditions and peak numbers as in Fig. 4. ——, Old sample (*ca.* 6 years old); – – –, new sample, manufactured from hydrogenated fish oil (shown by its fatty acid analysis).

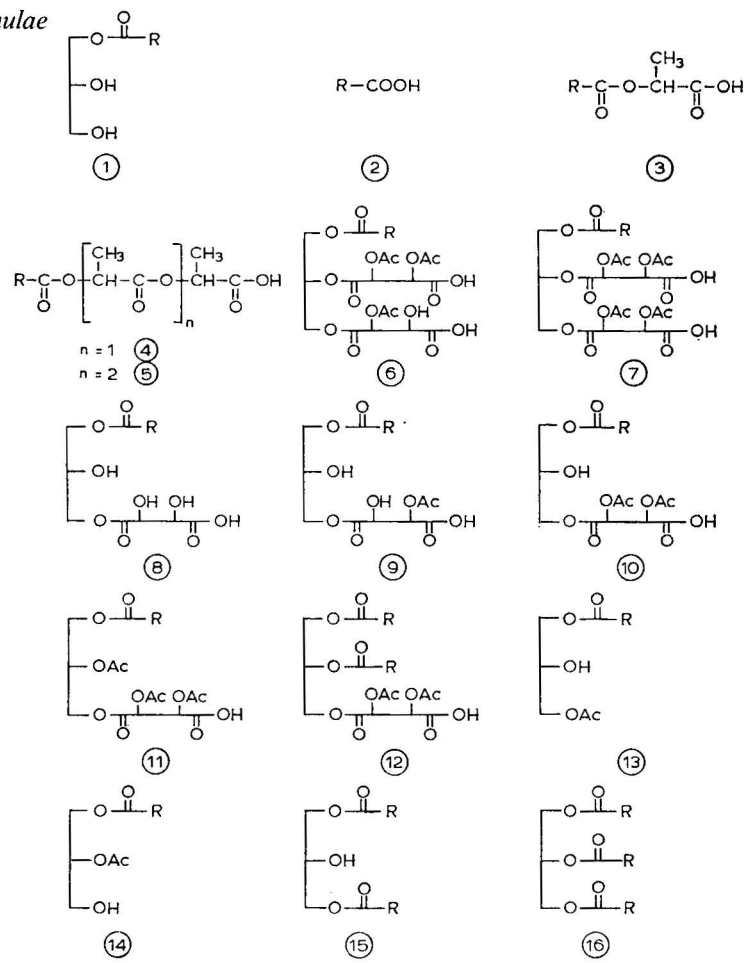
room temperature for several years. The emulsifier lost most of its acetic acid (Fig. 6) and higher-molecular-weight materials were formed (MW about 2500 Daltons). Such compounds may result from polycondensation of six to seven glycerol units linked by tartaric acid bridges with, for instance, the following structure:



Compounds with molecular weights higher than 1300 (after silylation) were also isolated by HPLC but not identified.

APPENDIX

Formulae



Only one isomer is shown. The actual structures and the number of isomers in each peak are not known. (a) R = $C_{13}H_{27}$; (b) R = $C_{15}H_{31}$; (c) R = $C_{17}H_{35}$; (d) R = $C_{19}H_{39}$.

CONCLUSION

Analysis of sodium or calcium stearoyllactylate (four samples from two manufacturers) shows that the major components are 2-stearoyl and 2-palmitoyllactic acid and their salts (or 2-stearoyloxypropionic acid and 2-palmitoyloxypropionic acid and their salts).

Acetylated tartaric acid esters of mono- and diglycerides are complex mixtures of which at least twelve different components, not including isomeric forms and homologues, were identified and isolated. On aging they lose acetic acid and form polymeric materials.

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CHROM. 13,228

IMPROVED SEPARATION OF TRIGLYCERIDES AT LOW TEMPERATURES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

GEORG W. JENSEN
Novo Research Institute, Novo Allé, DK-2880 Bagsvaerd (Denmark)

SUMMARY

A high-performance liquid chromatographic technique for the separation of triglycerides in natural mixtures is described. The influence of column temperature on separation is investigated, and a linear relationship between log k' and 1/T is demonstrated. Operating this reversed-phase technique with a LiChrosorb RP-18 packing at 14.5°C, separation of triglycerides with the same equivalent carbon numbers (ECN), but different degrees of saturation, is obtained.

The composition of an eluent which permits separation of saturated triglycerides with carbon numbers as high as 54 (*e.g.*, tristearin) is given.

INTRODUCTION

Interest in separating and analysing mixtures of natural fats is still growing, and high-performance liquid chromatography (HPLC) provides a powerful tool for the rapid analysis of fats. The use of argentation HPLC with silica particles loaded with silver ions permits separation of the positional isomers of triglycerides[1], but this normal-phase HPLC is difficult to run routinely. The literature contains many examples of triglyceride separations with HPLC in the reversed-phase mode[2–5]. All these references deal with separation at room temperature on C_{18} columns, and most of them lead to separation according to the equivalent carbon number (ECN). However, Herslöf *et al.*[3] and Vonach and Schomburg[5] have demonstrated the separation of the ECN 48 triglycerides containing oleic and palmitic acid, but so far no author has demonstrated elution of tristearin in reversed-phase HPLC. Plattner *et al.*[2] state that this triglyceride precipitates in the injector when their solvent system is used. Recently, Gilpin and Sisco[6] have investigated the effect of temperature on the retention times of some aromatic compounds; they found a linear relationship in the reversed-phase mode between ln k' and $1/T$ (where k' is the capacity ratio and T is the column temperature).

This paper demonstrates an improved separation of triglycerides from natural mixtures by reversed-phase HPLC at sub-ambient temperature and gives the composition of a solvent system that permits elution and separation of unsaturated and of saturated triglycerides containing fatty acids with chain lengths up to C_{18}.

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EXPERIMENTAL CONDITIONS

The HPLC was carried out using the following equipment: a Waters Assoc. pump (Model M 6000) delivering 1.5 ml/min, with a 25 cm × 4.6 mm stainless-steel column (Knauer) packed with LiChrosorb RP-18 (5 μm) (Merck, Darmstadt, G.F.R.). The column was packed in the laboratory in the upwards-slurry technique with the Waters pump.

The samples were injected as chloroform solutions by means of a U6K universal-loop injector (Waters), and a Waters differential refractometer (Model 401) was used as detector. The column was thermostatically controlled at sub-ambient temperature (routinely 14.5 ± 0.1°C) by means of Hetofrig cooling system (type 03PF623CB11).

The following solvent system was used: acetonitrile (Merck; art. 3)–tetrahydrofuran (Merck; art. 9731)–*n*-hexane (Merck; art. 4391) (224:123.2:39.6, w/w/w).

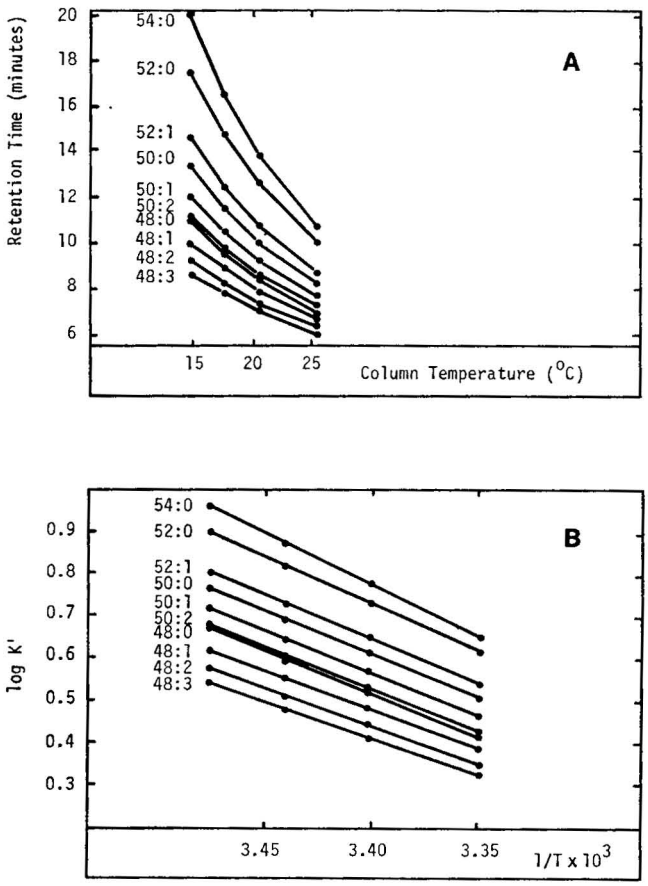


Fig. 1. A, Plots of retention time *vs.* column temperature for various triglycerides from vegetable oils. B, Plots of log k' *vs.* $1/T$ for the same triglycerides as in A. Eluent: acetonitrile–tetrahydrofuran–*n*-hexane; flow-rate 1.5 ml/min. The number at the end of each curve indicates the equivalent carbon number (ECN) of the fatty acids in the triglycerides as well as the number of double bonds, *e.g.*, 48:3 (ECN 48; 3 double bonds).

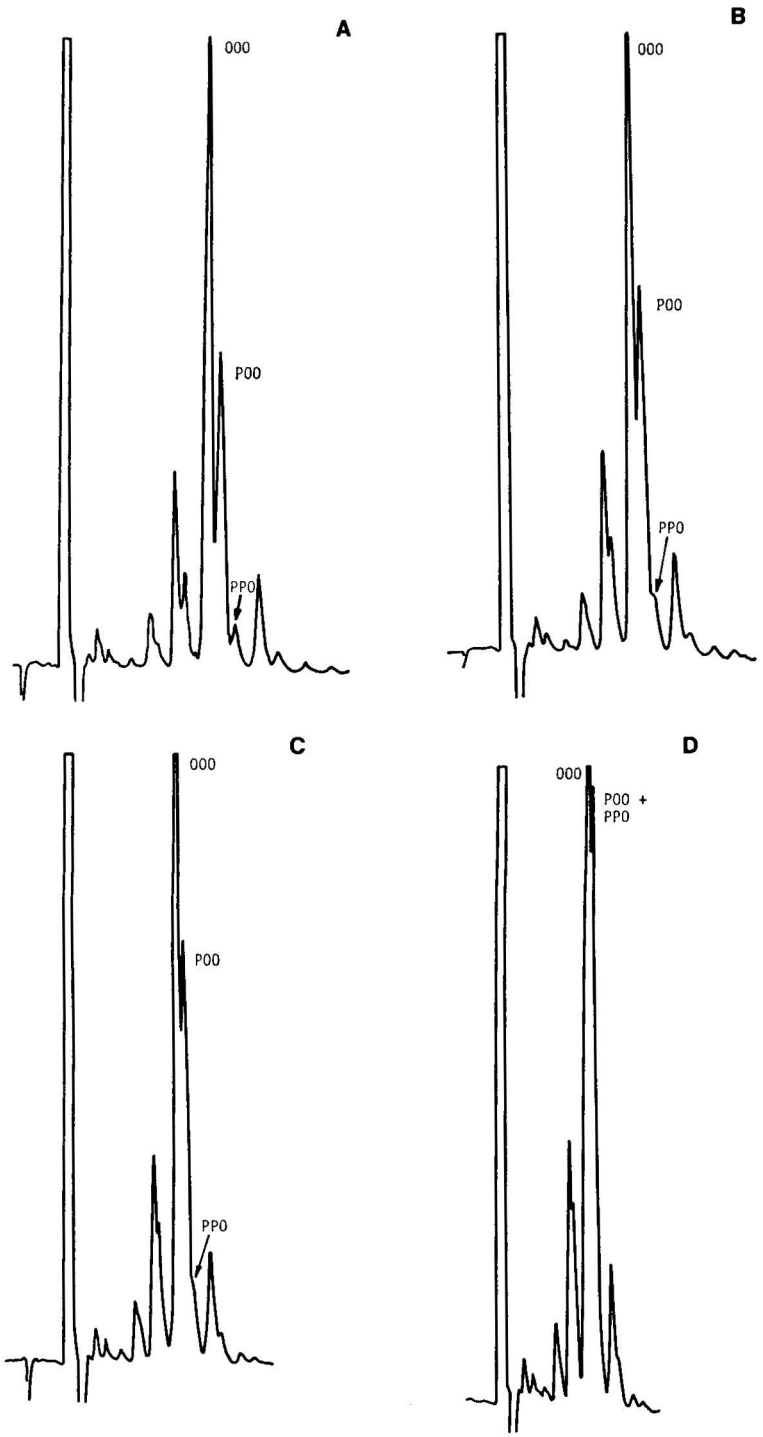


Fig. 2. Separation of triglycerides in olive oil at different temperatures. A, 14.5°C; B, 17.5°C; C, 20.5°C; D, 25.5°C. Chromatographic conditions as in Fig. 1.

It was necessary to weigh the components, as even small variations could cause shifts in the retention times. The samples used for investigating the influence of column temperature on the separation of triglycerides were olive oil, coconut oil and selected triglycerides, such as POS, POP, SOS, SSS and other standards (P = palmitic acid; O = oleic acid, and S = stearic acid); 5 μl of a 10% solution of the oil in chloroform and 5 μl of 4% standard triglyceride solution in chloroform were used.

RESULTS AND DISCUSSION

The purpose of this work was to establish an isocratic reversed-phase method for separating triglycerides in natural vegetable fats and oils, such as olive oil and coconut oil, and which permits elution of tristearin.

The influence of the column temperature on the separation was investigated by making several runs at four temperatures (14.5, 15.5, 20.5 and 25.5°C).

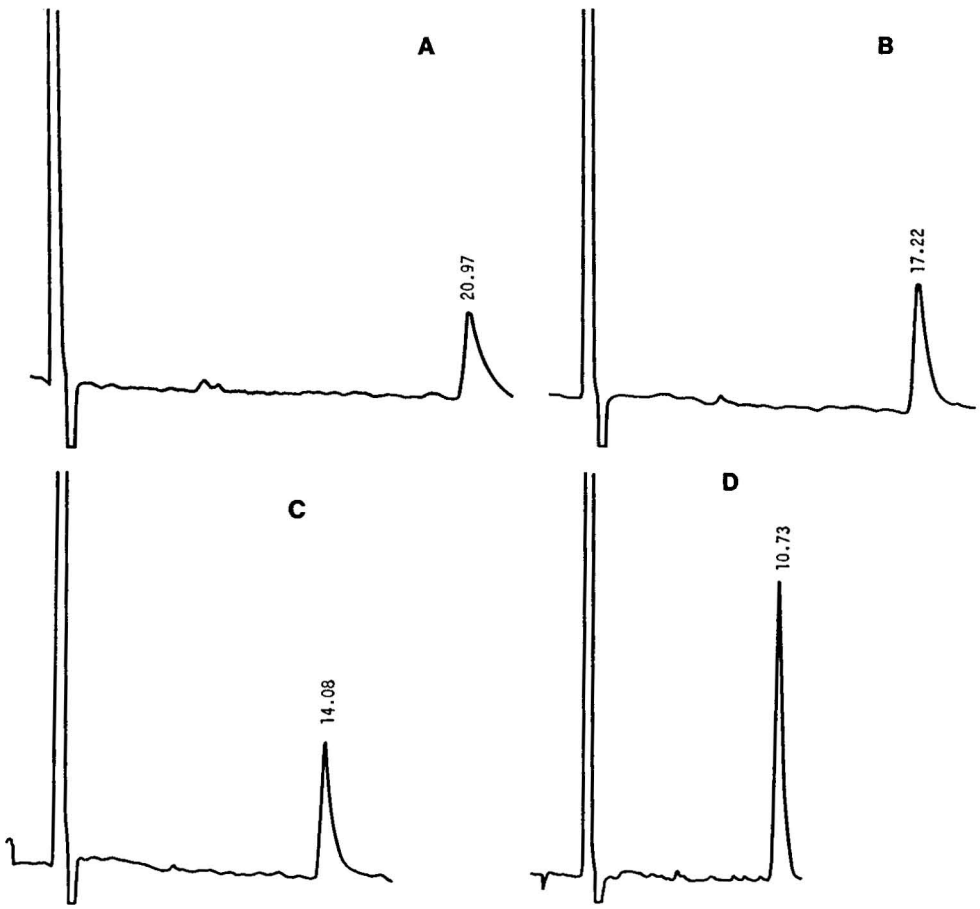


Fig. 3. Elution of tristearin at different temperatures. A, 14.5°C; B, 17.5°C; C, 20.5°C; D, 25.5°C. The number of the above the peak indicates the retention time (in minutes) at the temperature indicated. Chromatographic conditions as in Fig. 1.

From Fig. 1A it is obvious that the best separation is obtained at *ca.* 14.5°C, as the distance between the lines drawn for each triglyceride is here at its maximum (see also Fig. 2). At lower temperatures, the solubility, especially of tristearin (ECN 54:0), in the solvent falls drastically. The number at the end of each curve indicates the equivalent carbon number (ECN) of the fatty acids in the triglyceride as well as the number of double bonds within the triglyceride, *e.g.*, triolein is 48:3 (ECN 48; 3 double bonds).

Changes in column temperature have the greatest influence on retention time the longer and the more saturated the fatty acids in the triglycerides are. This effect is due to the more hydrophobic character of the long-chain saturated fatty acids and thereby a greater temperature-dependent affinity to the stationary phase.

Fig. 1B shows the relation between log k' and $1/T$ for the same triglycerides as in Fig. 1A. This relationship is linear and in good agreement with the results reported for aromatic compounds by Gilpin and Sisco[6]. None of the investigated triglycerides shows non-linear behaviour within the temperature range examined.

Fig. 2 shows four chromatograms of an olive oil separation at the four temperatures shown in Fig. 1; it demonstrates the chromatograms obtainable at these temperatures, and confirms that the best separation is obtained at *ca.* 14.5°C.

In Fig. 3, the effect of temperature on the elution of tristearin is demonstrated. At 25.5°C, tristearin is eluted as a sharp peak, virtually without tailing, but at lower temperatures it is eluted as a broader and skewed peak, the width increasing as the temperature decreases. This broadening effect is due to the low solubility of tristearin in the eluent and to its great affinity to the stationary phase, especially at low temperatures. Using a stronger eluent to overcome the boradening effect results in poorer resolution of the component preceding tristearin, so the eluent used is considered to offer a fairly good compromise.

CONCLUSION

Reversed-phase HPLC at low temperatures makes it possible to separate triglycerides, not only according to their ECN, but also according to their degree of saturation. The solvent system used permits elution of tristearin as a sharp peak at room temperature within 12 min, and within 22 min as a broader peak at 14.5°C.

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CHROM. 13,286

DETERMINATION OF INDIVIDUAL CHLORINATED BIPHENYLS IN AGRICULTURAL PRODUCTS BY AUTOMATED CAPILLARY GAS CHROMATOGRAPHY

DETERMINATION IN CATTLE FEED AND ITS RELATION TO MILK RESIDUES

L. G. M. Th. TUINSTRA*, W. A. TRAAG and A. J. VAN MUNSTEREN

State Institute for Quality Control of Agricultural Products, Bornsesteeg 45, 6708 PD Wageningen (The Netherlands)

SUMMARY

Polychlorinated biphenyls were determined by glass capillary gas chromatography with electron capture detection, with automatic splitless injection. Using a saponification step as clean-up procedure, the detection limit for individual polychlorinated biphenyls can be improved up to the sub-ppb* level for vegetabele material and up to the ppb level for fatty material. Quantification is carried out by comparison of separated chlorobiphenyl peaks with the corresponding individual chlorobiphenyl standards.

The results of monitoring programs of milkfat and cattle feed are summarized. Accumulation factors for several chlorobiphenyls, determined in an experiment with lactating cows, are used to show that contamination of the milkfat is not caused by contaminated cattle feed.

INTRODUCTION

The gas chromatographic (GC) determination of polychlorinated biphenyls (PCBs) in all kinds of samples is commonly accomplished by injecting the sample extract onto a packed column and comparing the chromatogram of the sample with the chromatogram of a technical Aroclor 1254 or 1260 mixture. Sometimes the total peak area of the sample is compared with the total peak area of an Aroclor mixture, or only a few similar peaks are used for quantitation. Especially with the last method, the results obtained on the same sample by different laboratories will differ considerably, because of the differences in the GC conditions (*e.g.*, stationary phase, column temperature). Within one laboratory, when analysing one type of sample, the quantitation (or better, estimation) is adequate for establishing trends. However, more frequently we are interested in relationships between different commodities in order to understand accumulation factors in food chains.

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

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The poor match between the peak patterns from the sample and the technical standard mixture has frequently been noted in the literature (*e.g.*, refs. 1–4). This implies that the significance of the quantitation of PCBs in different commodities giving different peak patterns will be, at least, questionable. For this reason we chose a method suitable for determining individual PCBs in all kinds of samples (fish, milk, dairy products, soil, vegetables, etc.), which also has a very low detection limit. In this paper attention is focused on the determination of PCBs in concentrated cattle feed and its relation to milkfat.

EXPERIMENTAL

Extraction of fatty material from cattle feed

Eighty grams of concentrated cattle feed were extracted for 24 h with 200 ml pentane in a stoppered flask using a shaking machine. A 100-ml volume of the pentane extract was then concentrated to 5 ml.

Fat extraction from milk[5]

A 200-ml volume of milk was centrifuged at 2000 *g*. The separated cream layer was placed into a beaker and enough anhydrous Na_2SO_4 added to give agranular mixture on stirring. A 50-ml volume of pentane was added with stirring. The pentane layer was then decanted through anhydrous Na_2SO_4 and glass wool. The pentane extraction was repeated several times until a yellow colour was no longer observed in the pentane. The solvent was then evaporated to obtain fat.

Extraction of PCBs

The PCBs in milkfat and in cattle feed extract were isolated by saponification of the fatty material as follows. A 5-ml volume of the cattle feed extract or 2.0 g of milkfat was placed into a flat-bottomed flask, 20 ml of alcoholic 0.6 *N* KOH were added and the mixtured allowed to saponify on a water-bath at 70°C for at least 30 min. A few drops of water were added followed by mixing. If the soap solution became turbid the saponification was allowed to continue. On completion of saponification the mixture was cooled and poured into a separator. A 30-ml volume of pentane and 20 ml water were added and shaken for 30 sec. The pentane layer was transferred into a separator.

The water layer was extracted three times with 15-ml portions pentane. The combined pentane fractions were washed several times with water until the water was neutral. The pentane layer should be clear. The pentane extract was then passed through anhydrous Na_2SO_4 and concentrated carefully to 2 ml on a water-bath (50°C) with a slow stream of nitrogen. Lower chlorinated biphenyls are volatile and may evaporate.

Clean-up

A 2-g amount of basic alumina (deactivated with 5% water) was placed in chromatographic tubing and washed with 5 ml pentane. The prewash was discarded and a measuring glass placed under the column. The sample extract (2 ml) was transferred quantitatively to the column, rinsing with 2 ml pentane, and eluted with 8 ml pentane. The eluate was concentrated to about 1 ml and made up with isooctane to 5.0 ml.

Gas chromatography

A Tracor 550 or Packard-Becker 429 gas chromatograph was used with a ^{63}Ni electron capture detector and a capillary injection system according to Grob and Grob[6]. The capillary column (25 m × 0.25 mm I.D.) was coated with CP-Sil 7, film thickness 0.4 μm (Chrompack, Middelburg, The Netherlands). Flow-rates: pressure-controlled mobile phase (He or H_2), linear velocity 30–40 cm/sec; make up (N_2 or Ar–CH_4), *ca.* 20 ml/min; detector purge, *ca.* 30 ml/min. Temperatures: injector, 210°C; detector, 300°C; column oven programmed from 100 to 220°C at 40°C/min, initial hold 4 min, final hold *ca.* 65 min, cooling time 4 min. Data system: Perkin-Elmer PEP 1 or Spectra-Physics 4000. Injection system: Precision Sampling 4200 or Varian 8000 autosampler. In case of the former, closing and opening of the splitter is remotely controlled by the three timers on the injection device as described elsewhere[7]. With the latter, the Spectra-Physics data system controls the timing of the splitter. Splitless injection was carried out automatically using 5 μl of the extract. Three minutes after injection the splitter opens automatically to flush the injector. The splitter closes 2 min before injecting the next sample.

Quantitation

To clarify the problems mentioned in the Introduction, the results for a cattle feed sample (Fig. 1) were obtained by comparison with a technical Aroclor 1260 mixture (Fig. 2). When peaks 6, 11, 17, 20, 22 and M (an unknown chlorobiphenyl) were used for calculation, the following results for each peak were obtained: 3.3; 7.5; 1.6; 3.1; 3.2 and 17.5 μg/kg in the cattle feed. However, when peaks 24, H, L (present

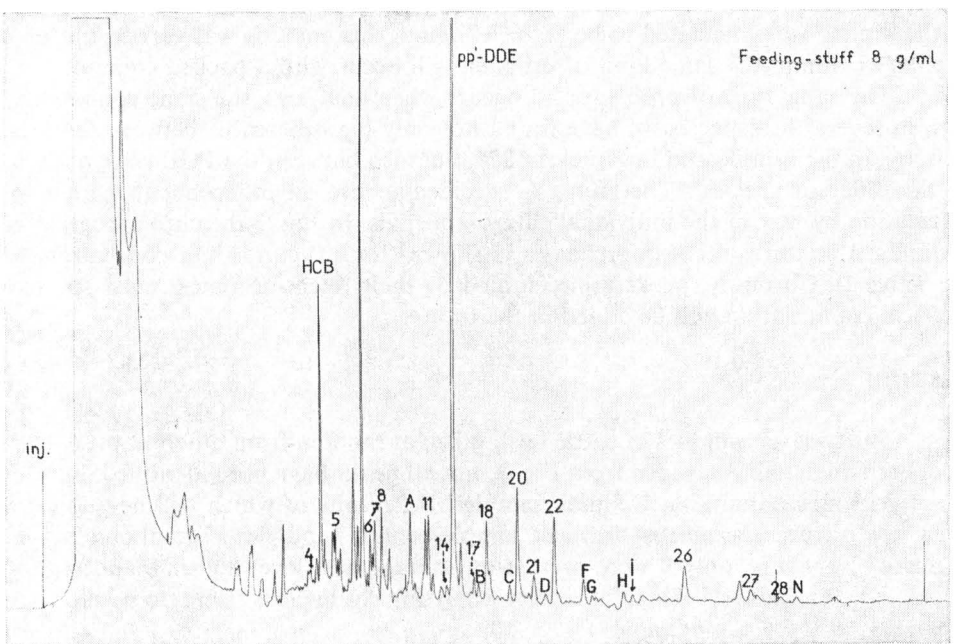


Fig. 1. Chromatogram of a cattle feed extract (8 g/ml) analysed on a CP-Sil 7 capillary (for structures see Table I).

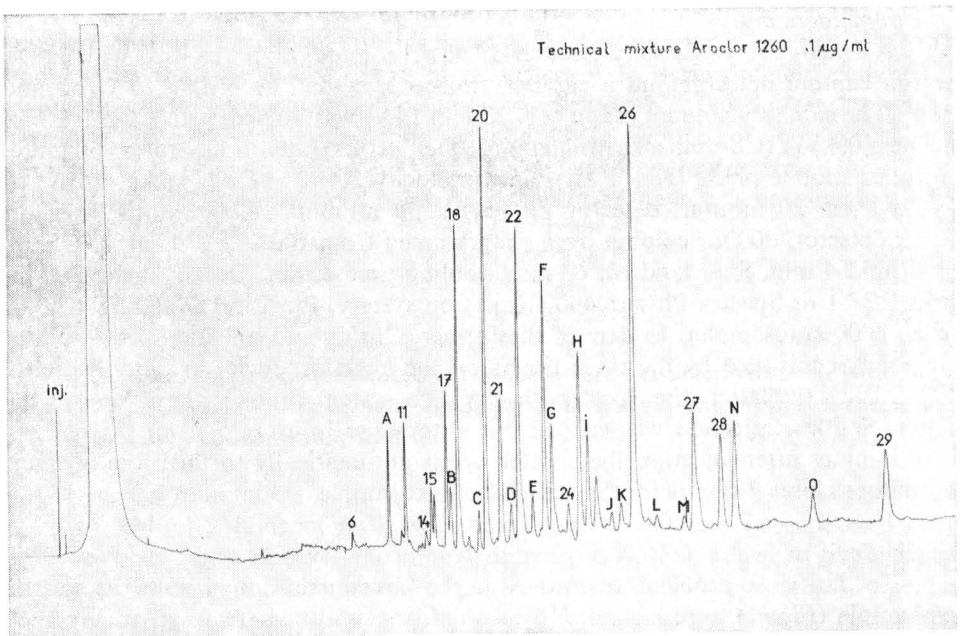


Fig. 2. Chromatogram of technical Aroclor 1260 mixture (0.1 µg/ml) analysed on a CP-Sil 7 capillary (for structures see Table I).

in the Aroclor 1260 mixture, but not in the sample) were employed the PCB content in the sample was calculated to be zero. Although this analysis was carried out on a capillary column, the same kind of problem will occur with a packed column.

The same type of problem also occurs when analyzing the same commodity: *e.g.*, in several fish species we have found not only big differences between the PCB patterns in the sample and in Aroclor 1254, but also between the PCB peak patterns of the different species[8]. Therefore, we decided to use an independent method of calculation by way of the individual chlorobiphenyls. In Fig. 3 the chromatogram of a standard mixture of chlorobiphenyls is shown (for structures and concentrations see Table I). Currently, peaks are identified by their retention times; mass spectrometrical confirmation will be added in the future.

RESULTS

Thirty-seven samples of cattle feed, taken at random from different producers, have been analysed. As is seen from Fig. 1, not all peaks have been identified, but this is not critical. In analysing 165 milk samples, the results of which will be published elsewhere[9], it was shown that in most samples only 17 individual compounds were detectable; of these, only 9 were present on average at a level higher than 2 µg/kg, calculated on a fat basis. Therefore, when analysing cattle feed, attention was focused especially on these compounds.

In cattle feed, the detection limit for polychlorinated biphenyls with two or more chlorine atoms in one phenyl ring is *ca.* 0.1 ppb. Only 4,4′-dichlorobiphenyl has

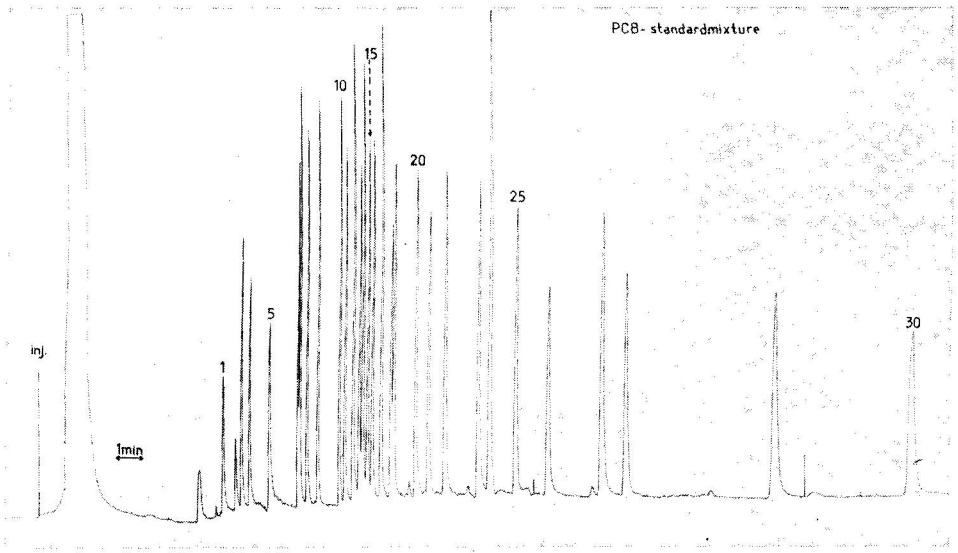


Fig. 3. Chromatogram of standard mixture of chlorinated biphenyls analysed on a CP-Sil 7 capillary (for structures see Table I).

TABLE I

COMPOSITION OF STANDARD MIXTURE OF PCB COMPOUNDS IN ISOOCTANE

Peak number	*Structure*	*Concn. (μg/ml)*	*Peak number*	*Structure*	*Concn. (μg/ml)*
1	2	2	16	2,4,5,2′,4′,6′	0.2
2	4	2	17	2,3,5,6,2′,5′	0.2
3	2,2′	2	18	2,3,6,2′,4′,5′	0.2
4	2,4	0.2	19	2,3,4,2′,4′,6′	0.2
5	4,4′	1	20	2,4,5,2′,4′,5′	0.2
6	2,5,2′,5′	0.2	21	2,3,4,5,2′,5′	0.2
7	2,4,2′,5′	0.2	22	2,3,4,2′,4′,5′	0.2
8	2,3,2′,5′	0.2	23	2,3,4,2′,3′,4′	0.2
9	2,4,6,2′,5′	0.2	24	2,3,4,5,6,2′,5′	0.2
10	2,4,6,2′,4′,6′	0.2	25	2,3,5,6,2′,3′,5′,6′	0.2
11	2,4,5,2′,5′	0.2	26	2,3,4,5,2′,4′,5′	0.2
12	2,4,6,3′,4′	0.2	27	2,3,4,5,2′,3′,4′	0.2
13	2,4,5,2′,3′	0.2	28	2,3,4,5,2′,3′,5′,6	0.2
14	2,3,4,2′,5′	0.2	29	2,3,4,5,2′,3′,4′,5′	0.2
15	2,3,6,2′,3′,6′	0.2	30	2,3,4,5,6,2′,3′,4′,5′	0.2

a detection limit of 1 ppb. In Table II the mean contents for the more important individual chlorobiphenyls in cattle feed are shown, together with the coefficient of variation (C.V.).

In another experiment, Aroclor 1260 was administered, together with the feed, to nine cows to establish the accumulation factor of individual chlorobiphenyls[10]. The accumulation factor for a given compound is defined as the ratio between the concentration in the milkfat and the concentration in the cattle feed on a dry matter

TABLE II

PERCENTAGE OF THE CONTAMINATION OF MILKFAT ACCOUNTED FOR BY CATTLE FEED

Compound	*Mean content of cattle feed*		*Accumulation factor* feed → milkfat*		*Theoretical content of milkfat*		*Actual mean content of milkfat*		*Contribution from the cattle feed contamination*	
	μg/kg	*C.V.*			*μg/kg*	*C.V.*	*μg/kg*	*C.V.*	*%*	*C.V.*
2,4	0.56	12	0.13	(19)	0.073	22	8.1	4	0.9	23
4,4′	3.50	19	0.14	(31)	0.49	36	22	10	2.2	38
2,5,2′,5′	0.28	14	1.7	(35)	0.48	38	2.1	2.6	23	38
2,3,2′,5′	0.35	25	1.2	(17)	0.42	30	0.9	4.3	47	31
2,4,5,2′,5′	0.24	16	0.44	(14)	0.11	21	3.1	3.5	3.4	22
2,3,5,6,2′,5′	0.12**	8	0.41	(3.2)	0.05	9	0.6	6.5	8.2	11
2,3,6,2′,4′,5′	0.32	11	0.46	(3.0)	0.15	11	2.5	5.3	5.9	13
2,4,5,2′,4′,5′	0.36	14	4.5	(3.2)	1.6	14	13.1	2.1	12	15
2,3,4,5,2′,5′	0.11**	6	0.48	(4.3)	0.05	7	0.6	8	8.8	11
2,3,4,2′,4′,5′	0.32	13	3.6	(3.6)	1.25	13	10.9	2.1	11	14
2,3,4,2′,3′,4′	0.13**	8	4.1	(4.2)	0.53	9	1.2	3.2	44	10
2,3,4,5,2′,4′,5′	0.15**	14	4.1	(3.0)	0.62	14	6.4	3.0	10	15
2,3,4,5,2′,3′,4′	0.10**	2.6	3.8	(3.0)	0.39	4	1.8	3.5	22	5

* C.V. value in parentheses.

** In most samples this compound is not detectable. This results in a mean content around the detection limit and causes a low C.V. Furthermore, this has consequences for the results in the last column. In fact the contribution will be lower than calculated.

basis. The values obtained from this experiment, together with the C.V., are shown in Table II, second column. By multiplying the mean content in feed by the accumulation factor[10], the expected mean content of a given chlorobiphenyl in milkfat is obtained (next column), assuming that the cattle feed is the only source for the contamination of milkfat with PCBs. However, the actual measured value for a compound in milkfat[9] (Table II) clearly shows that, in general, only a small part of the milkfat contamination with PCBs can be accounted for by contaminated cattle feed (last column). This conclusion is in agreement with the finding that there are only very small differences between the PCB contents of summer milk and winter milk[9].

ACKNOWLEDGEMENT

Some of the compounds used for quantitation were a generous gift from Professor C. A. Wachtmeister of Stockholm University (2,3,6,2′,4′,5′-hexachlorobiphenyl; 2,3,4,5,2′,4′,5′-heptachlorobiphenyl; 2,3,4,5,2′,3′,4′-heptachlorobiphenyl).

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DETERMINATION OF HYDROPHILIC VOLATILES IN GAS–AQUEOUS LIQUID SYSTEMS BY GROB'S CLOSED-LOOP STRIP/TRAP METHOD AND STANDARD-ADDITION CALIBRATION

J. NOVÁK*
Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, 61142 Brno (Czechoslovakia)
J. GOLIÁŠ
Institute of Fruit and Vegetable Technology, University of Agriculture, Brno, 69144 Lednice na Moravě (Czechoslovakia)
and
J. DROZD
Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, 61142 Brno (Czechoslovakia)

SUMMARY

The application of Grob's closed-circuit strip/trap technique to the determination of hydrophilic volatiles in aqueous matrices by the standard-addition method was investigated. Tenax GC was used as the trapping material. Both the conservation and equilibrium alternatives of trapping were assessed, employing butyl acetate and ethanol as model compounds, respectively. With both alternatives the experimentally found dependences of the amount of concentrate recovered from the trap on the total amount of the respective component in the system analyzed were fairly linear. The technique gives an extremely high selectivity of analysis. An application of the procedure to the headspace gas analysis of apple juice is demonstrated.

INTRODUCTION

Grob and co-workers[1–4] recently described an interesting variant of headspace gas analysis, in which volatile components of the condensed phase were trapped in a sorbent by pumping the headspace gas in a closed circuit via the trap and the condensed phase. Until now the method had been applied only to the determination of traces of hydrophobic compounds in water, the quantitation being carried out by means of a reference model system and internal standard; concentrations as low as 1–10 ng/l of hydrophobic organics in water were thus determined. This method is very suitable for such systems because of the low solubilities of hydrophobic compounds in water and the simplicity of the matrix material. With hydrophilic volatiles in aqueous media, however, the problem is more complicated. If, in addition, the matrix is a complex material of unknown composition, which almost always is the case with biological fluids, food products, beverages, etc., it is impossible to prepare an adequate reference model.

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The limitations on the use of the reference model method, combined, if necessary, with the internal-standard method, can be obviated by employing the standard-addition method, as with the latter the material under analysis serves as a reference matrix material in the calibration run. The aim of this paper is to show that the standard-addition method is a general means of calibration with the closed-loop strip/trap method, and to examine the applicability of the technique to the determination of hydrophilic volatiles in aqueous matrices. Model solutions of butyl acetate and ethanol and an apple juice were employed.

THEORETICAL

Considering the situation in the trap, Grob's method can be performed in two ways. In the early stage of the process, frontal chromatography occurs of the components being accumulated in the trap. If the volume of gas pumped through is such that it does not cause the zone of the least sorbed component to leave the trap, the proportions of the components deposited in the trap will be equal to their corresponding mean proportions in the gaseous phase. This case is regarded as conservation trapping. If the volume pumped though is large enough to bring the whole system into equilibrium then we have equilibrium trapping. In the latter case, the proportions of the components entrapped are given by the expression $c^*_{iG}\,(V_{Gt} + K_{SG}V_S)$, where c_{iG}, V_{Gt}, K_{SG} and V_S are, respectively, the final (equilibrium) concentration of solute i in the gaseous phase, the void volume within the trap, the actual sorbent–gas distribution constant of solute i and the volume of the sorbent in the trap.

From the analytical point of view it is important to know the relations between the total mass of component i in the entire system, *i.e.*, the quantity to be determined, W_i, and the mass of the component accumulated in the trap, W_{it}; the following relations were derived earlier[5] for conservation and equilibrium trapping, respectively

$$W_i = W_{it}\left[1 - \exp\left(-\frac{Ft}{V_G + K_{LG}V_L}\right)\right]^{-1} \tag{1}$$

$$W_i = W_{it}\left[\left(\frac{K_{SG}V_S}{V_{Gt} + K_{SG}V_S}\right)\left(\frac{V_G}{K_{SG}V_G} + \frac{K_{LG}V_L}{K_{SG}V_S} + 1\right)\right] \tag{2}$$

where K_{LG} is the liquid–gas distribution constant of solute i, V_L and V_G are the volumes of the liquid and gaseous phases, F is the volumetric gas flow-rate, as determined by the pump, and t is the stripping/trapping time. Both equations show that a linear dependence of W_i on W_{it} may be expected with both versions of trapping, provided the values of all the parameters in the bracketed terms are invariant. This qualification may well be true in the standard-addition method, as neither the distribution constants of the components of the system nor the volumes of its phases will change significantly upon adding a relatively small amount of the component to be determined. Under these conditions it is possible to employ either the single- or double-system procedure and calculate the results, respectively, by

$$W_i = (W_s - W_{it})/[(A'_i/A_i) - 1] \tag{3}$$

and/or

$$W_i = W_s/[(A'_i/A_i) - 1] \tag{4}$$

where W_s is the mass of the standard (component i) added to the system analyzed and A_i' and A_i are the chromatographic peak areas recorded for component i in the concentrates recovered from the trap after processing the systems with and without the added standard respectively.

EXPERIMENTAL

Apparatus

A schematic representation of the experimental set-up of the strip/trap circuit is shown in Fig. 1. The material to be analyzed was placed in a 350-ml serum bottle, through the septum of which passed two large-diameter injection needles, one of them (140 mm long) reaching into the liquid phase and the other (90 cm long) protruding only into the gaseous phase. The headspace gas was circulated by a modified MP 1 diaphragm pump (Chemoprojekt, Satalice at Prague, Czechoslovakia). The trap was a 120 × 3.5 mm I.D. glass tube with a layer of 51 mg of Tenax GC (Applied Science Labs., State College, PA, U.S.A.). In all instances the flow-rate of the headspace gas through the circuit was set to 100 ml/min by means of a fine needle valve and a flow meter, the latter then being removed from the circuit. The components of the circuit were connected by a copper capillary of 1 mm I.D. Both the serum bottle and the trap were kept at 42°C with the aid of a U-10 water ultrathermostat (Mechanik Prüfgeräte, Medingen, G.D.R.). Except for the experiments in which the time dependence of the amount of solute deposited in the trap was studied, the time of stripping/trapping was 5 min. There were no heaters or desiccators in the circuit.

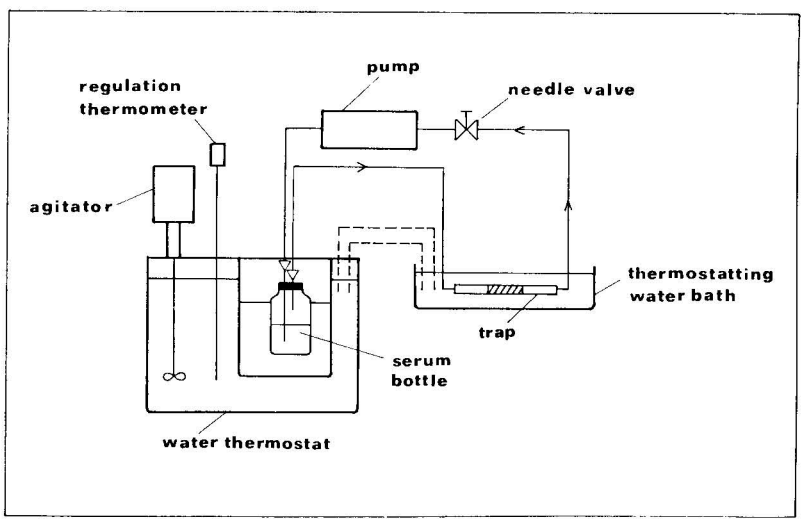


Fig. 1. Schematic representation of the experimental closed-loop strip/trap arrangement.

Preparation and processing of samples

Model solutions. A series of aqueous solutions of butyl acetate and ethanol in concentrations of 0.184–7.52 μg/ml and 0.0512–1.026 mg/ml, respectively, were prepared. In each experiment, 50 ml of the solution to be analyzed were pipetted into

the serum bottle and, after 6 min for stabilization of the temperatures of the bottle and the trap, the stripping/trapping procedure was carried out as described above.

Apple juice. A 30-ml volume of the juice from a Starkrimson apple squash was placed in the serum bottle and either 22.8 ml of distilled water (for runs without the addition of standard) or 20 ml of distilled water and 2.8 ml of a solution containing 39.8 μg/ml of butyl acetate and 18.4 mg/ml of ethanol (for runs with the standard) were added. Both materials were then processed as described above, employing the double-system method of analysis (*cf.*, eqn. 4).

Recovery and analysis of the concentrate from the trap

The substances deposited in the Tenax GC trap were recovered by thermal desorption and transferred into the gas chromatographic (GC) column using a stream of carrier gas. A three-way stopcock was connected by its two inlets in the carrier-gas line before the sample-inlet port of the gas chromatograph, and an oven, kept at 160°C, was situated above the septum. After disconnecting the trap from the strip/trap circuit, one end of it was provided with an injection needle and the other end was connected via a copper capillary to the free inlet of the stopcock. Then the trap was inserted in the oven and the needle was pushed half-way into the inlet-port septum, the carrier gas flow being conducted by the stopcock directly into the GC column. The heating period lasted 3 min, whereupon the needle was pushed through the septum and the carrier gas flow was diverted to pass via the hot trap into the GC column. This purging period lasted 2 min, after which the stopcock was returned to its initial position, and the trap was used for subsequent measurements.

A Chrom 41 gas chromatograph (Laboratory Instruments, Prague, Czechoslovakia) was employed, with a flame ionization detector and a 243 cm × 3 mm I.D. stainless-steel column packed with 10% (w/w) Carbowax 600 on Chromosorb W AW (80–100 mesh) (both components from Carlo Erba, Milan, Italy). The GC column was temperature programmed: after 2 min at 68°C the temperature was raised at 2°/min to 80°C and then at 4°/min to 120°C. The inlet port was kept at 110°C. The column-inlet excess pressure of the carrier gas (N_2) was 0.08 MPa at the initial temperature (68°C); the flow-rates of H_2 and air were 50 and 500 ml/min, respectively. Peak areas were determined from the graphical record of the chromatogram by the height × mid-width method.

RESULTS AND DISCUSSION

The choice of butyl acetate and ethanol as model compounds was made in order to provide conditions for simultaneous assessment of both the conservation and equilibrium versions of trapping. It is apparent from Fig. 2 that, after 5 min of stripping/trapping, ethanol is almost completely equilibrated while the frontal zone of butyl acetate has travelled about half the length of the Tenax trap. Fig. 2 also indicates that, with appropriate choice of the trapping sorbent, the method affords a very high selectivity of analysis, especially when performed in the equilibrium mode. Note that the peak areas of butyl acetate are about five times as large as those of ethanol, while the concentration of butyl acetate in the liquid phase is about three orders of magnitude lower than that of ethanol.

Figs. 3 and 4 show the experimentally determined dependences of the peak

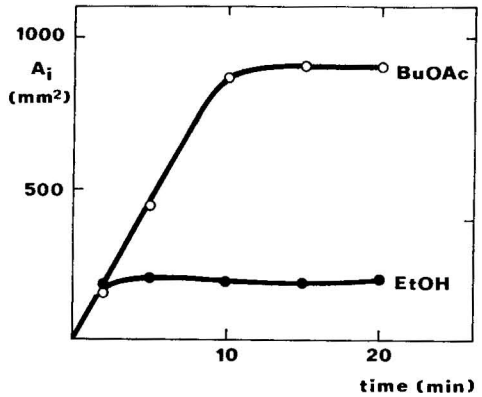


Fig. 2. Dependences of the peak areas of butyl acetate (BuOAc) and ethanol (EtOH) in the chromatogram of concentrate recovered from the trap on the time of stripping/trapping. The concentrations of butyl acetate and ethanol in the liquid phase were 7.1 μg/ml and 4.8 mg/ml, respectively.

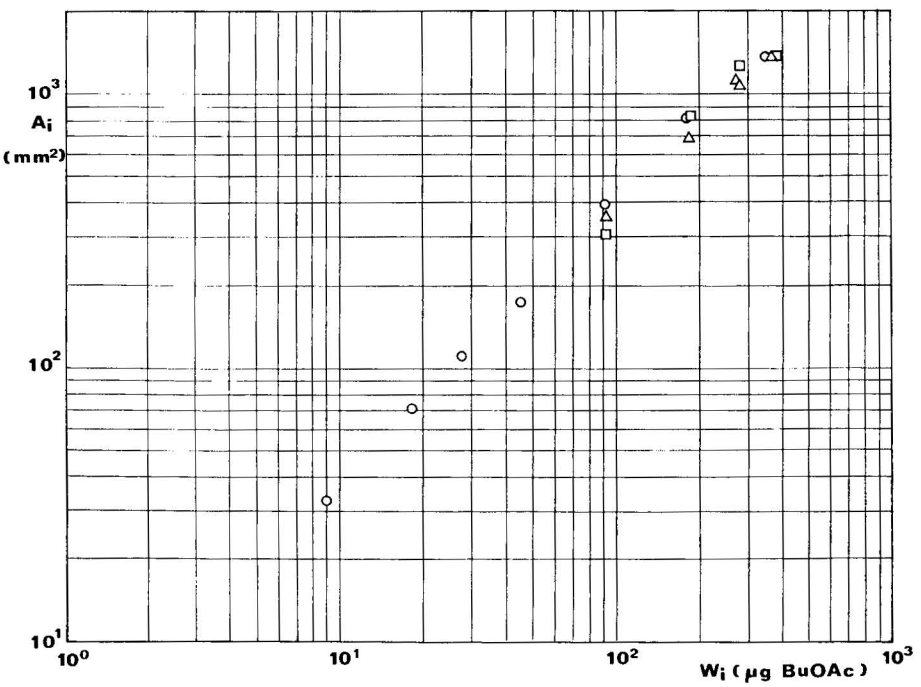


Fig. 3. Dependence of the peak area of butyl acetate in the chromatogram of concentrate recovered from the trap on the total initial mass of butyl acetate in the gas–liquid system analyzed.

areas of butyl acetate and ethanol on the total masses of these components in the system. The circles, squares and triangles refer to measurements carried out on different days. It is seen that both the conservation (butyl acetate) and equilibrium (ethanol) versions yield fairly linear dependences of A_i on W_i. As A_i in the above dependences actually represents W_{it}, the findings comply well with the predictions of eqns. 1 and 2.

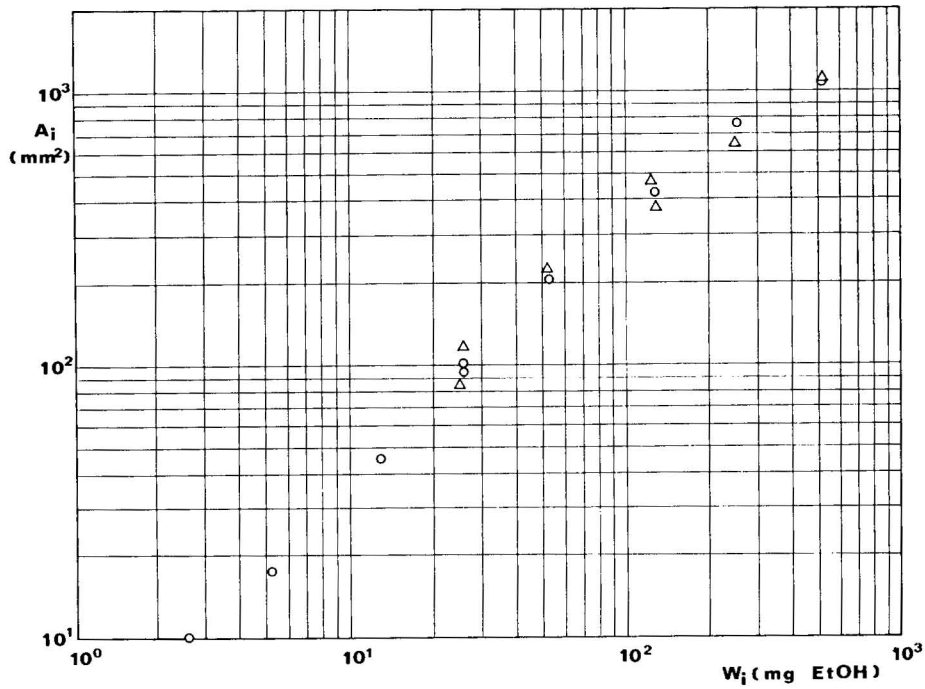


Fig. 4. Dependence of the peak area of ethanol in the chromatogram of concentrate recovered from the trap on the total initial mass of ethanol in the gas–liquid system analyzed.

It is possible to select from the actual data in Figs. 3 and 4 different pairs of $W_{i,j}$, $A_{i,j}$ and $W_{i,k}$, $A_{i,k}$ values with $k > j$, such that $W_{i,k} - W_{i,j} = W_s$, $A_{i,j} = A_i$ and $A_{i,k} = A'_i$, and employ eqn. 4 to calculate for given values of $W_{i,j}$ the corresponding "found" W_i values. Some results obtained in this way are shown in Table I; with butyl acetate and ethanol the average relative errors are 11.0 and 13.8%, respectively.

TABLE I

RESULTS OF THE DETERMINATION OF BUTYL ACETATE AND ETHANOL IN MODEL SYSTEMS

Δ = average | W_i (given) − W_i (found) |.

W_s	*Butyl acetate (μg in 50 ml of liquid phase)*			W_s	*Ethanol (mg in 50 ml of liquid phase)*		
	W_i *(given)*	W_i *(found)*	$100\,\Delta/W_i$ *(%)*		W_i *(given)*	W_i *(found)*	$100\,\Delta/W_i$ *(%)*
9.2	9.2	8.2		2.6	2.6	3.3	
9.2	18.4	16.1		7.7	5.1	5.0	
18.4	27.6	31.2	11.0	12.9	12.8	10.4	13.8
46.0	46.0	36.6		25.7	25.7	24.9	
92.1	92.1	85.4		77.1	51.4	57.5	
97.8	184.2	180.1					

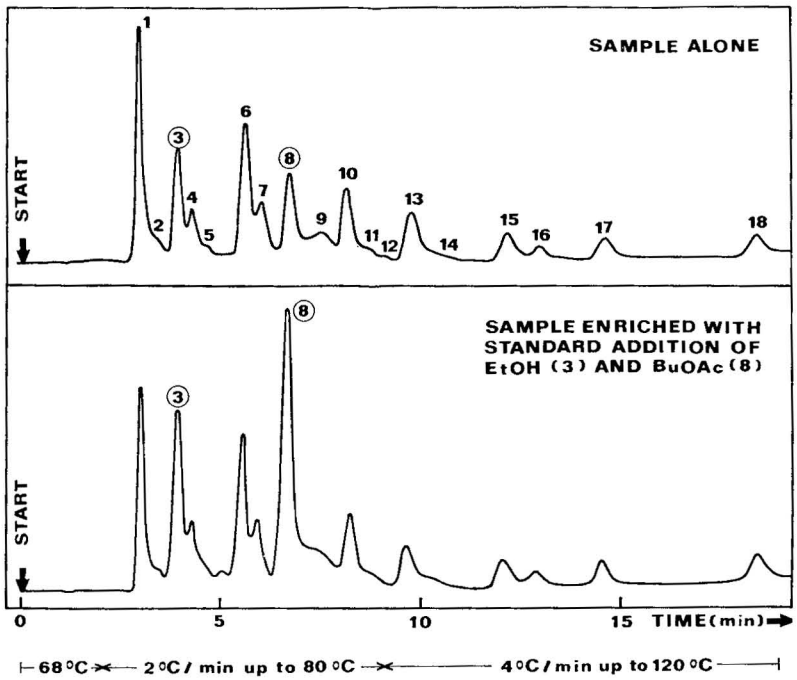


Fig. 5. Chromatograms of the concentrates recovered from the trap after processing a sample of Starkrimson apple juice alone and with addition of the standards butyl acetate and ethanol. Peaks: 1 = ethyl acetate; 2 = methanol; 3 = ethanol; 4 = propyl acetate; 5 = methyl butyrate; 6 = methyl isovalerate; 7 = propanol; 8 = butyl acetate; 9 = isobutanol; 10 = isoamyl acetate; 11 = ethyl valerate; 12 = butanol; 13 = amyl acetate; 14 = isoamyl alcohol; 15 = butyl butyrate; 16 = ethyl capronate; 17 = hexyl acetate; 18 = hexanol. Conditions: 10% Carbowax 600 on Chromosorb W AW; flame-ionization detection; electrometer sensitivity 10^{-11} A f.s.d.; sensitivity attenuation 1/50; recorder chart speed 2 cm/min.

Fig. 5 shows chromatograms obtained by processing a sample of Starkrimson apple juice without (upper chromatogram) and with the addition of the standards butyl acetate and ethanol. The results of five replicate determinations of the two components in the juice are summarized in Table II; the relative standard deviations of

TABLE II

RESULTS OF THE DETERMINATION OF BUTYL ACETATE AND ETHANOL IN STARKRIMSON APPLE JUICE

S = standard deviation of W_i/V_L; $\bar{X}$ = average W_i/V_L.

Butyl acetate			*Ethanol*		
W_s (μg)	W_i/V_L (μg/ml)	$100(S/\bar{X})$ (%)	W_s (mg)	W_i/V_L (mg/ml)	$100(S/\bar{X})$ (%)
111.4	1.22	10.6	51.4	2.62	14.0
	1.42			2.48	
	1.57			3.08	
	1.58			3.44	
	1.54			3.46	

the determination of butyl acetate and ethanol (Dean–Dixon method, five measurements) were 10.6 and 14.0%, respectively.

CONCLUSIONS

Grob's closed-loop strip/trap technique, combined with standard-addition calibration, gives fairly reliable results when applied to the determination of hydrophilic volatiles in complex materials having water as a major constituent. By appropriate choice of the sorbent in the trap and employing the equilibrium version of trapping, it is possible to control the degree of accumulation of different species of compound in the trap, thus enhancing markedly the selectivity of analysis.

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DETERMINATION OF BETAINE IN SUGAR AND WINE BY LIQUID CHROMATOGRAPHY

J. VIALLE* and M. KOLOSKY

Service Central d'Analyse, Echangeur de Solaize, B.P. 22, 69390 Vernaison (France)

and

J. L. ROCCA

Laboratoire de Chimie Analytique III, (E.R.A. C.N.R.S. 07.0474 M. Porthault) Université Claude Bernard Lyon I, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne (France)

SUMMARY

The analysis of betaine by high-performance liquid chromatography presents some difficulties owing to the quaternary ammonium moiety. This problem was investigated on polar stationary phases such as silica gel and amine-bonded silica gel. A method for titration is described and examples of betaine analysis in commercial beet sugars and wine are given. Pre-treatment of samples is required prior to the quantitative determination of betaine by liquid chromatographic analysis. Under particular conditions, it would be possible to detect or to determine the amount of sugar added to wines in order to increase their ethanol content.

INTRODUCTION

In France, as in some other countries, the addition of sugar to wines in order to increase their ethanol content is prohibited in some wine-growing areas, whereas in some other areas it is permitted in a given range. Saccharose is generally used for this purpose, and more especially refined saccharose from beet sugar. Because of the rapid hydrolysis of saccharose in wine a standard analysis of saccharose is not a convenient method for controlling either the fraudulent or the permitted addition of sugar to wines.

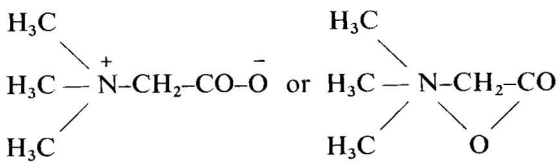
As betaine is a permanent component of beet sugar and as the initial content of betaine in wines is very low, the determination of betaine in wines could be a method determining the addition of saccharose.

Titration methods for the determination of betaine involved long and tedious chemical steps, and the first alternative chromatographic separations were carried out by thin-layer chromatography (TLC)[1–3]. Subsequently, by derivatization in order to obtain a volatile compound from the thermal degradation of the butyric ester of betaine, Dubois and Simand[4] made use of the great thermal sensitivity of gas chromatography (GC). Liquid chromatographic methods have been reported[5,6]. Because of the poor precision of TLC and the complexity of GC, which necessitates deriva-

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tization of the betaine, it was of interest to develop an improved method based on high-performance liquid chromatography (HPLC).

Betaine is the anhydride of carboxymethyltrimethylammonium hydroxide. It exists with either an open or an internally linked structure:



Owing to the presence of the trimethylamino group, it has the characteristics of a nitrogenous base. On the basis of its molecular structure, a method of separation similar to that used to separate amino acids[7,8] by ion-pair partition chromatography on a non-polar stationary phase was tried. However, the elution of betaine could not be properly controlled.

Considering the polarity of the molecule, we then performed the separation on polar stationary phases: firstly amino-bonded silica gel and secondly silica using chromatographic conditions derived from those used by Greving *et al.*[9] for the separation of quaternary ammonium compounds. Both methods are described in this paper.

EXPERIMENTAL

The pump was a Model 380 Chromatem (Touzart et Matignon, Vitry, France) and the detector an Optilab Multiref 902 interferometer (Touzart et Matignon), monitored in all experiments at a flow-rate of 1 cm^3/min.

Stainless-steel columns of length 15 or 5 cm and I.D. 4.7 mm were packed using the balanced density slurry method. According to the chromatographic method used, they were packed with silica (Partisil, 5 μm; Whatman, Ferrieres, France) or NH_2-bonded silica (LiChrosorb NH_2, 10 μm; Merck, Paris, France).

Samples were injected by means of a Rheodyne 7010 high-pressure valve (Touzart et Matignon) fitted with a 20-μl sample loop.

All solvents were of HPLC grade, purchased from various suppliers.

pH and potential values were measured by means of a Minisis millivoltmeter (Solea-Tacussel, Lyon, France), a working glass electrode (Solea-Tacussel) and a home-made Ag/AgCl/acetonitrile–methanol (97:3) saturated with KCl/$LiClO_4$ electrode as reference electrode. Using these electrodes, linearity of the calibration graph (potential *versus* pH) was verified with perchloric acid solutions in acetonitrile–methanol (97:3) solvent. The titration graphs for the various acids dissolved in the organic solvent were plotted using tetrabutylammonium hydroxide solutions as titrant. The potential of the half-neutralization point was related to the equivalent potential of the calibration graph and the corresponding pH value gave the pK_a value of the acid.

The extraction method was described many years ago by various authors[10,11]. However, the extraction conditions are defined here in order to ensure a good recovery of betaine. Pre-treatment of natural samples was carried out using glass columns of

length 15 cm and I.D. 1.8 cm: column I was packed with a cation-exchange resin (Dowex 50W-X8, 20–50 mesh) and column II with 1:2 (v/v) mixture of a cation-exchange resin (Amberlite IRC 50) and an anion-exchange resin (Dowex 1-X4, 20–50 mesh) (Rhône Alpes Chimie, Lyon, France).

For the determination of betaine in commercial refined sugar, a known volume of the solution to be analysed is, if necessary, purified on charcoal and evaporated in order to concentrate it to about 200 cm^3, the pH is adjusted to 3 with sulphuric acid and the solution is applied to column I (H^+ form). The column is then eluted with a large excess of distilled water in order to achieve a neutral pH of the eluate and to eliminate part of the sugar content of the sample. The betaine and amino compounds retained on the resin are then eluted using about 300 cm^3 of 4% ammonia solution and about 100 cm^3 of distilled water. The eluate is evaporated to dryness under vacuum and the residue is dissolved in 5 cm^3 of the mobile phase. This procedure was also used for the determination of betaine of commercial beet saccharose.

For the determination of betaine in wines, 1 l of wine is treated as described above on column I. The eluate is then concentrated under vacuum to about 100 cm^3 and adjusted to pH 7 by addition of 0.1 *N* hydrochloric acid. The solution is passed through column II. The betaine is eluted under these conditions whereas the main part of the amino compounds are retained. The column is then rinsed with 100 cm^3 of distilled water. The eluate is collected and evaporated to dryness under vacuum and the residue is dissolved in 5 cm^3 of the mobile phase.

RESULTS AND DISCUSSION

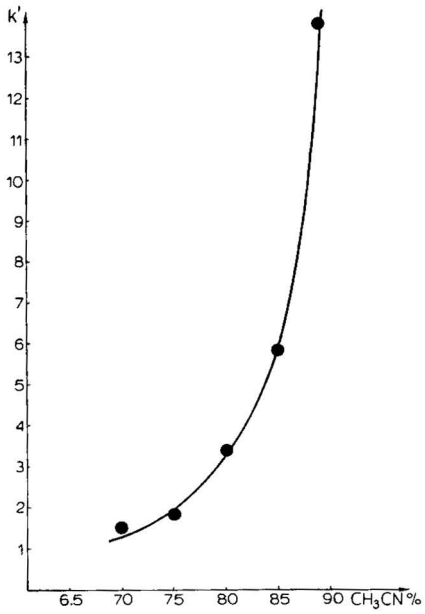
Chromatographic separation of betaine on amine-bonded silica

This stationary phase has already been used for sugar analysis[12] and has been mentioned for the chromatography of betaine in natural mixtures in the sugar beet industry[5]. The eluent is a binary mixture of acetonitrile and water. The dependence of the retention on the mobile phase composition is illustrated in Fig. 1. Fig. 2 shows the separation of a mixture of various oses and betaine. The peak corresponding to betaine was collected and identified by mass spectrometry. It should be noted that under the chromatographic conditions used, the betaine is eluted before the oses, which is different from the results obtained previously[5].

Chromatographic separation of betaine on silica

Quaternary ammonium compounds are eluted on silica by ion pairing with inorganic acid anions such as ClO_4^- and Br^-, and with methanol as the mobile phase[9]. With such a chromatographic system, the betaine shows a broad, tailing peak with a very long retention time. The addition of an inorganic acid allows the elution of the betaine as a sharp peak in a short period of time. However, the addition of counter ions does not improve the retention. In order to increase the retention time it was necessary to use a less polar mobile phase. Good results were obtained with acetonitrile–methanol (97:3).

A study of the elution with different acids showed that the acids that can lead to a good elution of betaine are weakly dissociated in the mobile phase. In order to establish their dissociation in such an organic medium (acetonitrile–methanol, 97:3),



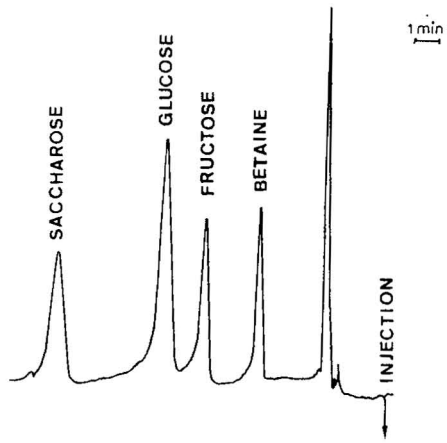


Fig. 1. Dependence of k' on the mobile phase composition. Column: 15 × 0.47 cm I.D. packed with NH_2-bonded silica; particle size, 10 μm. Eluent: acetonitrile–water.

Fig. 2. Separation of betaine and oses. Column: 15 × 0.47 cm I.D. packed with NH_2-bonded silica; particle size, 10 μm. Eluent: acetonitrile–water (75:25).

their pK_a values were measured by the classical potentiometric method. The measured pK_a values increase in the order 11 $\approx$ CH_3–COOH $\approx$ $CHCl_2$–COOH $<$ CCl_3–COOH $<$ HNO_3 $<$ HBr $<$ H_2SO_4 $=$ 3.2 $\ll$ $HClO_4$.

Perchloric acid, which is totally dissociated in the organic medium, elutes betaine without any retention whereas acetic acid is too weak to give a satisfactory elution.

Fig. 3 shows the influence of the acid concentration on the retention of betaine. Sulphuric and nitric acids have a similar influence and give the largest variation in k'. Trichloroacetic acid results in a broad and leading peak whereas hydrobromic acid, in spite of a suitable retention, corrodes parts of the chromatograph. Fig. 4 shows the variation of the retention with the composition of the mobile phase for sulphuric and nitric acids.

Detection level and betaine calibration graph

The use of the Optilab differential refractometer allows a considerable increase in sensitivity over other refractometric detectors. It was possible to detect down to a few tens of nanograms of betaine, and by optimizing the operating parameters it should be possible to lower the detection limit to a few nanograms.

Calibration graphs for betaine are drawn using the external calibration method. The linearity of the detector response (betaine peak height) *versus* concentration of betaine was determined over the entire working range, from 40 ng to 10 μg. The relative standard deviation of the calibration factor was less than 4%.

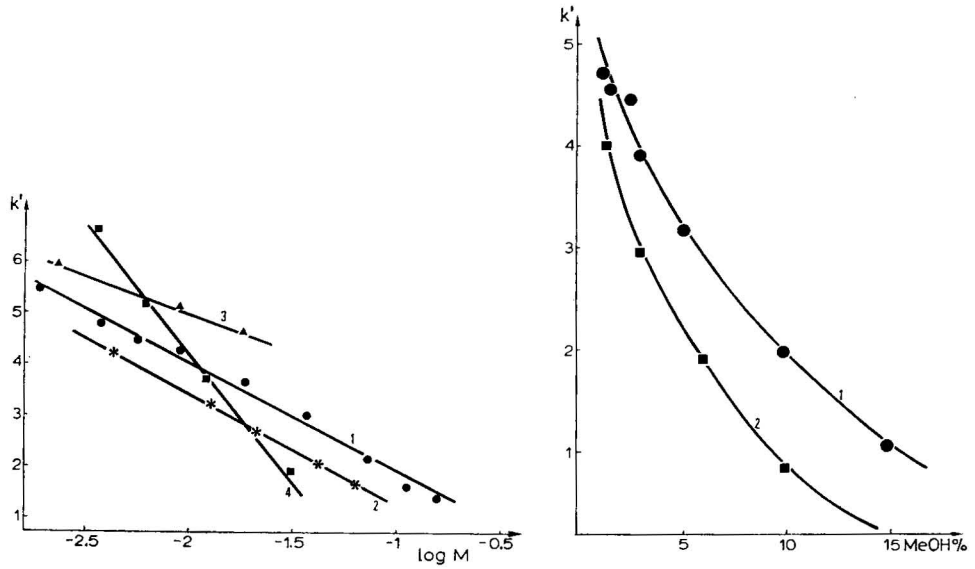


Fig. 3. Dependence of k' on acid concentration. Column: 5 × 0.47 cm I.D. packed with Partisil; particle size, 5 μm. Eluent: acetonitrile–methanol (97:3). 1 = H_2SO_4; 2 = HNO_3; 3 = HBr; 4 = CCl_3COOH.

Fig. 4. Dependence of k' on mobile phase composition. Column: 5 × 0.47 cm I.D. packed with Partisil; particle size, 5 μm. Eluent: acetonitrile containing different amounts of methanol. 1 = With H_2SO_4, $9.0 \cdot 10^{-3}$ M; 2 = with HNO_3, $5.6 \cdot 10^{-3}$ M.

Recovery and accuracy of betaine measurements

In order to test the recovery of betaine, standard solutions of betaine in water in the range 3–6 mg/l were prepared. They were treated according to the above procedures by using either column I or columns I + II. The chromatographic analysis of the residue was then performed on amino-bonded silica. The betaine was determined and the recoveries are given in Table I. In all experiments, the recovery of betaine was greater than 95%.

TABLE I

RECOVERY FOLLOWING PRE-TREATMENT OF BETAINE SOLUTIONS

Betaine content of standard solution (mg/l)	*Betaine recovered (mg/l)*	*Recovery (%)*	*Columns used for treatment (see text)*
6.0	5.7	95	I
6.0	5.9	98	I
3.0	2.9	97	I
3.4	3.5	103	I + II
6.05	6.1	101	I + II

Application to quantitative analysis of betaine in natural media

Betaine is a nitrogenous component in many natural media such as beet sugar, fruit juices and wine. Despite its low concentration in these complex mixtures, the

sensitivity of previously described analytical methods would theoretically allow the detection of betaine by the direct analysis of these liquids or solutions without any pre-treatment or pre- or post-column derivatization. However, these samples are extremely complex mixtures in which betaine is only a minor constituent and it will often be necessary to extract and concentrate the betaine prior to its chromatographic analysis in order to avoid interferences from compounds eluted with identical retention times.

Therefore, the samples are treated according to the described clean-up procedures and the betaine content of the residue is determined by chromatographic analysis using one of the previously described methods. As the residue of the samples studied is not always completely soluble in the acetonitrile–methanol mixture but is soluble in acetonitrile–water (75:25), the analyses were carried out with the latter mixture as the mobile phase and with amino-bonded silica as the stationary phase.

The determination of betaine in three different samples of commercial beet saccharose was performed. A 37-g amount of sugar was dissolved in 250 cm^3 of distilled water. The solutions were treated on column I and the chromatographic analysis gave values from 30 to 37 mg/kg of betaine in the sugar. This range is normal as it is well known that the betaine content of beet and consequently beet sugar widely varies from year to year and with the district of growth[5,13].

Three samples of wine were analysed. Sample A was a reference sample, *i.e.*, without any sugar added prior to the fermentation step. In samples B and C, the amounts of sugar had been added in order to increase the ethanol content of the wine. The betaine contents of samples A, B and C were found to be 0.8, 2.9 and 5.7 mg/l, respectively. The initial betaine content of wine depends on the wine-growing area and during the past few years there has been some controversy about this subject[14].

The particular kind of wine that we studied, as is shown with the analysis of sample A, contains a small amount of betaine and the addition of sugar to samples B and C is accompanied by increases in the betaine content of the wine of 2.1 and 4.9 mg/l, respectively. Hence, it is possible to determine the amount of added sugar in wine by analysing the corresponding commercial beet sugar used to increase the ethanol content of the wine.

CONCLUSION

The use of HPLC with amino-bonded silica as the stationary phase and water–acetonitrile as the mobile phase for the analysis of betaine in refined sugar and wine gave satisfactory precision and accuracy and the use of an interferometer as a detector could allow a considerable decrease in the detection level to a few nanograms of betaine.

The correlation between the betaine content in a particular wine and the corresponding amount of sugar added in order to increase the ethanol content is critical. Indeed, the betaine content of white beet sugar may widely vary[5] according to the year and the district of growth. Likewise, the betaine content of wine has given rise to controversy[14]. Thus, a knowledge of the betaine content can allow the determination of the amount of added sugar in a wine only if the amounts of betaine in the must on the one hand and in the sugar used to increase the ethanol content of the wine on the other are known.

The addition of sugar to wine is prohibited in some wine-growing areas, especially in France, but at present there is no means of measuring the amount added. In the absence of a rigorous method and without any blank allowing an exact determination, the method described here would allow fraudulent additions of sugar to wine to be disclosed when the betaine content in the wine is abnormally high. Obviously, many experiments would be necessary in order to determine precisely the betaine content in beet sugar and in wines according to the year and the growing area, and perhaps a statistical average content could be determined. Such a method could be a means of disclosing fraudulent additions of sugar in wine.

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APPLICATIONS IN ENVIRONMENTAL ANALYSIS

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GAS CHROMATOGRAPHIC INVESTIGATION OF RAW WASTEWATER FROM COAL GASIFICATION

S. K. GANGWAL
Research Triangle Institute, Research Triangle Park, NC 27709 (U.S.A.)

SUMMARY

Tars produced from a fixed-bed laboratory coal gasifier were characterized using two glass capillary gas chromatography systems. In one system, sulfur heterocyclics and polycyclic aromatic hydrocarbons were simultaneously monitored by splitting the column effluent to a sulfur specific dual-flame photometric detector and a flame ionization detector. In the other system, nitrogen heterocyclics and polycyclic aromatic hydrocarbons were simultaneously monitored using a nitrogen-specific detector and a flame ionization detector. Typical dual-detector profiles for tars produced from gasification of a bituminous coal and a lignite are presented and discussed.

INTRODUCTION

The shortage of crude oil and increased foreign dependence has triggered the development of synthetic fuel production processes in the United States. Coal conversion to clean liquid and gaseous fuels is receiving the maximum attention because of vast U.S. coal reserves. Side by side with the development of coal gasification and liquefaction processes, assessment of environmental problems associated with these processes is also receiving major emphasis.

The Research Triangle Institute (RTI) has undertaken a five-year study to determine pollution problems associated with coal gasification. Several coals ranging in rank from lignite to anthracite have been gasified in a laboratory reactor under a variety of conditions. Both coal type and operating conditions have been shown to influence pollutant production. Details of the reactor and the associated sampling system are available in various reports[1–4]. A variety of analytical techniques[1] including gas chromatography (GC), high-performance liquid chromatography (HPLC), GC–mass spectrometry (MS), atomic absorption spectroscopy, Fourier transform infrated spectroscopy, ion chromatography, and wet chemical procedures have been used to characterize the gaseous, liquid, and solid effluent streams from the coal gasifier.

In this paper, GC characterization of the tar portion of the liquid effluent stream that is produced from fixed-bed gasification is described. Typically, one gram of the liquid stream is produced per gram of coal gasified, of which *ca.* 2–4% is tar and the rest aqueous. Simulated GC distillation shows that this tar boils between

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150 and 650°C[1] and has a number-average molecular weight of *ca.* 180 to 200, as measured by vapor phase osmometry[5]. In the interest of space, only typical results are presented, with emphasis placed on experimental details. Detailed results are available elsewhere[2].

EXPERIMENTAL

Preliminary characterization of wastewater consisted of first separating the aqueous portion and tar by filtration. The aqueous portion was then exhaustively extracted using methylene chloride. At this point, mainly phenolics remained in the aqueous portion and were characterized by a reversed-phase HPLC technique[1]. The tar was subjected to a solvent partitioning scheme similar to one used by Novotny *et al.*[6]. This scheme separated the tar into five fractions including acids, bases, polar neutrals, non-polar neutrals, and polynuclear aromatic hydrocarbons (PAHs). Qualitative characterization of these fractions was carried out by GC–MS. The entire procedure was rather tedious; however, enough background information was now available about the nature of the tar sample so that a more direct and rapid GC technique could be used. This technique was based on high-resolution glass capillary GC and was initially only applied for the quantitation of the polynuclear aromatic fraction of the tar[1]. Recently, the technique has been extended for simultaneous detection of specific heterocyclic compounds and PAHs in tar directly; the technique is described below.

Two Varian 3700 glass capillary GC systems are used, one consisting of a flame ionization detector (FID) and a flame photometric detector (FPD), and the other consisting of a FID and a nitrogen–phosphorus detector (NPD). The systems are all-glass from the injector to the detector. Wall-coated OV-101 capillary columns are used in each system, and the column effluent is split 50:50 by means of a zero-dead volume splitter. All sample-wetted parts are made of glass. Helium is used as carrier gas as well as makeup gas. The FPD for specific detection of sulfur species is of a novel design consisting of two hydrogen-rich flames. The first flame is used to destroy the organic matrix so that a more uniform S-emission response can be produced from the second flame[7]. This reduces the problem of organic quenching of the response. The NPD is specific to organic nitrogen with a specificity of greater than 1000:1 for nitrogen *versus* carbon. The principle, based on an electrically heated alkali bead, is described in detail by Patterson and Howe[8]. These authors claim longer bead life and more stable response than for beads used in the past.

The tars are dissolved in methylene chloride at concentrations of *ca.* 15–20 μg/μl, and a 2–3-μl sample is injected following the "splitless technique" outlined by Grob and Grob[9]. Identification of peaks is carried out by combining information from GC–MS results, boiling point, and retention time of pure standards. Chromatographic conditions and typical results are described below.

RESULTS AND DISCUSSION

In Fig. 1, dual-detector FID–FPD chromatograms for tars from gasification of an Illinois No. 6 bituminous coal and a lignite coal are shown. In Fig. 2, the corresponding dual-detector FID–NPD chromatograms are shown. Compound identifica-

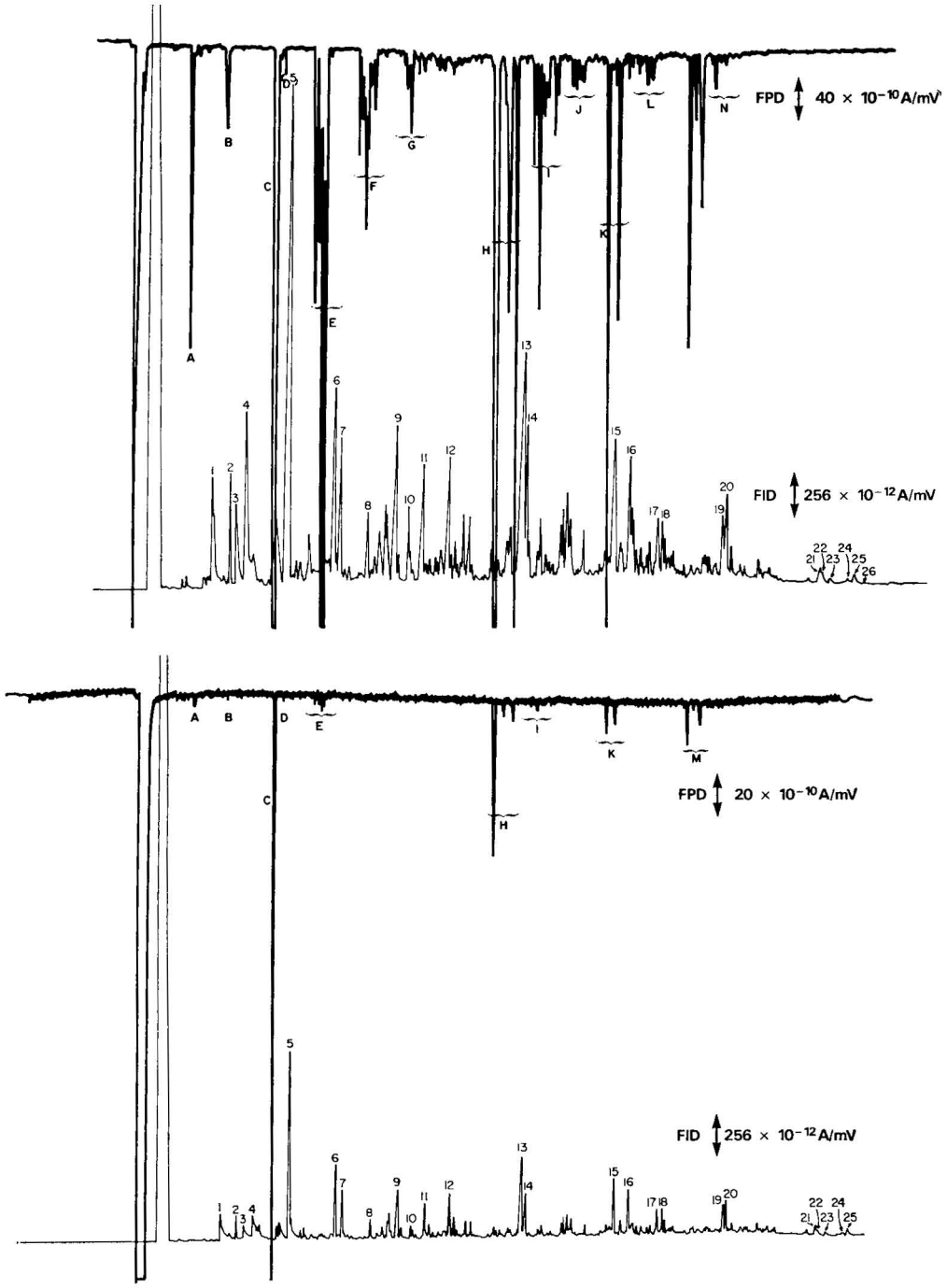


Fig. 1. Dual-detector FID–FPD chromatograms of Illinois No. 6 tar, 46 μg in methylene chloride (top) and N. Dakota lignite tar, 35 μg in methylene chloride (bottom). Conditions: instrument, Varian 3700 gas chromatograph; injector temperature, 250°C; detector temperature, 280°C; "Grob" splitless injection; column, 30 m × 0.25 mm OV-101 wall-coated open tubular; pressure, 21 p.s.i.; temperature, 70°C (1 min), programmed at 4°C/min to 260°C; FID–FPD split, 1:1.

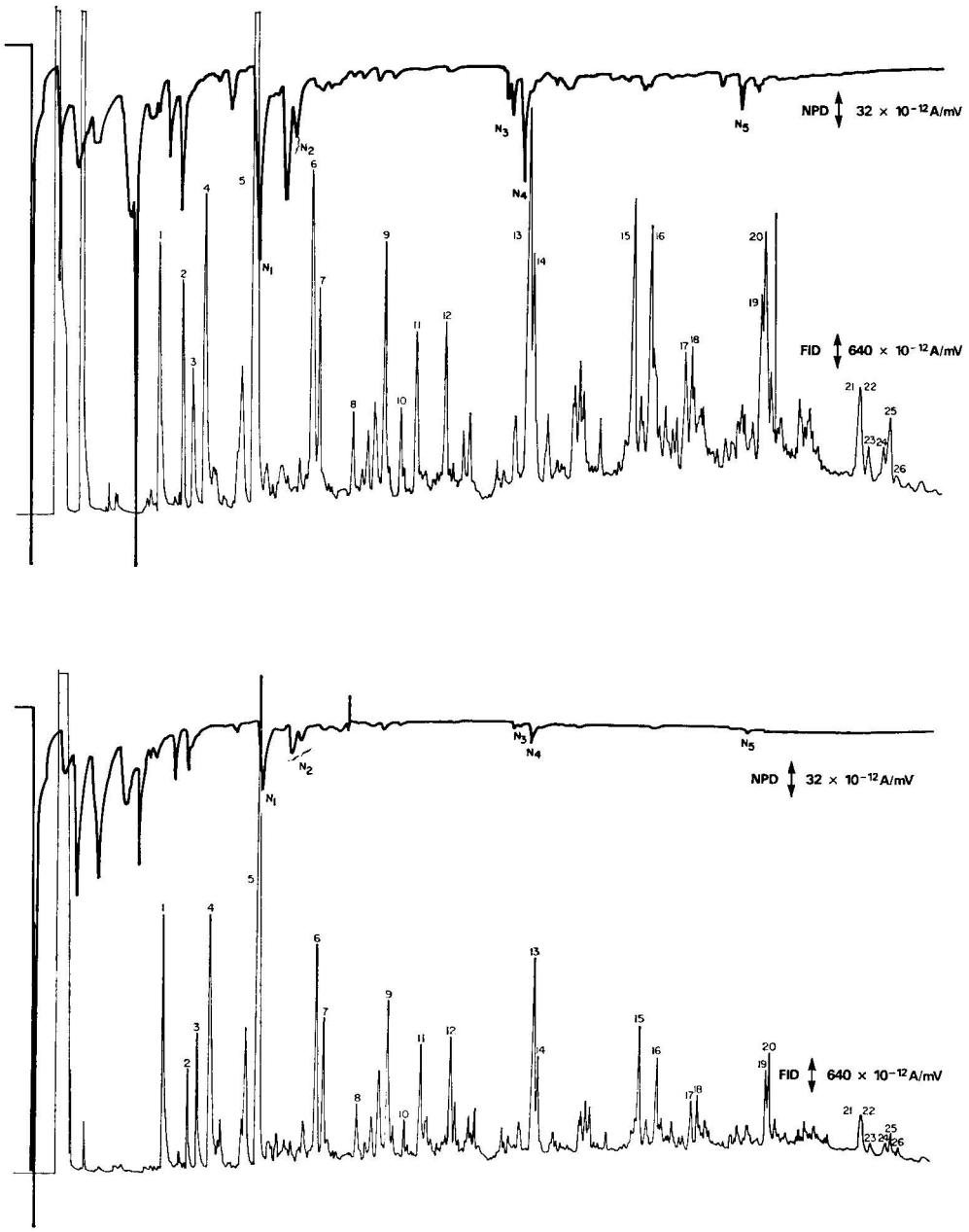


Fig. 2. Dual-detector FID–NPD chromatograms of Illinois No. 6 tar, 50 μg in methylene chloride (top) and N. Dakota lignite tar, 60 μg in methylene chloride. Conditions: column, 20 m × 0.25 mm OV-101 wall-coated open tubular; pressure, 16 p.s.i.; temperature, 50°C (1 min), programmed at 4°C/min to 260°C; FID–NPD split, 1:1, other conditions as in Fig. 1.

TABLE I

COMPOUND IDENTIFICATION

FID	*FPD*	*NPD*
1 Phenol	A Benzenethiol	N_1 Quinoline
2 Indene	B Toluenethiol or benzyl mercaptan (tent.)	N_2 Methylquinolines
3 O-Cresol	C Benzothiophene	N_3 Benzoquinolines
4 *m*- and *p*-Cresols	D Dihydrobenzothiophene (tent.)	N_4 Acridine
5 Naphthalene	E Methyl benzothiophenes	N_5 Benzoacridine (tent.)
6 2-Methylnaphthalene	F C_2 and dimethyl benzothiophenes	
7 1-Methylnaphthalene	G C_3 and trimethyl benzothiophenes	
8 Biphenyl	H Dibenzothiophene and naphthothiophene	
9 Acenaphthylene	I Methyl dibenzo- and methyl naphtho-thiophenes	
10 Acenaphthene		
11 Dibenzofuran	J C_2-dibenzothiophenes	
12 Fluorene	K Fluoranthene and pyrene sulfur derivatives (tent.)	
13 Phenanthrene		
14 Anthracene	L Methyl substituted (K) (tent.)	
15 Fluoranthene	M Naphthobenzothiophenes	
16 Pyrene	N Methyl naphthobenzothiophenes	
17 Benzo[*a*]fluorene		
18 Benzo[*b*]fluorene		
19 Benz[*a*]anthracene		
20 Chrysene + triphenylene		
21 Benzo[*b*]fluoranthene		
22 Benzo[*k*[fluoranthene		
23 Benzo[*f*]fluoranthene		
24 Benzo[*e*]pyrene		
25 Benzo[*a*]pyrene		
26 Perylene		

tions are presented in Table I. The Illinois No. 6 coal contained 3% sulfur and 1.38% nitrogen, and the lignite contained 0.56% sulfur and 0.73% nitrogen. The relative intensities of the nitrogen and sulfur profiles of the two tars reflects these differences. The profiles are generally similar qualitatively. The majority of the nitrogen heterocyclics are smaller than quinoline. These molecules are presumably (as indicated by GC–MS) mainly pyridine and methyl-, dimethyl-, and ethyl-substituted pyridines. The other major nitrogen peaks are quinoline, acridine, benzoacridine and methyl derivatives of these compounds. Sulfur compounds are mainly thiophenic, ranging from benzothiophene to naphthobenzothiophene. Small amounts of benzenethiol and toluenethiol are also present. A striking feature of the results is the absence of

TABLE II

COMPARISON OF PAH CONCENTRATIONS IN RAW WASTEWATER WITH THEIR CORRESPONDING $(DMEG)_w$ VALUES

PAH	*Concentration (μg/l)*	*$(DMEG)_w$ (μg/l)*
Phenanthrene	$8.76 \cdot 10^5$	$2.39 \cdot 10^4$
Benz[*a*]anthracene	$1.00 \cdot 10^6$	$6.72 \cdot 10^2$
Pyrene	$2.05 \cdot 10^5$	$3.45 \cdot 10^6$
Benzo[*a*]pyrene	$7.04 \cdot 10^4$	$3.00 \cdot 10^{-1}$
Dibenzo[*a*,*h*]anthracene	$5.50 \cdot 10^4$	1.40

aromatic compounds with long aliphatic side-chains, presumably because of their absence from the original coal structure. PAHs range from two to five rings, although some six-ring compounds have also been observed[1]. The PAHs are of major importance because of their adverse health effect and biological activity. In fact, sixteen PAHs ranging from naphthalene to benzo[*ghi*]perylene are on the priority pollutant list issued by the U.S. Environmental Protection Agency[10]. The concentrations of various PAHs in raw wastewater are compared with their corresponding $(DMEG)_w$ values in Table II. Here $(DMEG)_w$ is defined as the maximum concentration of the compound in an aqueous stream which is not expected to have adverse health effects[11]. The environmental severity of the wastewater stream is reflected by the fact that the concentrations for various PAHs far exceed, or are near to, their $(DMEG)_w$ value.

CONCLUSIONS

Dual detection with FID–FPD and FID–NPD of a capillary column effluent is feasible and represents a rapid method of characterization of tars produced from coal gasification. Background information from GC–MS on the nature of tars supplements identification by specific detection and retention time.

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CALIBRATION METHOD FOR THE GAS CHROMATOGRAPHIC ANALYSIS OF HALOCARBONS IN ATMOSPHERIC SAMPLES USING PERMEATION TUBES AND AN ELECTRON-CAPTURE DETECTOR

G. CRESCENTINI, F. MANGANI, A. R. MASTROGIACOMO and F. BRUNER*
Istituto di Scienze Chimiche, Università di Urbino, Piazza Rinascimento 6, 61029 Urbino (Italy)

SUMMARY

A method for calibrating the electron-capture detector for the analysis of halocarbons in atmospheric samples is presented. Permeation tubes are used as primary standards. The precision is evaluated under different operating conditions, and a standard deviation of 5% was the optimal. The difficulty of determining halocarbons present at very different concentrations is discussed.

INTRODUCTION

The accurate determination of halocarbons in the atmosphere became extremely important after Molina and Rowland[1] had concluded that these compounds destroy the stratospheric ozone layer. Their hypothesis is based on the assumption that halocarbons of anthropogenic origin (in particular CCl_2F_2 and CCl_3F) accumulate in the troposphere and reach the stratosphere after several years. An accuracy of a few percent is then necessary in order to follow the increase in atmospheric concentration with time, or to reveal the presence of any possible tropospheric sink. However, at present, the discrepancy among inter-laboratory comparative results is of the order of 30% in many instances[2].

This discrepancy can be attributed to the lack of reliable sources of known concentrations of halocarbons rather than to the well known difficulties connected with the routine use of the electron-capture detector (ECD). In this paper, a calibration method based on the coupling of permeation tubes with the ECD is described.

Permeation tubes, introduced by O'Keeffe and Ortman[3], have been employed as primary standards for the analysis of a number of atmospheric polluntants[4–9], and were first used by Singh and co-workers for the measurement of halocarbons in air samples[10,11]. The advantage of the use of permeation tubes over other calibration methods, such as static or dynamic dilution, is in that calibration is referred directly to gravimetric measurements, independent of the operator and ancillary techniques used.

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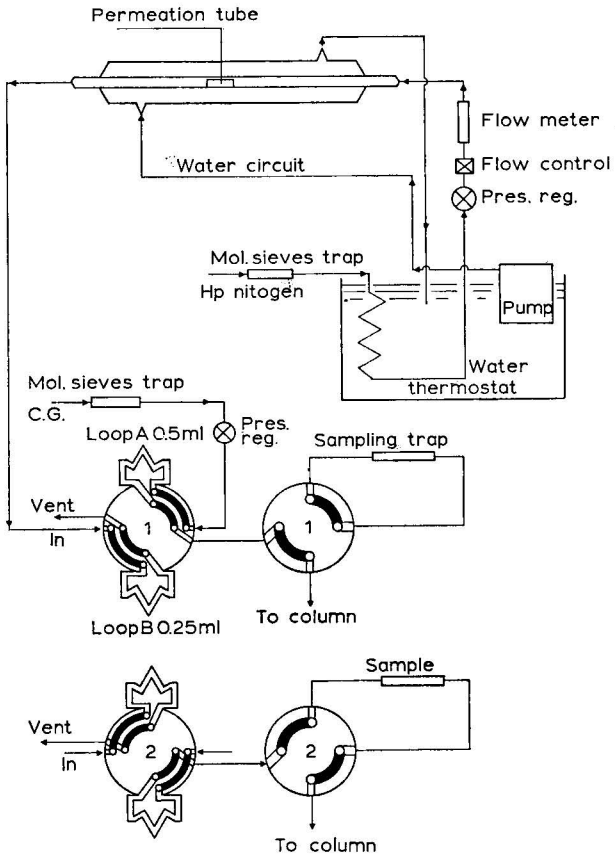


Fig. 1. Schematic diagram of the apparatus for the calibration and analysis of halocarbons in atmospheric samples. C.G. = Carrier gas.

EXPERIMENTAL

The apparatus used, shown schematically in Fig. 1, is designed to inject the calibration mixture following the same procedure as used for air sample injection, *i.e.*, trapping at −78°C on an adsorbent (Carbopack B) and injection by the heat-stripping technique[12].

A water thermostat kept at 25 ± 0.1°C feeds a double-walled glass holder, in the inner space of which are placed permeation tubes. High-purity nitrogen, further purified through a molecular sieves trap kept at −78°C (dry-ice and acetone slush) flows continously through the glass holder. The flow-rate can be varied in the range 10–150 ml/min. The two sample loops were calibrated by mercury weighing and allow the injection of 0.25 and 0.50 ml.

The calibration mixture is sampled through one of the two loops and the contents of the loop can be transferred into the trap kept at −78°C. This step can be repeated as many times as is required to obtain different amounts of various halocarbons according to the range of concentration needed. The contents of the trap are

then injected into the column through the zero-volume valve system (Valco Model 2-2274, 2-2278) by means of the heat-stripping technique. Alternatively, the contents of the loop can be injected directly into the column. When real samples have to be injected into the column, the trap used for calibration is replaced by the trap where air was sampled. In this way, the same procedure is followed for both calibration and the actual halocarbon mixture.

The gas chromatographic (GC) system is also equipped with a standard syringe injector. A Dani 3600 gas chromatograph equipped with a ^{63}Ni frequency-modulated ECD (Dani 68/30) is used. The detector is kept at 220°C and fed with an additional carrier gas flow-rate of 30 ml/min. High-purity nitrogen further purified as described above is used as the carrier gas.

The GC column, a 3 m × 2 mm I.D. glass column packed with Carbopack B (80–100 mesh), coated with 0.5% SP 1000 (Supelco, Bellefonte, PA, U.S.A.), and with the other conditions used have been described previously[12]. Only the temperature programme was modified and simplified, without affecting the overall separation (5 min at 40°C and then programmed at 6°C/min up to 230°C).

All permeation tubes are made of FEP Teflon (DuPont) and are sealed with stainless-steel spheres or glass plugs. Their dimensions and wall thickness (0.3, 0.9 and 3.0 mm) vary according to the permeation rate. The permeation tubes were calibrated by weighing them twice a week. Two to six weeks are required before a constant permeation rate is reached.

RESULTS AND DISCUSSION

Calibration of permeation tubes

The loss of weight with time for permeation tubes containing different halocarbons is shown in Fig. 2. The experimental points reported are those obtained only after linearity has been obtained. It can be seen that at least 15 days are needed after preparation before the graph becomes linear. However, after linearity has been reached, the permeation tubes behave as expected. The errors involved in the measurements do not exceed the thickness of the points on the graph. The lifetime of permeation tubes depends on the permeation rates of the individual halocarbons, which differ according to the nature of the compounds and the geometrical characteristics of the tube, such as thickness and surface area. In this way the slope of the graphs is not related to the physical characteristics of the individual compounds but represents the effect of all of the parameters involved.

Calibration graphs

Calibration graphs for various halocarbons are shown in Fig. 3. The most important feature of the graphs is that the more sensitive the detector, the less linear is its response to increasing concentration. This is perhaps the main disadvantage in the use of the ECD in the analysis of atmospheric samples of halcarbons. In fact, owing to the differences in the response factors and the mixing ratios, it is almost impossible to determine different halocarbons within the same sample. The example offered by F-11 and F-21 is one of best to illustrate the situation. F-11 is one of the most abundant halocarbons and the ECD is extremely sensitive to it, whereas F-21 is often present in ultra-trace concentrations and the detector shows a response that

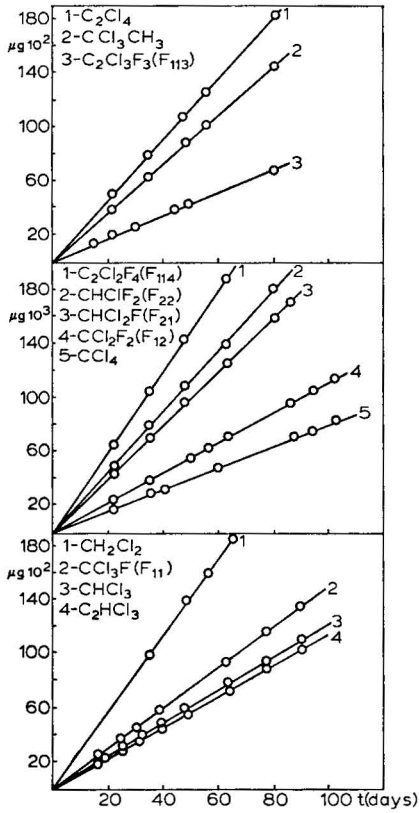


Fig. 2. Loss of weight with time of permeation tubes for various halocarbons.

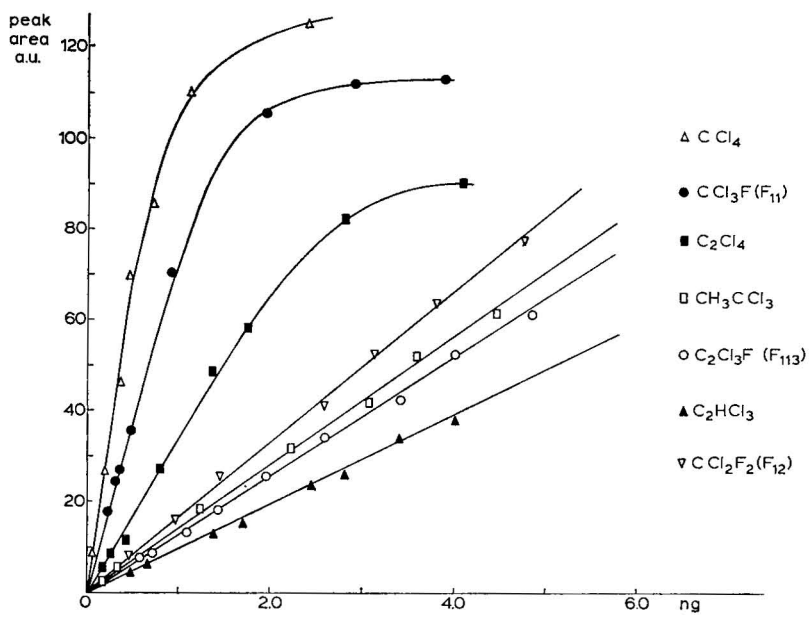


Fig. 3. Calibration graphs for several halocarbons.

is about 70 times lower. This means that unless very peculiar concentrations of the two compounds occur, the difference in peak areas between F-11 and F-21 is of the order of $7 \cdot 10^3$. This difference makes impossible the simultaneous determination of the two halocarbons in the same sample.

In Table I the volume range for a good quantitative evaluation of several halocarbons determined by the detector response, atmospheric abundance and linearity of calibration graphs is reported. Of course, the data are necessarily approximate and refer to the analytical procedure and detector used in our laboratory, but they should be of general interest. In order to have a linear response for CCl_4 the sample volume should not exceed 1.0 l. With such a volume of air F-11, F-12, F-113, 1,1,1-trichloroethane and tetrachloroethylene can be determined. In order to determine F-21, trichloroethylene and other possible minor components a larger volume of air should be sampled.

TABLE I

MINIMAL AND MAXIMAL SAMPLE VOLUMES FOR THE ANALYSIS OF SOME HALOCARBONS DIFFERING IN ATMOSPHERIC MIXING RATIOS AND DETECTOR RESPONSES

Compound	*Average concentration range in the atmosphere (ppt)*	*Maximal sample volume for linearity (l)*	*Minimal sample volume for detection (l)*
F-12 (CCl_2F_2)	200–1000	10–2	$8 \cdot 10^{-3}$–$1.6 \cdot 10^{-3}$
F-11 (CCl_3F)	70–300	3–0.7	$6 \cdot 10^{-4}$–$1.4 \cdot 10^{-4}$
CCl_4	100–180	1–0.6	$4 \cdot 10^{-4}$–$2.2 \cdot 10^{-4}$
$C_2H_3Cl_3$	80–130	14–9	$8 \cdot 10^{-3}$–$5 \cdot 10^{-3}$
C_2HCl_3	5–20	100–56	1.7–0.4
F-21 ($CHCl_2F$)	0.5–90	100–22	18–$9 \cdot 10^{-2}$
C_2Cl_4	20–40	17–8	$2 \cdot 10^{-3}$–$9 \cdot 10^{-3}$
F-113 ($C_2Cl_3F_3$)	5–20	100–39	$6 \cdot 10^{-2}$–$1.5 \cdot 10^{-2}$

Reproducibility of calibration

Calibration was carried out with particular care for F-21, because we are particularly interested in measuring this compound with a high degree of accuracy. Two different calibration graphs were constructed by (a) conditioning the ECD and the entire GC system for 1 week, then establishing the calibration graph in one working day; (b) obtaining calibration points on different days, with the detector turned off and the additional carrier gas flow to the detector stopped between different calibration experiments, the column flow-rate being maintained instead. Calibration runs were executed 2 h after the entire system had been re-set.

The obvious aim of such double procedure was to show the influence of the operating conditions has they occur in a typical monitoring programme.

In Fig. 4 the results obtained with procedure (a) are shown for F-21. The errors for the single points are shown by vertical bars. The errors on the abscissa are assumed to be smaller than the thickness of the points on the graph. An average standard deviation of 5 % along the entire graph was found. In Fig. 4 the top abscissa indicates the actual amount of F-21 injected (nanograms) and the lower abscissa the corre-

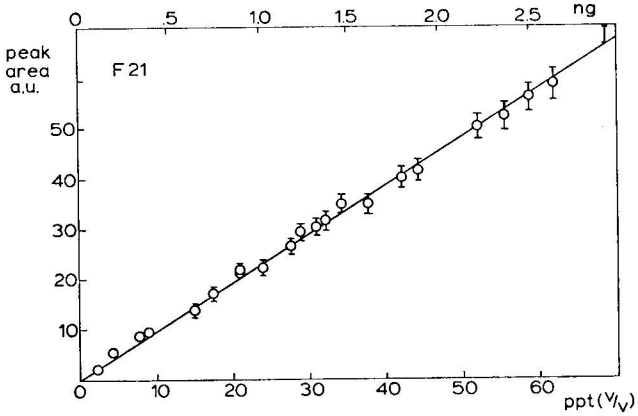


Fig. 4. Calibration for F-21 carried out in one working day.

sponding concentration (ppt*) referred to a 10-l air sample. This is usually the volume of air trapped in our sampling system for the analysis of F-21.

Fig. 5 shows the data points obtained during a calibration programme during 2 weeks. As expected, the data are more scattered than in Fig. 4, with a standard deviation of 20%. It is our opinion that such a value is more significant for checking the precision of the analytical method. However, the conditions found with procedure (a) are certainly met if a calibration run is made immediately before and after the elution of the actual air sample. It should be noted that the calibration run is made by injecting the standard mixture and eluting it following the temperature programme for the actual air sample.

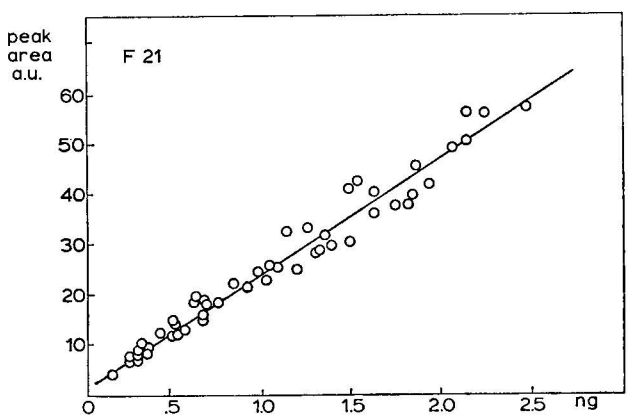


Fig. 5. Calibration for F-21 carried out in 1 week turning on and off the detector.

The most interesting feature of Figs. 4 and 5 is that the graphs are linear over the entire range examined. This is due to the circumstance that the concentrations hypothesized for F-21 in the troposphere are not high.

* Throughout this article, the American trillion (10^{12}) is meant.

In conclusion, the following statements can be made about the use of the ECD as a quantitative detector for the analysis of atmospheric samples for halocarbons. The overall method can have good precision, with a standard deviation of about 5%, if permeation tubes are used as reference standards.

The method has some disadvantages owing to the non-specificity of the ECD and to the high background that might occur as a result of other compounds present in the atmosphere. Also, the determination of halocarbons with greatly different abundances and detector responses within the same air sample is difficult. The mass spectrometer used in mass fragmentography is more specific and the linearity of the response is much higher[13], so that it is to be preferred if F-11 and F-21 have to be determined in the same air sample.

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SAMPLE COLLECTION AND FIELD ANALYSIS OF TRIS-(2-ETHYLHEXYL) PHOSPHATE USED IN A STUDY OF PESTICIDE SPRAY DRIFT

M. KRZYMIEN

National Research Council of Canada, National Aeronautical Establishment, Unsteady Aerodynamics Laboratory, Montreal Road, Ottawa, Ontario K1A OR6 (Canada)

SUMMARY

Tris-(2-ethylhexyl) phosphate vapour and aerosol were collected in glass adsorber tubes packed with a plug of fine platinum mesh coated with OV-101 and subsequently thermally released into a gas chromatograph for analysis. The capacity of the adsorber was determined to be *ca.* 700 l of air. Concentrations of pg/l were determined with a precision of better than $\pm$ 10%. The aerosol concentration and its drop-size distribution were determined at a picogram level with $\pm$ 5% precision using a cascade impactor.

INTRODUCTION

A number of papers have been published describing methods for determination of tris-(2-ethylhexyl) phosphate (TEHP) in plastics. Bloom[1] applied thin-layer and gas–liquid chromatography to the qualitative and quantitative analysis of phosphoric esters. Lerche and March[2] determined TEHP in poly(vinyl chloride) using gas chromatography (GC) with flame ionization detection. The separation of various plasticizers was achieved with silicone phases, QF-1 and OV-210[1] or OV-1[2]. Armstrong and Yule[3] determined TEHP deposited on foliage and twigs using GC (OV-1 column) with flame photometric detection.

However, the chemical literature lacks information on the determination of TEHP in ambient air. The need for such a method arose when it was decided to use TEHP as a non-toxic simulant in a study of the long-range drift of airborne pesticides from aerial spray operations. The method was required to provide information on the concentration of both vapour and aerosol in the atmosphere. The analytical procedures are similar in concept to those used for the determination of fenitrothion in ambient air[4]; however, several important changes in the GC and sampling procedures were made. The methods described here were successfully used for determination of TEHP vapour and aerosol in ambient air during an experimental spraying of New Brunswick forests in July 1979[6].

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EXPERIMENTAL

Preparation of adsorber

Samples of TEHP vapour and aerosol were collected in a 7.5 cm × 6.4 mm O.D. glass tube containing a 1.5-cm plug of tightly packed fine platinum mesh (*ca.* 0.5 g of Pt) coated with a thin layer of OV-101 silicone. The coating was applied by a single dip of the platinum mesh in a 10% chloroform solution of OV-101 and evaporation of the solvent by blowing an air stream over it. The adsorbers were conditioned overnight at 260°C in a nitrogen stream at a flow-rate of 20 cm^3/min. A chromatogram was recorded to check the purity of the adsorber. Neither peaks nor baseline drift were observed.

Collection of TEHP vapour and aerosol samples

Air was drawn through the adsorber by two kinds of samplers. The first one was a diaphragm pump ("Air Cadet" from Cole-Palmer Co.) modified to accept a 12 V d.c. automotive fan motor, capable of drawing air at a flow-rate of up to 8 l/min. The outlet of the pump was connected to a built-in flowmeter. The sampler was operated manually and was used to collect large volumes of air over relatively short periods of time, usually 15–40 min, so that the temporal variations of concentration could be determined.

The second type of sampler was operated automatically. Ambient air was drawn through the adsorber by means of a small diaphragm pump (Brailsford Co.) at a flow-rate of *ca.* 2.5 l/min. Designed for unattended operation, the pump could be preset to turn on up to 24 h ahead of time and to turn off after a selected time period by means of an electronic clock. The automatic sampler was powered by a rechargeable battery pack. It was used to monitor dosage of airborne TEHP over long periods when fluctuations of the concentration could be neglected. Automatic samplers were compact and lightweight; up to six could be suspended from a hydrogen-filled "advertising" balloon (4 m diameter) in a vertical array to determine the vertical profile of TEHP concentration.

Gas chromatographic analysis

TEHP collected on the platinum mesh adsorbers was analysed on a Tracor gas chromatograph which was modified to accommodate the adsorber. The instrument was equipped with a Perkin-Elmer nitrogen–phosphorus detector. A schematic view of the gas chromatograph is presented in Fig. 1. A sample was introduced into the gas chromatograph either by liquid injection through a septum or as a vapour thermally released from an adsorber. The adsorber tube with a sample of air trapped

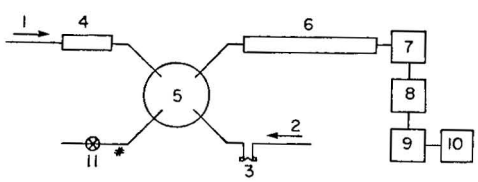


Fig. 1. Schematic view of the gas chromatograph. 1 and 2 = carrier gas (nitrogen) inlets; 3 = septum; 4 = adsorber; 5 = four-port switching valve; 6 = column; 7 = nitrogen–phosphorus detector; 8 = electrometer; 9 = computing integrator with printer; 10 = strip chart recorder; 11 = restriction.

on platinum mesh was reinstalled into the gas chromatograph and purged with a stream of carrier gas. After *ca.* 1 min the stream was redirected by a four-port switching valve (high temperature Valco valve) to a GC column and the adsorber was flash-heated to 250°C by surrounding it with a hot aluminium block. The nitrogen stream carried thermally released vapour to the GC column. The recorded TEHP peak area was compared with that of a TEHP standard solution deposited with a syringe directly on the platinum mesh and analysed in the same manner as an air sample. The concentration of the standard solution was $1.14 \cdot 10^{-10}$ g/μl. Usually 5 μl of the standard solution were injected to obtain *ca.* 50% full scale deflection of the recorder pen.

The chromatographic conditions were as follows: column, 1 m × 3.2 mm O.D. nickel tube packed with 3% OV-1 on Chromosorb W HP 80–100 mesh; carrier gas, nitrogen at a flow-rate of 40 cm^3/min; oven temperature, 240°C; adsorber heater temperature, 250°C; detector temperature, 240°C; detector hydrogen and air flow-rates 3 cm^3/min and 100 cm^3/min, respectively, rubidium glass bead electrically heated at a setting of 740.

A signal from the detector was amplified by the electrometer and recorded on a Gould-Brush 105 strip chart recorder. Peak areas were integrated by an LDC 304-50 computing integrator and printed out together with retention times. To scale down large peaks the integrator's output attenuator was used.

Collection and analysis of aerosol

Eight-stage Anderson 2000, Inc. impactors were used to collect TEHP aerosol. The aerosol was trapped on consecutive plates according to drop size. Deposited aerosol was washed off each plate with *ca.* 3 cm^3 of acetone. The solution was concentrated to a volume of 100 μl by evaporation of the solvent by blowing a small stream of high purity nitrogen over the surface of the solution while heating to *ca.* 50°C. Then 5-μl aliquots of the concentrate were injected directly into the gas chromatograph via the septum, *i.e.* without preconcentration on an adsorber. Recorded peaks were integrated and their areas compared with that of a standard injected the same way.

RESULTS AND DISCUSSION

A number of materials were examined as possible adsorbents for trapping TEHP vapour and aerosol. Tenax GC and polar silicones of the OV series supported on Chromosorb required too high a temperature to release trapped TEHP. Moreover, these packings created a significant flow restriction in the adsorber tubes, and therefore it was not possible to achieve the desired sampling rate of 1–1.5 l/min. The adsorbent without these drawbacks was found to be fine platinum mesh coated with OV-101 silicone. Even when packed very tightly to ensure many collisions of vapour and aerosol particles with the adsorbing surface, the adsorber created little restriction to the sample flow. Flow-rates of the order of 2.5 l/min with the automatic sampler and 7.5 l/min with the manual one were easily achieved. The desorption temperature was low enough to deliver a narrow plug of vapour when the heating block temperature was maintained at *ca.* 250°C. Its adsorption capacity was still big enough to trap TEHP from large volumes of air.

The capacity of an adsorber is described as a maximum sample volume, *i.e.*, the volume of sampled air at which trapped material begins to elute. This parameter, fundamental to the accuracy of the method, was determined by a direct measurement. Validity of the direct measurement relies on the ability to deliver a continuous stream of TEHP vapour at constant concentration. This can be easily achieved with a continuous-flow trace vapour source[5].

A continuous stream of air containing a constant concentration of TEHP vapour, say $3 \cdot 10^{-12}$ g/l, was generated by a continuous-flow vapour source. The air was sampled on a platinum mesh adsorber at a constant flow-rate of *ca.* 1–1.5 l/min which was similar to that used during ambient air analysis. Samples were collected for various periods, ranging from 7.5 min to 16 h, to obtain sample volumes from 12 to 1200 l. Each sample was analysed as described above, and TEHP peak areas were plotted *versus* sample volume (Fig. 2).

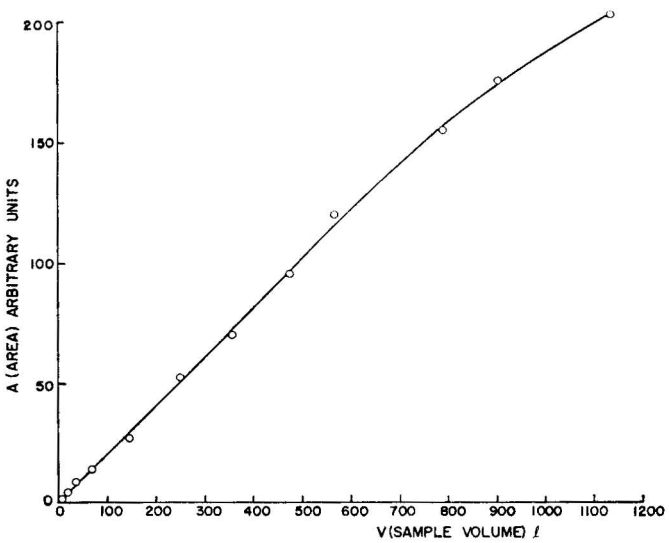


Fig. 2. Maximum sample volume.

Since the concentration of TEHP vapour in sampled air remained constant, the peak area should be directly proportional to the sample volume; if so, a plot of peak area *vs.* volume should be a straight line starting at the origin. The plot (Fig. 2) actually remained a straight line to *ca.* 650–700-l sample volume, and curved slightly for sample volumes over 700 l to 1200 l, owing to elution of TEHP from the adsorber. Therefore, the maximum sample volume can be estimated as being *ca.* 700 l at ambient temperature. The critical point on the curve, 700-l sample volume, was also determined using 8 l/min sampling rate. The obtained peak area agreed with that for 1.5 l/min sampling rate to within $\pm$ 10%.

Efficiency of the adsorbers in trapping the TEHP aerosol was determined in another laboratory test. The TEHP aerosol was released from a large annular jet at a rate of *ca.* 1 mg/min into "still" laboratory air. Eight adsorbers, including manual and automatic samplers, symmetrically positioned over a plume cross-section and a cascade impactor on the plume axis, were used to measure the cross-sectional con-

centration distribution. The concentration determined with the adsorbers and the impactor were the same to within 5%, confirming that the adsorbers trapped aerosol as efficiently as the cascade impactor. Mass budget calculations revealed that more than 80% of the released TEHP could be accounted for. Most of the remaining 20% represents the turbulent flux of aerosol, which could not be measured. The TEHP "far-downwind" concentration fluctuations in the actual field trials are believed to have been lower than in the jet, so that the trapping efficiency of the adsorbers may reasonably be expected to have been at least 90%[6].

Aerosol samples were collected on the plates of cascade impactors. The material trapped on a metal plate was exposed to a large volume of air flowing over it at high flow-rate. This could cause a significant loss of material by evaporation and/or decomposition, as was the case with fenitrothion[4]. However, the loss of TEHP was, as proved in the experiment, negligible because of its low vapour pressure and chemical stability. TEHP formulation (5% solution of TEHP in fuel oil No. 585) was diluted with hexane to a concentration of 0.63 ng/μl. Then 20 μl of the solution (12.6 ng TEHP) were deposited by syringe on each plate of a cascade impactor in the smallest possible drops and air was drawn through the impactor for 5 h at a flow-rate of *ca.* 30 l/min. The plates were then analysed by the method described above. The results are presented in Table I. Relative standard deviation of eight measurements was less than $\pm$ 5%. The results are scattered more or less symmetrically around the true value, showing that the method was not burdened by systematic error. The very good recovery rate proved that TEHP was not lost either during collection of aerosol or during analysis of the plates. The integrator was programmed not to print out peaks below 100 area units, corresponding to *ca.* 1 pg of TEHP. Since a 5-μl aliquot injected into the gas chromatograph is only 5% of the total TEHP deposited on the plate, 20 times that, *i.e.* 20 pg, is the minimum amount of TEHP that can be quantitatively determined with a precision of *ca.* $\pm$5% (Table I).

TABLE I

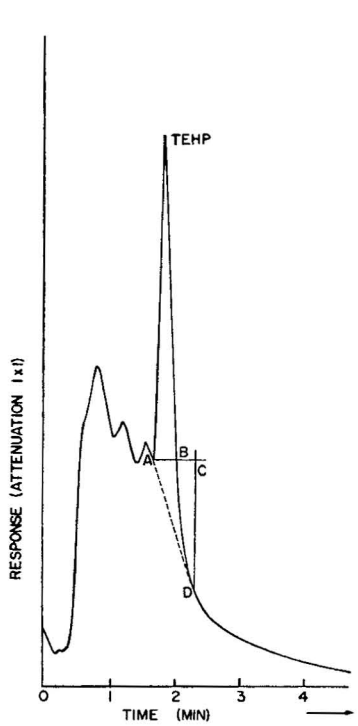
RECOVERY OF TEHP DEPOSITED ON CASCADE IMPACTOR PLATES

Amount deposited (ng)	*Amount recovered (ng)*	*Recovery (%)*
12.60	12.5	99.2
12.60	12.2	96.8
12.60	12.3	97.6
12.60	13.3	105.6
12.60	11.7	92.9
12.60	11.9	94.4
12.60	13.2	104.8
12.60	12.0	95.2
	average: 12.4	
	standard deviation: 0.59	
	relative standard deviation: 4.76%	

A typical chromatogram of TEHP preconcentrated on OV-101 coated platinum mesh is presented in Fig. 3. The retention time of TEHP is 111 sec when a sample is introduced as a vapour thermally released from an adsorber, and 94 sec for samples injected as a liquid (Fig. 4). The difference occurs because it takes time to heat the

adsorber to the release temperature, and also because the adsorber acts as a chromatographic column and as such retains TEHP for a noticeable period. The retention time was measured from the moment when a heating block was placed around the adsorber. The short retention time is very advantageous when a great number of samples are to be analysed. During the field experiment as many as 100 samples were analysed daily. In some instances, as presented in Fig. 3, when contaminated air was analysed some overlap of the TEHP peak with background peaks occurred. The peak, however, was well shaped and suitable for quantitative analysis, providing the right integration procedure was applied. The case was treated as a peak above a negative sloping baseline. In this situation as soon as the tail of the peak falls below the microvolt level at which it was first detected, the integrator calculates the peak width AB, doubles it and projects to time C where the integration ends. No peak has been detected before time C, the integrator then draws a baseline from A to D. By doing so, it eliminates the overlapping background peaks, so the integrated area represents only the TEHP peak. This procedure allows keeping analysis time short without sacrificing accuracy.

Before the experimental TEHP spray operation began, a number of air samples were collected in the forest area designated for spraying. Analysis of these samples



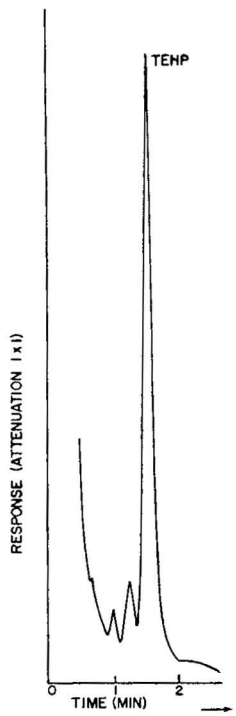


Fig. 3. Chromatogram of an air sample, containing 0.86 ng of TEHP. Sample volume, 165.25 l; TEHP concentration, 5.24 ng/m^3; sampling time, 25 min with manual sampler. The TEHP peak is integrated.

Fig. 4. Chromatogram of TEHP solution washed off a cascade impactor plate and containing 1.11 ng of TEHP.

showed that no TEHP was present in the air. Chromatograms of the samples, together with chromatograms of TEHP standard, were used to identify the presence of TEHP in air samples taken during aerial spraying and shortly after.

The analytical method presented here provided the experimental data for evaluation of the effect of atmospheric stability on long-range pesticide drift[6] and of the mathematical model of long-distance transport of vapour and aerosol generated during aerial spraying of pesticides over forests[7].

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UNTERSUCHUNGEN MIT HILFE DER HOCHLEISTUNGS-FLÜSSIGCHROMATOGRAPHIE ZUM VERHALTEN VON METRIBUZIN BEI EINIGEN WASSERAUFBEREITUNGSMETHODEN

WOLFGANG MAASFELD, WERNER DÜLME und ANTONIUS KETTRUP*

Fachbereich Naturwissenschaften II, Angewandte Chemie, Universität-Gesamthochschule Paderborn, D-4790 Paderborn (B.R.D.)

SUMMARY

High-performance liquid chromatographic determination of the behaviour of metribuzin during some water treatment processes

An extraction procedure with subsequent high-performance liquid chromatography for the determination of herbicide metribuzin in water samples is described. For concentrations from 2.5 μg/l to 10 μg/l the recovery was 77.7%–85.5% with standard deviations of 4.5%–13.3%.

In further investigations concerning the behaviour of this herbicide during some water treatment processes no elimination of metribuzin was observed during slow sand filtration or chlorination whereas adsorption on activated carbon resulted in a quantitative removal. Metabolites which were formed during slow sand filtration and chlorination were identified by UV spectroscopy and mass spectrometry.

EINLEITUNG

In einigen Regionen Deutschlands wird in verstärktem Masse zur Trinkwassergewinnung das Verfahren der künstlichen Grundwasseranreicherung angewendet. Bei diesem Prozess wird Oberflächenwasser, z.B. Flusswasser, einem Langsamsandfilter zugeleitet. Das auf diese Weise aufbereitete Wasser versickert dann in den Untergrund, wo es schliesslich als Grundwasser zu Sammelbrunnen geleitet wird. Da einige Arbeiten[1,2] gezeigt haben, dass durchaus mit der Präsens von Herbiziden in einigen unserer Flüsse zu rechnen ist, ist eine grössere Kenntnis über ihr Verhalten und Verbleiben von grosser Wichtigkeit. Ziel dieser Arbeit war eine Studie über das Verhalten eines Herbizids während der Wasseraufbereitung.

Der herbizide Wirkstoff Metribuzin (4-Amino-3-methylthio-6-*tert.*-butyl-1,2,4-triazin-5-(4H)-on) ist die wirksame Komponente des Handelspräparats Sencor®. Dies findet Verwendung bei der Unkrautbekämpfung in Kartoffeln, Tomaten und Sojabohnen.

Alle bisherigen Bestimmungsmethoden für Metribuzin (vornehmlich in Pflanzen und Böden) beinhalten eine gaschromatographische Detektion[3–8], und nur zwei dieser Arbeiten berichten über eine gleichzeitige Erfassung von Metribuzin und seinen

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Metaboliten[7,8]. Eine Methode zur Bestimmung dieses Herbizids im Wasser ist unseres Wissens nach nur von Jarczyk[8] publiziert. Daher könnte eine einfache und empfindliche Bestimmung mittels Hochleistungs-Flüssigchromatographie (HPLC) eine Alternative oder gar Verbesserung darstellen. Um dies zu zeigen, wurden alle chromatographischen Messungen mit Hilfe der HPLC durchgeführt.

EXPERIMENTELLER TEIL

Geräte und Materialien

Als Flüssigchromatograph diente ein Gerät der Firma Hewlett-Packard (Böblingen, B.R.D.), Modell 1084 A mit einem eingebauten Festwellenlängendektor (254 nm). Alle Chromatogramme wurden bei einer Temperatur von 40°C Säulentemperatur durchgeführt. Die verwendete Stahlsäule hatte eine Länge von 150 mm, einen Innendurchmesser von 4.6 mm und wurde jeweils mit Swagelok® Reduzierstücken, die eine 2 μm Fritte enthielten, verschlossen. Die Säulenfüllung erfolgte mit LiChrosorb RP-18 5 μm (E. Merck, Darmstadt, B.R.D.) nach der Methode von Cassidy *et al.*[9]. Als Eluens wurde ein Wasser–Methanol-Gemisch (60:40) verwendet. Der Fluss betrug 1 ml/min, das Injektionsvolumen 10 μl. Alle Lösungsmittel waren zur Rückstandsanalyse geeignet. Metribuzin (99.8 % Reinheit) sowie drei Metaboliten (95 % Reinheit) wurden von der Firma Bayer (Leverkusen, B.R.D.) zur Verfügung gestellt. Die Aktivkohle, pulverisiert, Reinheitsgrad "zur Analyse", wurde von der Firma E. Merck bezogen.

Methodenentwicklung

Zur Bestimmung des Wirkstoffs in Wasser wurde eine Extraktion mit Dichlormethan gewählt. Dazu wurde die Wasserprobe (1 l) dreimal mit dem Lösungsmittel ausgeschüttelt (100 ml, 50 ml, 50 ml). Die organische Phase wurde jeweils über eine Glasfritte mit Natriumsulfat in einen 250-ml Birnenkolben abgelassen und abschliessend (nach der dritten Extraktion) mit Hilfe eines Rotationsverdampfers bis zur Trockene eingeengt (Wasserbadtemperatur, 50°C).

Der Rückstand wurde dreimal mit jeweils 10 ml Methanol in einen 50-ml Kolben überführt und anschliessend wiederum bis zur Trockene eingeengt. Abschliessend erfolgte die Aufnahme in 2 ml Methanol.

Zur Überprüfung, ob diese drei Ausschüttelungen den Wirkstoff quantitativ aus der Probe extrahieren, wurden alle Extraktionsschnitte einzeln dem oben beschriebenen Verfahren unterworfen und zusätzlich noch eine vierte Extraktion mit 50 ml Dichlormethan durchgeführt. Ferner wurde nach der Überführung des Wirkstoffs aus dem 250-ml Kolben in den 50-ml Kolben der 250-ml Kolben am Rotationsverdampfer nochmals zur Trockene gebracht, ein eventuell verbliebener Rückstand in 2 ml Methanol aufgenommen und chromatographisch untersucht. Die Ergebnisse sind in Tabelle I wiedergegeben. Die Wiederfindungen aller Extraktionsschritte sind dabei zu 100 % aufsummiert.

In Tabelle II sind die Wiederfindungen als auch die Reproduzierbarkeit des Verfahrens bei verschiedenen Konzentrationen dargestellt. Da es unserer Ansicht nach durchaus sinnvoll ist, die jeweiligen Standardabweichungen der einzelnen Schritte des Verfahrens (Extraktion, Chromatographie) als auch die Gesamtstandardabweichung zu kennen, wurden diese in folgender Weise berechnet[10].

TABELLE I

ÜBERPRÜFUNG DER EINZELNEN EXTRAKTIONSSCHRITTE (2 PARALLELPROBEN)

Anmerkung: Die in dieser Tabelle zu 100% aufsummierte Gesamtextraktion entspricht jeweils in der Wiederfindung dem Wert "Mittel" in Tabelle II.

Konz. (µg/l)	*Extraktionsanteile der Gesamtextraktion (%)*				
	1.	2.	3.	4.	1.'*
2.5	90.5	7.4	2.1	—	—
	93.8	5.6	0.6	—	—
5	97.5	2.3	0.2	—	—
	97.3	2.2	0.5	—	—
10	95.5	4.5	—	—	—
	98.6	1.4	—	—	—

* Rückstand in 250-ml Kolben nach Überführung.

TABELLE II

WIEDERFINDUNG VON METRIBUZIN (FÜNF PARALLELPROBEN)

Konz. (µg/l)	*Wasser*	*Bereich (%)*	*Mittel (%)*	S_1 *(%)*	S_2 *(%)*	*S (%)*
2.5	Dest.	75.2–80.4	77.7	2.8	4.7	5.5
	Ruhr	66.1–88.5	79.6	12.2	5.2	13.3
5	Dest.	79.5–89.8	84.0	6.6	5.1	8.4
	Ruhr	73.2–95.6	85.5	10.8	4.3	11.6
10	Dest.	76.7–85.9	82.8	5.0	1.6	5.2
	Ruhr	77.0–81.1	79.9	2.4	3.8	4.5

Die Standardabweichung S_1 errechnet sich nach

$$S_1 = \sqrt{\frac{\sum_{a=1}^{N} (x_a - \bar{x})^2}{N - 1}}$$

wobei N der Anzahl Parallelproben entspricht, x_a den Mittelwert der Analysenwerte einer Probe und $\bar{x}$ den Mittelwert aller x_a-Werte darstellen. S_1 ist also die Standardabweichung des Extraktionsverfahrens.

Die Standardabweichung des chromatographischen Verfahrens S_2 wird berechnet nach

$$S_2 = \sqrt{\frac{\sum_{i=1}^{N} \delta^2}{n - N}}$$

wo δ die Differenz zwischen dem Analysenwert einer Injektion der Probe und dem Mittelwert aller Analysenwerte dieser Probe, n die Gesamtzahl der Injektionen und N die Anzahl der Parallelproben sind.

Die Gesamtstandardabweichung S wird dann bestimmt als[11]

$$S = \sqrt{S_1^2 + S_2^2}$$

Anwendungsbeispiel der entwickelten Methode

Um das Verhalten von Metribuzin während der künstlichen Grundwasseranreicherung mittels Langsamsandfiltration zu untersuchen, benutzten wir eine halbtechnische Versuchsanlage, die folgendermassen aufgebaut war: sie bestand aus einer 50 cm starken Sandschicht, gefolgt von einer 2 m starken Kiesschicht, die wiederum durch eine 80 cm Schicht grober Kiesel gestützt wurde. Die Oberfläche des Beckens betrug 1 m^2 und es wurde kontinuierlich mit Ruhrwasser gespeist. Die Infiltrationsgeschwindigkeit betrug 10 cm/h. Die Dosierung der wässrigen Metribuzinlösung erfolgte direkt in den Filtereinlauf, so dass dort eine Konzentration von ≈ 5 μg/l Metribuzin vorhanden war. Die Probenahme erfolgte einmal täglich im Überstau, in 10 cm und 20 cm Sandtiefe mit Hilfe eingebauter Fritten, sowie am Ablauf des Beckens. Die Dosierphase dauerte sieben Tage.

Die Durchführung einer Chlorung erfolgte durch Zugabe von hypochloriger Lösung zu 1 l dest. Wasser, das 5 μg bzw. 5 mg Metribuzin enthielt. Der Gehalt an wirksamen Chlor betrug 0.3 mg/l bzw. 30 mg/l (entsprechend 0.2 mg/l bzw. 20 mg/l freiem wirksamen Chlor). Die Lösungen wurden mit Salzsäure (1 *M*) neutralisiert und nach einer Reaktionsdauer von 3 h extrahiert.

Die Adsorptionsversuche erfolgten durch Zugabe von jeweils bekannten Mengen Aktivkohle zu definierten Herbizidkonzentrationen. Nach einer Schütteldauer von einer Stunde wurden die Proben zentrifugiert und die Herbizidkonzentration im Überstand photometrisch bestimmt.

ERGEBNISSE UND DISKUSSION

Die Ergebnisse der Langsamsandfiltration zeigt Fig. 1. Eine Eliminierung findet nicht statt. Bereits am Tag 1 (die Dosierung begann nach der Probenahme des Tages 0) konnte Metribuzin im Ablauf des Filters nachgewiesen werden. Nach Beendigung der Dosierphase am Tag 7 wurde kein Metribuzin mehr nachgewiesen. Fig. 2 zeigt ein Chromatogramm einer Probe des Tages 2 (20 cm Sandtiefe). Nach Metribuzin wird noch eine weitere Substanz eluiert. Sowohl das UV-Spektrum als auch die Retentionszeit liessen darauf schliessen, dass es sich bei dieser Substanz um Desaminometribuzin handelte. So haben Rosen und Siewierski[12] und Pape und Zabik[13] gezeigt, dass in wässrigen Lösungen die Einwirkung von UV Licht zu einer Desaminierung von Metribuzin führt. Da es an einigen Tagen während der Dosierphase ebenfalls sehr sonnig war, könnte die Sonneneinstrahlung die Bildung dieses Metaboliten bewirkt haben.

In Fig. 3 sind zwei Chromatogramme der Chlorung wiedergegeben. Während im Chromatogramm a keine statistisch signifikante Abnahme an Metribuzin festgestellt werden konnte, ist im Chromatogramm b Metribuzin nicht mehr vorhanden. Vielmehr wird zu einer geringeren Retentionszeit ein grosser Peak eluiert. Eine massenspektrometrische Untersuchung ergab Signale mittlerer und hoher relativer Intensität von Ionen mit m/z 169 (M^+), 127, 111, 83 und 57. Ein Vergleich mit dem Massenspektrum des Desaminodiketometribuzins, das die gleiche Retentionszeit aufweist wie die neue Substanz, zeigte die gleichen Bruchstücke mit entsprechenden Intensitäten. Das UV-Spektrum bestätigte schliesslich ebenfalls die Bildung von Desaminodiketometribuzin.

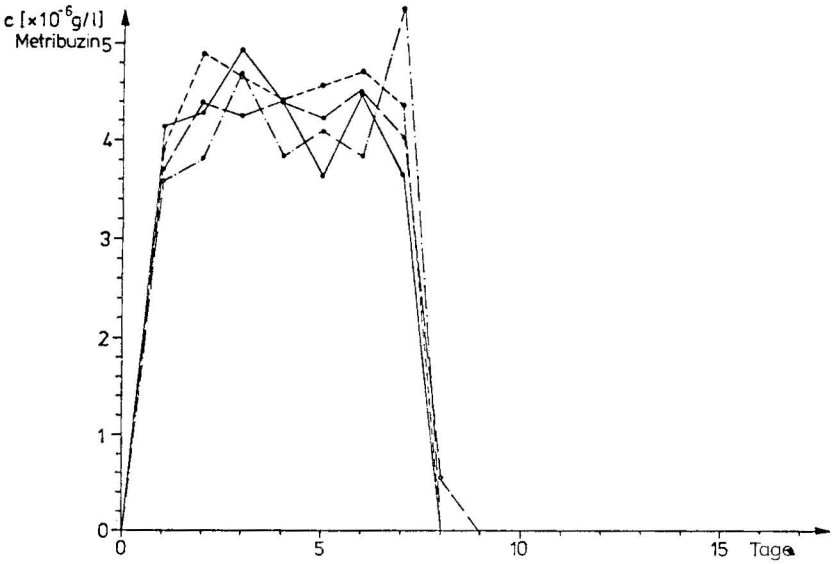


Fig. 1. Verhalten von Metribuzin während der Langsamsandfiltration. —·—·—, Überstau; ———, 10 cm; - - -, 20 cm; – – –, Ablauf.

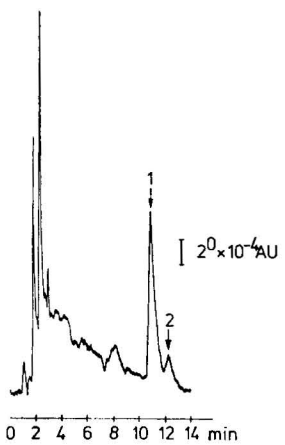


Fig. 2. Chromatogramm von Metribuzin während der Langsamsandfiltration. Chromatographische Bedingungen siehe Text, 1 = Metribuzin, 2 = Desaminometribuzin.

Als Ergebnis der Chlorung bleibt aber dennoch festzuhalten, dass unter realistischen Bedingungen eine Eliminierung des Herbizids nicht stattfindet. Lediglich mit Hilfe der Adsorption an Aktivkohle gelang bei geringen Herbizidkonzentrationen eine quantitative Eliminierung.

Mit dieser Studie wird gezeigt, dass die HPLC als geeignete und empfindliche Methode auch im Bereich der Wasseranalytik gut anwendbar ist.

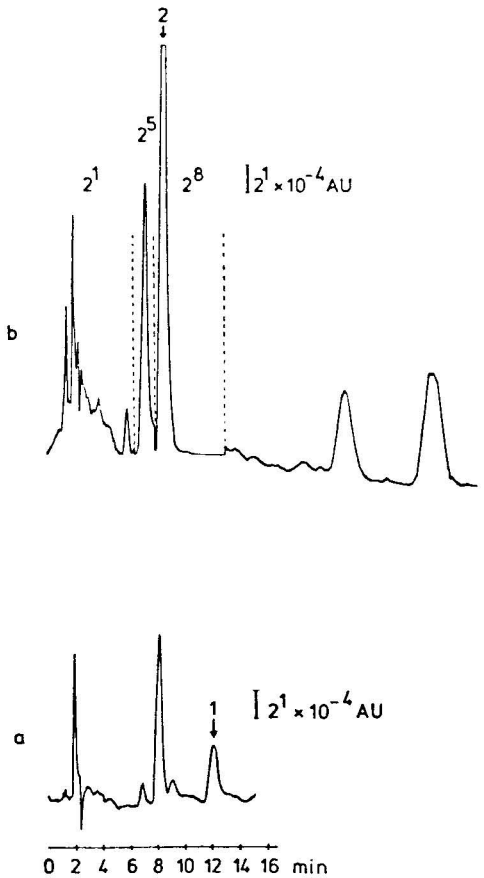


Fig. 3. Chromatogramm von Metribuzin nach der Chlorung. Die gestrichelten Linien zeigen einen Wechsel der Detektorabschwächung an. Chromatographische Bedingungen: siehe Text, 1 = Metribuzin, 2 = Desaminodiketometribuzin. (a) = 5 µg/l Metribuzin, 0.3 mg/l wirksames Chlor; (b) = 5 mg/l Metribuzin, 30 mg/l wirksames Chlor.

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ZUSAMMENFASSUNG

Es wird eine Extraktionsmethode für die flüssigchromatographische Bestimmung des Herbizids Metribuzin aus Wasser beschrieben. Die Wiederfindung lag für

Konzentrationen von 2.5 μg/l bis 10 μg/l zwischen 77.7% und 85.5%. Die Standardabweichungen betrugen 4.5%–13.3%.

Weiterhin wurde das Verhalten von Metribuzin bei der Wasseraufbereitung untersucht. Weder bei der Langsamsandfiltration noch bei einer Chlorung konnte eine Eliminierung festgestellt werden; dagegen erfolgte eine Adsorption an Aktivkohle. Metaboliten, die während der Langsamsandfiltration bzw. Chlorung gebildet wurden, konnten mit Hilfe der UV-Spektroskopie sowie der Massenspektrometrie identifiziert werden.

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EVALUATION OF BONDED PHASES FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN EFFLUENT WATERS

R. AMOS
Esso Petroleum Co. Ltd., Esso Research Centre, Abingdon, Oxfordshire (Great Britain)

SUMMARY

A wide range of chemically-bonded stationary phases have been evaluated to assess their potential usefulness for the determination of polycyclic aromatic hydrocarbons in refinery effluents by high-performance liquid chromatography. Of the 30 columns tested, no more than six achieved the target value of a resolution of 1.5 for perylene–benzo[*a*]pyrene within an analysis time of 30 min using isocratic elution with aqueous acetonitrile as mobile phase. Under these conditions, a column packed with HC-ODS-Sil-X (Perkin-Elmer Corp.) was the only one to separate completely all the components of interest.

INTRODUCTION

The analysis of polycyclic aromatic hydrocarbons (PAHs) in environmental samples has been the subject of several hundreds of papers during the last thirty years and many more will undoubtedly be published before common agreement on standard methods will be achieved. Many combinations of analytical techniques have been used usually involving various types of chromatography with molecular spectroscopy. Most procedures are time-consuming and complex, requiring expensive equipment and considerable analytical skills.

To obtain a complete analysis, a sophisticated analysis such as one based on capillary gas chromatography (GC) with mass spectrometry and UV absorption or fluorescence spectroscopy is necessary. However, for many purposes the determination of a limited number of representative PAHs is quite sufficient and provided reference samples are available, high-performance liquid chromatography (HPLC) offers a relatively simple but very powerful means of effecting this analysis.

In 1971, the World Health Organisation (WHO) set an upper limit of 200 ng/l for the total concentration of six PAHs in potable water (Table I). More recently, in 1976 the U.S. Environmental Protection Agency (EPA) drew up a list of 16 PAHs whose presence should be monitored and limited in effluent waters in the U.S.A. Reversed-phase HPLC has been examined by several workers[1–15] for this analysis, but with conflicting reports on the effectiveness of the separation. Others[16,17] have suggested the use of normal-phase chromatography using polar bonded phases. Only

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TABLE I

DESCRIPTION OF POLYCYCLIC AROMATIC HYDROCARBONS

Name	*Abbreviation*	*EPA list*	*WHO list*	*Blend concentration (μg/ml)*
Fluorene	Fl	+		11.2
Phenanthrene	Phen	+		11.2
Anthracene	Ant	+		8.8
Fluoranthene	Ft		+	17.6
Pyrene	Pyr	+		19.2
Benz[*a*]anthracene (1,2-benzanthracene)	B*a*A	+		19.2
Chrysene	Chrys	+		22.4
Perylene	Per			—
Benzo[*b*]fluoranthene (3,4-benzofluoranthene)	B*b*F	+	+	16.0
Benzo[*k*]fluoranthene (11,12-benzofluoranthene)	B*k*F	+	+	20.0
Benzo[*e*]pyrene (1,2-benzpyrene)	B*e*P			—
Benzo[*a*]pyrene (3,4-benzpyrene)	B*a*P	+	+	19.2
Dibenz[*a*, *h*]anthracene (1,2,5,6-dibenzanthracene)	DiBa	+		24.0
Benzo[*g*, *h*, *i*]perylene (1,12-benzoperylene)	*ghi*	+	+	17.6
Indeno[1,2,3,*c*,*d*]pyrene (2,3-*o*-phenylene pyrene)	123*cd*	+	+	17.6

recently has it been recognised[18] that nominally similar stationary phases exhibit different selectivities towards PAHs.

During the course of our work, we have evaluated a large number of stationary phases for a wide variety of analyses, including PAH analysis. From the data reported here, we have successfully developed a chromatographic system for the analysis of PAHs, down to at least the 0.01 μg/l level, in refinery effluent water.

EXPERIMENTAL

Equipment

A Perkin-Elmer Series 2 liquid chromatograph with LC 55 UV detector was used throughout this work. Samples were injected by microsyringe via a septum injector from hetp, Macclesfield, Great Britain, excepting columns 8 and 11 for which a Rheodyne Model 7120 injection valve with 20-μl sample loop was used.

Reagents

Fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene were purchased from BDH (Poole, Great Britain); benz[*a*]anthracene and dibenz[*a*,*h*]-anthracene from R. N. Emanuel Ltd. (Alperton, Great Britain); perylene from Koch-Light Labs. (Colnbrook, Great Britain); benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene and benzo[*e*]pyrene from Phase Separations (Queensferry, Great

Britain); and benzo[*g,h,i*]perylene and indeno[1,2,3,*c,d*]pyrene from Aldrich (Milwaukee, WI, U.S.A.).

Standard solutions were prepared by dissolving about 0.01 g of each solute in about 5 ml chloroform and making up to 50 ml in a volumetric flask with acetonitrile. Some solutions needed to stand 24 h before dissolution was complete. A PAH blend was made by mixing some of the above solutions. The composition of the final blend is shown in Table I.

Acetonitrile (HPLC grade) was purchased from Rathburn (Walkerburn, Great Britain); all other solvents and chemicals were obtained from BDH.

Binary test mixtures: 1% each of dimethyl (DMP) and dibutyl phthalate (DBP) in tetrahydrofuran; 0.02% each of pyrene and chrysene in acetonitrile; 0.02% each of perylene and benzo[*a*]pyrene in acetonitrile; 0.02% each of naphthalene and phenanthrene in heptane.

Columns

The columns are described in Tables II–IV. All home-made columns were slurry-packed in either 1,1,2,2-tetrabromoethane–dioxane–carbon tetrachloride (20:15:15) or trichloroethylene–ethanol (1:1) at a pressure of 5000 p.s.i.g. Except for the commercial pre-packed columns, the same column (25 cm × 4.6 mm I.D.) and end fittings were used throughout this work.

TABLE II

DESCRIPTION OF OCTADECYL REVERSED-PHASE COLUMNS

Column No.	*Name*	*Particle size (μm)*	*Packing method*	*Source*
1	LiChrosorb RP-18	5	Prepacked	Jones Chromatography Llanbradach, Glamorgan, Great Britain
2	LiChrosorb RP-18	10	Slurry	E. Merck, Darmstadt, G.F.R.
3	Spherisorb ODS	5	Prepacked	Jones Chromatography
4	Spherisorb ODS	10	Slurry	Phase Separations, Queensferry, Great Britain
5	Partisil ODS-1	10	Slurry	Whatman, Maidstone, Great Britain
6	Partisil ODS-2	10	Slurry	Whatman
7	Partisil-5 ODS	5	Prepacked	Whatman
8	HC-ODS-Sil-X-1	—	Prepacked	Perkin-Elmer, Norwalk, CT, U.S.A.
9	Hypersil ODS	7	Slurry	Shandon Southern Products, Runcorn, Great Britain
10	Zorbax ODS	5	Slurry	DuPont, Wilmington, DE, U.S.A.
11	μBondapack C_{18}	10	Prepacked	Waters Assoc., Milford, MA, U.S.A.
12	C_{18} modified silica	10	Slurry	H.S.C.P., Bourne End, Great Britain

TABLE III

DESCRIPTION OF OTHER ALKYL REVERSED-PHASE COLUMNS

Packing method: slurry.

Column No.	*Name*	*Particle size (μm)*	*Source*
13	LiChrosorb RP-2	10	E. Merck
14	Hypersil SAS	7	Shandon Southern Products
15	C_5 modified silica	10	H.S.C.P.
16	Spherisorb hexyl	5	Phase Separations
17	Spherisorb phenyl	5	Phase Separations
18	Zorbax C_8	5	DuPont
19	LiChrosorb RP-8	10	E. Merck
20	Magnusil C_{22} supergrade	5	HPLC Technology, Wilmslow, Great Britain
21	LiChrosorb SI 60, standard grade C_{22}	5	HPLC Technology
22	Partisil-5-C_{22}	5	HPLC Technology
23	Hypersil C_{22}	7	HPLC Technology

TABLE IV

DESCRIPTION OF POLAR BONDED-PHASE COLUMNS

Packing method: slurry.

Column No.	*Description*	*Particle size (μm)*	*Source*
24	Partisil PAC	10	Whatman
25	Nucleosil CN	10	Macherey, Nagel & Co., Düren, G.F.R.
26	Spherisorb nitrile	5	Phase Separations
27	Nucleosil NO_2	5	Macherey, Nagel & Co.
28	Hypersil APS	7	Shandon Southern Products
29	LiChrosorb NH_2	10	E. Merck
30	LiChrosorb DIOL	10	E. Merck

Procedure

Reversed-phase columns were conditioned by pumping acetone, methanol, acetonitrile and acetonitrile–water mixtures for 15 min each at 3 ml/min. When operating conditions were steady, the flow-rate was set at 1.0 ml/min, and detector wavelength set to 254 nm at an appropriate sensitivity to obtain 30–80% full scale deflection. Portions of 1–5 μl of each of the first three binary test mixtures and the PAH blend were injected and from the chromatograms, values for the retention time (t_R), efficiency (N), plate height (H), and resolution (R_s) were measured.

Normal-phase columns were conditioned by pumping ethyl acetate, dichloromethane and heptane for 15 min each. The columns were evaluated by injecting the last three binary test mixtures and the PAH blend.

RESULTS AND DISCUSSION

Tables V and VI show the performance of the reversed-phase columns with the phthalate test mixture. Evaluations similar to this are frequently provided by commercial suppliers as a back-up to their products. Our results are in line with those generally reported in the literature. At this stage, the apparently poor performance of

TABLE V

EVALUATION OF OCTADECYL REVERSED-PHASE COLUMNS WITH DMP AND DBP

Column No.	Mobile phase (% acetonitrile in water)	DMP			DBP			R_s
		t_R (min)	N (plates)	H (mm)	t_R (min)	N (plates)	H (mm)	
1	85	4.5	21140	0.012	10.4	7056	0.035	22.7
2	80	3.0	4956	0.050	6.8	4199	0.060	12.88
3	80	3.5	6790	0.037	5.4	5184	0.048	8.09
4	80	3.1	4733	0.053	6.4	3598	0.069	12.00
5	60	2.8	1866	0.13	4.3	1340	0.19	4.11
6	80	2.6	2704	0.092	6.9	2830	0.088	11.94
7	80	3.4	3844	0.057	7.9	6922	0.032	15.00
8	60	1.8	784	0.32	4.7	1414	0.18	7.87
9	80	3.7	6084	0.041	7.4	8538	0.029	14.51
10	80	3.3	4844	0.052	9.4	3944	0.063	15.44
11	80	4.9	4251	0.059	8.7	3364	0.074	8.44
12	60	5.0	3894	0.064	7.6	3697	0.068	6.34

TABLE VI

EVALUATION OF OTHER ALKYL REVERSED-PHASE COLUMNS WITH DMP AND DBP

Column No.	Mobile phase (% acetonitrile in water)	DMP			DBP			R_s
		t_R (min)	N (plates)	H (mm)	t_R (min)	N (plates)	H (mm)	
13	60	3.5	3136	0.080	7.4	3505	0.071	10.40
14	60	4.5	4147	0.060	10.4	5127	0.049	13.72
15	50	3.1	1600	0.16	4.6	1936	0.13	4.11
16	80	3.4	2043	0.12	5.4	3226	0.077	5.88
17	60	3.7	4516	0.055	5.3	3091	0.081	5.33
18	80	2.5	2079	0.12	5.6	4900	0.051	11.48
19	70	2.9	2788	0.090	7.1	2500	0.10	10.59
20	80	4.6	8464	0.030	7.3	8836	0.028	10.59
21	60	4.1	3003	0.083	7.2	3181	0.079	7.65
22	80	3.3	1205	0.21	5.3	2540	0.098	5.00
23	80	4.1	6724	0.037	5.3	5013	0.050	4.80

TABLE VII

EVALUATION OF POLAR BONDED PHASES WITH NAPHTHALENE AND PHENANTHRENE

Mobile phase, *n*-heptane.

Column No.	Naphthalene			Phenanthrene			R_s
	t_R (min)	N (plates)	H (mm)	t_R (min)	N (plates)	H (mm)	
24	8.6	3272	0.076	21.7	2343	0.11	10.92
25	3.2	3364	0.074	3.7	5476	0.046	2.38
26	5.2	8911	0.028	7.3	10161	0.025	8.24
27	5.2	2704	0.092	10.2	1665	0.15	7.14
28	3.7	3505	0.071	5.4	1866	0.13	4.53
29	5.0	5127	0.049	6.9	6209	0.040	6.03
30	2.9	3047	0.082	3.5	2500	0.10	2.46

column 8 is noteworthy. Table VII shows the conventional evaluation of normal-phase columns with the naphthalene–phenanthrene test mixture.

Tables VIII–XIII present the data obtained from the evaluation of all columns with the pyrene–chrysene and perylene–benzo[*a*]pyrene test mixtures. In general, the efficiencies measured on PAHs are lower than those on phthalates.

TABLE VIII

EVALUATION OF OCTADECYL REVERSED-PHASE COLUMNS WITH PYRENE AND CHRYSENE

Column No.	*Mobile phase (% acetonitrile in water)*	*Pyrene*			*Chrysene*			R_s
		t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
1	85	13.8	3745	0.067	18.7	6922	0.036	5.44
2	85	8.2	2059	0.12	10.8	1798	0.14	3.02
3	60	14.5	5535	0.045	18.3	8391	0.030	4.81
4	70	13.2	Poor peak shapes		18.0	—	—	—
5	60	5.0	495	0.51	6.1	493	0.51	1.10
	50	9.7	1397	0.18	12.8	655	0.38	2.07
6	85	8.2	1121	0.22	10.8	1600	0.16	2.52
7	80	9.4	2500	0.088	11.0	3457	0.064	2.13
8	70	5.2	1024	0.24	11.0	1345	0.19	6.27
	80	3.5	647	0.39	6.5	1381	0.18	4.80
	85	3.0	900	0.28	4.7	1414	0.18	3.78
9	80	8.4	2304	0.11	9.7	2342	0.11	1.73
10	80	14.5	664	0.38	18.0	939	0.27	1.52
	85	10.5	784	0.32	12.4	1351	0.19	1.33
11	80	10.8	2581	0.097	12.5	2788	0.090	1.89
	70	14.4	4096	0.061	18.4	4900	0.051	4.10
12	60	10.2	1156	0.22	12.0	846	0.30	1.26

TABLE IX

EVALUATION OF OTHER ALKYL REVERSED-PHASE COLUMNS WITH PYRENE AND CHRYSENE

Column No.	*Mobile phase (% acetonitrile in water)*	*Pyrene*			*Chrysene*			R_s
		t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
13	60	6.3	697	0.36	7.3	1183	0.21	1.11
14	60	8.8	1530	0.16	10.6	1665	0.15	1.86
15	50	6.4	—	—	6.9	—	—	<1
16	60	12.9	3272	0.076	15.4	3600	0.069	2.59
17	60	5.4	—	—	5.9	—	—	≪1
18	70	9.9	1568	0.16	11.8	1832	0.14	1.81
19	70	7.7	949	0.26	8.7	1211	0.21	1.00
	60	12.5	1276	0.20	15.3	1901	0.13	2.00
20	80	8.4	3745	0.067	9.5	3411	0.073	1.83
	70	11.9	4624	0.054	14.4	5184	0.048	3.33
21	70	6.3	1764	0.14	7.1	1515	0.17	1.20
	60	8.7	2153	0.11	10.5	1832	0.14	2.08
22	70	8.5	2343	0.11	10.4	2153	0.12	2.38
	60	13.6	1764	0.14	17.7	2540	0.098	3.03
23	70	9.7	1936	0.13	11.7	1731	0.14	1.99

TABLE X

EVALUATION OF POLAR BONDED PHASE COLUMNS WITH PYRENE AND CHRYSENE

Mobile phase, *n*-heptane; the mobile phase for columns 24 and 27 contained 1% ethyl acetate.

Column No.	*Pyrene*			*Chrysene*			R_s
	t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
24	9.5	2959	0.084	14.5	2788	0.090	5.56
25	3.7	3505	0.071	4.3	3272	0.076	2.18
26	6.0	900	0.28	7.3	944	0.26	1.49
27	6.4	3226	0.077	8.8	3697	0.068	4.66
28	5.2	2460	0.10	6.7	718	0.35	2.11
29	7.2	1697	0.15	9.4	1360	0.18	2.56
30	3.7	1242	0.20	4.5	662	0.38	1.43

TABLE XI

EVALUATION OF OCTADECYL REVERSED-PHASE COLUMNS WITH PERYLENE AND BENZO-[*a*]PYRENE

Column No.	*Mobile phase (% acetonitrile in water)*	*Perylene*			*Benzo[a]pyrene*			R_s
		t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
1	85	23.0	3317	0.075	28.9	2153	0.12	2.88
2	85	15.3	1900	0.13	18.8	1730	0.14	2.19
3	60	24.5	6529	0.038	27.0	6922	0.038	1.99
4	70	28.0	poor peak		32.8	poor peak		—
	80	15.0	shape		17.3	shape		—
5	50	16.7	1543	0.16	18.7	1058	0.24	1.00
6	85	15.7	4356	0.057	18.3	6593	0.038	2.81
7	80	14.4	4096	0.054	15.7	5476	0.040	1.49
8	70	17.4	595	0.42	27.1	753	0.33	2.85
	80	9.3	1697	0.15	13.7	1764	0.14	4.00
	85	6.6	1600	0.16	9.4	1731	0.14	3.59
9	80	12.5	2500	0.10	13.6	1833	0.14	0.76
10	85	18.2	2343	0.11	20.1	2007	0.12	1.15
11	80	15.6	5535	0.045	16.6	4570	0.055	1.10
	70	22.7	4303	0.058	25.0	4303	0.058	1.59
12	60	15.2	—	—	15.9	—	—	$\ll 1$

If we were to set, as a criterion for a successful column for PAH analysis, an R_s value of at least 1.5 for perylene–benzo[*a*]pyrene achieved in an analysis time of less than half an hour, then the number of potentially useful columns falls to about six. The performance of column 8 is again noteworthy. Despite its poor efficiency, it achieved the highest R_s values for both PAH test mixtures. Although, as would be expected, the octadecyl reversed phases performed better than those with lower alkyl chains, the performances of the C_{22} bonded phases were somewhat disappointing. Of the polar bonded phases, only columns 24, 26 and 27 seemed interesting, indicating potential use in pre-separation techniques.

TABLE XII

EVALUATION OF OTHER ALKYL REVERSED-PHASE COLUMNS WITH PERYLENE AND BENZO[*a*]PYRENE

Column No.	*Mobile phase (% acetonitrile in water)*	*Perylene*			*Benzo[a]pyrene*			R_s
		t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
13	60	8.4	—	—	8.6	—	—	$\ll 1$
14	60	11.6	5595	0.045	12.5	3894	0.064	1.27
15	50	5.2	—	—	5.4	—	—	$\ll 1$
16	60	17.9	4251	0.059	19.0	3697	0.068	0.94
17	50	5.9	—	—	6.3	—	—	$\ll 1$
18	70	15.3	1901	0.13	16.3	1901	0.13	0.69
19	60	20.0	—	—	21.1	—	—	<1
20	70	18.3	5069	0.049	20.0	4570	0.055	1.54
21	60	13.6	1971	0.13	14.7	1832	0.14	0.85
22	60	22.8	3697	0.068	24.9	4147	0.060	1.38
23	70	15.3	3091	0.081	16.8	3411	0.073	1.33

TABLE XIII

EVALUATION OF POLAR BONDED PHASE COLUMNS WITH PERYLENE AND BENZO-[*a*]PYRENE

Mobile phase, *n*-heptane; mobile phase for columns 24 and 27 contained 1% ethyl acetate.

Column No.	*Benzo[a]pyrene*			*Perylene*			R_s
	t_R *(min)*	*N (plates)*	*H (mm)*	t_R *(min)*	*N (plates)*	*H (mm)*	
24	18.6	3844	0.065	21.0	4303	0.058	1.94
25	4.4	2153	0.12	4.6	1936	0.13	$\ll 1$
26	9.6	871	0.29	11.3	3648	0.069	1.66
27	11.2	3745	0.067	12.6	2830	0.088	1.67
28	7.6	924	0.27	7.6	924	0.27	0.00
29	10.4	1381	0.18	11.6	1378	0.18	1.01
30	4.9	496	0.50	5.3	449	0.56	<1

Finally, Table XIV lists the retention times of all the PAHs listed in Table I obtained on the most promising columns. The PAH blend contains three groups that are difficult to separate: chrysene–benzo[*a*]anthracene; perylene–benzo[*b*]fluoranthene–benzo[*k*]fluoranthene–benzo[*e*]pyrene–benzo[*a*]pyrene; and dibenz[*a,h*]anthracene–benzo[*g,h,i*]perylene–indeno[1,2,3,*c,d*]pyrene. Column 8 was the only column to separate all components using isocratic elution with aqueous acetonitrile.

CONCLUSIONS

Of the 30 columns we have tested, no more than six are potentially useful for PAH analysis. As a result of this work we now use a semi-preparative column packed with Partisil-PAC to effect a preliminary clean-up of a cyclohexane extract of refinery effluents, followed by a quantitative analysis using the HC–ODS–Sil-X column with variable UV wavelength detection. This allows us to see at least 0.01 μg/l of each PAH on the WHO and EPA lists.

TABLE XIV

RETENTION TIMES OF INDIVIDUAL POLYCYCLIC AROMATICS

Column No.	*Mobile phase (% acetonitrile in water)*	*Fl*	*Phen*	*Ant*	*Ft*	*Pyr*	*BaA*	*Chrys*	*Per*	*Bbf*	*Bkf*	*Bep*	*BaP*	*DiBA*	*ghi*	*123dc*
1	90	5.0	6.2	6.9	8.5	9.7	12.5	12.5	—	17.0	18.5	—	19.0	24.4	31.8	31.8
2	80	5.2	5.7	6.2	7.2	8.3	11.0	11.4	16.7	16.3	18.1	16.2	20.9	23.9	31.0	32.5
3	60	7.2	10.0	11.0	13.7	14.5	18.3	18.3	24.5	25.1	26.1	—	27.0	34.8	36.8	36.8
7	80	5.1	6.7	7.3	8.7	9.4	11.0	11.0	14.4	14.3	14.8	—	15.7	17.9	20.3	20.3
8	80	2.1	2.3	2.5	3.2	3.7	5.5	6.6	9.3	9.7	12.6	—	14.4	22.2	28.2	30.2
9	80	6.1	6.3	6.7	7.5	8.4	9.7	9.7	11.9	11.8	12.0	—	12.9	14.6	16.5	16.5
10	85	5.2	6.8	7.6	9.5	10.5	12.4	12.4	18.2	17.5	18.2	—	20.1	22.7	28.5	30.1
11	70	10.3	11.3	12.0	13.8	14.8	18.0	18.7	22.7	23.7	24.7	—	25.7	31.7	34.0	34.0
16	60	6.8	10.3	11.2	13.3	12.9	16.1	15.4	17.9	18.4	19.3	—	19.0	22.2	22.8	22.8
18	70	4.9	7.0	7.7	9.4	10.0	11.8	11.8	15.3	15.3	15.9	—	16.3	19.6	20.9	21.3
20	70	7.2	8.7	9.5	11.2	11.9	14.2	14.4	18.3	18.5	19.0	—	20.0	24.1	26.0	27.7
21	60	5.8	6.3	6.9	8.1	8.7	10.5	10.5	13.6	13.7	13.7	—	14.7	17.8	19.4	19.4
22	65	7.7	8.5	8.9	10.2	10.7	13.1	13.7	—	17.3	17.8	—	18.3	23.1	24.2	24.2
23	70	6.9	7.9	7.8	9.0	9.7	11.6	11.7	15.3	16.0	16.3	—	16.8	22.1	24.8	25.4
24	2% Ethyl acetate in heptane	5.4	7.0	6.8	8.5	8.5	11.9	12.4	15.5	14.8	—	—	13.9	21.3	18.4	17.7
27	1% Ethyl acetate in heptane	4.2	4.9	4.7	6.0	6.4	8.3	8.8	12.6	11.5	—	12.1	11.2	14.3	14.4	13.3
29	*n*-Heptane	5.7	6.9	6.3	7.3	7.3	9.4	9.0	11.6	10.6	—	10.6	10.4	13.2	11.7	11.6

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APPLICATIONS IN GEOCHEMICAL ANALYSIS

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CORRELATION OF CRUDE OILS AND SOURCE ROCKS IN THE GERMAN MOLASSE BASIN

APPLICABILITY OF CHROMATOGRAPHIC TECHNIQUES

H. WEHNER and M. TESCHNER*

Federal Institute for Geosciences and Natural Resources, P.O. Box 510153, D-3000 Hannover 51 (G.F.R.)

SUMMARY

Chromatographic techniques can be applied successfully in organic geochemical research. Taking the oil–oil and oil–source rock correlations in the German Molasse basin as examples, it is demonstrated how classical column chromatography can help in oil–oil correlation work. Secondary alteration of oils can be detected by analysing the fraction of saturated hydrocarbons by means of capillary column gas chromatography. Aromatic hydrocarbons are less affected by biodegradation. The high-performance liquid chromatographic separation of this group of compounds is also demonstrated. Final conclusions in oil–oil correlations can be drawn from gas chromatographic–mass spectrometric (GC–MS) analyses of biomarker components (steranes, triterpanes).

Preliminary results of oil–source rock correlations were obtained by using thermochromatographic methods (pyrolysis–GC) for pre-selecting potential source rocks and GC–MS investigations of steranes.

INTRODUCTION

Owing to the increasing demand for fossil fuels it is becoming more and more important to intensify exploration for crude oils and to investigate sedimentary basins with advanced geophysical and geochemical techniques. Organic geochemical methods are powerful tools for evaluating the hydrocarbon potential of a sedimentary sequence.

The aim of this paper is to show how chromatographic techniques can be used successfully in crude oil–crude oil and crude oil–source rock correlation work. Taking the South German Molasse basin as an example, it is demonstrated how the crude oils found in this region can be grouped together. Further, some preliminary results are presented on the correlation of these crude oils with possible source rocks. Such investigations may reveal important information on migratory pathways of crude oils, which in turn may lead to an increase (or decrease) in the exploration efforts in the basin investigated. Because drilling boreholes is very expensive, such analyses have a considerable economic importance.

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EXPERIMENTAL

Column chromatography

Crude oils were topped at 40°C for 6 h. The deasphaltation procedure involved pouring the sample into a mixture of dichloromethane–light petroleum (b.p. 40–60°C) (1:30) and centrifugation at 1500 *g* for 20 min. After removal of the solvent the samples were redissolved in *n*-heptane and then chromatographed on a 50 cm × 4 mm I.D. column, of which the top one third was filled with alumina (neutral, activated at 700°C for 2 h) and the bottom two thirds with silica gel (activated at 400°C for 6 h, 70–230 mesh). The saturated hydrocarbons (SAT) were eluted using *n*-heptane, the aromatic hydrocarbons (ARO) with dichloromethane and the hetero-compound fraction (HET), containing N-, O- and S-substituted molecules, with dichloromethane–methanol (2:1). For a higher sample throughput an automated instrument was constructed using a desktop computer (Commodore PET 2001) as a controlling device, electrically controlled/pneumatically actuated Valco valves, a Waters M 45 solvent delivery pump and a fraction collector. A detailed description of the instrument, which is manufactured by R. Fromholz (Hannover, G.F.R.), is available from the authors on request.

Sedimentary material was ground to less than 100 μm, Soxhlet extracted with dichloromethane for 18 h and then deasphalted and fractionated as described above.

Gas chromatography and gas chromatography–mass spectrometry

The SAT fraction was further investigated by gas chromatography (GC) using a Hewlett-Packard 5985 A instrument composed of a 5840 A gas chromatograph, a flame-ionization detector (FID), a quadrupole mass spectrometer and a 21 MXE computer. For separation a 30 m × 0.28 mm I.D. capillary column coated with SP-2100 was used. Samples were injected at 100°C and after 1 min the column temperature was programmed at 10°C/min to 170°C, then at 4°C/min to 250°C. After an isothermal period of 22 min the column temperature was further increased to 270°C at 2°C/min. The injector and detector temperatures were set at 220 and 280°C, respectively. Helium was used as the carrier gas. The column effluent was split in the ratio 1 (to the FID) to 2 (to the mass spectrometer). The coupling to the mass spectrometer was direct. For mass spectrometric (MS) analysis the selected-ion monitoring mode was preferred, measuring 200 msec at each mass point (*m/e* 191.2, 217.2 and two others) with an electron energy of 70 eV.

High-performance liquid chromatography

The ARO fraction was further separated by means of high-performance liquid chromatography (HPLC) in the reversed-phase mode with a DuPont 830 instrument, equipped with a UV detector (254 nm), a 25 cm × 4.6 mm I.D. and a 15 cm × 4.6 mm I.D. Zorbox-ODS column and a 5 cm × 1 mm I.D. guard column packed with Spherisorb ODS. For elution of compounds a hyperbolic gradient from 65% aqueous methanol to pure methanol within 100 min was used. Working at constant pressure, the flow-rate at the initial composition was 0.9 ml/min. The column temperature was 65°C.

Thermochromatography (pyrolysis–GC)

The amount and composition of the sedimentary organic material was investigated with a Spex MP 3 thermochromatograph. A few milligrams of the sample were pyrolysed under helium in the temperature range 25–600°C at 40°C/min. An aliquot of the volatiles was measured with an FID and the remainder of the material was trapped on a 1 ft. × 1/8 in. O.D. column packed with 4% SE-30 on Porapak Q (80–100 mesh). Heating of the trap to 220°C transferred all volatiles on to a 6 ft. × 1/8 in. O.D. column packed with 3% Dexsil 300 GC on Chromosorb W HP (80–100 mesh). GC was then performed from 30 to 300°C at a rate of 12°C/min.

RESULTS AND DISCUSSION

Oil–oil correlation

The column chromatographic separation of crude oils is shown in Table I. For comparison purposes these data are plotted in a triangular plot (Fig. 1b). This coarse separation allows the crude oils found in the German Molasse basin to be subdivided into three groups: firstly the oils found in reservoirs of Triassic age in the Western part of the basin (area I; Fig. 1a), secondly oils that have accumulated in reservoirs of Tertiary age (area II, Western part; area III, Eastern part; Fig. 1a), and thirdly oils that were found in the North-Eastern part of the Molasse basin (area IV; Fig. 1a). The differentiation of the oils is based mainly on the variation of the saturated hydrocarbon content.

The composition of crude oils may be altered by various factors. In addition to thermal destruction and removal of components by washing with water, biodegradation also has to be considered. Bacterial attack on a crude oil starts with the reduc-

TABLE I

COLUMN CHROMATOGRAPHIC SEPARATION OF CRUDE OILS FROM THE GERMAN MOLASSE BASIN

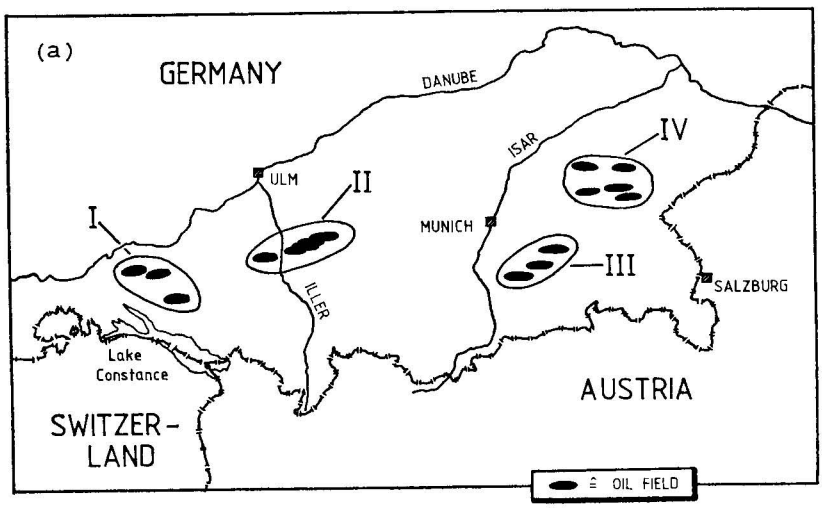
Field	*Area*	*Age of reservoir*	*Composition of deasphalted oil (%)*		
			SAT	*ARO*	*HET**
Ilmensee	I	Triassic	50.9	18.4	30.7
Pfullendorf	I	Triassic	48.7	20.2	31.1
Wald	I	Triassic	49.7	19.3	31.0
Mönchsrot	II	Tertiary	55.6	15.9	28.5
Heimertingen	II	Tertiary	56.4	18.0	25.6
Lauben	II	Tertiary	56.2	14.8	29.0
Lauberhart	II	Tertiary	55.6	11.9	32.5
Arlesried	II	Tertiary	56.7	21.1	22.2
Darching	III	Tertiary	55.8	15.5	28.7
Höhenrain	III	Tertiary	52.7	14.1	33.2
Assling	III	Tertiary	58.3	22.9	18.8
Mühldorf	IV	Tertiary	24.6	23.8	51.6
Teising	IV	Tertiary	29.2	38.5	32.3
Velden	IV	Tertiary	27.9	25.6	46.5
Ampfing	IV	Tertiary	31.9	31.2	36.9
Isen	IV	Tertiary	23.8	25.3	50.9

* HET = 100% − SAT − ARO.

tion of aliphatic compounds, especially with the removal of *n*-alkanes[1,2]. Hence looking only at the triangular plot and neglecting the composition of the SAT fraction may lead to incorrect results.

Typical traces from the GC investigation of the SAT fraction are shown in Fig. 1c. The most abundant peaks represent *n*-alkanes. A direct correlation of the oils from areas II and III can clearly be seen. Triassic oils from area I possess a pronounced *n*-alkane pattern, but this pattern is completely different from the pattern of the area II/III oils, best deduced from the maximum of the *n*-alkane distribution. The gas chromatogram from crude oil from area IV shows a lack of *n*-alkanes. This trace is typical of a biodegraded oil.

Considering these results, there is no possibility of correlating the crude oils



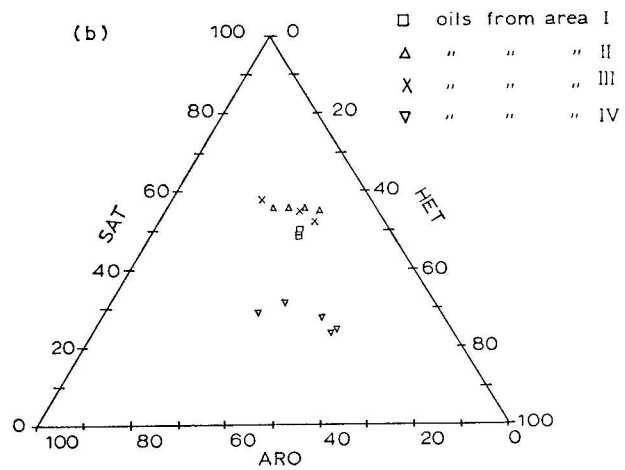


Fig. 1.

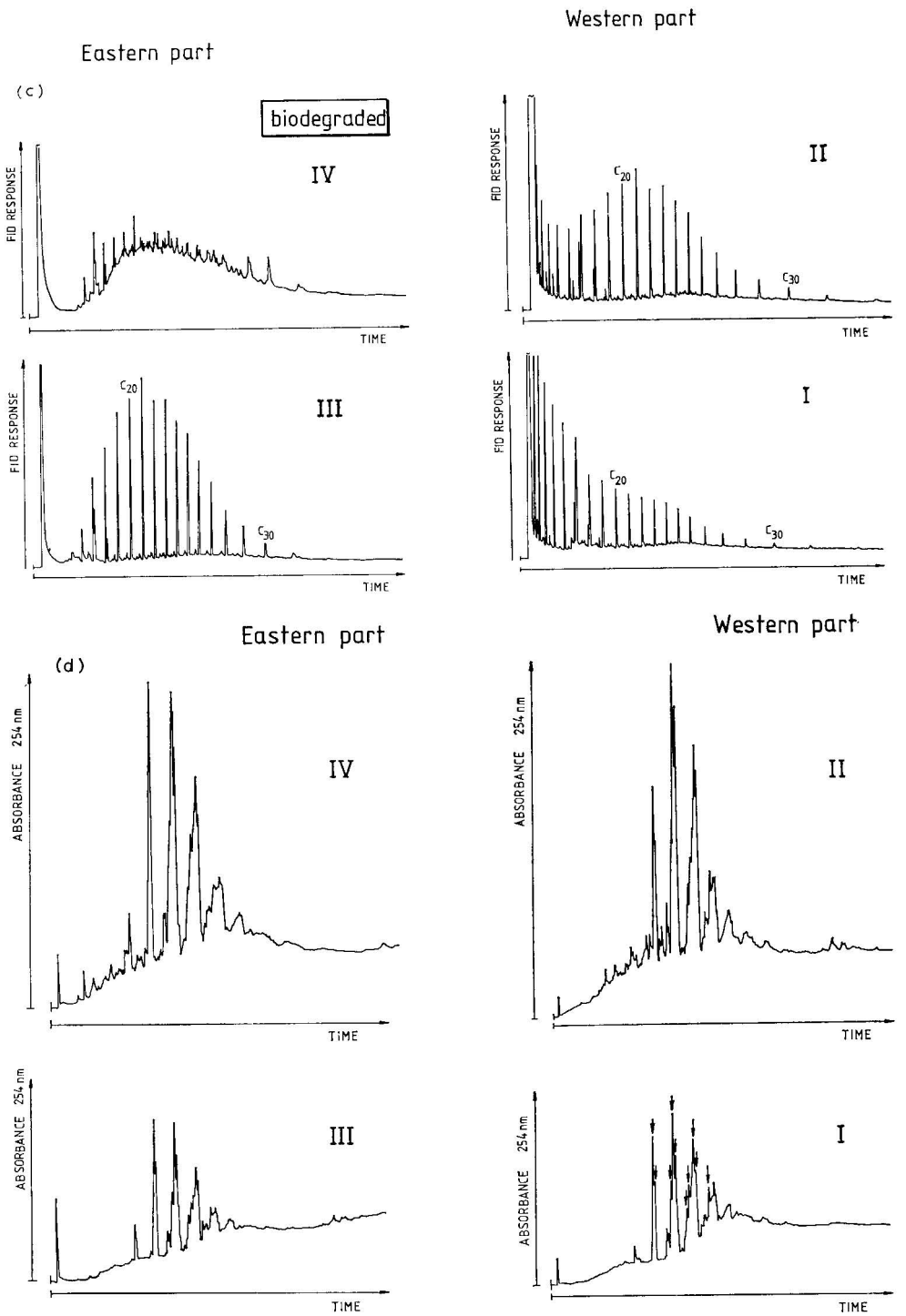


Fig. 1. (a) Map of the South German Molasse basin. (b) Triangular plot of the results of column chromatographic analyses of crude oils. (c) GC traces of SAT fractions of crude oils. (d) HPLC traces of ARO fractions of crude oils. Locations are indicated by I–IV.

from area IV with the other oil groups based only on the GC investigations. The aromatic hydrocarbon distribution is not so easily influenced by bacterial action because the nuclei are substituted with short alkyl substituents, mostly methyl groups, and direct action on the aromatic ring was observed only in some special cases[3].

We investigated the distribution of aromatic hydrocarbons in the ARO fraction by means of HPLC. Typical traces for oils from all four areas are shown in Fig. 1d. These complex chromatograms without tracer compounds are easily compared with one another by using a simple equation derived in detail elsewhere[4]. Briefly, the heights of a set of peaks are measured and the height ratios of adjacent peaks are calculated, thus eliminating quantification problems. The set of ratios obtained is considered as a description of a vector. To compare two samples the distance of the vector tips, D, is then calculated by means of the equation

$$D = \left[\sum_{a=1}^{n} (h_{ai} - h_{aj})^2\right]^{1/2}$$

where h_{ai} and h_{aj} are corresponding peak-height ratios and n is the number of ratios. To deduce a positive correlation, this D value has to be less than, *e.g.*, 1.5 when using 10 heights in this calculation. In Table II the results for the four oils shown in Fig. 1d are presented. The heights taken for this calculation are indicated by arrows (compare Fig. 1d, bottom trace). All D values calculated indicate a positive correlation. In detail there are only slight differences between the chromatograms obtained. The findings of this investigation are contradictory to those of the GC and column chromatographic analyses. We feel that further conclusions can be drawn only by considering not only total fractions of a crude oil but also suitable substance classes within those fractions.

TABLE II

D VALUES FOR THE HPLC SEPARATION OF CRUDE OILS FROM THE GERMAN MOLASSE BASIN (AREAS I–IV)

Field	*Area*	*I*	*II*	*III*	*IV*
Pfullendorf	I	0.00	1.30	0.86	1.42
Arlesried	II		0.00	0.91	1.02
Darching	III			0.00	0.65
Isen	IV				0.00

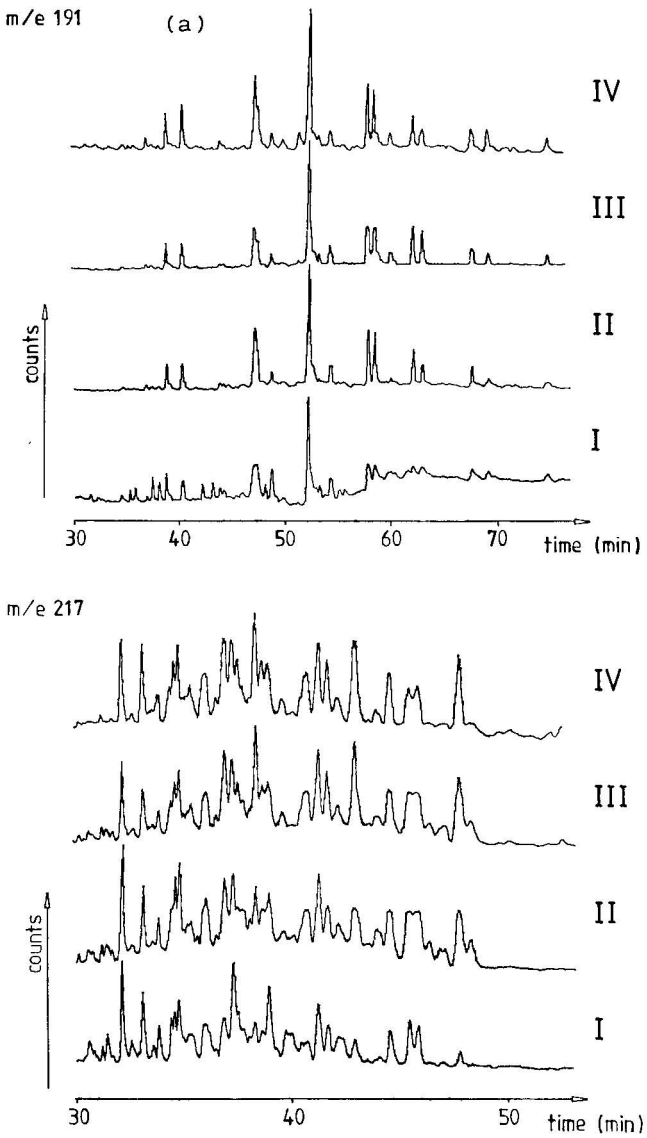
Biological markers are molecules synthesized by organisms. The molecules are found today in crude oils and sediments and are unchanged or have suffered only minor subsequent changes with preservation of the carbon skeleton. Tetracyclic steranes and pentacyclic triterpanes are suitable biomarkers[5]. Using GC–MS the distribution of these molecules can be analysed without prior separation from the SAT fraction by looking at the mass chromatograms of $m/e = 217$ (steranes) and $m/e = 191$ (triterpanes). In Fig. 2a mass fragmentograms are shown for crude oils from all areas of the German Molasse basin. For a positive oil–oil correlation these traces should be as similar as possible.

From Fig. 2a it can be deduced that there are only two oil groups in the Molasse basin. One consists of the crude oils found in Triassic reservoirs (area I;

Fig. 1a), and all of the other oils belong to the second group. Biodegradation seems to have little influence on the distribution pattern of steranes and triterpanes. Hence this technique is a real improvement in geochemical correlation work.

Oil–source rock correlation

The correlation of a crude oil with a sedimentary extract is difficult. First, sediments that may be possible source rocks are pre-selected according to the following rules: (1) the organic material, which is embedded in the sediments, must be of a type



(a)

Fig. 2.

(Continued on p. 488)

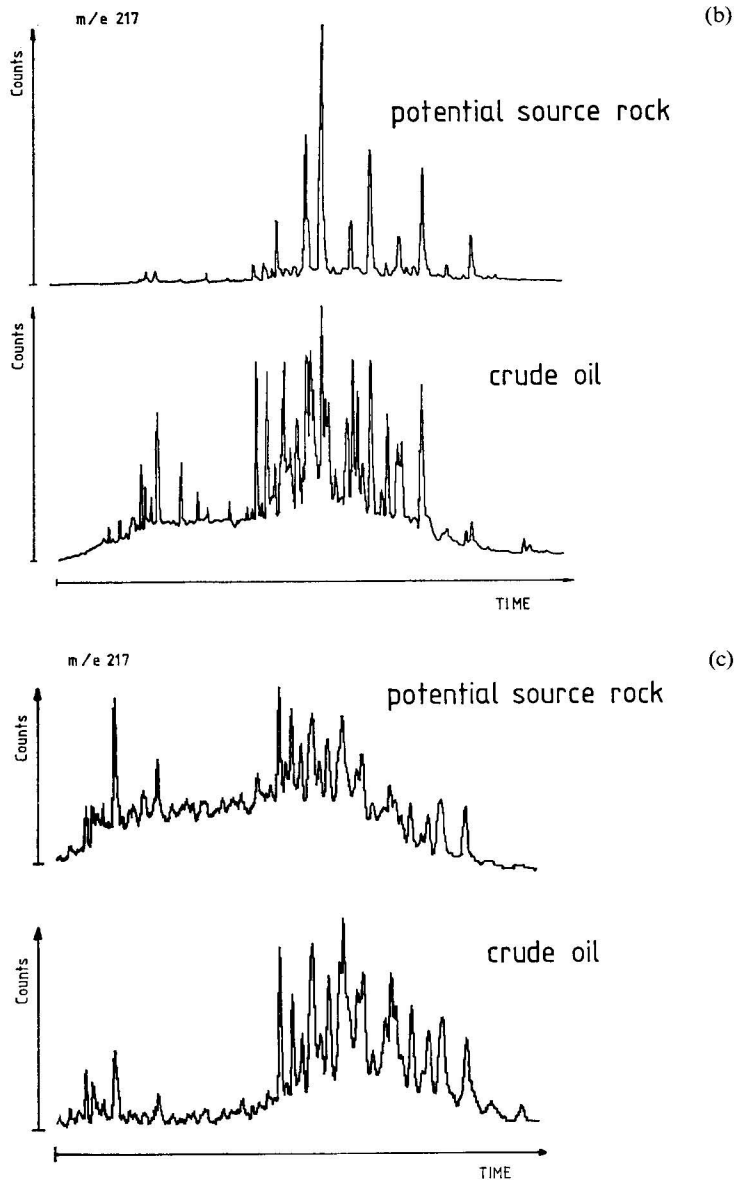


Fig. 2. (a) GC–MS investigation of crude oils; triterpane distribution pattern (m/e = 191) and sterane distribution pattern (m/e = 217). (b) Comparison of sterane distributions from a crude oil and a potential source rock in the Eastern part of the Molasse basin. (c) Comparison of sterane distributions from a crude oil and a potential source rock in the Western part of the Molasse basin.

that is able to generate oily material; (2) the sediment must be in a maturity stage that is suitable for oil generation, *i.e.*, it must have been buried deep enough; and (3) sediments that are only impregnated by an oil must be excluded. The maturity of a sediment is usually determined by petrographic methods, *e.g.*, measurement of the

vitrinite reflection[6]. Impregnated sediments may be detected by comparing the extract yield with the total organic carbon content.

The type of organic material can be determined by thermochromatographic methods[7]. Sediments containing bituminous material can act as a source rock for oils.

Such sediments show a larger amount of pyrolysable material in comparison with the total organic carbon content than do sediments with humic material, provided that the degrees of maturity are similar. Additionally, the GC trace of the volatiles is of importance[8]. In the Eastern part of the Molasse basin an organic-rich shale occurs at the base of Oligocene. In the past this "Fischschiefer" was considered as a potential source rock[9]. The degree of maturity of the organic material is just at the lower border of what is considered to be necessary for source rocks. However, considering the reconstructed mass chromatograms for steranes ($m/e = 217$, Fig. 2b), the distribution pattern is very different. For this reason the "Fischschiefer" must be ruled out as a source rock for the oils from area III of IV.

Another example can be shown from the Western part of the Molasse basin. Considering the degree of maturity of sediments, a strong increase is observed on going from the oil deposits in area II (Fig. 1a) to the South[10]. About 30 km from area II material was available from the "Elbsee" location. In Fig. 2c the $m/e = 217$ mass fragmentogram of an extract of an Upper Jurassic shale is compared with that of an oil from area II. The traces show good agreement, indicating that this sedimentary layer may act as a source rock. A positive correlation of this sediment with an oil from area II has to be checked further, considering the geological situations in the past. Moreover, other potential source rocks in the Western part of the German Molasse basin have to be investigated. The results of our further studies will be published elsewhere.

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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC IDENTIFICATION OF GEOCHEMICALLY SIGNIFICANT ISOALKANE HYDROCARBONS

J. ALBAIGÉS* and J. BORBÓN
Instituto de Química Bio-Orgánica (CSIC), Jorge Girona Salgado, Barcelona-34 (Spain)
and
M. GASSIOT
Instituto Químico de Sarriá, Barcelona-17 (Spain)

SUMMARY

Computerized gas chromatography–mass spectrometry has been used for fingerprinting C_{15}–C_{40} methyl- and polymethylalkanes in geological extracts. With proper selection of the key ions, three different series of isoprenoid hydrocarbons, exhibiting *iso*- and *anteiso*- head-to-tail and tail-to-tail structures can be observed. The regularities displayed by these series in the fragmentograms have been rationalized on the basis of the chromatographic behaviour of their components. In this respect, the possibility of predicting retention indices either by retention increments or by empirical relationships is discussed. The elution order of the isoalkanes identified is correctly established by the first-order molecular connectivity (χ), and a retention relationship giving mean absolute errors of 1.1 and 2.7 index units for Apiezon L and Dexsil, respectively, has been obtained for the C_{15}–C_{40} regular isoprenoid series.

INTRODUCTION

Isoalkane hydrocarbons are widely distributed in geological materials such as sediments and petroleum because they are already present as such in living organisms or because they may constitute the final step in the diagenesis and maturation of biolipids. In addition, they are stable enough to survive over long geological periods. Consequently, the analysis of these hydrocarbons, and especially the comparison of their carbon skeletons with those of contemporary biolipids, can furnish much information about the origin, depositional conditions and geochemical evolution of sedimentary organic matter.

In this class of hydrocarbons, homologous series of *iso*- and *anteiso*-alkanes (I) and acyclic isoprenoids with head-to-head (II), head-to-tail (III) and tail-to-tail linkages (IV) are of special significance because they have been recognized as the remains of bacterial activity in sediments[1] and the relative distribution of certain homologues as an indication of the paleoenvironmental conditions of deposition[2].

However, geological extracts generally afford very complex isomeric mixtures whose identification is far from complete, thus making difficult the direct character-

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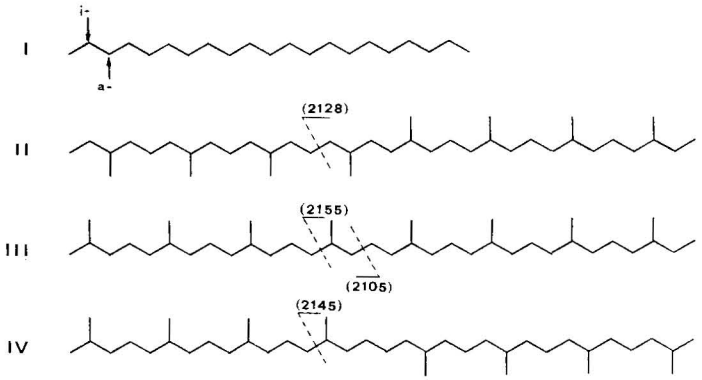


Fig. 1. Isoalkane hydrocarbon types occurring in geological samples. Dotted lines indicate a series of C_{24} isomers with their Kováts retention indices in parentheses.

ization of these components. Identifications made from gas chromatograms or mass spectra are not always unequivocal for isomeric isoalkanes. There are several examples in the literature of isoalkanes eluted separately which display very similar mass spectra[3] and, also, of co-eluting hydrocarbons that exhibit very different mass spectra[4]. Therefore, the use of multiple ion monitoring in gas chromatography–mass spectrometry (GC–MS) seems to be a suitable approach for the characterization of such hydrocarbons. Moreover, bearing in mind that many of these compounds occur as a part of homologous series, the regularities observed in the mass fragmentographic profiles may be of diagnostic value, provided that the chromatographic behaviour of the suspected compounds is known or may be predicted.

It is the main purpose of this paper to give some insight into the analytical methodology that can be used for such identifications, which, in turn, can contribute to a better knowledge of these fractions and to the discovery of new series of biological markers. Owing to its particular geochemical interest, the paper will be focused on the long-chain isoalkane range ($>C_{20}$).

EXPERIMENTAL

Concentrates of isoalkane hydrocarbons were obtained from sediment extracts and deasphaltened crude oils (in 40 volumes of *n*-pentane) by chromatography in a silica gel column (50 × 1 cm I.D.) using an adsorbent to sample ratio of 100:1. Then, saturated hydrocarbons were eluted with *n*-pentane and isoalkanes isolated by conventional procedures. Typically, the saturated fraction was treated with molecular sieve 5 Å and then subjected to adduct formation three times each in methanolic urea at 0°C and methanolic thiourea. The adducts, after decomposition in hot water, afforded fractions enriched in methyl- and polymethylalkanes, respectively.

Analysis by GC of pure compounds and purified extracts[5,6] was performed on a Perkin-Elmer 900 instrument, equipped with a flame-ionization detector using helium as the carrier gas, with 2 m × 0.2 cm I.D. columns packed with 3% Dexsil on Gas-Chrom Q (100–120 mesh) programmed from 150 to 300°C at 6°C/min, and 50 m × 0.25 mm I.D. open-tubular capillary columns coated with Apiezon L, programmed from 120 to 280°C at 6°C/min. Retention indices were calculated by means

of an HP 9830 desk calculator from retention times obtained with a digital integrator (Autolab 6300) in the range 200–270°C. The operating conditions were optimized as reported previously[7] and the mean absolute errors for the whole C_{10}–C_{40} series were found to be 1.4 and 0.9 index units for packed and capillary columns, respectively. As this error included the variation of retention index with temperature in the range studied, a mean index value is given for each compound (see Table I).

Fingerprinting of isoalkane hydrocarbons was carried out on the saturated fractions by computerized GC–MS using an LKB 9000 S-PDP 11E10 system. The gas chromatograph was operated with glass columns, either packed with 1% Dexsil on Gas-Chrom Q (100–120 mesh) (2 m × 0.2 cm I.D.) and temperature programmed from 200 to 300°C at 4°C/min or wall coated with SE-30 (25 m × 0.25 mm I.D.) and temperature programmed from 100 to 290°C at 4°C/min, using splitless injection. The jet separator was maintained at 290°C.

RESULTS AND DISCUSSION

It is well known that isoalkane hydrocarbons exhibit characteristic fragment ions at m/e C_nH_{2n+1}, corresponding to the cleavage of bonds adjacent to tertiary carbon atoms. When these ions are used in the mass fragmentographic analysis of geological extracts, relatively constantly recurring patterns of peaks appear, as is shown in Figs. 2 and 3. Then, suitable selection and grouping of these key ions may

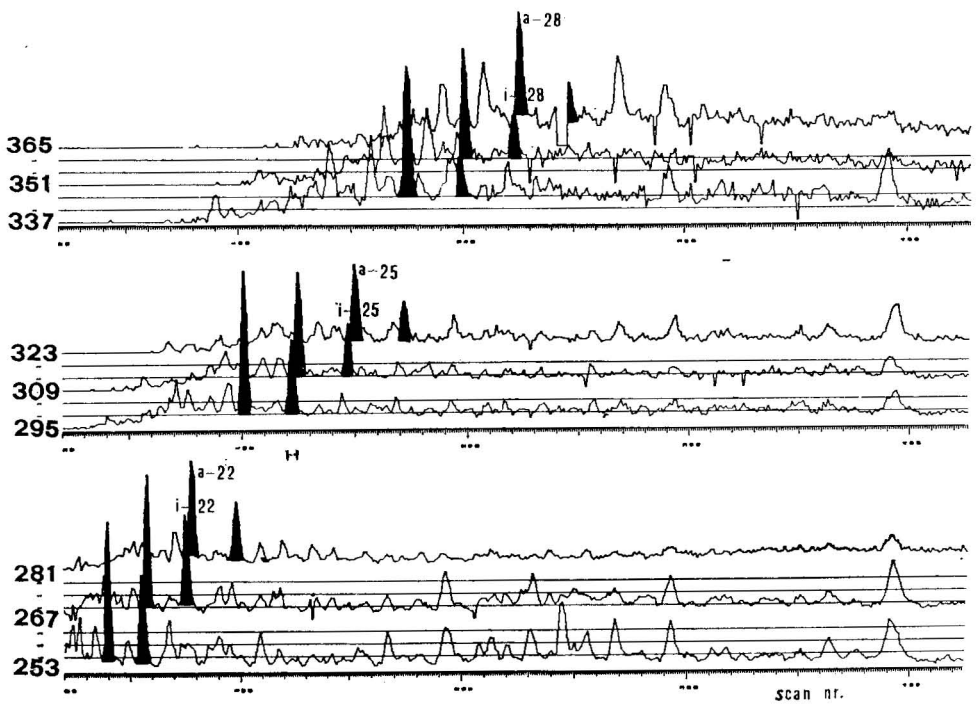


Fig. 2. Computer-reconstructed fragmentograms of methylalkanes (I) in an oil sample (branched + cyclic fraction).

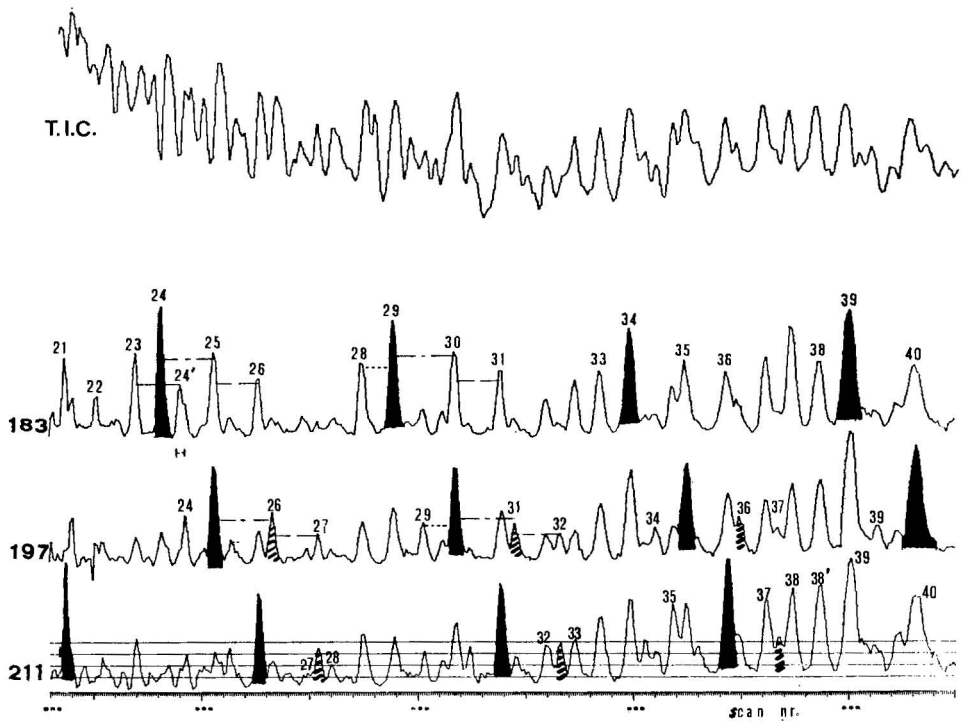


Fig. 3. As Fig. 2, for isoprenoid alkanes. The number of carbon atoms of the homologous series III, V and IV are indicated on the *m/e* 183, 197 and 211 profiles, respectively.

allow the characterization of homologous series, thus overcoming the possible interference between series exhibiting common diagnostic ions.

Methylalkanes (I) produce only one major fragment of the series C_nH_{2n+1}. According to the position of the single methyl branching, the same fragment ion can be exhibited by several alkanes, the most abundant being those of *iso*- and *anteiso*-series (see Fig. 2). On the other hand, regular isoprenoid alkanes (III) display intense peaks at *m/e* 113 + 70*N* and 113 + 14*n*, where *N* and *n* depend, respectively, on the number of isoprenoid units and the number of carbon atoms in the molecule (*e.g.*, C_{10} : $N = 0$, $n = 1$). Finally, tail-to-tail isoprenoids should present prominent peaks at *m/e* 113, 183, 239, 267 or 113, 183, 253, 309, 337, depending on which is the precursor, namely squalane (C_{30}) or lycopane (C_{40}) (IV), whilst head-to-head series (II) should present peaks at *m/e* 127, 197, 267, 323 or 113, 183, 253, 309, 379, according to the pristane or phytane molecular moieties.

In this way it is possible to discriminate the above series of isoalkanes, as is shown in Figs. 2 and 3. In addition, careful examination of Fig. 3 revealed the presence of another series of peaks giving rise to higher responses in the 127 + 70*N* and 127 + 14*n* profiles, as can be seen partially in more detail in Fig. 4.

Although much work is in progress on the unequivocal identification of this series, various evidence points to a regular anteisoprenoid structure (V, Fig. 4). Such an assumption is supported by the mass spectra of individual peaks and their positions in the chromatogram, with retention displacements paralleling those of the *iso* series

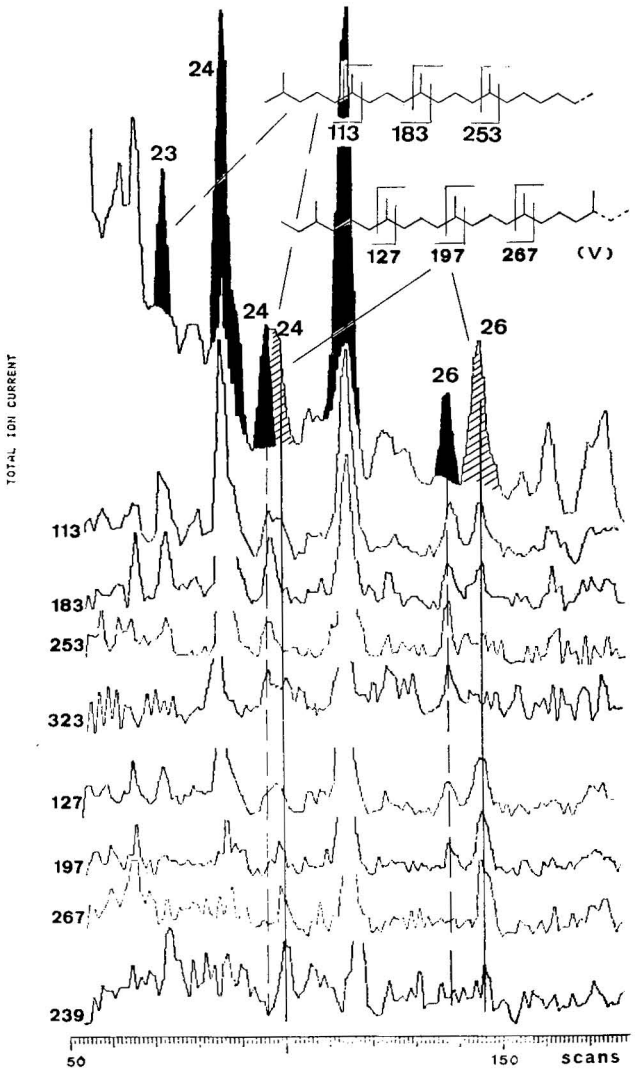


Fig. 4. Extended mass fragmentographic characterization of the C_{24}–C_{26} range shown in Fig. 3.

(III) for the elongation of the chain or the transfer of methyl groups to the *anteiso*-position (*i*-$C_{24} \rightarrow$ *a*-C_{24}) (see the dotted lines in Figs. 2 and 3).

In order to substantiate these correlations, retention increments on Apiezon L and Dexsil were calculated for the whole C_{10}–C_{40} isoprenoid series (Table I) and further applied to the determination of retention indices of other series, in a similar manner to that described by Shlyakhov *et al.*[8] for the lower C_{20} homologues. The results obtained for the regular *anteiso*- series (3, 7, 11, 15, . . . polymethylalkanes) showed fair agreement with those determined experimentally, as reported elsewhere[6].

In an additional application, the calculated retention indices for the different C_{24} isomers, originating from the higher homologues (see Figs. 1 and 4), provide confirmation of the proposed structures for those isomers.

TABLE I

KOVÁTS RETENTION INDICES OF C_{10}–C_{40} ISOPRENOID HYDROCARBONS

Retention increments corresponding to methyl and isoprenoid unit chain elongations are indicated by squares and circles, respectively.

Apiezon L	*Carbon number*											
n	*10 + n*		*15 + n*		*20 + n*		*25 + n*		*30 + n*		*35 + n*	
0	931	(434)	1365	(424)	1789	(419)	2208	(419)	2627	(413)	3040	
	[86]		[84]		[83]		[85]		[84]		[85]	
1	1017	(432)	1449	(423)	1872	(421)	2293	(418)	2711	(414)	3125	
	[92]		[89]		[88]							
2	1109	(429)	1538	(422)	1960		-		-		-	
	[96]		[94]		[92]							
3	1205	(427)	1632	(420)	2052	(418)	2470	(415)	2885	(413)	3298	
	[56]		[53]		[53]		[53]		[52]		[52]	
4	1261	(424)	1685	(420)	2105	(418)	2523	(414)	2937	(413)	3350	
	[104]		[104]		[103]		[104]		[103]		[104]	
5	1365	(424)	1789	(419)	2208	(419)	2627	(413)	3040	(414)	3454	

A general feature displayed by isoalkanes is that their retention times decrease when methyl branches migrate towards the ends of the chain. Hence, for example, the elution order of the above C_{40} hydrocarbons should be IV $<$ III $\approx$ II, and this trend can be highly useful for the characterization of these fractions if it can be adequately established.

Bearing in mind that topological parameters are especially suitable for the characterization of molecular branching, we try to establish an empirical relationship that would be able to predict the chromatographic behaviour of such hydrocarbons. In this respect, it has been shown that retention indices are strongly correlated with the first-order molecular connectivity (χ)[9], although until now methods have been developed only for low-molecular-weight isoalkanes[10]. Thus, the following retention–connectivity relationships were established for the isoprenoid series:

$$\text{Apiezon L: } RI = 99.8 + 180.7\,\chi - 0.2\,\chi^2 + 52.8\,(\Delta\chi - 0.5);\ |\varepsilon| = 1.1 \text{ index units}$$

$$\text{Dexsil: } RI = 92.2 + 183.7\,\chi - 0.3\,\chi^2 + 42.3\,(\Delta\chi - 0.5);\ |\varepsilon| = 2.7 \text{ index units}$$

where $\Delta\chi$ represents the difference between the isoalkane and its preceding homologue. This factor was introduced to improve the correlation for the deviations displayed by the more branched isoalkanes, namely those of the C_{14+5n} series.

The results obtained (Table II) afford striking evidence for the adequacy of this approach, which will be extended to other series.

TABLE II

EXPERIMENTAL AND CALCULATED RETENTION INDICES FOR THE REGULAR ISOPRENOID SERIES, USING THE MOLECULAR CONNECTIVITY (χ) RELATIONSHIP

Carbon No.	χ	*Apiezon L*			*Dexsil*		
		RI_{exp}	RI_{calc}	ε	RI_{exp}	RI_{calc}	ε
14	6.5198	1261	1260.1	−0.9	1270	1269.8	−0.2
15	7.0578	1365	1365.1	0.1	1375	1373.9	−1.1
16	7.5578	1449	1451.7	2.7	1457	1461.8	4.8
17	8.0578	1538	1540.2	2.2	1550	1551.1	1.1
18	8.5578	1632	1628.5	−3.5	1644	1640.2	−3.8
19	8.9137	1685	1683.7	−1.3	1700	1697.4	−2.6
20	9.4516	1789	1788.2	−0.8	1804	1800.7	−3.3
21	9.9516	1872	1874.2	2.2	1884	1887.8	3.8
22	10.4516	1960	1962.1	2.1	1975	1976.3	1.3
23	10.9516	2052	2049.9	−2.1	2068	2064.7	−3.3
24	11.3076	2105	2104.7	−0.3	2122	2121.4	−0.6
25	11.8455	2208	2208.5	0.5	2225	2223.9	−1.1
26	12.3455	2293	2294.0	1.0	2304	2310.2	6.2
27	12.8455	—	2388.0	—	—	2402.4	—
28	13.3455	2470	2468.5	−1.5	2490	2485.5	−4.5
29	13.7014	2523	2522.9	−0.1	2543	2541.6	−1.4
30	14.2394	2627	2626.1	−0.9	2645	2643.3	−1.7
31	14.7394	2711	2711.0	0.0	2722	2728.8	6.8
32	15.2394	—	2807.1	—	—	2823.0	—
32	15.2394	—	2807.1	—	—	2823.0	—
33	15.7394	2885	2884.4	−0.6	2906	2902.5	−3.5
34	16.0953	2937	2938.3	1.3	2957	2958.1	1.1
35	16.6332	3040	3040.9	0.9	3060	3058.9	−1.1
36	17.1332	3125	3125.2	0.2	3137	3143.7	6.7
37	17.6332	—	3223.9	—	—	3238.1	—
38	18.1332	3298	3297.4	−0.6	3319	3315.9	−3.1
39	18.4891	3350	3351.0	1.0	3370	3370.9	0.9
40	19.0271	3454	3453.0	−1.0	3473	3470.9	−2.1

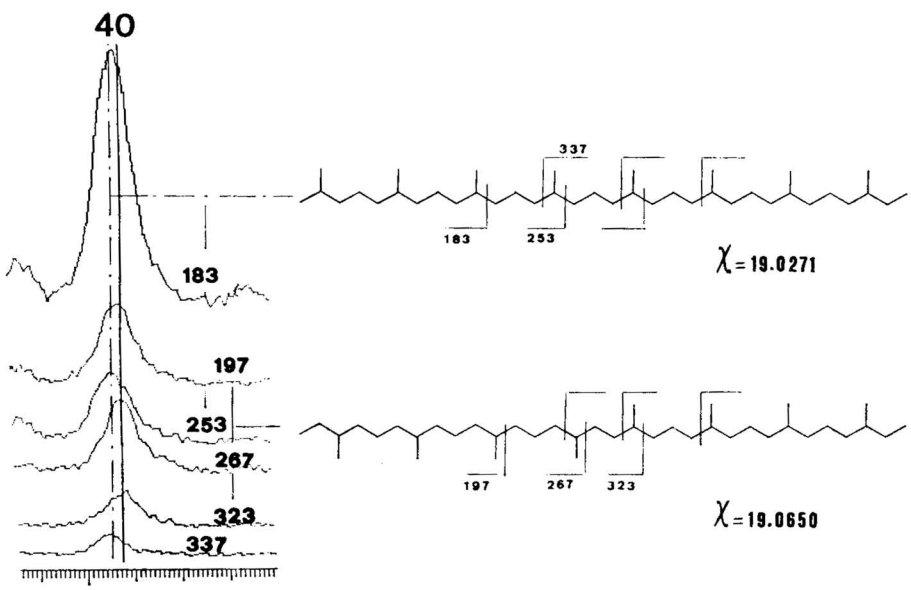


Fig. 5. As Fig. 4, for the C_{40} peak.

It is worth mentioning that molecular connectivity has been found satisfactory for the prediction of the elution order of all of the isoalkanes considered.

Fig. 5 shows an example for C_{40} isomers, which can be only partially resolved by mass fragmentography. Consequently, mass fragmentograms can be advantageously exploited with such information. In this manner, these profiles can be used as complementary tools in oil–oil, oil–source roch and oil–pollutant source correlations, and this will be considered elsewhere[6].

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PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3		The publication schedule for further issues will be published later.								
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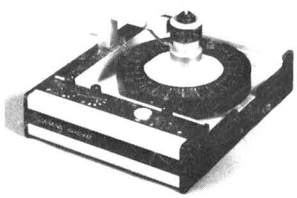
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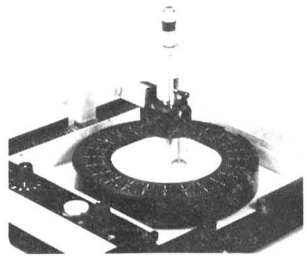
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either in combination with fixed volume Nanopipette 100 or 200 nl, reproducibility 1%

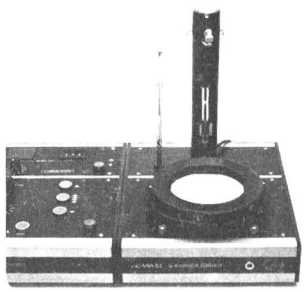
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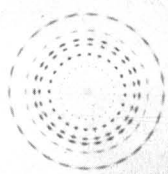
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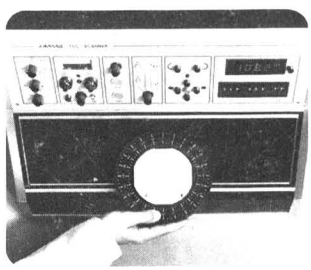
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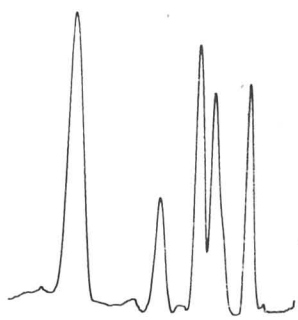




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