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FUSED-SILICA NARROW-BORE MICROPARTICLE-PACKED-COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

   Narrow-bore fused-silica liquid chromatography columns with I.D. $\leqslant 500$ μm and packed with $\leqslant 10$-μm particles in lengths from 10 cm to a few meters can be efficiently prepared. A 1-m fused-silica column with 110,000 theoretical plates can be packed with 3-μm C_{18} bonded-phase particles. Narrow-bore microparticle-packed columns can be used in conjunction with a constant-pressure piston pump, a splitter injector and an on-column UV absorbance detector. A 45 cm × 300 μm I.D. column packed with 3-μm C_{18} bonded-phase particles allows baseline separation of an Environmental Protection Agency priority pollutant mixture containing sixteen polynuclear aromatic hydrocarbons. The use of the effect of solvent modifier(s) in the sample solution to improve solute resolution, peak detection, and column selectivity and to reduce analysis time of the narrow-bore microparticle-packed-column high-performance liquid chromatography is proposed and demonstrated. The practical importance of the solvent effect in narrow-bore packed-column high-performance liquid chromatography is also discussed.

INTRODUCTION

   Recent advances in high-performance liquid chromatography (HPLC) column technology center on open tubular columns[1–6] and microparticle-short-packed columns[7–10]. The interest in open tubular columns is due to the following reasons: (1) a 10 to 10^4 fold saving in solvent consumption compared with conventional packed-column HPLC using columns of 1–5 mm I.D.; (2) higher resolution can be achieved using long microbore columns; (3) minimum sample requirements provide the potential for trace analysis in cases of limited available sample volume, such as in the analysis of biological fluids; and (4) the low flow-rates should facilitate direct interfacing to mass spectrometers and flame-based chromatographic detectors[10]. The advantages of directly interfacing with flame-based detectors are particularly important due to the fact that these detectors offer greater sensitivity and selectivity (elemental specificity) than do conventional LC detectors. As discussed previously[11], with the use of open tubular columns of I.D. less than 30 μm, the resolving power, speed of analysis and column peak capacity can be equal to or better than that achieved with

the state-of-the-art 5-μm-particle-packed columns having a reduced plate height of 2 and a reduced velocity of 5 in a given analysis time. However, the stringent requirements imposed by microbore open tubular columns on the stability of the hydraulic pumps at low flow-rates, nanoliter sample volume injection, submillimeter connector tubing and subnanoliter detector flow cells present substantial impediments to the full use of available column performance[11]. In addition, columns with I.D. less than 30 μm are difficult to prepare and have limited partition ratios (e.g., k less than 3), sample capacities (e.g., less than 1 μg), column selectivity and ranges of applicable sample mass and concentration.

Compared with conventional 25–30 cm × 4–5 mm I.D. LC columns, microparticle-short-packed columns (10 cm × 1–5 mm I.D.) packed with 3–10-μm particles offer some savings in solvent consumption, a decreased analysis time and enhanced detection of solutes. However, the application of microparticle-*short*-packed columns is limited to samples containing not more than several dozen components, due to a limited column-peak capacity and theoretical plate number. In addition, the direct interfacing of such microparticle-packed columns to a mass spectrometer or a flame-based chromatographic detector for solute detection and characterization cannot be obtained at the flow-rates used (1–10 ml/min).

It is the purpose of this paper to propose and to examine the use of columns with I.D. less than 500 μm and packed with $\leqslant$10-μm particles in lengths from 10 cm to a few meters. *Narrow-bore microparticle-packed columns*★, as suggested here, are normally operated at the same linear velocity as conventional packed columns, 0.5–2 mm/sec, dependent upon the particle size used. Because of the small I.D., the volumetric flow-rates are between 0.1 and 10 μl/min. This low mobile-phase flow-rate allows easy, direct interfacing of the column to a mass spectrometer[12,13] and/or a flame-based chromatographic detector[11] so that more sensitive and specific LC detection can be obtained. The proposed narrow-bore microparticle-packed column LC provides both the advantages of open tubular column and short microparticle-packed column LC in terms of solvent-saving, fast analysis, and minimum sample size requirement. In addition, the packed bed in the narrow bore column is stable due to the difficulty of rearrangement and resettling of the tightly packed particles in a very small cross-sectional area. The use of long columns (0.5–2 m) provides the high resolving power and peak capacity required for complex sample analysis.

In this report, the chromatographic performance and practical aspects of narrow-bore microparticle-packed columns of I.D. ranging from 57 to 376 μm and packed with 3-, 5- and 10-μm C_{18} bonded reversed-phase particles are evaluated in conjunction with a constant-pressure piston pump, a splitter injector[1] and an on-column★★ UV absorbance detector[14].

EXPERIMENTAL

A Varian Model 8500 high-pressure piston pump was used in the constant-pressure mode. Flow-rates as low as 0.1 μl/min were obtained without any modification of the pump. A 0.5-μl Valco (Houston, TX, U.S.A.) internal sample loop valve was used for sample injection. The splitting of the injected sample was achieved by

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★ Patent pending.
★★ U.S. patent application S.N. 223,445.
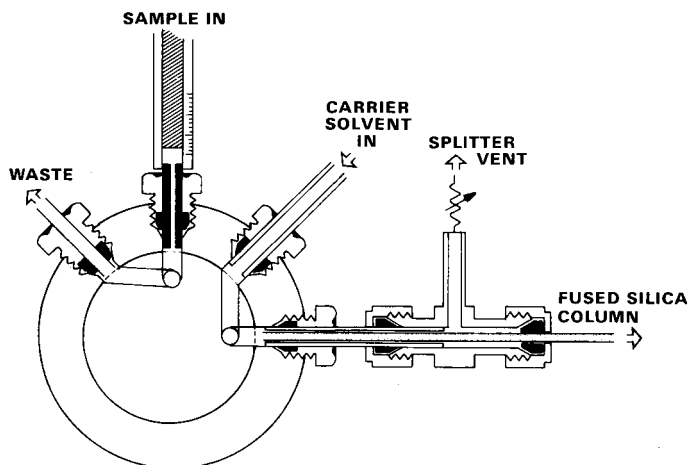
Fig. 1. The modified Valco 0.5-μl internal loop sampling valve for split injection in narrow-bore packed column HPLC.

utilizing the inlet of the narrow-bore microparticle-packed column as a split point, which is located inside the housing of the Valco valve as shown in Fig. 1. Fused-silica tubing (courtesy of Mr. Ernest Dawes, Scientific Glass Engineering) with I.D. ranging from 57 to 376 μm and with lengths up to 2 m was packed with 3-, 5- and 10-μm C_{18} bonded-phase particles using a slurry-packing technique. The reversed-phase octadecylsiloxane was chemically bonded onto the microparticulate silica before packing into the microbore columns. The mobile phase used in the study was 70:30 acetonitrile–water under isocratic conditions.

A Jasco UVIDEC-III UV spectrometer (Jasco, Tokyo, Japan) with a time constant of 0.5 sec in conjunction with on-column flow cell detection[14] was used as shown in Fig. 2.

Experimental data collected in real time using a conventional chromatographic data system were processed using a chromatographic column evaluation routine[16] in which chromatographic performance parameters such as peak width, retention time, partition ratio, plate height, peak area, peak height, statistic moments, peak skewness and peak asymmetry are calculated.
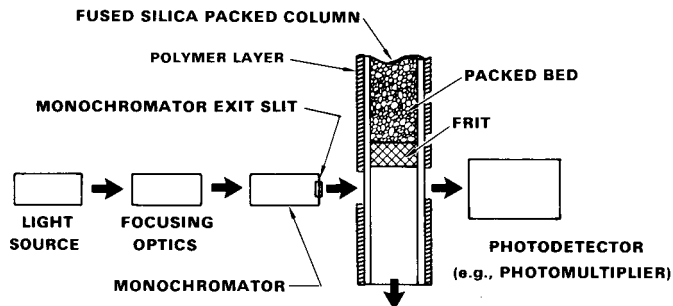
Fig. 2. Schematic diagram for on-column detection using narrow-bore fused-silica microparticle-packed column.

RESULTS AND DISCUSSION

Fused-silica columns were used as both the separation column and as the flow cell for on-column UV absorbance detection. The use of the column end as an on-column absorbance flow cell eliminates the need for column-to-detector connections and provides a sub-nanoliter detector flow cell which contributes no significant band broadening to the eluted peaks. The use of thin-wall flexible fused-silica tubing as the LC column material provides the following advantages:(1) good mechanical strength, flexibility and, thus, ease of handling; (2) inlet pressure ranges up to 800 atm may be applied and, thus, long columns can be prepared and used; (3) a smooth inner surface minimizes solute zone-spreading due to wall effects; (4) good chemical inertness and low metal content minimize chemical and physical interactions between solute molecules and the column walls; (5) good optical transparency allows visual observation of the column packed bed as well as on-column absorbance detection in the UV region; (6) small outside diameters of the fused silica columns allow easy interfacing into injector and detector with minimum extra-column effects; (7) no connectors or interface tubing are required if split injection and on-column detection are used; and (8) columns can be coiled and thus the design of a column oven is simplified.

In addition, the temperature effect[15] as normally observed due to the temperature gradient across the column cross-sectional plane and along the column axial direction is minimized for fused-silica narrow-bore microparticle-packed columns. The narrow column diameter, the thin fused-silica wall, and the low volumetric flow-rates insure that heat generated by friction at high operating pressures can be rapidly equilibrated with the ambient or column oven temperature. The absence of a significant thermal effect allows the use of microparticles ($\leqslant 10 \ \mu$m) and long column lengths at high operating pressures for high column efficiency and resolving power.

Instrumental extra-column zone-spreading effects

Extra-column zone-spreading effects arise, in general, from three major sources: injectors, connectors and detectors. Because of the small elution volumes (small peak widths) and flow-rates in narrow-bore microparticle-packed column LC, the design requirements for a narrow-bore microparticle-packed column liquid chromatograph are quite stringent. In this work, no column-to-injector and detector[14] interface tubings were used. Split injection utilizes the narrow-bore packed-column inlet as a splitter such that a fraction of sample is split to the column and the remaining fraction is purged to waste. Injector dead-volume effects are minimized at high injector flow-rates and split ratios.

On-column detection[14] uses the column end as a detector flow cell for optical detection. The extra-column zone-spreading contribution to the percentage loss in peak resolution, $\%\varDelta R_s$, can be expressed as

$$\%\varDelta R_s = \left(1 - \left[1 + \frac{H'L'}{HL} \right]^{-1/2} \right) \times 100\% \tag{1}$$

where H' and H are the height equivalent to a theoretical plate for the flow cell and the column, respectively, and L' and L are the entrance/exit slit length for the detector flow cell and the column length, respectively. For a column of circular cross-section, as an example, H' can be calculated according to eqn. 2:

$$H' = \frac{2\pi \, r^2 \, D_m}{F} + \frac{F}{24\pi \, D_m} \tag{2}$$

where r, F, and D_m are the column radius, the mobile-phase volumetric flow-rate, and the solute–mobile phase binary molecular diffusion coefficient.

Assuming a 1 m × 200 μm I.D. narrow-bore packed column of 5-μm C_{18} bonded-phase particles, a reduced plate height, h, of between 2 and 8 can be obtained at a flow-rate of 1 μl/min. The percentage loss of resolution, $\%\Delta R_s$, against reduced plate height, h, using on-column detection with a 0.3-cm entrance/exit slit optical cell height and a 200-μm light path-length is calculated and given in Fig. 3.
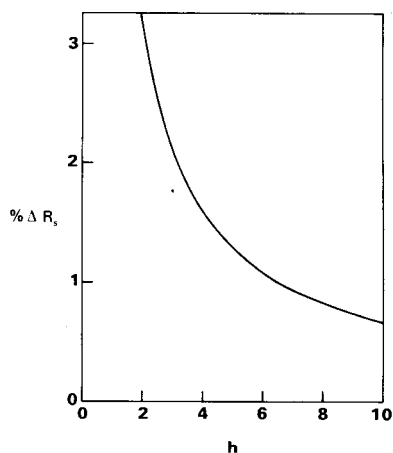
Fig. 3. Theoretically calculated percentage loss in peak resolution using on-column detection for a 5-μm particle 1 m × 200 μm I.D. packed column. A 300 × 200 μm I.D. on-column detector flow cell was assumed.

Fig. 3 indicates that a 300 × 200 μm I.D. cylindrical on-column flow cell contributes *ca.* 3% to the loss of peak resolution obtained by a 1 m × 200 μm I.D. narrow-bore microparticle-packed column having a reduced plate height of 2 and particle diameter of 5 μm. The loss in peak resolution is greatly reduced for columns of lower efficiency. For example, the same on-column detector contributes less than 1% loss to the peak resolution if the column has a reduced plate height of greater than 6.5. Experimentally observed reduced plate heights for the narrow-bore micoparti-cle-packed columns reported here ranged from 3 to 6, and so the on-column detector contribution to the loss of peak resolution was *ca.* 1–2%.

The injector could become a major source of extra-column zone-spreading if the internal volume of the injector cannot be effectively swept by the mobile solvent. Effective sweeping of the internal volume of the injector can be achieved either with high split ratios or with high mobile-solvent flow-rates.

Fig. 4 shows a comparison of the plate heights, H, *vs.* partition ratios, k', for polynuclear aromatic hydrocarbon (PAH) components eluted from a 30 cm × 376 μm I.D. narrow-bore column, packed with 5-μm C_{18} bonded-phase silica gel par-ticles. Direct injection was obtained by using a Valco loop valve with a 0.5-μl internal
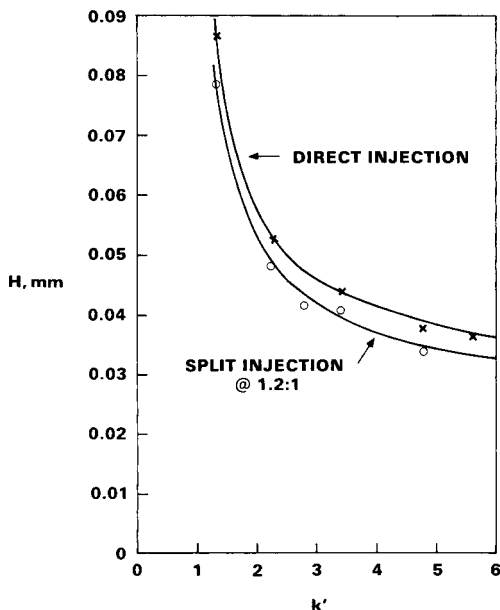
Fig. 4. A comparison of injector dead-volume effect on zone-spreading for PAH samples: benzene, $k' = 1.26$; toluene, $k' = 2.25$; naphthalene, $k' = 2.75$; fluorene, $k' = 3.4$; phenanthrene, $k' = 4.8$; pyrene, $k' = 5.7$. Column mobile solvent (70:30 acetonitrile–water) and splitter vent flow-rates were 9.24 and 10.9 μl/min, respectively. A 30 cm· × 376 μm I.D. column packed with 5-μm particles was used.

loop volume, where the injector mobile flow-rate equals the column flow-rate at 9.24 μl/min. Split injection was obtained by splitting the injector flow-rate of 20.3 μl/min at 1.2 to 1, and it allowed a 9.24 μl/min mobile-phase flow-rate in the column. Plate heights obtained by using direct injection were nearly 10 % higher than that obtained by using split injection.

At low column mobile-phase flow-rates, the injector dead-volume contribution to solute zone-spreading could be substantial if a long residence time in the injector were allowed for the sample. Fig. 5 shows the injector dead-volume effect on the measured plate heights for the same sample and conditions as that for Fig. 4, but the column flow-rate is 3.5 μl/min. The plate heights obtained by using direct injection ranged from 25 to 75 % higher than those obtained by using a 2.1 to 1 split injection. A conventional direct injector (loop valve) is obviously not satisfactory if narrow-bore microparticle-packed columns are to be operated at their optimum efficiencies. A 2.1 to 1 splitter has a significantly reduced injector dead-volume effect at low mobile-solvent flow-rates. However, the negative slopes of H vs. k' on Figs. 4 and 5 indicate that a significant degree of solute zone-spreading is the result of the injector dead-volume effect. High split ratios (e.g., $\geqslant 10$ to 1) are desirable if no zone-spreading contribution from the injector is allowed and if low column mobile-phase flow-rates are used.

Column efficiency versus column inner diameters

Column efficiency in terms of plate height is compared in Fig. 6 for columns
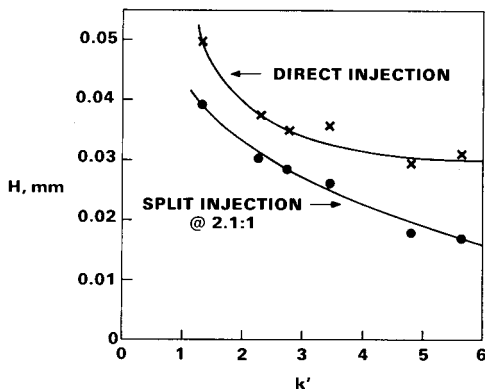
Fig. 5. The effect of injector dead volume on the measured column efficiency in terms of plate height, H. Column and mobile solvent used were the same as for Fig. 4. Column mobile phase and splitter vent flow-rates were 3.52 and 7.27 μl/min, respectively.

with 130-, 210- and 310-μm I.D. columns packed with the same 5-μm C_{18} bonded-phase particles under similar slurry-packing conditions. All three columns were 1 m in length. Three of five columns of each I.D. size were studied.

The performance of the 210-μm column at low flow-rates is superior to that obtained from both 130- and 310-μm columns. A reduced plate height of 4.4 was measured for the 210-μm column and a reduced plate height of 5.5 was obtained for the 130- and 310-μm columns. The reduced plate heights reported here, however, were two to three times higher than the commonly assumed optimum value of 2. The column packing procedure thus requires further optimization. Even so, long columns can be used and good reproducibility in column efficiency was obtainable, under the same conditions.
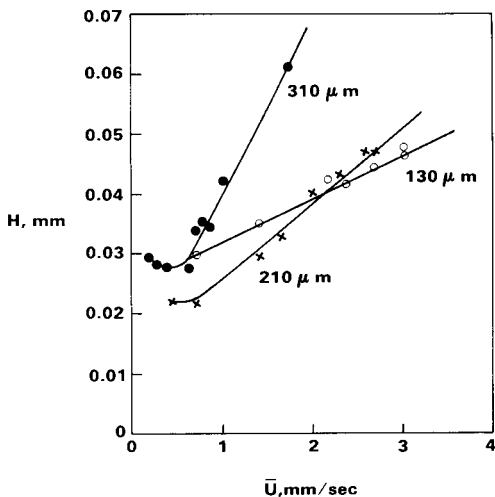
Fig. 6. Performance of 5-μm MicroPak MCH-5 reversed-phase packing in narrow-bore packed columns. Test compound was pyrene at $k' = 5.7$.

Fig. 6 also shows that the slope of H vs. $\bar{u}$ for the 310-μm column is the highest among the three studied here. This could indicate a non-uniform packed bed due to the non-optimized packing technique used here. Columns of 130 μm I.D. had the lowest slope of H vs. $\bar{u}$. This indicates that the smaller column I.D., the smaller the flow-rate effects. The short narrow-bore microparticle-packed column of 130 μm I.D. would thus be the best among the three for operating at high flow-rates for fast analysis.

Resolving power of microbore packed columns

The resolving power of conventional packed columns is limited by the total available column theoretical plate number. Conventional 5-μm particle packed columns with a length of 30 cm can generate 15,000 to 25,000 plates. Recently developed 3-μm particle packed columns, available in 10–15 cm lengths, generate 10,000 to 20,000 plates. Complex sample analysis with baseline resolution of most components can be difficult using conventional packed columns limited to less than 25,000 plates.

The van Deemter plot for 1 m $\times$ 330 μm I.D. columns packed with spherical 3 μm C_{18} bonded silica particles is shown in Fig. 7. (RoSil, courtesy of Dr. M. Verzele, State University of Ghent, Belgium). A minimum plate height of 9.7 μm corresponding to a reduced plate height of 3.2 was obtained. The total column plate number exceeded 110,000 for the flow-rate range studied. The flow-rate did not significantly affect plate height in the range 0.5–1.5 mm/sec.
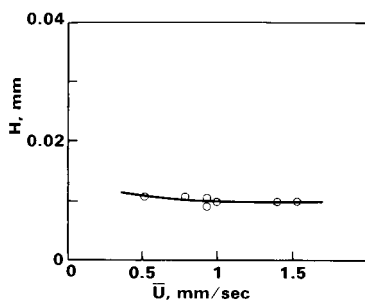
Fig. 7. Performance of a 3-μm C_{18} bonded-phase particle 330 μm I.D. packed column. Test compound was pyrene.

Application of the high plate count available in such a long, narrow-bore packed column is demonstrated in the isocratic separation of an Environmental Protection Agency (EPA) priority pollutant mixture of sixteen PAH components, shown in Fig. 8. Baseline resolution of the PAH mixture was obtained using a 50,000-plate, 45 cm $\times$ 330 μm I.D. column packed with the 3-μm C_{18} material (RoSil) discussed in the preceding paragraph. The mobile phase was 70:30 acetonitrile–water and the flow-rate was 3.7 μl/min ($\bar{u}$ = 1.2 mm/sec). The high plate count of the long microcolumn was necessary to achieve baseline resolution of the PAH pair benzo[a]anthracene/chrysene (peaks 9, 10).

Solvent usage in the PAH separation was 600 μl for the 180-min separation. This represents a solvent use reduction of 65 fold relative to a conventional 15 cm $\times$ 4.6 mm I.D. column packed with 3-μm C_{18} material, generating only 20,000 plates.
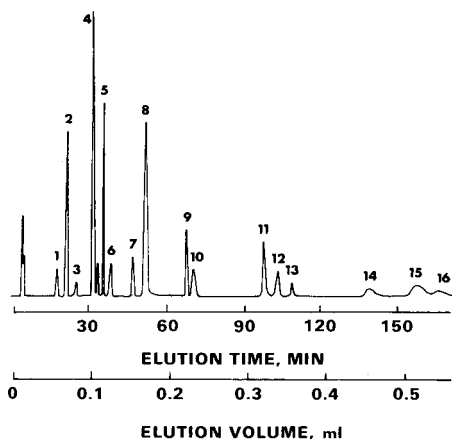
Fig. 8. Separation of sixteen-component PAH mixture on a 50,000-plate, 45 cm × 330 μm I.D., 3-μm C$_{18}$ packed column. Peak identifications are: 1, naphthalene; 2, acenaphthalene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene; 9, benzo[a]anthracene; 10, chrysene; 11, benzo[b]fluoranthene; 12, benzo[k]fluoranthene; 13, benzo[a]pyrene; 14, dibenzo[a,h]anthracene; 15, benzo[ghi]perylene, 16, indeno[1,2,3-cd]pyrene.

Solvent effect in narrow-bore column HPLC

In column LC, the use of solvent and solvent modifier(s) to improve column selectivity and resolution of sample components and to reduce the analysis time of well-retained components are practised either by premixing the solvent and its modifier(s) in isocratic operations or by step or linear solvent programming in programmed elution chromatography. To date, there have been few reports[17,18] on the use of solvent or solvent modifier(s) in the sample solution for the improvement of solute resolution, column selectivity and reduction in analysis time. In general, because of the large column cross-sectional area and mobile-solvent flow-rates used in conventional column (4–6 mm I.D.) HPLC, a large volume of solvent modifier(s) in the sample would be necessary in order to exert an appreciable solvent effect. This large volume of solvent modifier(s) in the sample solution may precipitate the sample components and disturb the equilibrium of the phase system at the head of the column. In practice, the same solvent as the mobile phase is used to dissolve the sample solutes and to prevent such problems. For trace analysis, on-column trace enrichment utilizing weak solvent for trace-component preconcentration at the inlet of the column has been reported[19]. However, its application has been limited to enhancement of sample detectivity, and the use of step or linear solvent programming was necessary.

Narrow-bore packed column HPLC as proposed here utilizes columns of I.D. less than 500 μm, and packed with ≤ 10 μm particles in lengths from 10 cm to a few meters. Because of the narrow bore of the columns, low mobile-solvent flow-rates in the range 0.1–10 μl/min are used. This low flow-rate and the limited surface area of the narrow-bore column packed bed allow easy and effective modification of the mobile-solvent system by the injection of a small amount of solvent modifier(s) into the column. The solvent modifier(s), in practice, can be injected as one of the solvent components in the sample solution or as a sample into the column at the time the modification of mobile solvent system is desirable.

An example of this technique is shown in Fig. 9, where pure methanol at a flow-rate of 3.1 μl/min was used as the mobile solvent for the elution of a sample mixture containing *ca.* 10–50 ng each of benzenethiol, hexanethiol, octanethiol and dodecane-thiol in methanol solution, or in methanol plus a modifier (methylene chloride or water) from a C_{18} chemical-bonded reversed-phase on 5-μm-silica-gel-particle, 50 cm × 250 μm I.D. narrow-bore packed column. The mobile-solvent hold-up volume measured for the column was 14.8 μl. Sample sizes injected were 0.6 μl for all experiments.
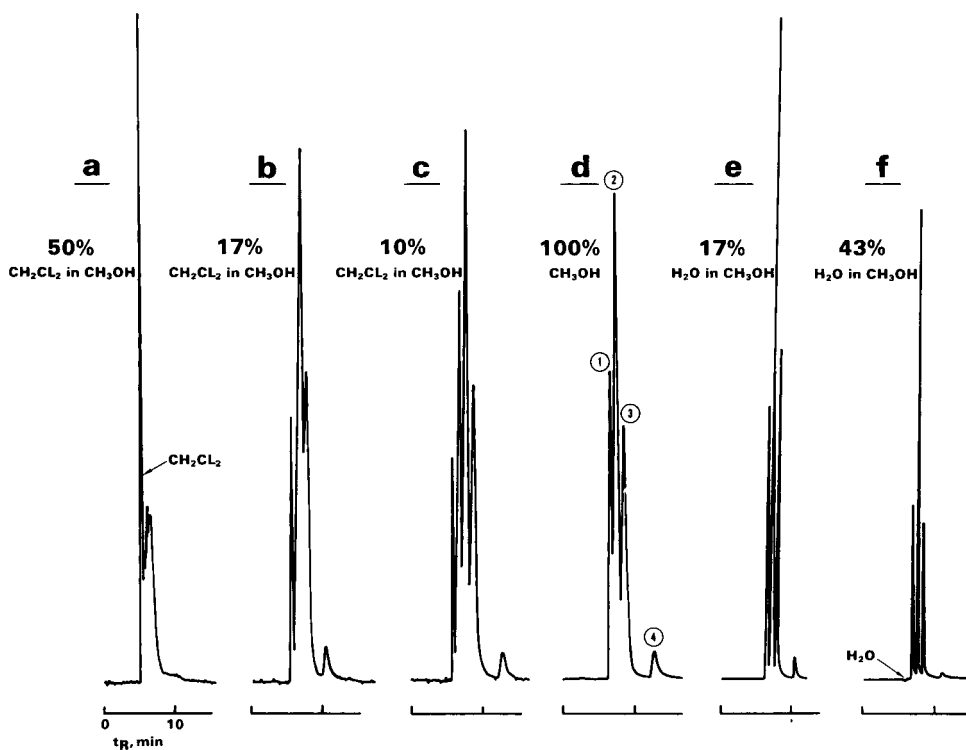
Fig. 9. Solvent effect in narrow-bore column HPLC. Solutes are 1, benzenethiol; 2, hexanethiol; 3, octanethiol and 4, dodecanethiol. Solvent modifier peaks are identified in Figs. 9a and 9f. Solvent modifiers and their concentrations are shown. The sample volume was 0.6 μl for all experiments. Methanol was used as mobile solvent. A C_{18} chemical-bonded reversed-phase 5-μm silica-gel particle packed column (50 cm × 250 μm I.D.) was used.

The example demonstrated in Fig. 9 shows the effects of a strong and a weak solvent modifier on both the retention and resolution of the thiol components. First, with the use of a strong solvent modifier such as methylene chloride in the sample solution, the solubility of the solvents into the mobile solvent is increased. The effects of the strong solvent modifier are thus a reduction in the analysis time and the loss of resolution for those components eluted right after the solvent modifier(s). The effectiveness of the strong solvent modifier on the loss of resolution and the reduction in the analysis time depends on the amount of the solvent modifier injected into the column. As shown in Fig. 9c, a 10% methylene chloride (modifier) in methanol

sample does not have a significant effect on either solute retention times or resolutions from that obtained (Fig. 9d) using pure methanol as solvent for both mobile phase and sample solution. However, as shown in Fig. 9b, a 17 % methylene chloride in methanol sample solution was observed to reduce the retention times of the thiol compounds by more than 20 % and to reduce the resolution of benzenethiol, hexanethiol and octane-thiol. Near complete loss of resolution of thiols as shown in Fig. 9a was observed when a 50 % methylene chloride methanol sample solution was injected.

In the case of the use of a weak solvent modifier (e.g., water) in the sample solution, an on-column focusing of the injected solute zone is obtained due to reduced solubility of sample solutes in the mobile solvent. This on-column focusing of the initial solute sample zone results in a significant improvement in peak resolution and allows a large sample volume to be injected into the narrow-bore packed column with minimum injector-dead-volume and sample-volume effects on the column efficiency. Fig. 9c shows the resolution and peak shape of the thiol compounds obtained under the same conditions as that for Figs. 9a–d, except that the injected 0.6-μl sample mixture contains 17 % water in methanol. A dramatic improvement in solute resolu-tion, a reduction in solute peak width and an enhancement in solute sample detection due to a narrower peak width and less mobile solvent dilution were demonstrated.

A comparison of Figs. 9d and 9e in terms of the thiol retention times suggests that 0.1 μl (i.e., 17 % of 0.6-μl sample injected) of water injected into a 50 cm × 250 μm I.D., 5-μm particle packed column at a mobile solvent (methanol) flow-rate of 3.1 μl/min is just sufficient to allow solute initial zone focusing at the inlet of the column. There is no significant effect on the retention of those thiols eluted near the solvent modifier (water in this case) peak.

By increasing the amount of solvent modifier injected into the column, the mobile-solvent system in the front portion of the column is modified and the sol-ubility of the solute compounds in the mobile solvent can be adjusted to achieve higher capacity ratio (k), selectivity, and resolving power (R) for the solutes eluted right after solvent modifier. Fig. 9f shows a baseline resolution of benzenethiol, hex-anethiol and octanethiol obtained by increasing the water concentration in the sample mixture to 43 %. The amount of water injected into the column in this case is 0.26 μl, or 1.7 % of the column free volume. This relatively high volume of water injected into the column provides not only the on-column focusing of this initial solute zone but also the effective modification of the mobile-solvent system during the time that solutes remain in solution in water–methanol mixed solvents. As a result, better resolution and near 10 % increases in retention times for those thiols eluted near the water peak were observed. It should also be pointed out that the use of weak solvent modifier in the injected sample will affect the peak shape (due to on-column focusing) but not the retention time of those well-retained sample components be-cause the time spent by those well-retained solutes in solution in the solvent-solvent modifier mobile-phase system is short relative to their retention times. The total analysis time for solutes with partition ratio higher than 2 is normally not affected by the utilization of the weak solvent modifier for improving solute resolution, column selectivity, peak width and detection. The solvent effect demonstrated above in nar-row-bore-column HPLC could also be applied to conventional packed column HPLC by scaling up the amount of solvent modifier needed according to flow-rate and column cross-sectional area ratio to be injected to achieve the solvent effect.

The practical importance of the suggested solvent effect in narrow-bore-column HPLC are: (1) the injection of a strong solvent modifier at the time of sample injection or at any desirable time reduces the analysis time of a well-retained sample without using a multicomponent solvent programming system, and thus, a single-pump hydraulic system could be utilized in narrow-bore-column HPLC; (2) the use of strong solvent modifier allows better peak detection due to less dilution of solute zones; (3) the injection of a weak solvent modifier at the time of sample injection or at any desirable time enhances peak resolution, and thus difficult and complex sample separation can be obtained; (4) the use of a weak solvent modifier allows on-column focusing and thus improves solute peak width and detection; (5) the use of a weak solvent modifier will not significantly increase the analysis time of well-retained compounds; and (6) a single mobile solvent or mixture of solvents in the pumping system minimizes solvent waste, exchange solvent difficulties, and cross-contamination of the narrow-bore-column HPLC system.

CONCLUSIONS

High-efficiency fused-silica narrow-bore columns packed with $\leqslant 10$ μm particles in lengths of up to a few meters can be prepared. The long length narrow-bore packed columns allow extremely high resolving power and peak capacity for the analysis of complex samples. A 1-m column with 110,000 theoretical plates was prepared and used in conjunction with a constant-pressure piston pump, a splitter injector and an on-column UV detector.

The use of fused-silica tubing for column material allows on-column optical detection and eliminates the column-to-detector connector and interface tubing. Optimum performance of the narrow-bore microparticle-packed columns can, therefore, be obtained.

Narrow-bore packed columns with 3- and 5-μm particles have minimum plate heights of *ca.* 10 and 20 μm, respectively. Further study in the optimization of column packing techniques may allow more efficient columns to be prepared. Narrow-bore columns packed with 3-μm spherical C_{18} bonded-phase particles achieved over 100,000 theoretical plates for a 1-m column. A 0.45 m $\times$ 330 μm I.D. 3-μm particle packed column allowed baseline separation of an EPA priority pollutant mixture containing sixteen PAH components.

The use of solvent effect in narrow-bore packed column HPLC, by the injection of a solvent modifier at the time of the modification of mobile-solvent system, is desirable in that it allows a single-pump hydraulic system to be utilized in narrow-bore microparticle-packed column HPLC for enhancing speed of analysis, solute detection and solute resolution.

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UNIVERSAL LIQUID CHROMATOGRAPHY METHODS

II. SENSITIVE, LOW-WAVELENGTH, GRADIENT REVERSED-PHASE METHODS*

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SUMMARY

A reversed-phase liquid chromatography "universal method" is presented that reduces expensive manpower costs for industrial laboratories by substituting automation for operator-attended runs and by eliminating method development for each new separation problem. Successful, fast (15–30 min), "first-run problem-solving" and quantitation of even trace components of completely unknown wide-polarity mixtures are obtained with the near universal detection possible with 210 nm UV detection. The reversed-phase method uses gradients at both acid and alkaline pH values (3–9) that allow low-wavelength detection at 210 nm with high detector sensitivity (0.1 a.u.f.s.) and baseline drift of less than 5 % and no ghost peaks. The techniques that make possible this new realm of gradient work are presented.

Finally, a discussion is presented of the interaction of detector components, UV absorbance of eluents, and sample absorption spectra; factors that become critical at 190–210 nm detection.

INTRODUCTION

For industrial analytical laboratories that are faced with heavy daily loads of many different heat-labile or non-volatile materials that must be analyzed for main components or impurities, there is a real need for "universal" liquid chromatography (LC) methods. Such universal methods should be able, rapidly, accurately, precisely, and automatically, to quantitate both main components and trace impurities of almost any organic compound, while covering wide polarity ranges of elution. "General detection" of most organic compounds can be obtained to low levels (detectable with 0.1 a.u.f.s.) with low-wavelength UV detection (210 nm). Separation of components of widely different polarity is classically accomplished by gradients, column

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switching, flow programming, and temperature programming. The use of sequential isocratic step (SIS) LC to cover as wide a polarity range as gradients but without the problems of baseline drift, ghost peaks, and the need for clean eluents was presented recently[1].

This paper describes a new approach to "universal" LC that fulfills the above criteria: sensitive, low-wavelength reversed-phase gradient elution. This method covers the full polarity range from full aqueous to full organic elution, without baseline problems, and allows general detection of most organic compounds to low levels.

Some earlier work with sensitive, low-wavelength gradients has been reported, although gradients were not as "universal" in that they did not cover as wide a polarity range, did not have as low a detection wavelength, or did not use as high a detector sensitivity. Also, previous work did not systematically study the cause of gradient baseline problems or methods for eliminating them.

The most universal gradients have been in the field of LC of amino acids, peptides, and proteins. Rivier first introduced to high-performance liquid chromatography (HPLC) the advantages of trialkylamine phosphate buffers[2] that give high recoveries and high UV transparency to less than 200 nm for peptide bond detection (vs. acetate and formate buffers[3]).

Hearn and Grego[4,5] show gradients at 210 nm with 0.2 a.u.f.s. and detection using gradients from water with 0.1 % phosphoric acid to 50 % acetonitrile for protein digests. Bennett et al.[6] show gradients at 210 nm with 0.4 a.u.f.s. detection using linear gradients from 0.01 M perfluorinated carboxylic acids to the acid and 58 % acetonitrile with good baselines and high protein recoveries.

Majors and co-workers[7,8] have used an approach to "universal" LC separation systems for aqueous samples in which the emphasis was on separating the lower-molecular-weight (MW) components from higher-MW complex matrices by the techniques of multi-dimensional LC using coupled columns.

Some early approaches to "universal" LC methods used normal-phase silica LC. Building on earlier suggestions by Snyder[9], Rabel[10] showed that the 254-nm UV detector could be used with medium sensitivity to limit baseline drift when used with a series of eluents covering the relatively wide polarity range from hexane to methanol. The "incremental gradient elution" approach of Scott and Kucera[11] used a moving wire with a flame ionization detector and a clever system to generate a gradient from heptane through eleven intermediate polarity solvents up to water with a normal-phase silica column. However, this system did not detect materials with modest volatility, the detector and gradient system were difficult to use (and are no longer commercially available), and the normal-phase chromatography did not give good separation of ionic or polar components.

This paper presents an approach to universal LC methods that can rapidly, accurately, and precisely quantitate most of the organic components that can be detected with low-wavelength UV (210 nm) and can be eluted with acetonitrile from a reversed-phase LC system. Three sensitive, low-wavelength (0.1 a.u.f.s., 210 nm) gradient methods are described that give little baseline drift (less than 0.005 a.u.) and no detectable ghost peaks: one buffered at pH 3 with triethylamine phosphate solubilizing agent for acidic samples; one at pH 9 for basic samples; and one unbuffered system. The techniques used to eliminate and determine the source of ghost peaks and baseline shifts (the "mid-gradient hump" and UV mismatch between initial and final

eluent) are applicable to other eluent systems that may be required for other separation problems. In a last section, precautions with 210-nm low-wavelength universal detection that are necessary for selecting the right wavelength to optimize sensitivity and give linear detection are presented.

EXPERIMENTAL

The "eluent conditioner column" sequence in the "acid" and "alkaline" systems that proved to give such low drift and ghost-free baselines were made from 25 × 0.46 cm I.D. DuPont columns (Wilmington, DE, U.S.A.). An 80–200 mesh acid alumina Brockman activity I eluent conditioner column was installed in this line to remove amine-like materials from the acetonitrile that cause the "mid-gradient hump", as explained later. This column had to be repacked when the mid-gradient hump re-appeared (after *ca.* 2–3 gallons of acetonitrile). A porous polymer eluent conditioner column was installed in the aqueous eluent line to remove impurities that cause ghost peaks, as explained later. This column was dry tap-packed with 80–100 mesh Chromosorb 101 styrene–divinylbenzene porous polymer (Supelco, Bellefonte, PA, U.S.A.). Before the first use and each morning, this column was cleaned by turning a valve arrangement so that *ca.* 15–25 ml (5 min) of acetonitrile flushed the aqueous line. A silica eluent conditioner column of 37–53 μm Whatman silica was installed after the gradient mixer and before the in-line filter in front of the injector to diminish silica dissolving from the analytical columns, especially a problem with the pH 9 alkaline system[12]. The 2-μm outlet frit of this column required periodic changing because of pressure build-up, presumably because of silica "fines" released from dissolved silica.

A 1084B Hewlett-Packard (Avondale, PA, U.S.A.) LC was used with Zorbax C_{18} octadecyl columns (25 × 0.46 cm I.D.; DuPont) preceded by Brownlee Labs "Spheri 5" RP-18 guard column (3 × 0.46 cm I.D.; Rheodyne, Berkeley, CA, U.S.A.). The best system used recent 6-μm d_p Zorbax columns (of serial number 8000 or higher) which have *ca.* 30–40 % reduced permeability compared to earlier columns of *ca.* 7-μm d_p particles[13] (d_p = particle diameter).

Typical solvents were Burdick & Jackson acetonitrile for HPLC (Muskegon, MI, U.S.A.) (absorbance at 190 nm was 0.8 a.u. and at 210 nm was 0.01 a.u.) and water, UV irradiated for at least 3 h in a No. 816 HPLC Reservoir from Photronix Co. (Medway, MA, U.S.A.) just before use.

Gallon (3.81 l) lots of the "acid" buffer of 7.5 mM triethylamine phosphate (TEAP), pH 3, were prepared by titrating 2.891 g of purified triethylamine in irradiated water with *ca.* 20 ml of phosphoric acid diluted to *ca.* 8.5 % (1 in 10). The final acid eluent typically had a UV absorbance of 0.34 a.u. at 190 nm, 0.06 a.u. at 200 nm, and 0.05 a.u. at 210 nm. The dilute phosphoric acid (Mallinckrodt, St. Louis, MO, U.S.A.) was made from reagent-grade 85 % phosphoric acid selected from several lots so as to have less than 2.5 a.u. at 190 nm. The triethylamine (Fisher, Fair Lawn, NJ, U.S.A.) was purified by passing *ca.* 1 l through a 50 × 2.5 cm I.D. column of Fisher basic alumina, Brockman Activity 1. Triethylamine so purified had the 190 nm absorbancy reduced from 1.2 a.u. to 0.64 a.u. and the 210 nm absorbancy reduced from 2.5 a.u. to 1.2 a.u. Gallon lots of the "alkaline buffer", 2.5 mM ammonium borate, pH 9, were prepared by titrating 0.589 g of Mallinckrodt (reagent-grade)

boric acid with *ca.* 12 ml of a reagent-grade ammonium hydroxide (Baker, Phillipsburg, NJ, U.S.A.) diluted to *ca.* 2.8 % (1 in 10). The final alkaline buffer typically has a UV absorbance of 1.0 a.u. at 190 nm, 0.17 a.u. at 200 nm, and 0.05 a.u. at 210 nm.

PROCEDURES

Experiments were done with acrylamide monomer to determine the "highest linear sample size" (in micrograms) at each detector wavelength for the TEAP eluent system as follows. Serial dilutions of acrylamide were prepared from 0.06 to 60 mg/ml. For each wavelength from 190 to 260 nm in 10 nm increments, a sample solution was selected so that for the 2–200 μl range of the Hewlett-Packard injector, that concentration would show a "response curve" with linear and non-linear portions in a "best fit" plot of area counts *vs.* amount injected. For the monomer example, 254 nm required a concentration of 60 mg/ml; 240–220 nm required 0.6 mg/ml; and 200–190 nm required 0.06 mg/ml. From the response curve at each wavelength, the highest linear load point (in micrograms) is the intersect between the response curve and a line drawn through the origin and any point 10 % lower than the linear part of the response curve, paralleling the ASTM method[14].

Experiments such as that described above indicate that peaks not going off the printer-plotter paper at a detector sensitivity of 2^9 (*i.e.* less that 1 a.u. high) are within the linear range of the Hewlett-Packard variable-wavelength detector when using the acid (pH 3) 7.5 m*M* TEAP buffer at all wavelengths from 190 nm up. For the alkaline (pH 9) 2.5 m*M* ammonium borate buffer, peaks are within the linear range of the detector if they are below 0.25 a.u. at 210 nm.

RESULTS AND DISCUSSION

Achieving sensitive, low-wavelength gradients
Introduction. Fig. 1 shows the type of clean and flat baseline that can be obtained with high sensitivity detection (0.1 a.u.f.s.) and very general detection (210 nm) while covering the polarity range from full aqueous to acetonitrile elution, and using additives to control the buffer pH or to elute strongly retained components. Note that one system uses a triethylamine additive, which is well documented as a good eluent[2], and the second system is alkaline at pH 9. The power of these high- and low-pH systems together, to separate acidic and basic compounds, will be illustrated below. The gradient shape is shown in the lower part of the figure.

A practical "acceptable baseline" is illustrated by the dashed curve between the aqueous and acetonitrile (Fig. 2). With this definition, the highest sensitivity that can be achieved readily would come from a 50 % shift in baseline (up or down), since this is the lowest sensitivity setting in which the whole chromatogram will be on scale.

A number of problems can cause the acceptable baseline to deviate from the experimental gradient baseline, as illustrated in Fig. 2. The three primary problems are (1) the "mid-gradient hump", (2) UV absorbance mismatch between initial and final eluents, and (3) aqueous-eluent-derived ghost peaks. The source of these different types of problem, methods for determining their causes, and methods to prepare eluents with flat gradient baselines will now be presented.

While Fig. 1 illustrates the "clean" noise-free baseline that was ultimately
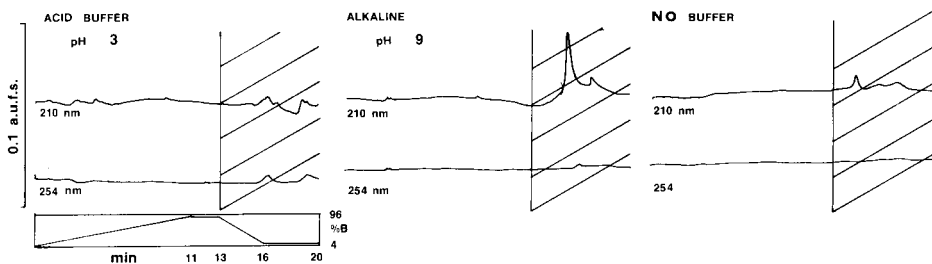
Fig. 1. Typical low-noise baselines for sensitive, low-wavelength, full gradients using an "acid buffer" of pH 3, 7.5 mM triethylamine phosphate (left); an "alkaline buffer" of pH 9, 2.5 mM ammonium borate (middle); and a "no buffer" system of irradiated distilled water. A flow-rate of 3 ml/min is used with a reversed-phase, long column of a 3-cm Brownlee C_{18} guard column followed by a 25-cm 6-μm Zorbax C_{18} column. The 20-min gradients are from 4 to 96% acetonitrile using the gradient shape shown at the bottom. This "clean" baseline system uses eluent components selected or purified by methods described in the text plus the following 0.46-cm diameter "eluent conditioner columns": a 25-cm 150–200-μm Chromosorb 101 porous polymer column in the aqueous eluent line; a 25-cm 75–200-μm acid alumina column in the acetonitrile line; and a 15-cm 37–53-μm Whatman "silica saturator" column after the gradient mixer and before the injector. The hatched portions of the baselines are in the return gradient and are used only to monitor the cleanliness of the system.

achieved in a routine fashion, the other chromatograms shown in this section generally evolved over a period of time, before the methods for eliminating the various baseline problems were developed. To evaluate the three problems about to be discussed, the time between runs must be reproducible, as is possible with a microprocessor-controlled LC, and replicate runs must be made until reproducible chromatograms are obtained. Thus, the first chromatogram must usually be discarded.

The "mid-gradient hump" problem. The shaded zone between the acceptable
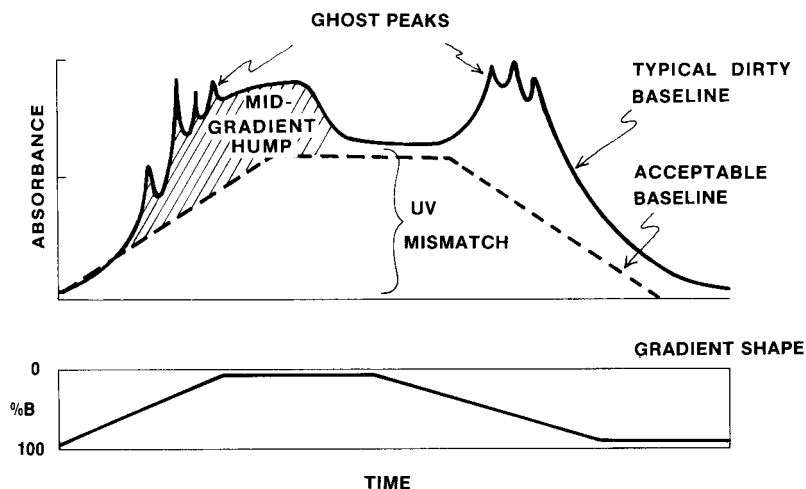
Fig. 2. Illustration of a typical "dirty" gradient baseline showing (1) the "mid-gradient hump" problem, (2) the problem of UV absorbance mismatch between initial and final eluents, and (3) the ghost peak problem. The "acceptable baseline" (dashed line) results if the mid-gradient hump and ghost peak problems are eliminated.
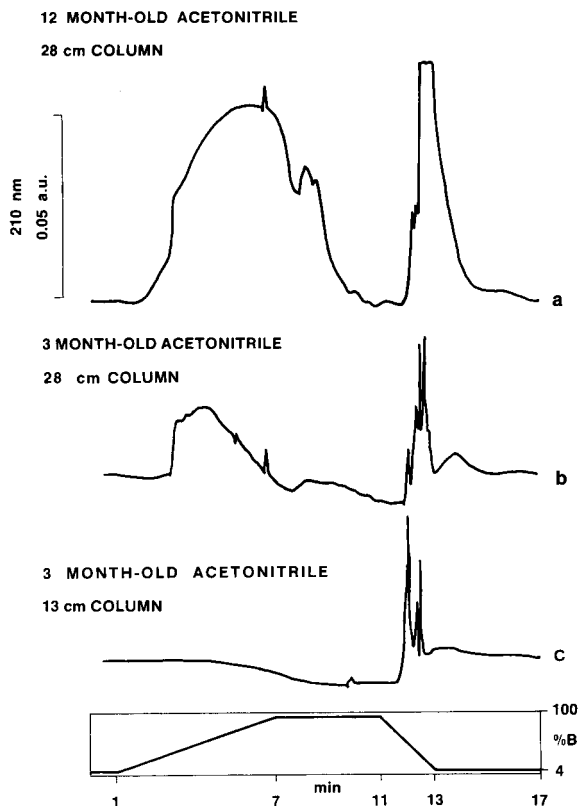
Fig. 3. Sensitive, low-wavelength gradients showing that the "mid-gradient hump" diminishes with newer lots of HPLC grade acetonitrile [(a) *vs.* (b)] and with shorter columns [(b) *vs.* (c)]. The gradient is from irradiated and degassed water to acetonitrile at 4 ml/min with a 3-cm Brownlee C_{18} guard column and 25-cm Zorbax C_{18} column (a) and (b) or a 3-cm Brownlee C_{18} guard column and a 10-cm Brownlee C_{18} column (c).

baseline and the experimental baseline in Fig. 2 is the "mid-gradient hump" that was found to originate from the acetonitrile. A large mid-gradient hump can be seen in Fig. 3, chromatogram a, originating from a lot of acetonitrile distilled *ca.* 12 months earlier. A more recently distilled lot of acetonitrile, 3 months old, gives a reduced mid-gradient hump (compare chromatograms a and b).

        The source and elimination of the mid-gradient hump was discovered by modeling experiments. Rabel[15] has suggested that contaminants in the acetonitrile will cause sharp ghost peaks during a gradient. The possibility was investigated that such acetonitrile contaminants might give broad peaks and be the cause of the mid-gradient hump. Fig. 4 shows that the initial mid-gradient hump (the shaded portion) remains unchanged as the acetonitrile is made 6 and 12 μM with the uncharged additive benzene (chromatograms b and c). As expected, the mismatch of initial and final eluent UV absorbance from *ca.* 7 to 9 min is seen, and this increases linearly with concentration of benzene in the acetonitrile.

        With this modeling experiment, as benzene is added, two sharp peaks appear at
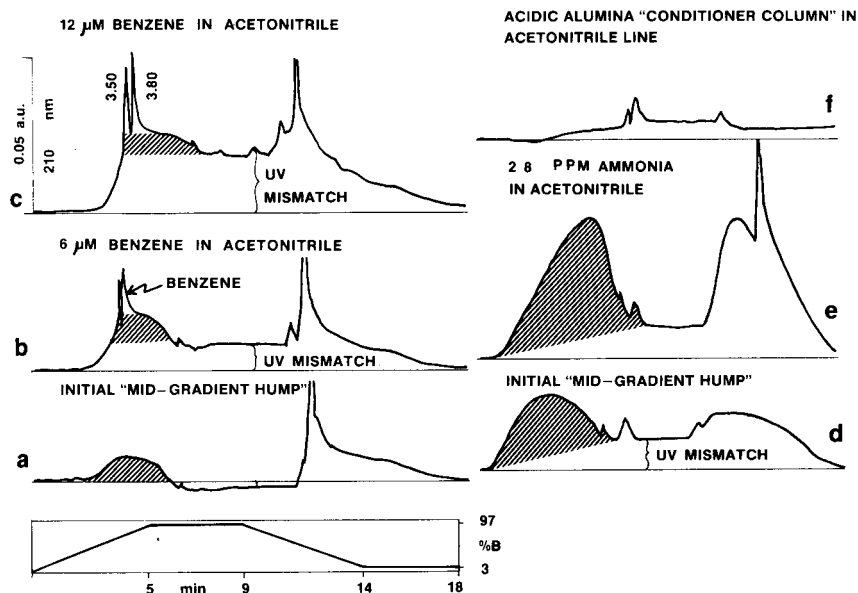
Fig. 4. Modeling experiments that led to the elimination of the "mid-gradient hump" (hatched areas). The initial hump (a) is constant with increasing benzene from 6 to 12 μM in acetonitrile (b) and (c) giving sharp peaks at 3.8 min (benzene elution position) and 3.5 min. In another experiment, the initial hump in (d) increases with the addition of 28 ppm ammonium hydroxide in acetonitrile (e). The mid-gradient hump is eliminated by an acidic alumina "conditioner column" in the acetonitrile line (f). The gradient is from 12-month-old acetonitrile at 4 ml/min with a 3-cm Brownlee C_{18} column and 25-cm Zorbax C_{18} column with an aqueous eluent conditioner column of porous polymer added in (f).

3.5 and 3.8 min, and the second peak is at the elution position of benzene. The area of the second peak increases linearly with benzene concentration in acetonitrile. This is evidence that a contamination of a single uncharged pure component in the acetonitrile will not cause a mid-gradient hump. It is possible that oligomeric contaminants can cause a series of fused peaks to produce a hump, under some conditions.

Other work using pyridine and picoline as model samples showed that these amines give peaks 20–40 times broader than uncharged molecules on an unbuffered water-to-acetonitrile reversed-phase gradient. However, they give sharp peaks with good retention when the aqueous eluent is 0.1 % in ammonia, and sharp but un-retained peaks when the eluent is acid (pH 3, 15 mM TEAP). Also, early amino acid analyzer work was plagued with a mid-run hump that proved to be ammonia[16]. The addition of ammonia to the acetonitrile (28 ppm) doubles the area of the mid-gradient hump (chromatogram e). Under the possibility that amines such as ammonia in the acetonitrile can cause the mid-gradient hump, an "acetonitrile conditioner column" of acidic alumina was placed in the acetonitrile line. This column eliminates the mid-gradient hump (chromatogram f) and potentially allows even older lots of acetonitrile to be used.

Before discovering that the acetonitrile conditioner column of acidic alumina could eliminate the mid-gradient hump, other methods were found that reduced the size of the hump. Fig. 3 shows that reducing the column length from 28 cm (chromato-

gram b) to 13 cm (chromatogram c) nearly eliminates the hump at the cost of reduced separation. Note that the C_{18} loading is reduced to a greater extent than the proportion to column length since the Brownlee column has a lower C_{18} loading than Zorbax C_{18} (7 *vs.* 15%). In other work, for identical columns, with an acetonitrile gradient from 3 to 70%, the mid-gradient hump was reduced to *ca.* one-half the 3–97% gradient level. However, a shortened gradient moves away from universal LC in not utilizing the fullest extent of the gradient so as to separate and elute as wide a sample polarity as possible.

UV absorbance mismatch between initial and final gradient eluents. The second type of baseline problem with gradients is the mismatch of UV absorbance between the initial aqueous eluent and the final acetonitrile eluent. The gradient illustrated in Fig. 3 gives a sufficiently long final isocratic run at 100% acetonitrile so that the baseline shift due to UV mismatch between the aqueous and the acetonitrile eluents can be seen and can be distinguished from the mid-gradient hump problem. By the 11-min point, the column has reached equilibrium with acetonitrile. Note that for the lot of acetonitrile giving even a very bad hump (chromatogram a), the UV absorption mismatch is very low, below 0.005 a.u., using these very clean eluents. Allowing the
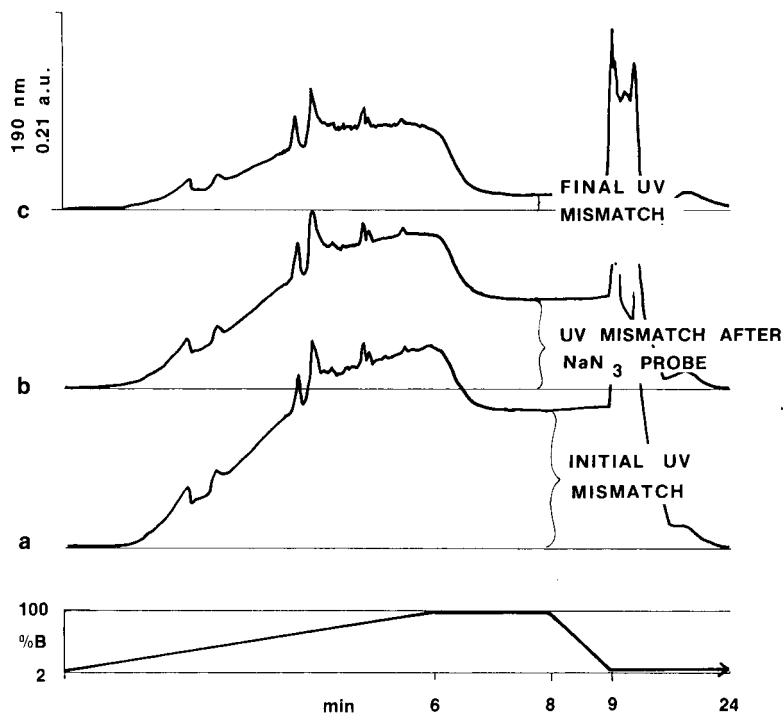
Fig. 5. Example of reducing the UV mismatch between the initial and final gradient eluents by adding an unretained component, 0.2% sodium azide, to the lower UV absorbing aqueous eluent. The 6-cm UV mismatch in (a) is reduced to 4 cm with a "probe" addition of 10 ml of sodium azide per liter of aqueous eluent in (b) and to 0.4 cm with a final addition of 20 ml/l in (c). The pH 2 aqueous uses 10 mM sodium phosphate buffer to acetonitrile gradient at 5 ml/min using a 25-cm Zorbax C_{18} column with no eluent conditioner columns.

column to come to equilibrium with the large volume of acetonitrile allows the mid-gradient hump problem and UV mismatch problem to be distinguished and each clearly assessed.

Were the acid alumina column not used to eliminate the mid-gradient hump as described in the previous section, a practical fast cost-effective gradient might end at some point at the top of the gradient hump, perhaps at 7 min in Fig. 3, chromatogram a. This would suggest a UV mismatch between initial and final eluents that could not be confirmed in a static spectrophotometer measurement of the eluent UV absorbances.

Using a gradient in which the final composition is held at 100 % will allow the UV mismatch between the initial and final eluents to be readily assessed. If the UV mismatch is such that the baseline shifts down, then a cleaner initial eluent must be selected; perhaps by decreasing the concentration of additives. Alternatively, adding an unretained UV absorber to the acetonitrile might be used to raise the UV absorption of the final eluent so as to give a flat baseline. The technique for using additives is illustrated in the following situation.

If the UV mismatch between the initial and final eluent is such that the baseline shifts up with the gradient, the UV absorption of the initial solvent can be increased so as to give a nearly flat baseline by adding an unretained UV absorber to the aqueous eluent, as described in Fig. 5. This example is for an aqueous (pH 2, 10 mM sodium phosphate) to acetonitrile gradient (3–100 %) in which the techniques were not used to minimize the mid-gradient hump problem or eliminate ghost peaks. Note that the sodium azide did not change the retention of any of the ghost peaks or contribute any ghost peaks itself. Additionally, sodium azide will protect the aqueous system from microbial growth.

Several other approaches can be used to minimize the UV mismatch between the initial and final eluents in a gradient. Usually going to higher wavelengths will minimize the UV mismatch. Fig. 6, chromatograms a and b, show a nearly flat baseline at 220 nm and 210 nm compared to the 200 and 190 nm baselines. While higher wavelength detection can minimize the UV mismatch, this works against the general detection possible at the lower 190–210 nm wavelengths. An alternative approach to match initial and final UV absorbance is to increase the UV absorbance of the aqueous eluent by increasing the concentration of the usual additives as TEAP or ammonium borate.

Eliminating aqueous derived ghost peaks. The third problem found with 210 nm, 0.1 a.u. sensitivity gradients is the presence of ghost peaks in going from the aqueous to non-aqueous eluents. The source of these peaks is from the initial or final eluent in reversed-phase chromatography[15]. A method for determining the ghost peak levels is illustrated in Fig. 7, and has been discussed by Bristol *et al.*[17]. Gradients show the ghost peaks, and the sizes of the ghost peaks diminish as the volume of aqueous phase is reduced. Thus, a first and classical method for reducing ghost peaks from the aqueous eluent is to shorten the equilibration time between runs.

A second and recently popular method for eliminating ghost peaks from the aqueous eluents is to use UV irradiation to photo-oxidize organics in water. The principle of photo-oxidation is established as an LC halide photoconductivity detector (Model 965; Tracor Instruments, Austin, TX, U.S.A.)[18], as an organic carbon analyzer[19], and as a means to prepare water for LC[20,21].
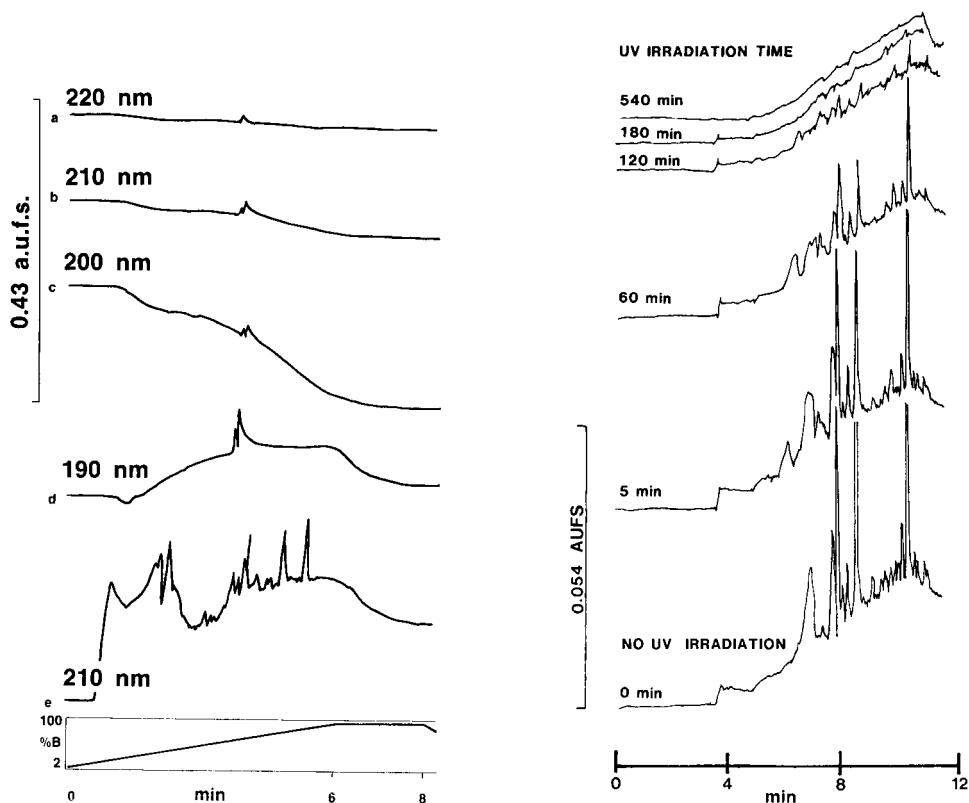
Fig. 6. Gradient chromatograms showing that higher wavelength detection at 220 nm (a) and 210 nm (b) give flatter baselines than lower wavelengths, as 200 nm (c) and 190 nm (d). The effect of additives to increase ghost peaks, as the ion-pairing tetrabutylammonium phosphate (pH 2, 4 mM) is determined by comparing chromatograms with no additive [(e) vs. (b)]. A 3-cm Brownlee C_{18} guard column and a 25-cm Zorbax C_{18} column is used at 5 ml/min with a gradient from water with $2.8 \cdot 10^{-4}$ g per liter of sodium azide to acetonitrile for (a) to (d) to minimize the UV mismatch between initial and final eluent. Sensitivity is low and no eluent conditioner columns are used.

Fig. 7. Gradient liquid chromatograms showing the UV irradiation method for preparing LC-grade water. The initial level of impurities in commercial distilled water is eliminated by UV irradiation from 0 to 540 min (bottom to top chromatograms) in a Photronix HPLC reservoir. The gradient is from 0 to 95% in 9 min; isocratic at 95% for 3 min; return to 3% in 1 min; and initial isocratic water for 28 min (73 ml) to concentrate impurities. A flow-rate of 5 ml/min is used with a 25-cm Zorbax C_{18} column.

The effect of a Model 816 UV irradiator LC reservoir (Photronix, Medway, MA, U.S.A.) for removing ghost peaks originating from the water can be seen in Fig. 7. After 3 h the ghost peaks from the water are undetectable.

Besides water, the second major source of ghost peaks is the additives used to buffer pH, form ion-pairs, or solubilize and elute samples (as trialkylamines). The effect of impurities in additives can be determined by comparing the chromatogram of water to that of water with additives. For example, Fig. 6 shows that the ion-pairing agent tetrabutylammonium phosphate, pH 2, 4 mM (chromatogram e), shows many peaks at 210 nm compared to the water (chromatogram b).

UV irradiation of water containing additives is not useful. Irradiation of 10 mM potassium phosphate buffer produced high UV-absorbing materials that broke through the column after *ca.* 3 l of irradiated buffer were pumped. Thus the water should be purified with UV irradiation before adding buffers, amines, etc.

The third major method of reducing ghost peaks from additives is to clean and/or select components. For example, phosphoric acid lots with minimum UV absorption were selected simply by comparing their UV spectra, and triethylamine lots were cleaned through a column of basic alumina. Both methods are described in the Experimental section.

Despite alumina cleaning of triethylamine and selecting phosphoric acid lots, ghost peaks from the TEAP were still evident, as can be seen in Fig. 8, chromatogram a. A novel and fourth approach was successfully used to eliminate the mid-polar ghost peaks arising from the aqueous TEAP. An "eluent conditioner column" of styrene–divinylbenzene polymer (Chromosorb 101) was used in the aqueous line before the mixing chamber, as described in the Experimental section. Chromatogram b shows that the mid-polar ghost peaks are completely eliminated by the aqueous eluent conditioner column. The aqueous conditioner column cleans more than 1 gallon of the TEAP buffer, and is regenerated by a 5 min (15–25 ml) purge with acetonitrile each morning.

Octadecyl and octyl silica columns have been used off-line[22] to remove ghost peaks from water, but the porous polymer columns offer the advantages of on-line
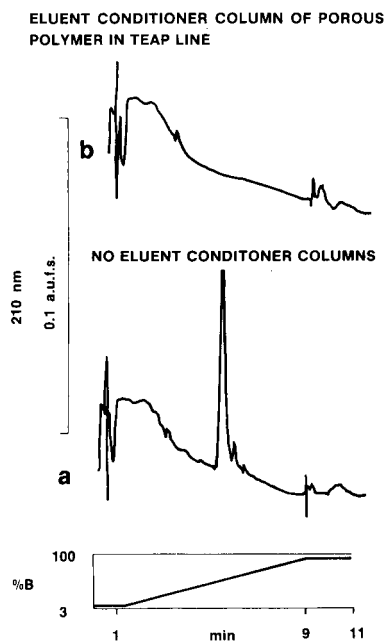
Fig. 8. Elimination of ghost peaks at 5 min (a) by an "eluent conditioner column" of styrene–divinylbenzene "porous polymer" in the aqueous TEAP (pH 3, 15 mM) line (b). A 3-cm Brownlee C_{18} guard column and 10-cm Brownlee C_{18} column are used at 5 ml/min with no acetonitrile eluent conditioner column to eliminate the initial hump from 0 to 3 min.
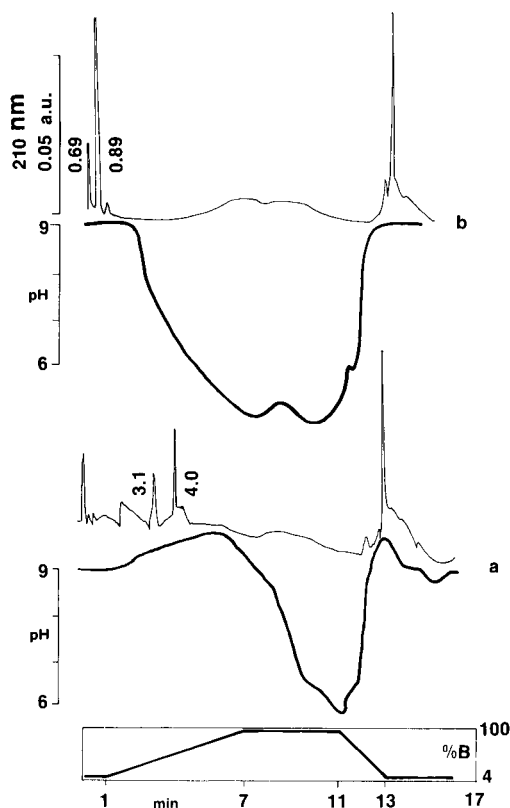
Fig. 9. Reduction in retention of model amine, 2-methylthiazolidine in 0.4 *M* KOH in pH 9 alkaline system from 4.0 min (a) to 0.89 min (b) when an open lot of 2.5 m*M* ammonium borate buffer absorbed atmospheric carbon dioxide over a weekend. The heavy line shows the delayed fall in pH in the fresh (a) *versus* the older eluent (b). A 25-cm Zorbax C_{18} column at 4 ml/min is used with an aqueous eluent conditioner column.

conditioning of the solvent just before use as well as much increased capacity and convenience.

Precautions with alkaline buffers for gradients

With alkaline buffers in LC systems some special precautions were found necessary for successful long-term chromatography. Fig. 9 shows that retention of samples can be affected by absorbed atmospheric carbon dioxide. Chromatogram a shows that the model amine, 2-methylthiazolidine, had a retention of 4.0 min in a freshly prepared pH 9 buffer. The corresponding pH trace as determined by the eluent flowing over a pH electrode is shown as the heavy line. Chromatogram b shows that the retention of the amine diminishes to 0.89 min with the same 1 gallon lot of buffer sitting open to the atmosphere over the weekend. Note that the pH trace begins to fall at *ca.* 2 min in this run, as against 6 min in the run with the fresh eluent. A fresh lot of eluent restored the retention and pH trace to that shown in chromatogram a. Purging the head of the eluents with a slow stream of helium or simply capping the buffer and
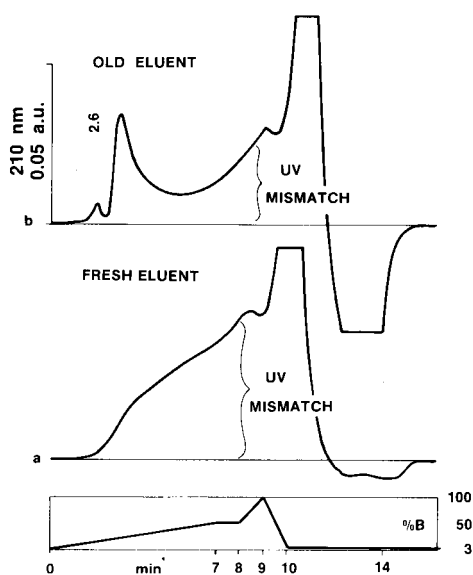
Fig. 10. Fresh eluent (a) *versus* "old" eluent stored at 6°C for 4 days (b) showing broad microbial con-
taminants at 2.6 min and a decrease in UV mismatch between the initial and final eluent due to build-up of
microbial products in the aqueous pH 9, 2.5 mM ammonium borate eluent. A 3-cm Brownlee C_{18} guard
column and a 25-cm Zorbax C_{18} column are used at 5 ml/min with a silica-saturator column.

acetonitrile tightly with aluminum foil eliminated amine retention changes with buffer
age.

A second precaution with the alkaline buffer system is that a silica-saturator
column of large particle, high-permeability silica must be used for successful long-
term chromatography[12]. With alkaline buffers, the silica-saturator column must be
after the mixer and before the injector and thus in the gradient stream. Initial experi-
ments with pH 9 buffers made of ammonium borate and ammonium phosphate
showed that if the silica-saturator column is used on the aqueous eluent line alone,
after 10–20 gradients, sufficient gelatinous silica would precipitate on the filter after
the mixer and before the injector to overpressure the system.

Fig. 10 illustrates another precaution necessary with all aqueous buffers when
using sensitive, low-wavelength detection. Microbial growth can cause ghost peaks.
In Fig. 10 a broad peak is seen at 2.6 min that appeared in a 6°C refrigerated
ammonium borate buffer after 4 days storage. Ghost peaks from microbial growth
are often of such low molecular weight materials that they cannot be removed by the
porous polymer aqueous eluent conditioner column. Similar levels of ghost peaks
were noticed eventually in all buffers except those in which 0.04 % sodium azide was
added. Thus buffers must be made fresh and usually cannot be stored for more than 2
to 3 days even with refrigeration.

Precautions with sensitive, low-wavelength detection and universal LC system

The sensitive reversed-phase gradient "universal method" described above
makes it easy to detect and quantitate all components to low levels on a first run by
using low-wavelength detection (at 210 nm). However, this low-wavelength detection

PLOT I

DETECTOR LINEAR LIMIT

(ESTIMATED)

ABSORBANCE

ELUENT ABSORBANCE

ALKALINE BUFFER

ACID BUFFER

PLOT II

SYSTEM LINEAR LIMIT

ACID SYSTEM

ALKALINE SYSTEM

ABSORBANCE

190   210   230   250

nm
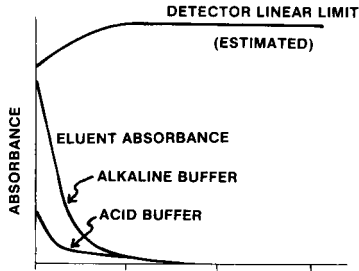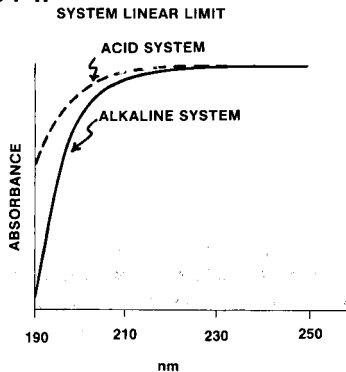
Fig. 11. Plot I shows the "detector linear limit" (lamp, detector, optics and sample cell) and the absorb-
ance of the eluents. Plot II shows that the difference between the "detector linear limit" and the eluent
absorbances gives a "system linear limit" that is higher for the acid system than for the alkaline system.

requires some special precautions compared to the usual 254-nm detection in order to
be useful. Compounds with moderate absorptivities at 254 nm frequently have very
high absorptivities at low-wavelength detection, and low concentrations can often
exceed the maximum system linear limit.

Fig. 11 illustrates some important relationships between the detection system,
the eluent, and the sample. Note that the "detector linear limit" is actually the entire
detection system linear limit which is determined by the lamp output and age, the
detector characteristics and age, stray light, detector bandwidth, characteristics and
cleanliness of the optics and detector cell, as well as the electronic conditioning that
may be used to make linear the response of the detection system *versus* wavelength.
The upper line shown in Plot I is an estimate of the detector linear limit, which can
vary from instrument to instrument and with time. The relative eluent absorbance
spectra of the acid and alkaline buffers are correct, with the alkaline buffer giving 1.0
a.u.f.s. at 190 nm, in a 1-cm cell as mentioned in the Experimental section.

For the alkaline system, the "system linear limit" is the difference between the
detector linear limit and the eluent absorbances (Fig. 11, Plot I). This system linear
limit is shown in Plot II. Note that the system linear limit is usually important only at
low wavelengths where the eluent absorbance and detector non-linearity may become
significant. The system linear limit is wider for the acid buffer system *versus* the
alkaline system, since the acid eluent has the lower UV absorbance. Thus, if 190-nm

detection is used (and zero percent acetonitrile) any peak eluting with an absorbance between the curves in Plot II would be in the non-linear zone of the detection system for the alkaline buffer, but in the linear zone for the acid buffer. Decreasing the sample size would give a proportional decrease in area counts for the acid system.

Accurate specific absorbances of materials are generally not known in the low UV range since low-wavelength detectors and clean materials are only now available with the advent of the LC coupled with sensitive spectrophotometers. Thus little can be done, at present, to predict concentrations of samples that fall in the system linear limit for a specific wavelength. In fact, the next section will show the inverse approach, in which the system linear limit for a particular detection system and eluent is estimated experimentally from the "highest linear sample size".

The wide range in absorptivities of different components in a mixture (leading to poor proportionality to weight-percent); the high absolute level of absorptivities (leading to sensitive detection but possible detector overload); and the nearly universal detectability of all components (leading to complex chromatograms) when used with the universal chromatography methods described above requires that some special precautions be used, as will now be presented.

Sensitive, low-wavelength detector precautions: wavelength selection for sample size

In classical spectrophotometry, it is good practice when developing a colorimetric analysis to select a photometer wavelength in the middle of a broad absorption band, provided no interferences absorb at that point. This minimizes errors in reselecting the detector wavelength, or changes during a test due to the photometer heating, vibrating, etc. A typical method then adjusts the concentrations of samples so they are below the "highest linear concentration" for the spectrophotometer, although quantitation is possible in the response curve zone where the detection system is non-linear but still not saturated.

In LC analyses using variable-wavelength detectors, other considerations may make it desirable in some cases to select detection wavelengths far from the peak absorption band maxima. For example, to quantitate both the trace level components and major components in the same sample, wavelengths of high absorption might be
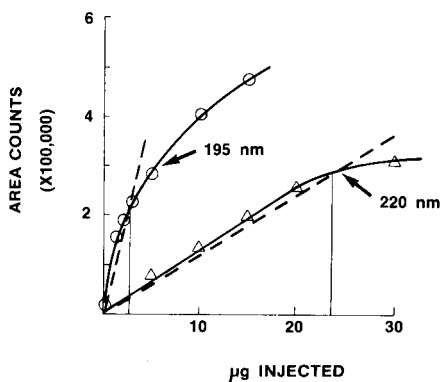
Fig. 12. Peak area counts *versus* sample size injected for methacrylamide showing the "highest linear sample size" of *ca.* 24 μg at 220-nm detection but less than 2 μg at 195-nm detection.

necessary to detect the trace components. However, the major component might grossly overload the detector at this wavelength. With the new generation of rapid-scanning UV-visible detectors now appearing, as well as the microprocessor-controlled Hewlett-Packard detector used in this work, it is possible to quantitate both major and minor components by changing the detection wavelength. The relationship between the UV absorption spectra of compounds and the "highest linear sample size" is illustrated below.

The highest linear sample size at a particular detection wavelength is defined here as the amount of sample that causes a 10% lower value than expected from a plot (the response curve) of area counts *versus* amount of sample injected. Details for determining the highest linear sample size are described in the Experimental section. Fig. 12 shows a typical plot of area counts *versus* amount injected using both 195- and 220-nm detection for methacrylamide monomer. Curve a shows that for this monomer the highest linear sample size is only 2 μg at 195 nm, but it is 24 μg at 220 nm.

Data from plots similar to this were used to determine the highest linear sample size at wavelengths between 190 and 254 nm. This is shown along with the UV absorption spectra for acrylamide in Fig. 13. The UV absorption spectra was determined by the automated "gradient wavelength scan" mode[1] by plotting the area counts *versus* wavelength resulting from a 0.2 μg sample injected at each wavelength. This sample size is below the highest linear sample size for all wavelengths.

The plot of wavelength *versus* highest linear sample size shows the experimentally determined relationship between the UV absorption of acrylamide monomer and the highest linear sample size at each wavelength. The highest linear sample size decreases at lower wavelengths for three reasons: (1) the UV absorption of the sample increases; (2) the eluent UV absorption increases; and (3) the detection system has lower linearity. Points (2) and (3) determine the *system* linear limit, as described above.
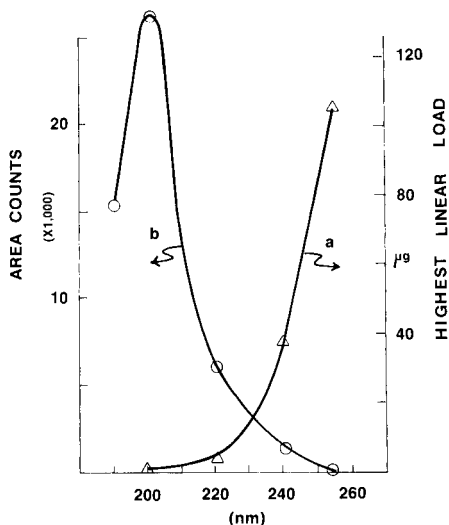
Fig. 13. Experimentally determined "highest linear sample size" as amount injected *versus* detector wavelength (a), and UV absorption spectra as area counts *versus* detector wavelength (b) for acrylamide monomer. Methods are described in the Experimental section.

A figure such as Fig. 13 suggests an important advantage of variable-wavelength detectors over fixed-wavelength detectors, such as those now offered with 214-nm and 229-nm detection. If the situation exists where the largest sample volume is being injected to maximize detector sensitivity for the samples available, then it will be desirable to change the detection wavelength depending on the problem. In the example shown in Fig. 13, if the problem were to quantitate trace levels of acrylamide below 0.5 μg (in the sample volume injected), then the best detection wavelength is the absorption maximum, near 200 nm. On the other hand, if the problem were to quantitate larger levels of monomer, ranging from trace levels to a maximum of 35 μg, then the side of the absorption band at a wavelength of 240 nm is best, since trace level samples give the largest peaks and the highest level samples just fall on the sample linear range. A fixed-wavelength detector at 254 nm would give no detectable levels for many of the trace level samples, and a fixed-wavelength detector at 214 nm would quantitate the trace level samples but require dilution or a reduction in injected sample volume to quantitate samples with higher monomer levels.

CONCLUSION

Industrial laboratories can realize considerable costs savings with "universal LC methods" that substitute automation for expensive manpower and allow "first-run problem-solving". The universal method of full-gradient reversed-phase LC is described that gives sensitive, low-wavelength detection (0.1 a.u.f.s. at 210 nm) with less than 5% baseline drift and no ghost peaks. Techniques for minimizing baseline problems by using aqueous and acetonitrile "eluent conditioner columns", solvent and additive selection and cleanings, reduced column length or column loading, reduced gradients or equilibration times, UV irradiation of water, and addition of components to eliminate UV mismatch, potentially can be applied to other modes of chromatography.

The low-wavelength detection (190–210 nm) that gives the above universal methods the power to quantitate most all components to low levels brings a new realm of problems. As rapid-scanning UV detectors become common in LC, one can anticipate extensions of the discussions begun here of the interaction of (1) the detector components linear limit, (2) the UV absorbance of the eluents, and (3) the absorption spectra of the sample. The combined effect of points (1) and (2) at low wavelengths give a "system linear limit" that determines a wavelength-dependent "highest linear sample size" that must be considered in order to obtain the benefits of nearly universal detection of even trace level components.

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DEPENDENCE OF THE SPLITTING RATIO ON COLUMN TEMPERATURE IN SPLIT INJECTION CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

The amount of sample entering a capillary column during split injection increases considerably if the major component (*e.g.*, the solvent) recondenses in the column inlet. Owing to the reduction in volume during the recondensation, additional sample vapour is sucked into the column. The resulting true splitting ratio may deviate from the pre-set ratio by a factor exceeding 30. Recondensation occurs at column temperatures below the boiling point of the major component (usually the solvent). It is favoured by a short distance between the tip of the syringe needle and the column entrance, large sample volumes, narrow glass inserts in the injector and other factors that limit the dilution of the sample vapour with carrier gas prior to the splitting process. The deviation of the true from the pre-set splitting ratio is an important source of error in quantitation based on the external standard method because this deviation may depend sensitively on critical parameters. It may cause high standard deviations, and also the true splitting ratio may be different for, *e.g.*, the calibration mixture and the sample, creating systematic errors that are difficult to detect.

INTRODUCTION

We consider it still to be important to investigate the processes involved in split injection because we do not know of a replacement for this injection technique. For many applications split injection is the most convenient sampling method, as it allows one to inject mixtures nearly regardless of the solvent, at any column temperature, with little risk of disturbing solvent effects[1,2] or of band broadening due to slow sample transfer from the injector to the column. For a number of samples it may even be nearly impossible to replace split injection.

The apparent simplicity of the split sampling method conflicts with the many problems that arise as soon as accurate analytical results from other than the easiest samples are required[3]. One of the problems is concerned with the splitting ratio. The pre-set splitting ratio[4], adjusted by the ratio of the carrier gas flow-rates passing by and entering the column, is seldom equal to the true splitting ratio, *i.e.*, the proportion of the sample reaching the column. The true splitting ratio, obtained by division

of the peak area resulting from a splitless injection by the peak area resulting from a split injection, is generally lower than the pre-set splitting ratio, so that more sample enters the column than expected. This deviation of the true from the pre-set splitting ratio is important for the following reasons:

(1) In general it is not possible to calculate the amount of substance entering the column by division of the total sample size by the pre-set splitting ratio. Errors of factors up to 50 may occur.

(2) As the deviation of the true from the pre-set splitting ratio is often poorly reproducible, quantitation by the external standard method is severely hindered. There is a considerable risk of experiencing systematic errors, *e.g.*, if the true splitting ratio of the calibration mixture is different from the splitting ratio obtained for the sample.

(3) Discrimination, *i.e.*, non-linearity of the splitting process, is partly due to a splitting ratio that fluctuates during the period of time during which the sample is split, thus due to the mechanism which also causes the deviation of the true from the pre-set splitting ratio[3]. When using the internal standard method, the true splitting ratio might be considered to be of little importance. However, it is important as soon as a change in the splitting ratio alters the discrimination pattern of mixtures with a wide range of boiling points.

Although we do not have a solution to offer for these problems, we consider it to be important to describe the sources of errors and their dependence on various parameters in order to keep them under control. Even if using a calibration method that supposedly corrects for all errors, it is important to know about the possible sources of errors in order to know the critical parameters. Reproducibility, the factor which determines the accuracy of results obtained by calibration procedures, is usually tested by re-injecting the same mixture of standards. This method does not give any information about the extent of the deviations involved and it is not known whether these deviations are kept constant when injecting the real sample, possibly including a slight change in a number of unrecognized critical parameters.

In a previous paper[4] we reported on the effects on the splitting ratio caused by the pressure wave initiated upon introducing a large amount of sample vapour into a relatively small injector cavity. The fluctuation of the pressure inside the injector changes all of the gas flow-rates, including the column and the split flow-rates, which determine the true splitting ratio. These flow-rates do not change simultaneously or by the same ratio and therefore they change the splitting ratio in a complex manner. In that paper we did not consider another cause which results in a deviation of the true from the pre-set splitting ratio, *viz.*, recondensation of the sample in the column inlet.

EXPERIMENTAL AND RESULTS

Recondensation of the sample

Some experimental results showing the dependence of the splitting ratio on the column temperature during the injection are given in Fig. 1. We injected a test mixture containing *n*-octadecane diluted 1:5000 in various solvents at a pre-set splitting ratio of 100:1, adjusted at a column temperature of 30°C. The temperature of the column during the injection was varied between 30 and 200°C. Ten seconds after the injection the oven was heated to elute the sample at 200°C.
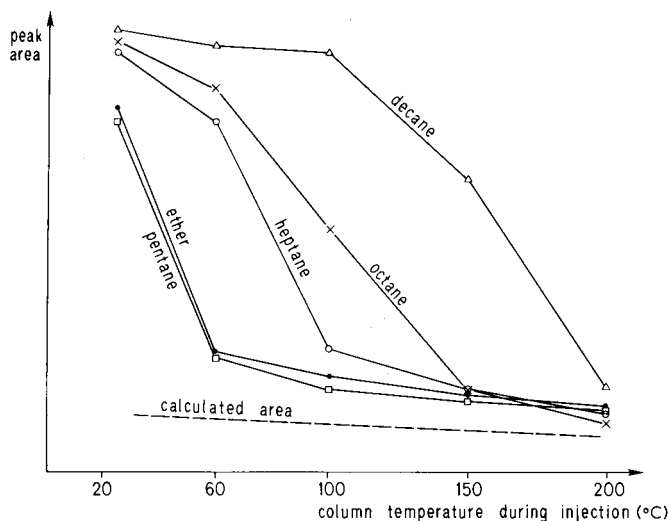
Fig. 1. Dependence of peak areas obtained for *n*-octadecane in the various solvents indicated at a constant pre-set splitting ratio on column temperature during sampling. The peak area expected from the pre-set splitting ratio was calculated by dividing the peak area obtained from a splitless injection of the same sample by the pre-set splitting ratio of 100:1. As the carrier gas flow-rate is reduced at increased column temperature, the pre-set splitting ratio increased by about 25 % between 30 an 200°C (broken line). At column temperatures near or below the b.p. of the solvent the peak areas increase, *i.e.*, the true splitting ratio decreases owing to the recondensation of the solvent in the column inlet. The conditions chosen moderately favour recondensation.

The instrument was a Model 4160 (Carlo Erba, Milan, Italy) with an injector at 300 C, equipped with a glass liner of 2 mm I.D.; 2 µl of sample were injected by a syringe with a needle of length 7.5 cm, leaving 2 cm between the needle exit and the column entrance.

The peak area obtained by a splitless injection of the above mixture was divided by the pre-set splitting ratio (100:1) to calculate the peak area expected from the pre-set splitting ratio. As shown in Fig. 1, the peak areas determined for injections at high column temperatures were relatively close to this calculated area. The deviations, *i.e.*, the differences between the true and the pre-set splitting ratio, of a factor of 1.8–2 were assumed to be due primarily to the pressure wave. However, at column temperatures decreasing below the boiling point (b.p.) of the solvent, the peak areas increased many-fold. When using decane as a solvent (b.p. 175°C), the peak areas increased between 200 and 100°C and reached a moderately stable value below 100 C.

The decrease in the splitting ratio (or the increase in the peak area) with decreasing column temperature is explained by the recondensation of the solvent in the cool column inlet. The recondensation greatly reduces the vapour volume of the sample, which creates a zone of reduced pressure in the column inlet, thus sucking in further amounts of sample vapour. Recondensation becomes important at a column temperature close to the b.p. of the solvent. At a column temperature 50–80°C below the b.p. of the solvent, the recondensation is virtually complete and a further decrease in the column temperature hardly alters the splitting ratio.

As recondensation is the cause of the decreasing splitting ratio with decreasing column temperatures, it might be expected that the splitting ratio would be influenced by the coating of the column inlet with stationary phase. To investigate this aspect we used an extremely thick-filmed OV-73 column (film thickness 2 μm) with the following inlet sections:

(a) the complete inlet section coated with 2 μm of OV-73;

(b) with a 1-m length of persilylated but uncoated column connected to the front of the column;

(c) with a 1-m length of column coated with 0.1 μm of Carbowax 1000.

These three configurations were tested by injections of a 0.1 % solution of n-octadecane in n-octane at a constant pre-set splitting ratio (100:1), varying the column temperature between 30 and 230°C. The results are summarized in Fig. 2. Conditions were chosen such that the peak areas changed drastically in the range of the b.p. of the solvent (125°C). However, these changes depended little on the characteristics of the column inlet. Thus the stationary phase seems to be of little importance for the recondensation. Recondensation is just a matter of the volatility of the solvent in its own environment, as was observed for recondensations creating the solvent effect during splitless injections.

The increase in the carrier gas flow-rate into the column by recondensation has been observed previously. In a recent paper[5] we reported on the increase in the flow-rate into the column during splitless injections when choosing conditions that favoured the recondensation of the solvent, thus creating a solvent effect. Especially
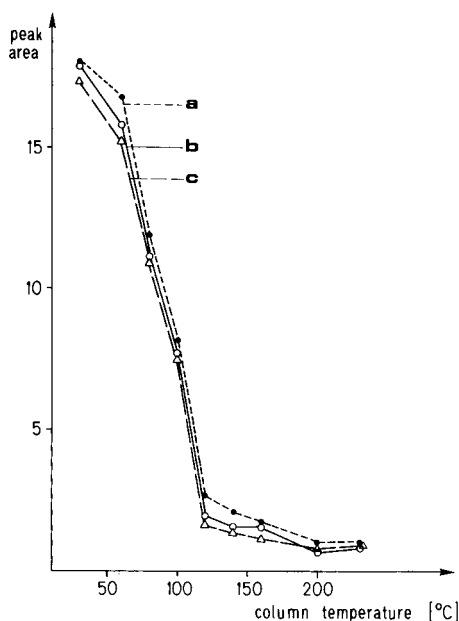
Fig. 2. Recondensation effects on a capillary column with different inlet sections. Injections of 3 μl of 0.1 % of n-octadecane in n-octane; pre-set splitting ratio 1:100; injector temperature, 300°C; glass insert of 2 mm I.D.; long syringe needle to release the sample 2 cm above the column entrance. (a) Column inlet coated with 2 μm of OV-73; (b) column inlet uncoated; (c) inlet coated with 0.1 μl of Carbowax 1000. It is concluded that the recondensation is hardly dependent on the stationary phase in the column inlet.

during the first few seconds of the splitless period far more sample entered the column than would have been possible with the normal flow-rate, even when the dilution of the sample vapour with carrier gas is neglected. In this instance the recondensation in the column inlet is visible.

Factors that influence recondensation

Some factors that influence the recondensation and hence determine the deviation of the true from the pre-set splitting ratio were investigated. These are considered below in some detail to show which parameters have an important influence on the true splitting ratio. As contradictory arguments do not allow simple general working rules to be derived, the reader will have to draw his own conclusions for his samples on how to minimize and to control the recondensation effect.

Width of the injector cavity

We determined the amount of sample (n-pentadecane diluted 1:5000 in n-octane) entering the column at a pre-set splitting ratio of 100:1 at various column temperatures, using injectors with different inner diameters. Table I gives the ratios of the peak areas obtained at 30 and 200°C, *i.e.*, factors by which the recondensation decreased the true splitting ratio ("recondensation effect").

TABLE I

RATIOS OF PEAK AREAS AT 30 AND 200°C ("RECONDENSATION EFFECTS") FOR DIFFERENT WIDTHS OF THE INJECTOR CAVITY

Distance of the syringe needle from the column entrance 2 and 6 cm, with or without a dense packing of glass-wool in the injector cavity; pre-set splitting ratio, 100:1.

I.D. of injector cavity (mm)	2 cm distance, glass-wool		2 cm distance, no glass-wool		6 cm distance, glass-wool	
	2 μl	4 μl	2 μl	4 μl	2 μl	4 μl
2	28	20	14	8	2	2.5
3.6	3	9	4	6	3	2.5
6	4	7	4	8	2.5	3

The recondensation was most effective for the narrow (2 mm) glass insert, especially if combined with the use of the long syringe needle, leaving a distance of 2 cm between the tip of the needle and the column entrance. The true splitting ratio was decreased by factors of up to 30, which means that the true splitting ratio was close to 2:1 instead of 100:1 according to the pre-set ratio (the true splitting ratio at a column temperature of 200°C was about 60:1). This is nearly splitless injection. In fact, the reconcentration effect may be used to introduce a large proportion of a sample without creating a band broadening effect (at a pre-set splitting ratio of 2:1 the sample enters the column so slowly that the peaks are broadened). The recondensation effect is markedly reduced when using an injector wider than 2 mm. However, there was a surprisingly small difference whether the glass insert of 3.6 mm I.D. was used or the glass liner was taken out of the injector, leaving the metal body of 6 mm I.D. For the 6 mm injector cavity, the recondensation still decreased the splitting ratio by factors of between 2.5 and 7.

Distance between the needle tip and the column entrance

The ratios of the areas at column temperatures of 30 and 200°C in Table I show that the distance between the needle tip of the syringe and the column entrance is a dominating parameter influencing the recondensation effect. When using the 2 mm glass insert, the 2 cm distance gave ratios between 20 and 30 whereas for the 6 cm distance they were only between 2 and 2.5. For the wider injectors the differences between the recondensation effects determined by using the long and the short distance were only of the order of a factor of 2.

Both the width of the glass insert and the distance between the needle and the column influence the dilution of the sample vapour with carrier gas. Increasing dilution of the vapour leads to a double reduction of the recondensation effect. Under critical conditions (column temperature) the dilution influences the extent of the recondensation (depending on the partial vapour pressure). At the same time the recondensation of the vapour from a diluted gas phase creates only a weak reduction in the pressure, sucking in only small amounts of additional vapour (which is again diluted). Both parameters are well known also to influence the maximal column temperature for creating a solvent effect.

Sample volume

Table II shows peak areas (average integration counts divided by 1000) obtained for different sample volumes. The test sample was *n*-pentadecane diluted 1:5000 in *n*-octane. The experimental conditions favoured recondensation as the long syringe needle (giving the 2 cm distance) and the 2 mm glass liner were used. The glass insert was filled with glass-wool. At low column temperatures the peak areas show an unregular dependence on the sample volume. Instead of being doubled on changing from a 1- to a 2-μl sample size (always considering the needle volume of the syringe), the peak area was multiplied by a factor close to 15. However, again doubling the sample volume from 2 to 4 μl increased the peak area by only 30%. Above the b.p. of the solvent (125°C) the relationships normalized, although not yielding the accurate data as desirable.

The data in Table II show that the strong recondensation effect requires a concentration of sample vapour in the carrier gas stream which exceeds a critical limit. This is confirmed by the ratio of the peak areas at 30 and 200°C. This ratio was relatively small for the 1-μl sample size (with the small peak area at 30°C), which is in

TABLE II

DEPENDENCE OF PEAK AREAS (INTEGRATOR COUNTS $\times$ 10^{-3}) ON SAMPLE VOLUME AT VARIOUS COLUMN TEMPERATURES

Temperature (°C)	Sample volume			
	1 μl	*2 μl*	*3 μl*	*4 μl*
30	35	510	640	690
60	27	360	490	530
100	21	180	330	420
140	15	26	48	76
200	11	19	28	32

agreement with relatively little recondensation. The many-fold larger peak area obtained for 2 μl of sample at 30 C was due to a concentration of sample vapour in the carrier gas which exceeded the critical limit to cause strong recondensation. This is confirmed by the fact that the difference in the peak areas obtained at 30 and 200°C was large.

Pre-set splitting ratio

When considering the influence of the sample size on the recondensation effect (Table II), it is not surprising that the deviation of the true from the pre-set splitting ratio also depends on the pre-set splitting ratio itself. The data given in Table III show that the true splitting ratio may change by a factor of 15 when the pre-set splitting ratio is changed by a factor of only 5 (6 cm distance at 30°C). Apparently the reduced splitting ratio promoted the recondensation effect. The recondensation effect involved is confirmed by the fact that the change in the true splitting ratio between a column temperature of 30 and 200°C is increased when decreasing the pre-set splitting ratio. However, as the data in Table III show, this observation cannot be generalized as the strong deviations occur only under critical conditions.

TABLE III

PEAK AREAS AND DIFFERENCES IN PEAK AREAS AT LOW AND AT HIGH COLUMN TEMPERATURES AS A FUNCTION OF PRE-SET SPLITTING RATIO

Distance between the needle tip and column entrance, 2 and 6 cm; sample size, 2 μl; 2-mm glass insert packed with glass-wool; injector at 300°C.

Column temperature (°C)	Splitting ratio			
	6 cm distance		2 cm distance	
	100:1	20:1	100:1	20:1
30	41	700	410	2100
200	22	70	14	100
Difference	×2	×10	×30	×20

Injector packed with glass-wool

For some of the experiments the glass insert of the injector was packed with silanized glass-wool. Glass-wool hinders the large droplets of sample from passing the splitting point without prior evaporation or at least fragmentation into small droplets. Large droplets may fall on to the capillary entrance on one occasion or they may pass it on another, thus not providing reproducible results. Further, large droplets are not likely to be split according to the gas flow-rates.

The true splitting ratio and the reproducibility of the results were little dependent on whether the injector was packed or empty, provided that volatile solvents were used (pentane, hexane, diethyl ether; injector at 300°C as throughout this work). However, with *n*-octane as the solvent the recondensation effect differed with and without glass-wool by a factor of up to 2. Such differences were found to be related to

the absolute peak areas. In those instances where the glass-wool caused an increased amount of sample to enter the column, the recondensation effect was reinforced.

n-Octane is only partially evaporated on being introduced into an empty injector at 300°C. The absolute peak areas were mostly smaller than for injections into a glass insert packed with glass-wool. Further, the reproducibility of the peak areas was sometimes extremely poor. Table IV gives the first few peak areas for a series of injections obtained under conditions giving pronounced differences (narrow glass liner, sample volume 4 μl and high column temperature). When using the short syringe needle leaving a distance of 6 cm to the column entrance, the peak areas varied by a factor of 20, between 30 and 1000 % of the area obtained with glass-wool (ten replicate injections). The peak areas give the impression that sometimes a large droplet fell into the column, giving exceedingly high peak areas, but more often the droplets passed by the column, resulting in too small a peak. The peak areas varied by less than by a factor of 2 for the long syringe needle (2 cm distance). Considering the reproducibility of the absolute peak areas for the sample dissolved in n-octane, the use of glass-wool in the injector was shown to be of great advantage.

TABLE IV

TYPICAL PEAK AREAS FROM INJECTIONS INTO AN INJECTOR WITH A 2 mm I.D. GLASS LINER WITH AND WITHOUT GLASS-WOOL

Short and long syringe needles were used to release the sample 6 and 2 cm from the column entrance; column at 200°C.

Distance (cm)	With glass-wool	No glass-wool
2	3260, 3209, 3298, 3251	5915, 6216, 6912, 4873, 5502
6	6075, 6009, 6063, 6105	5658, 1444, 13,850, 1367, 4028

DISCUSSION

The user of the split injector may aim for two different goals:
(a) to eliminate the deviation of the true splitting ratio from the pre-set ratio; and
(b) (the far more modest goal) to establish working rules that allow one to work with today's deficient systems with the intention of minimizing deviations and to use conditions that allow one to reproduce them (non-reproducibility of results is a consequence of non-reproducibility of the deviations).

Obviously it would be most useful to eliminate all mechanisms that cause deviations of the true from the pre-set splitting ratio or, more precisely, to avoid fluctuations of the splitting ratio during sampling. However, during the study reported here we could not find conditions that allowed a general elimination of the recondensation effects. Recondensation was reduced by diluting the sample vapour with carrier gas. The mixing of the sample vapour with carrier gas required by Ettre and Purcell[6] to achieve linear splitting probably also aims for a stable splitting ratio during the splitting process. The dilution with carrier gas is improved by increasing the effective injector volume, *i.e.*, by an enlarged injector cavity and by an increased

distance between the needle and the column. However, our attempts to eliminate recondensation by dilution of the vapour had only limited success. For a distance of 6 cm between the needle and the column and for an injector cavity of 6 mm I.D., the splitting ratio still increased by factors exceeding 2 on increasing the column temperature from below to above the b.p. of the solvent. The difference between the true and the pre-set splitting ratio easily reached a factor of 3. Further, a comparison of the results obtained with an injector of 3.6 and 6 mm I.D. did not indicate that a substantial improvement could be achieved by a further enlargement of the injector. During these experiments we found no evidence that glass-wool would improve the mixing of sample vapour with carrier gas.

On the other hand, dilution of the sample vapour with carrier gas is undesirable from the point of view of the initial band width of the sample deposited in the column. Peak broadening as a result of a slow transfer of a large (diluted) vapour cloud into the column can be avoided only by choosing a high flow-rate in the injector, *i.e.*, by a high splitting ratio. However, for many applications it is desirable to use a pre-set splitting ratio down to 10:1 or 5:1.

There remains the option of injecting small sample volumes. This seems attractive as the pressure wave is weak and the recondensation effects are small owing to the high dilution of the small amounts of vapour. However, small sample volumes cause problems with the syringe because of premature and selective elution out of the needle[7,8] (especially when using a 1-μl syringe).

Recondensation of the sample is avoided if the column temperature is kept at least about 20°C above the b.p. of the major constituents of the sample (*e.g.*, solvent). Unfortunately, for our applications, this is possible only for a few samples.

A split injector that minimizes the deviation of the true from the pre-set splitting ratio would probably have a cavity about 10–12 cm long with an I.D. of 5–6 mm. The pressure wave would be negligible and the recondensation effect would probably cause the true splitting ratio to deviate by less than a factor of 2 from the pre-set ratio. However, the matter becomes complicated when some contradictory requirements for this injector are included:

(a) It should be possible to work at a splitting ratio below 10:1.

(b) Conditions for minimizing discrimination often require a minimal evaporation with the intention of splitting the sample in the liquid (droplet) phase[3]. This calls for a rapid transfer of the sample from the syringe to the column and therefore, for a narrow liner and a short distance between the needle and the column.

(c) It should be possible to carry out splitless injections with the same sampling device. Splitless injections require a minimum of dilution of the sample vapour. The transfer of the vapour does not allow the use of an injector volume exceeding about 1 ml. Further, the syringe should release the sample vapour close to the column entrance, which calls for extremely long syringe needles (the column is bound to be situated on the bottom of the injector to minimize dead volumes).

At present the operator has to work with a split injector which, in general, does not provide a predictable splitting ratio. In this situation it is greatly preferable to quantitate on the basis of internal standards, which renders an accuracy that is fairly independent of the splitting ratio, because the accuracy of the analytical results depends only on the ratio of the sample parts to the internal standard (at least if one does not consider the dependence of the discrimination phenomena on the splitting

ratio). However, if using, *e.g.*, selective detectors or gas chromatography–mass spectrometry and single-ion detection, the operator is often forced to use the external standard method or the method of standard addition[9] based on absolute peak areas. These techniques rely on a contant true splitting ratio, *i.e.*, on the reproducibility of its deviation from the pre-set splitting ratio. As long as the injection of a sample has not been investigated, we recommend that the following working rules should be considered:

(1) The pre-set splitting ratio must be kept constant. A change in this ratio does not always change the true splitting ratio in the same proportion.

(2) The column temperature during the injection must be reproduced, especially if the column temperature is close to the b.p. of the solvent or of the major constituent.

(3) The external standard must be dissolved in the same solvent as the sample in order to reproduce the evaporation characteristics, the pressure wave and the recondensation effect.

(4) The sample volume has to be reproduced. From Table II it may be estimated that a change in the sample volume of 0.1 μl may double the peak area if the sample volume happens to be in a critical range depending on the recondensation effect.

The last rule not only requires a constant sample volume to reduce standard deviations but also disallows some fairly common methods. It is not possible to change the sample volume and to correct the peak area as may be desirable because the column is overloaded or the peaks are too small to be detected. An attractive version of the method of standard addition also is not allowed. It would often be of advantage to add the standard mixture to the sample only in the syringe, *i.e.*, by taking the normal sample volume and adding a known volume of the standard mixture on top of it. However, the total sample volume changes and therefore it cannot be assumed that the splitting ratio remains constant.

There is a single useful aspect of the recondensation effect: it is possible to work at very small true splitting ratios without the risk of obtaining broadened peaks.

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CHROM. 14,429

SYSTEMATIC ERRORS OCCURRING WITH THE USE OF GAS-SAMPLING LOOP INJECTORS IN GAS CHROMATOGRAPHY

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SUMMARY

Gas sampling valves, involving a fixed-volume loop, are widely used as injection devices in gas chromatography. It is shown that adsorption of the substance under consideration on the inner wall of the loop tube will cause a systematic error in the injected amount. This effect can in many practical cases be significant as is shown by experiments and theoretical calculations.

INTRODUCTION

The simple arrangement of a sampling loop valve for gas-phase injections has been used in gas chromatography (GC) for a long time, and it has been described in most standard texts of gas chromatographic praxis[1,2]. It makes use of a piece of tubing of known volume, which can be connected alternately to a sample gas stream and to the carrier gas stream. Such devices are offered as accessories to almost every type of commercial gas chromatograph. In process GC, this is the standard injection technique. Also in liquid chromatography[3] and in flow-injection analysis[4], similar arrangements are widely used.

When recently using a gas-sampling loop in an apparatus for measurements of gas–aqueous partition coefficients for hydrocarbons[5], we noted systematically higher concentrations from analysis of the hydrocarbon gas stream using the loop injection compared with the concentration expected from the parameters of the generating apparatus. The latter agreed with measurements performed with other types of gas-phase analysis: injection with a gas-tight syringe and sampling via an adsorption tube.

It is clear that if the substance under consideration adsorbs on the inner wall of the loop tube when it is connected in the sample stream, it will desorb when clean carrier gas phase passes through. The result is an injection into the chromatograph of a larger amount of analyte than is expected from the volume of the tube and the gas-phase concentration. Should this effect be significant there is a systematic error, the

magnitude of which should depend on the tube material and geometry and on the sample type and concentration. No references to this problem were found in the literature.

As the precision of the injections with the loop injector is superior to that which is possible with other techniques, a systematic investigation of this phenomenon was performed.

In this work we report a simple theoretical treatment of the problem and some experimental data, showing that the effect is real and in practical cases often significant, especially for substances with low vapour pressures.

THEORY

As a model for the adsorption behaviour, we use the so called B.E.T. equation[6]:

$$\frac{x}{x_m} = \frac{c \cdot p/p_0}{(1 - p/p_0) [1 + (c - 1) \cdot p/p_0]} \tag{1}$$

where p is the partial pressure of the adsorbate, p_0 is its saturation vapour pressure, x is the amount adsorbed per unit area, x_m is the amount adsorbed in a monolayer of unit area and c is a constant. The amount of analyte, adsorbed on the inner wall of the sample loop is $x \cdot S_{loop}$ (S_{loop} is the inner surface area of the loop tube). Thus the relative error, f, caused by this adsorption is:

$$f = \frac{x \cdot S_{loop} \cdot R \cdot T}{p \cdot V_{loop}} \tag{2}$$

where V_{loop} is the volume of the loop, and R and T are the gas constant and absolute temperature, respectively. Ideal behaviour of the sample vapour is assumed. With eqn. 1 and the relation $V_{loop}/S_{loop} = r/2$, where r is the radius of the tube, we get:

$$f = \frac{x_m \cdot c \cdot 2 \cdot R \cdot T}{(1 - p/p_0) [1 + (c - 1) \cdot p/p_0] \cdot p_0 \cdot r} \tag{3}$$

For a given loop and vapour, eqn. 3 can be written:

$$f = \frac{d}{(1 - p/p_0) [1 + (c - 1) \cdot p/p_0]} \tag{4}$$

where d is a constant.

It can be seen that if $p = 0$ then $f = d$, and if $c > 2$ then f has a minimum value:

$$f_{min} = \frac{4d(c - 1)}{c^2} \tag{5}$$

at $p = 1/2 \cdot p_0 \cdot (c - 2)/(c - 1)$, approximately equal to $1/2 \cdot p_0$ at large values of c. Also, $f \rightarrow \infty$ when $p \rightarrow p_0$ (which is a hypothetical extrapolation, and does not apply
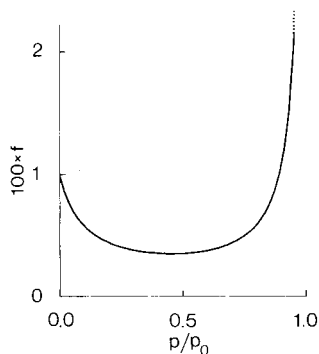
Fig. 1. Plot of f versus p/p_0 according to eqn. 4. Parameters: $c = 10$, $d = 10^{-2}$.

to the systems which were studied.) In Fig. 1, f is plotted *versus* p/p_0 in a typical case.

In principle it is possible to calculate the error which is made by using a sample loop from the equations given above. However, very few values of c can be found in the literature for systems of interest in this context. Thus it is possible only to give rough estimations of the effects involved.

CALCULATIONS

To calculate values of f from the equations given above, values of x_m are required. These were calculated from $x_m = 1/(\sigma \cdot N_A)$, where N_A is Avogadro's number and σ is the cross-sectional area of the adsorbate molecule, calculated by Hill's formula[7], using critical constants readily available[8]. Saturated vapour pressures were calculated from Antoine equations given in ref. 8. The values found for these physical constants are summarized in Table I.

TABLE I

PHYSICAL CONSTANTS AT 25°C

Substance	p_0 (atm)	σ (Å^2)	x_m (moles m^{-2})
Hexane	0.199	42	$4.0 \cdot 10^{-6}$
Benzene	0.123	32.5	$5.1 \cdot 10^{-6}$
Toluene	$3.75 \cdot 10^{-2}$	38.5	$4.3 \cdot 10^{-6}$
Ethylbenzene	$1.25 \cdot 10^{-2}$	41.3	$4.0 \cdot 10^{-6}$

EXPERIMENTAL

Sample vapour, generated with an apparatus, described earlier[9], was pumped through a six-port valve to which were connected loops made of different materials and of varying size (see Fig. 2). The loops were kept at ambient temperature, and protected against draughts by a polystyrene insulation. In position A, clean nitrogen passed through the loop and further to a gas chromatograph (Hewlett-Packard, Model 402, Avondale, PA, U.S.A.) equipped with a Porapak P column (Waters Assoc., Milford, MA, U.S.A.), a flame ionization detector and a digital integrator

(Infotronics, Model CRS 101, Shannon Airport, Ireland). In position B of the valve, the sample vapour passed through the loop. Samples were injected by switching the valve to position B for a defined period of time (see below) and then back to position A. The area of the resulting peak is denoted A_{loop}.
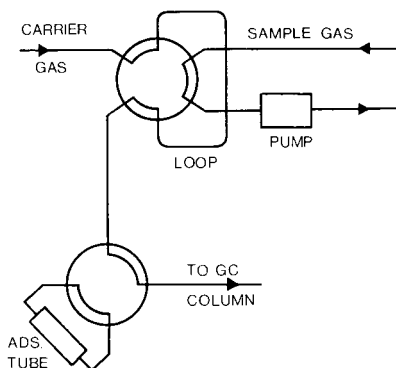
Fig. 2. Experimental arrangement.

Also, as a reference measurement, the vapour was sampled by adsorption on a porous polymer (Porapak Q, Waters Assoc.). Using a gas-tight syringe, a known volume of vapour, V_a (5–10 ml) was drawn through a tube (50 × 3 mm I.D.) filled with the polymer. The tube was connected to a four-port valve (see Fig. 2), and the adsorbed sample was thermally desorbed into the same gas chromatograph as the sample from the loop, giving the peak area A_a. The parameters of this analysis, such as desorption time and temperature, vapour volume and speed of sampling, were determined individually for each substance, and the sampling efficiency and recovery were checked as described below.

A quantity F, expressing the difference between the amounts of substance sampled in the two different ways, was calculated from eqn. 6:

$$F = \frac{A_{loop} \cdot V_a}{A_a \cdot V_{loop}} - 1 \tag{6}$$

RESULTS AND DISCUSSION

Measurements were performed with four substances using three different loops. The results, expressed as F values according to eqn. 6 are summarized in Table II. The uncertainty of the F values is $ca.$ 0.03. To compare the experimental results with the theory, values of d (the maximum value of the expected error f), f_{min} (the minimum value) and $f_{0.01}$ (the value of f at $p/p_0 = 0.01$) were calculated for the current values of r and for different values of c. This is presented in Table III.

According to the very sparse literature data[10,11], c for hexane and benzene when adsorbed on clean glass or SiO_2 is $ca.$ 10. From the tables it can be seen that a value of c $ca.$ 100 ($i.e.$ stronger adsorption) gives a better agreement with the experimental results for the GLT loop and the glass loop. For toluene and ethylbenzene, c values between 10 and 100 seem to give the observed effects. With the copper loop a very high adsorption was observed, corresponding to a c value of $ca.$ 1000, which

TABLE II

EXPERIMENTAL RESULTS

Loops: GLT, glass-lined stainless-steel tubing, $r = 0.35$ mm, length 300 mm; glass, glass tube, with short copper connections, $r = 1.5$ mm, length 108 mm; Cu, copper tube, $r = 1.0$ mm, length 180 mm.

Substance	p/p_0	F	Loop
Hexane	$7.3 \cdot 10^{-4}$	0.16	GLT
	$9.0 \cdot 10^{-3}$	0.25	GLT
	$1.1 \cdot 10^{-2}$	0.25	GLT
	$5.0 \cdot 10^{-2}$	0.26	GLT
	$7.3 \cdot 10^{-4}$	0.05	Glass
	$9.0 \cdot 10^{-3}$	0.11	Glass
	$1.1 \cdot 10^{-2}$	0.11	Glass
	$5.0 \cdot 10^{-2}$	0.08	Glass
Benzene	$2.2 \cdot 10^{-2}$	0.20	GLT
	$7.0 \cdot 10^{-3}$	0.23	GLT
	$2.0 \cdot 10^{-2}$	1.5	Cu
Toluene	$2.3 \cdot 10^{-2}$	0.35	GLT
	$6.5 \cdot 10^{-3}$	0.33	GLT
Ethylbenzene	$2.9 \cdot 10^{-2}$	0.53	GLT

probably is caused by charge-transfer interactions (applicable with the aromatics only).

These comparisons can be made only half-quantitative owing to the uncertainties involved: the nature of the glass (especially the metal content of the GLT lining), the possibility of small cracks in the GLT lining, the presence of short copper connection tubes (attached with shrinkable PTFE) on the glass loop, the applicability of the B.E.T. equation to these systems (especially at lower pressures), the scantiness of literature data applicable to this problem, and the experimental uncertainty.

TABLE III

CALCULATED VALUES

Substance	c	r (mm)	d	f_{min}	$f_{0.01}$
Hexane	10	0.35	0.028	0.010	0.026
	100	0.35	0.28	0.011	0.14
	100	1.5	0.065	0.003	0.033
Benzene	10	0.35	0.058	0.021	0.054
	100	0.35	0.58	0.023	0.29
	100	1.0	0.20	0.008	0.104
	1000	1.0	2.04	0.008	0.188
Toluene	10	0.35	0.16	0.058	0.148
	100	0.35	1.60	0.063	0.81
Ethylbenzene	10	0.35	0.45	0.161	0.41
	100	0.35	4.47	0.177	2.27

By variation of the time during which the sample gas stream passed through the loop, rough information about the kinetics of the process was obtained. The peaks increased in size with this time, up to a limiting value which was reached after *ca.* 5 sec for the GLT loop, 15 sec for the glass loop and 60 sec for the copper loop. These times, which were used for the measurements of *F*, are much longer than what is needed to flush the loop with the gas, which is an additional indication that the adsorption indeed takes place.

The reference analysis, by adsorption on porous polymer and subsequent thermal desorption, was thoroughly checked. Two identical adsorption tubes were connected in series during the adsorption step. The second tube gave no peak at desorption. Various volumes of gas were drawn through the adsorption tube. The resulting peak areas formed, when plotted *versus* volume, a straight line through the origin. Different flow-rates were used with no significant difference. Also, some experiments were made with an adsorption tube containing Tenax-GC, with the same results as with Porapak Q. Two successive desorptions on the same tube showed that the desorption was complete. These experiments show that both the adsorption and desorption steps are performed with full efficiency, and the procedure can be trusted to give a result which is free of major systematic errors.

The adsorption in the six-port valve itself is negligible, as shown by the following experiment: another loop made from the same GLT tubing as the one which was used in most experiments, but shorter, gave peak areas which were smaller by the same factor as between the lengths of the tubes.

CONCLUSIONS

We have shown that systematic errors due to adsorption of the sample on the inner walls of gas-sampling loops can occur and will in many cases be significant and seriously influence the accuracy of this type of gas analysis. The error is most important for less volatile substances and for low concentrations. To decrease the errors, the loop should be made from an inert material and calibrations with known gas concentrations (approximately equal to that of the sample) should be made. The loop might be heated, to increase the vapour pressure. In that case, the gas stream should be preheated to the same temperature to avoid errors from expansion effects.

The precision (repeatability) of this injection technique is not impaired by the adsorption if the gas stream is flushed through the tube for long enough to allow the adsorption equilibrium to be completely attained.

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ALKYLARYLKETONES AS A RETENTION INDEX SCALE IN LIQUID CHROMATOGRAPHY*

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SUMMARY

A retention index scale based on the relative retentions of the alkylarylketones (PhCOR) has been evaluated for reversed-phase high-performance liquid chromatography. The standards were readily detected by absorbance at 254 nm and showed a linear relationship between log k' and carbon number. The retention index values for a number of test compounds were determined using different column materials and solvent systems.

INTRODUCTION

The acceptance of high-performance liquid chromatography (HPLC) in official and standard methods has been hindered by wide variations in the properties of reversed-phase columns with nominally the same composition[1]. These differences are caused largely by the different techniques used to prepare the bonded phase and to cap residual silica hydroxy groups[2]. It is therefore difficult to specify elution conditions for a particular type of column, such as ODS-silica, without recommending or defining a manufacturer or grade of stationary phase to be used. However, if the retention performance of a column could be determined and expressed in a readily comparable form, this could be used as in a specification. Any column and conditions satisfying the criteria would then be suitable for a particular separation, as long as the efficiency of the column was also appropriate.

In order to be able to make comparisons between chromatographic systems, it is necessary to have a means of expressing retentions which is largely independent of column efficiency, variations in solvent composition, flow-rate, and temperature. For simple assays the retention of an analyte relative to a single standard can be used but no one compound is suitable in all cases.

A retention index system based on a series of standards covering a range of polarities would avoid many of these problems and could form the basis of a guide to

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column activity. In gas–liquid chromatography (GLC) the Kovats retention index[3] based on the retention times of n-alkanes has been widely accepted as a reference scale, independent of individual laboratory standards. By also acting as the basis of the Rohrschneider[4] and McReynolds[5] constants, the Kovats index has enabled the comparative retention behaviour of different liquid stationary phases to be compared, and for most chromatographers this is its most valuable function. Recent studies aimed at the standardisation of essential oil analysis have extended its use to the determination of g-pack values characteristic of the performance of individual columns[6]. However, so far no comparable index system has been adopted for liquid chromatography (LC).

The n-alkane scale cannot be adopted directly for LC because of the low polarity and thus limited retention range of the alkanes. They also lack a significant chromophore and would be inconvenient or impossible to detect on most instruments using absorbance detectors. Recently Baker and co-workers proposed a retention index scale based on the alkan-2-ones[7] and examined the possibility of predicting the index value for a number of pharmaceuticals[8–10]. However, although they have a wide polarity range the alkan-2-ones have only a limited absorbance at 254 nm and the higher homologues are not widely available.

The use of retention index values would also enable the retentions of samples to be more easily compared. The present nearly universal use of capacity factors, while eliminating effects due to differences in column size and eluent flow-rate, cannot overcome the changes caused by small differences in solvent composition, and capacity factors, (k') reported by one laboratory are difficult to reproduce exactly even using the same column material. The trend in recent years to short microparticulate columns has also made the accurate determination of absolute values of $k' = (t - t_0/t_0)$ (where t = retention time and t_0 = retention time of an unretained compound) more difficult because of the uncertainty in the measurement of t_0 values of 1 min or less, compared to retention times of up to 30 min.

In the present paper we report the consideration of alternative retention index standards, which could have wide applicability, and discuss the detailed evaluation of the alkylarylketones as potential standards using different solvent and column systems.

EXPERIMENTAL

Materials

All standard and test compounds were reagent grade. Alkylarylketones used as standards were acetophenone (BDH, Poole, Great Britain), propiophenone (Hopkin and Williams, Great Britain), butyrophenone and valerophenone (Koch-Light, Poole, Great Britain), hexanophenone, heptanophenone and octanophenone (Aldrich, Poole, Great Britain).

Chromatography

Separations were carried out using Shandon columns (10 cm × 5 mm I.D.) which had been slurry-packed with 5-μm packing materials, ODS-Hypersil and SAS-Hypersil (Shandon Southern, Runcorn, Great Britain), C_{22}-Magnusil (Magnus Scientific, Sandbach, Great Britain) or Spherisorb-Phenyl (Phase Separations, Queensferry, Great Britain).

Solvents were made up using HPLC grade methanol (Fisons, Loughborough, Great Britain) and distilled water and were pumped at 1 ml min^{-1} using a Pye-Unicam XPS Pump or LDC DK Pump. The samples (usually 10 μl) were injected using a Rheodyne 7125 syringe valve and detected with an Altex 153 absorbance detector at 254 nm and sensitivity 0.08 a.u.f.s.

Standard test mixtures

Retention index standards were made up using 25 or 50 μl of alkylarylketone in 10 ml of methanol, which was diluted 100-fold in methanol–water (50:50) before use.

Aliphatic ketones were examined at a concentration of 100 μl in 10 ml of methanol, and test compounds at 1–10 μl in 10 ml of methanol.

Retention index (I) determinations

Reported capacity factors are the mean values of repeated determinations. Using a least-squares correlation, log k' values for the standards were compared with the number of carbon atoms $\times$ 100 (*i.e.* acetophenone $I = 800$). The retention index values of test compounds were determined from the equation log $k' = aI + b$ using the values of a and b derived from the standard correlation curve. The values could also have been obtained from a semi-log plot of k' and $C_n \times 100$ (Fig. 1).
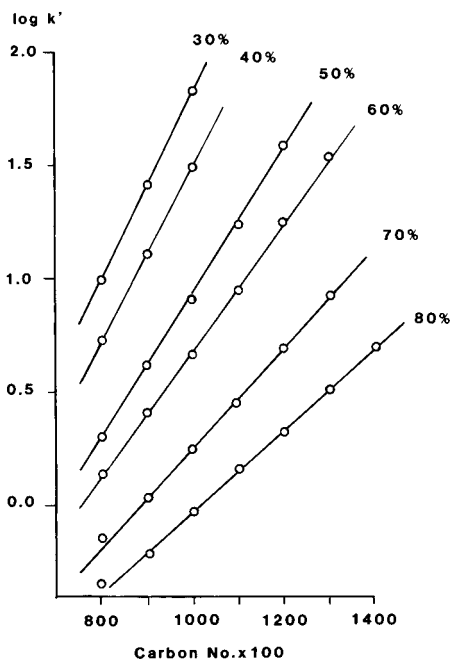
Fig. 1. Capacity factors for alkylarylketones compared with retention index (carbon number $\times$ 100) using different percentages of methanol as eluent.

TABLE I

CAPACITY FACTORS AND RETENTION INDEX VALUES FOR ALKYLARYLKETONES AND TEST COMPOUNDS ON ODS-HYPERSIL WITH DIFFERENT SOLVENTS

Compound	Capacity factors, k' Solvent (methanol, %)						Retention index Solvent (methanol, %)						Nominal
	30	40	50	60	70	80	30	40	50	60	70	80	
Retention index standards													
Acetophenone	9.96	5.54	2.00	1.35	0.72	0.45	800	800	804	803	816	816	800
Propiophenone	25.9	13.1	4.04	2.58	1.07	0.61	900	899	900	904	894	891	900
Butyrophenone	67.9	31.3	8.00	4.59	1.74	0.93	1000	1000	993	993	991	995	1000
Valerophenone			17.3	8.79	2.88	1.42			1098	1094	1091	1099	1100
Hexanophenone			38.0	17.5	4.91	2.09			1205	1202	1198	1194	1200
Heptanophenone				33.6	8.32	3.25				1303	1302	1302	1300
Octanophenone					14.1	4.97					1407	1407	1400
Samples													
Acetone	0.53	0.50	0.27	0.26			494	522	531	546			
Butan-2-one	1.19	0.96	0.50	0.51			579	600	614	651			
Pentan-2-one	3.09	2.13	0.95	0.78	0.55		678	690	703	718	761		
Hexan-2-one	8.78	5.53	2.00	1.47	0.77		787	800	804	816	829		
Octan-2-one			10.1	5.73	2.11	1.16			1025	1028	1029	1049	
Undecan-2-one					10.3	3.84					1344	1343	
Benzyl alcohol	3.84	2.57	1.08	0.83	0.48		701	711	720	727	735		
Benzaldehyde	6.67	4.16	1.58	1.14			758	767	772	776			
2-Phenylethanol	7.61	4.65	1.75	1.21	0.65		772	780	786	786	795		
p-Cresol	9.78	5.81	2.04	1.41	0.73	0.50	798	806	807	810	818	842	
Nitrobenzene	11.5	7.38	2.67	1.82	0.94	0.64	815	834	843	845	869	903	
Methyl benzoate	26.2	15.1	4.33	2.66	1.26	0.82	901	916	909	909	926	963	
Phenetole	42.9	26.1	7.08	4.75	2.03	1.18	952	979	976	999	1022	1053	
Toluene		30.6	9.08	5.63	2.31	1.39		998	1010	1025	1047	1094	

RESULTS AND DISCUSSION

Selection of retention index standards

For a series of compounds to be widely applicable as retention index standards, they should if possible satisfy the following criteria:

(1) they should have a strong chromophore at 254 nm so that they can be added to unknown samples in small amounts to act as internal standards;

(2) they should not be readily ionised to avoid changes in retention because of pH variations or the presence of ion-pairing reagents;

(3) a range of members of the series should be readily available at reasonable cost;

(4) the most polar member of the series should be eluted with a similar retention to water-soluble pharmaceuticals;

(5) the standard compounds must be unreactive and stable in common LC solvents.

Although in GLC a homologous series of compounds is used as retention standards, in LC, which is based on polarity rather than volatility, either a homologous series or a series of compounds with repeating functional groups can be considered as possible standards. The latter could be based on systems such as the carbohydrates $H(CH_2OH)_nOH$ or poly-substituted aromatic systems, *i.e.* nitrobenzenes $Ar(NO_2)_n$. However, in neither case is a sufficiently wide range of compounds available, and the directive effect of aryl substituents means that the polarity effects are not culmulative. The carbohydrates would also require the use of a refractive index detector.

On the other hand the capacity factors of members of a homologous series have previously been shown to possess a simple relationship to carbon number, $\log k' = aC_n + b$, in which the values of a and b vary with the solvent[11–13]. However, most simple monofunctional aliphatic compounds, including the alkan-2-ones used by Baker and Ma[7], have only a weak chromophore (acetone[14]: λ_{max}, 265 nm; ε, 17 l mol^{-1} cm^{-1}) and in the present study a concentration of 1 % of the alkanones had to be used to obtain reasonable signals. Conjugated aliphatic compounds such as the 2-alkenals might be suitable, but series of homologues are not readily available.

Aromatic compounds, particularly those with conjugated systems, have medium-to-strong chromophores and would be easy to detect. The need for a fairly polar smallest homologue, in order to cover the wide range of retentions of samples of interest in HPLC, excludes the use of the relatively non-polar simple aryl hydrocarbons, ethers, halogen or nitro compounds, based on a comparison of their capacity factors[15,16]. More polar systems such as carboxylic and sulphonic acids, amines and phenols are also excluded because of their sensitivity to pH changes. The remaining groups include alcohols, benzoate and phenyl alkanoate esters, amides and alkylarylketones. Of these only the phthalate esters and ketones are readily available although if needed the benzoates should be easy to synthesise. The phthalates, unfortunately, increase by two methylene groups between members and rapidly become very non-polar. It was therefore decided to examine the methyl to heptylphenylketones as the basis of a possible retention index system. Previously these compounds, which were all readily available, have been suggested as generally applicable relative retention standards although their use to form a scale was not examined[17].

Alkylarylketones as a retention index scale

Using an ODS-Hypersil column and varying compositions of methanol–water (from 30 % to 80 %), the k' values of the alkylarylketones were compared with those of the alkan-2-ones and a number of test compounds (Table I). Using the k' values for the alkylarylketones, a retention index scale was derived based on number of carbon atoms $\times$ 100 (*i.e.* acetophenone = 800) using a least-squares correlation. As expected from previous work the homologues showed a close correlation between log k' and C_n $\times$ 100 (Fig. 1), and the slope and intercept of the curve changed with the composition of the solvent (Table II and Fig. 1). Using these calibrations retention index values were determined for all the compounds examined (Table I). Interestingly, benzaldehyde clearly does not behave as the zero member of the ketone series. The small deviations in the index values of the alkylarylketones from their nominal values are a guide to the precision of the results. The alkan-2-ones also followed a linear relationship between log k' and C_n but the slope was different from that determined for the aromatic ketones. The retention index values of the test compounds showed only a small but general increase with increasing proportion of methanol in the solvent, (Fig. 2), in contrast to a marked change of up to 300 units found by Baker and Ma[7] for selected drugs when the alkan-2-one scale was used. Small variations in solvent composition would therefore have little effect on index values.

TABLE II

CORRELATION FACTORS FOR ALKYLARYLKETONE REFERENCE STANDARDS UNDER DIFFERENT CHROMATOGRAPHIC CONDITIONS AND COLUMNS*

Solvent (methanol, %)	Slope $\times$ 10^3	Intercept	Correlation
ODS-Hypersil			
30	4.165	-2.33	0.9999
40	3.76	-2.27	0.9999
50	3.19	-2.27	0.9995
60	2.79	-2.11	0.9997
70	2.18	-1.92	0.9991
80	1.77	-1.79	0.9992
SAS-Hypersil			
30	3.35	-2.041	0.9997
C_{22}-Hypersil			
30	2.921	-1.8857	0.9986
Spherisorb-phenyl			
30	2.379	-1.4532	0.9987

* For the equation log $k' = a (n \times 100) + b$.

Comparison of the alkylarylketones and alkan-2-ones shows that the lower members of the aliphatic series were more polar, the index value for acetone (*ca.* 500) requiring an extrapolation of the aromatic scale. Although this might suggest a limitation of the aromatic ketone scale when water-soluble drugs are being examined, in the previous study of barbiturates and propranolol analogues most of the compounds had retention index values on the aliphatic scale greater than 500, acetophenone having values of *ca.* 600[8]. Thus the alkylarylketone scale would largely cover these relatively polar compounds.
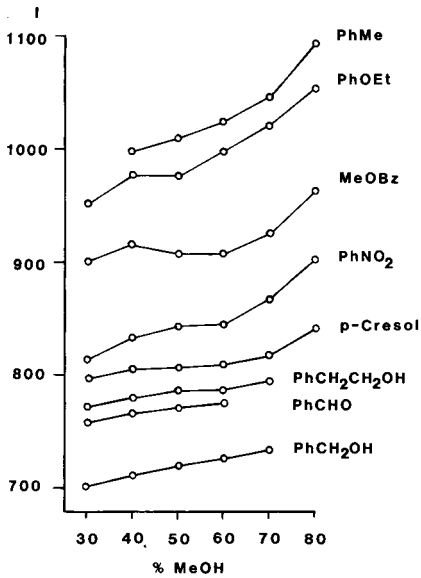
Fig. 2. Variation of retention index values of test compounds with eluent composition. Me = Methyl; Et = ethyl; Ph = phenyl; Bz = PhCO.

TABLE III

CAPACITY FACTORS AND RETENTION INDEX VALUES OF ALKYLARYLKETONES AND TEST COMPOUNDS ON SAS-HYPERSIL, C_{22}-MAGNUSIL AND SPHERISORB-PHENYL

Compound	SAS-Hypersil 30% methanol–water		C_{22}-Magnusil 30% methanol–water		Spherisorb-phenyl 30% methanol–water	
	k'	I	k'	I	k'	I
Retention index standards						
Acetophenone	4.40	801	2.92	805	2.96	809
Propiophenone	9.24	897	5.43	897	4.76	895
Butyrophenone	20.6	1001	10.3	992	8.00	990
Valerophenone			22.2	1106	14.4	1098
Hexanophenone					26.3	1208
Heptanophenone						
Octanophenone						
Test compounds						
Acetone	0.36	477	0.33	481	0.38	436
Butan-2-one	0.71	565	0.57	562	0.61	521
Pentan-2-one	1.56	667	1.03	650	1.02	614
Hexan-2-one	3.57	774	2.13	758	1.73	711
Octan-2-one			11.47	1008	4.88	900
Benzyl alcohol	1.71	677	1.08	657	0.69	543
Benzaldehyde	3.07	755	1.97	746	2.11	747
2-Phenylethanol	2.90	747	1.80	733	1.08	625
p-Cresol	3.69	779	1.90	741	1.04	618
Nitrobenzene	4.93	816	2.70	793	2.85	802
Methyl benzoate	9.06	895	5.13	889	3.68	848
Phenetole	10.46	914	5.36	895	2.96	809
Toluene	8.88	892	4.43	867	2.08	745

The aromatic system is considerably more sensitive to absorbance detection (acetophenone[14]: λ_{max}, 199, 240, 278 nm; ε, 19,953, 12,589, 1071 l mol^{-1} cm^{-1}), only 0.0025% (v/v) solutions being needed compared to 1% solutions of the aliphatic compounds. This is particularly useful for the longer chain compounds, which have a low solubility in methanol–water mixtures. Thus a set of standards could be added to an analyte sample in low concentration, without significantly altering the test solution.

Using only a single solvent system in each case all the compounds were also examined using SAS-Hypersil (Short Alkyl Silyl), C_{22}-Magnusil and Spherisorb-phenyl columns (Table III). In each case the calibration curve was again linear (Table II), but the values of the retention indexes of the test compounds differed between the columns.

Further studies making use of these differences as a method of characterising column materials in a similar way to the Rorhschneider constants in GLC, will be reported in the following paper[18].

As the majority of LC analyses are carried out at ambient temperature, the effect of different column temperatures has not yet been examined but it would not be expected to have a dramatic effect over small temperature ranges.

CONCLUSION

The alkylarylketones represent an easy way to determine a retention index value for test compounds, which is relatively intensitive to changes in solvent com-position. The standard ketones show a linear relationship between log k' and carbon number, and are readily detected using an absorbance detector. They cover the ex-pected retention range of all but the most polar compounds, and have the potential to be used as the basis of tests of column polarity.

ACKNOWLEDGEMENT

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COMPARISON OF REVERSED-PHASE LIQUID CHROMATOGRAPHY COLUMNS USING "ROHRSCHNEIDER" TYPE CONSTANTS

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SUMMARY

Using a set of aromatic reference compounds, the retention characteristics of reversed-phase high-performance liquid chromatographic columns can be expressed in terms of "Rohrschneider" type constants. These are based on the differences in retention indices of the reference compounds using the alkylarylketone scale, between the test column and solvent conditions and an ideal hexane–water partition system. The system can be used to quantitatively differentiate between phases coated with different alkyl chains and phenyl groups, and could be used to define the retention specifications of a column.

INTRODUCTION

It is often difficult to compare the results of reversed-phase liquid chromatography obtained in different laboratories, because of differences in columns coated with nominally identical bonded phases, particularly octadecylsilyl (ODS silica, and in variations in the ways results are expressed either as capacity factor, k', which is very susceptible to solvent composition, relative retention times to standards, or retention times at a particular flow-rate. Part of this problem could be largely solved by the use of a retention index scale based on a widely accepted homologous series. Two alternative systems using alkylarylketones[1] or alkan-2-ones[2] have been proposed and in both cases index values have been found to vary only slightly with solvent composition but to be dependent on column bonded phase.

It is harder to compensate for differences between columns because these often result from the different methods used to bond the stationary phase to the silica and the extent to which unreacted silica hydroxyl groups are capped[3]. It is therefore very difficult to define columns for use in a particular application such as an official method without specifying a particular make or brand or to select alternatives to a column reported in the literature. It would be highly desirable if the polarity and absorption of a column material could be determined in some way similar to the Rohrschneider[4] or McReynolds[5] constants used in gas–liquid chromatography (GLC), which could then be used to define the retention performance required to

carry out an assay and to compare columns of different makes or coated with different phases.

The present paper proposes a method for defining retention characteristics based on the use of the alkylarylketone-retention index-scale and a set of reference compounds the index values of which are compared to a standard system. The outline of a similar proposal based on the alkan-2-one scale and a standard ODS-column was mentioned by Baker but has not been examined further[2].

EXPERIMENTAL

Retention index values

Index values for reference compounds were determined as described previously[1].

Partition coefficients

Hexane–water partition coefficients were measured at $25 \pm 1°C$ using HPLC-grade hexane (Fisons Scientific, Loughborough, Great Britain) and distilled water. The concentration of the test compound in the hexane phase was determined by UV spectroscopy.

RESULTS AND DISCUSSION

In order to be able to produce a set of factors characteristic of the retention behaviour of a chromatography column, two components are needed. Firstly in order to eliminate differences in retention due to flow-rate, column length, and if possible solvent composition, a set of retention index standards are needed to provide a scale independent of absolute k' values. Secondly, a set of retention-test reference compounds are required, chosen so that they are characteristic of a range of structural types to test different possible interactions with the column. The index values of the test compounds could in themselves serve as retention factors but as with the Rohrschneider constants in GLC[4] the difference in index values compared to a standard column of optimum low polarity would be a more useful guide.

As reported in the previous paper, the alkylarylketones, which can be readily detected by UV detectors, can be used to provide an index scale which is largely independent of solvent compositions[1] and this scale was adopted as the basis of the present work.

Set of test compounds

In their choice of test compounds for use in GLC, Rohrschneider[4] and McReynolds[5] selected compounds, which were characteristic of typical sample groups. The final set of test substances, benzene, ethanol, methyl *n*-propyl ketone, nitropropane, pyridine, and in some cases 2-methyl-2-pentanol and iodobutane, would not be suitable for HPLC as most have only weak chromophores and could not easily be detected at 254 nm.

Initially therefore a number of aromatic compounds, benzaldehyde, benzyl alcohol, 2-phenylethanol, *p*-cresol, methyl benzoate, phenetole, nitrobenzene, and toluene were examined, the choice of compounds, *i.e.* methyl rather than ethyl ester,

being chosen so that 2 or 3 compounds could be examined in the same run. An attempt to include as a basic compound either ethylpyridine or β-phenylethylamine in the set was unsuccessful as without specially adjusting the solvent conditions only broad and distorted peaks were obtained. Because of their sensitivity to pH changes carboxylic acids were also not included. The capacity factors and retention index values for the test compounds on four reversed-phase columns using methanol–water (30:70) as eluent were determined earlier (Table I)[1].

TABLE I

RETENTION INDEX VALUES OF TEST COMPOUNDS*

From ref. 1, solvent methanol–water (30:70).

Test compound	Column			
	ODS-Hypersil	SAS-Hypersil	C_{22}-Magnusil	Spherisorb-phenyl
Benzaldehyde	758	755	746	747
Phenetole	952	914	895	809
Toluene	998*	892	867	745
Methyl benzoate	901	895	889	848
Nitrobenzene	815	816	793	802
Acetophenone	800	800	800	800
Benzylalcohol	701	677	657	543
2-Phenylethanol	772	745	733	625
p-Cresol	798	779	741	618

* Solvent methanol–water (40:60).

2-Phenylethanol and benzyl alcohol behaved in a very similar way, the difference between their index values remaining nearly constant on all four columns, the former was therefore not studied further. The index value of benzaldehyde remained virtually constant relative to the alkylarylketone scale suggesting that similar factors effected the aldehyde and ketone groups. The remaining six compounds, toluene, phenetole, methyl benzoate, nitrobenzene, benzyl alcohol and p-cresol, could be classified using the differences between their partition coefficients (log P) in octanol–water and hexane–water (Table II). This is a guide to their polarities and roughly follows the elution order (Table I). The set of test compounds thus includes compounds both more and less polar than the ketone index scale. The differences between the log P values of the test compounds in the two systems emphasises the effect of sample polarity on the distribution into the non-aqueous phase.

Standard reference-partition system

Although the index values of the test compounds could themselves be used to characterise the retention properties of different columns or packing materials, they cannot be related directly to the properties of the column. This is because the index value reflects both the polar interaction of the functional group of the compounds with the stationary phase and the lipholicity of the hydrocarbon grouping which depends on the size and shape of the molecule. As it is the former interaction which is

TABLE II

PARTITION COEFFICIENTS FOR TEST COMPOUNDS AND ALKYLARYLKETONES IN OCTANOL–WATER AND HEXANE–WATER

Compound	Log P*		$Log\ P_{octanol-water}\ -$ $Log\ P_{hexane-water}$
	Octanol–water	Hexane–water	
Phenetole	2.51	3.05**	−0.54
Toluene	2.58	2.87	−0.29
Methyl benzoate	2.17	2.19**	−0.02
Nitrobenzene	1.84	1.47	0.37
Benzylalcohol	1.02	−0.76	1.78
p-Cresol	1.95	−0.35	2.30
Acetophenone	1.66	1.15	0.51
Propiophenone	2.20	1.97**	0.23
Butyrophenone	(2.70)***	2.74**	(−0.04)

   * From ref. 6 unless stated.
  ** Measured in this study.
 *** Predicted value as addition of CH_2 usually increase log $P_{octanol-water}$ by 0.5.

primarily of interest it is necessary to compare the index values with a standard system in which the polar interaction with the stationary phase is minimised.

Any attempt to use as a standard an alkane-bonded silica column/mobile phase system, such as the ODS-silica/water system proposed by Baker and Ma[2], will be difficult to implement because of a lack of a guaranteed degree of coating or capping of the column. There will almost always be some residual polar interaction present.

The alternative is a reproducible two-phase liquid–liquid partition system, the distribution coefficients of which for the test compounds can be determined. These values can then serve as a standard against which the effective distribution coefficients of the column systems can be compared. Although a lot of work has been carried out using the octanol–water partition system because of its similarities to biological systems, octanol has considerable polarity and would interact with the polar test compounds. Test column systems could then have both greater and lesser interactions and interpretation of the results would be confused.

It would therefore be desirable to use an alkane–water reference partition system to represent an extreme non-polar–polar interaction system. In much the same way Rohrschneider and McReynolds in devising constants for GLC used squalane as their standard stationary phase, because it was the least polar stationary phase in common use.

Because the distributions coefficients for many of the test compounds are already available it is therefore proposed to use as a baseline standard the hexane–water partition system. In order to make comparisons easier to determine, rather than using distribution coefficients of the standard system and test columns, the theoretical retention index values for the test compounds will be calculated as if they had been measured on a hexane–water liquid–liquid partition column.

Since the capacity factor k' of a partition chromatography system is proportional to the distribution coefficient P (eqn. 1), the relationship eqn. 2 can be derived.

$$k' = P \frac{\text{(Volume of stationary phase)}}{\text{(Volume of mobile phase)}} \tag{1}$$

$$\log k' = \log P + \text{constant} \tag{2}$$

It has been shown for a homologous system that the capacity factor depends on the number of carbon atoms (eqn. 3)[7]. Thus for all compounds in the same system, k' must depend on their retention index (I) values (eqn. 4).

$$\log k' = a\, C_n + b \tag{3}$$

$$\log k' = a'\, I + b' \tag{4}$$

Combining eqns. 2 and 4 gives eqn. 5 and hence if two compounds are compared we obtain eqn. 6.

$$I = \alpha \log P + \text{constant} \tag{5}$$

$$\Delta I = \alpha\, \Delta \log P \tag{6}$$

The partition coefficients of homologous alkylarylketones in hexane–water (Table II) give a mean value for $\Delta \log P = 0.793$ and hence $\alpha = 126$. Although in octanol–water systems $\Delta \log P$ for different homologous series is approximately 0.5, the reported values for hexane–water partitions are very variable, e.g. n-alkanols 0.58, ω-arylalkanols ($Ph(CH_2)_nOH$) 0.43, alkylpyridines 0.66 and alkylamphetamines 0.91[6].

Using the experimental value for α and the partition coefficients of the test compounds (Table II) their theoretical retention indices in hexane–water can be calculated from eqn. 6 (Table III).

Although it might seem that compounds with the same carbon skeletons should have similar index values, on the assumption that hexane would show little or no polar interaction with the test compounds, different interactions will occur with the aqueous phase.

Retention constants of reversed-phase columns

Comparison of the hexane–water indices of the test compounds with their retention indices from Table I gives a set of constants which represent the relative interaction of the test compounds with the four columns in comparison with the interaction of the alkylarylketones (Table IV). Because of its long retention the index value for toluene on ODS-silica was not measured and the value for methanol–water (40:60) has been used. From the earlier study this is expected to be very similar[1].

As can be seen the different groups interact as expected, greater retentions being found for the more polar compounds, implying some residual polarity in the alkyl-bonded reversed-phase columns.

TABLE III

CALCULATION OF THEORETICAL INDEX VALUES FOR HEXANE–WATER SYSTEMS

Test compound	Log P	Δ Log P★	ΔI★★	I
Phenetole	3.05	1.90	239	1039
Toluene	2.87	1.72	217	1017
Methyl benzoate	2.19	1.C4	131	931
Nitrobenzene	1.47	0.32	40	840
Acetophenone	1.15	0.00	0	800
Benzylalcohol	−0.76	−1.91	−241	559
p-Cresol	−0.35	−1.50	−189	611

★ log P − log P (acetophenone).
★★ From $ΔI = αΔ \log P$: $α = 126$.

Each column gives a different set of constants characteristic of the retention properties of the column-eluent combination, variations between columns such as the change in relative interaction of toluene and methyl benzoate, very similar on ODS-Hypersil but markedly different on SAS-Hypersil, being clearly indicated.

The considerable differences observed for the phenyl-bonded phase have been examined further and will be discussed in more detail in a subsequent paper[8].

Using indices reported previously[1] the variations of ODS-Hypersil with solvent composition was calculated (Table V). There is a slow but steady change on moving to higher percentages of methanol presumably as a result of differences in the interactions with the mobile phase, a problem not faced with the GLC constants. Therefore comparisons between columns should always be made using the same eluent solvent in each case, ideally this should be water but for most columns this would be impractical as k' of the reference compounds would be too long.

TABLE IV

RETENTION CONSTANTS FOR TEST COMPOUNDS ON FOUR COLUMNS

Retention constant = retention index − retention index (hexane–water). Mobile phase methanol–water (30:70).

Test compound	Column			
	ODS-Hypersil	SAS-Hypersil	C_{22}-Magnusil	Spherisorb-phenyl
Phenetole	−87	−125	−144	−230
Toluene	−19★	−125	−150	−272
Methyl benzoate	−30	−36	−42	−83
Nitrobenzene	−25	−24	−47	−38
Acetophenone	0	0	0	0
Benzylalcohol	142	118	98	−16
p-Cresol	187	168	130	7
$Σ_6^1$ constant	168	−24	−155	−632
$Σ_6^1$ $\overline{\text{constant}}$	490	596	611	646

★ Mobile phase methanol–water (40:60).

TABLE V

VARIATION IN RETENTION CONSTANTS WITH COMPOSITION OF MOBILE PHASE FOR ODS-HYPERSIL

Based on retention index values in ref. 1.

| Test compound | Retention constants | | | | | |
| | Methanol (%) | | | | | |
	30	40	50	60	70	80
Phenetole	−87	−60	−63	−40	−17	14
Toluene	−	−19	−7	8	30	77
Methyl benzoate	−30	−15	−22	−22	−5	32
Nitrobenzene	−25	−6	3	5	29	63
Acetophenone	0	0	0	0	0	0
Benzylalcohol	142	152	161	168	176	−
p-Cresol	187	195	196	199	207	231
$\sum_6^1$ constant	−	247	268	318	420	−
$\sum_6^1$ $\overline{\text{constant}}$	−	447	452	442	462	−

In order to be able to see if a simplification of the set of constants could be used as a rough guide to the overall retention ability of the column a number of ways were examined to relate the retention constants. The span of constants between phenetole and p-cresol varied from 274 to 224 on ODS-Hypersil but on the other three columns was 293, 274 and 237, and thus was little help. The summation of the constants, $\sum_6^1$ constant, was dependent on solvent (Table V) but varied markedly between stationary phases (Table IV) and therefore is a guide at constant solvent composition.

The one value, which was relatively intensitive to solvent composition was the summation of the absolute values of the constants, $\sum_6^1$ $\overline{\text{constant}}$, but the reason for this is not clear, although the other columns gave different values and it could be used as a guide.

It should be emphasised that these values and constants are relative retention values, the most retentive column (highest k' values) being the ODS-Hypersil. Similarly the change in the constant of toluene between ODS-Hypersil and C_{22}-Magnusil is more probably due to a change in the index scale, caused by a change in the RCOPh interaction, rather than a large difference in the toluene interaction.

The variations in retention indices of the test compounds on different stationary phases, emphasise a problem in the use of HPLC to directly determine log $P_{\text{octanol–water}}$ for structure activity studies, unless the column is impregnated with octanol[9]. Similarly attempts to predict retention indices on ODS-silica from log $P_{\text{octanol–water}}$ values or Hansch π additivity constants will be difficult if polar groups are present. If the change is in an alkyl chain prediction is easier and Baker obtained a good correlation for calculated relative k' values for the alkyl barbiturates[10] and N-substituted 3-propananilidonortropane analogues[11]. However attempts to predict the retentions of propranolol analogues[10] and of narcotics and related compounds[11] gave only poor correlations, in the latter case discrepancies between observed and calculated values varying from +200 to −422 index units. Polar and in particular

ionisable groups caused the largest difficulties. Equivalent changes to different molecules such as the metabolic formation of glucuronides have been shown to cause similar relative changes in retention index[12], even though the additional group is very polar.

As is clear from the present study $\log P_{octanol-water}$ and $\log P_{hexane-water}$ can differ greatly and both can differ from the apparent $\log P_{ODS-silica/methanol-water}$.

CONCLUSIONS

Using the alkylarylketones as a reference scale the retentions of a set of test compounds can be compared with a theoretical hexane–water partition chromatograph to give a set of retention constants which are a guide to the retention properties of a column eluent combination. The retention constants differ between columns and change with eluent composition.

ACKNOWLEDGEMENT

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THERMODYNAMICS OF SOLUTIONS OF POLYCYCLIC AROMATIC HYDROCARBONS STUDIED BY GAS–LIQUID CHROMATOGRAPHY WITH A NEMATIC AND AN ISOTROPIC STATIONARY PHASE

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SUMMARY

Thermodynamic solution parameters of the isomeric polycyclic aromatic hydrocarbons phenanthrene and anthracene at infinite dilution in the nematic liquid crystal N,N′-bis(*p*-methoxybenzylidene)-α,α′-bi-*p*-toluidine (BMBT) and in its isotropic analogue N,N′-bis(*p*-methoxybenzylidene)-4,4′-diaminodiphenylmethane (BMBDM) have been derived by gas–liquid chromatography. The unique selectivity exhibited by BMBT towards isomeric polycyclic aromatic hydrocarbons is explained in the light of a current solution theory in terms of differences in the partial molar enthalpies and entropies of solution. Differences in solution behaviour between BMBT and BMBDM are examined with infinite-dilution solute activity coefficient, partial molar excess enthalpy and entropy data.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in nature, being present in diverse environmental situations. Some of the most potent experimental carcinogens are members of this class of compounds[1]. Gas–liquid chromatography (GLC), particularly with open tubular columns coated with polymeric stationary phases such as SE-52, SE-30 and the Dexsil series, has been used, with various degrees of success, for the analysis of complex PAH mixtures[2,3]. However, the GLC separation of isomeric PAH mixtures has proved to be a difficult task. In particular, the separation of isomers such as phenanthrene/anthracene, benz[*a*]anthracene/triphenylene/chrysene and benzo[*a*]pyrene/benzo[*e*]pyrene/perylene/benzo[*k*]fluorenes were not satisfactory[4].

Nematic liquid crystals, on the other hand, show pronounced selectivities towards PAH isomers on the basis of differences in solute molecular shape. This unique solvent property of such phases is closely related to the long-range parallel alignment of their rod-like molecules, whereby rod-like solute molecules are preferentially accommodated while bulky solute molecules are discriminated against. Considerable interest has recently been shown in the use of liquid crystal stationary phases for practical analytical separations of PAH isomers and other classes of compounds[5-7].

The theoretical interpretation of the separation mechanisms has not recieved as much attention since the prevailing "rule of thumb" that the more rod-like solute isomer should be retained longer in nematic phases does explain the order of retention in the majority of systems studied. It is to be emphasized that, while differences in solute shape are more or less the predominant factor in liquid crystal selectivity, other factors contributing to solubility (e.g., dipole–dipole, dipole-induced dipole and charge transfer interactions if present) also play a part in the retention mechanisms. For example, the observed order of retention of isomeric methylbenz[a]anthracenes on a nematic stationary phase cannot be unambiguously explained solely on the basis of solute length-to-breadth ratio differences[8]. Moreover, the behaviour of PAH solutions in nematic solvents is more complex than that of rod-like molecules[9], because PAHs are biaxial plate-like molecules with two, rather than one, principal long molecular axes. It is, therefore, evident that detailed solution thermodynamic studies are needed in order fully to characterize the mechanism of retention of PAH solutes in nematic stationary phases.

GLC is a particularly attractive method for the investigation of solution thermodynamics of non-mesomorphic solutes in liquid crystal solvents. The ease with which data are obtained and the high level of accuracy make it the preferred technique for this type of study. The method has been successfully applied to solutions in nematic[10–15], smectic[16,17] and cholesteric[17,18] stationary phases. The results were generally interpreted according to a solution model originally proposed[10] and subsequently developed[19] by Martire and co-workers. In most of the studies cited the solutes were mainly low-molecular-weight aliphatics and disubstituted benzenes.

To our knowledge, the thermodynamics of solution of polycyclic aromatic hydrocarbons have never been studied on non-polymeric stationary phases, mesomorphic or otherwise. In this paper, GLC derived thermodynamic data, infinite-dilution activity coefficients, γ_2^∞, molar enthalpies, ΔH_2^s, and entropies, ΔS_2^s, of solution, partial molar excess enthalpies, H_2^e, and entropies, S_2^e, are reported for phenanthrene and anthracene on the nematic liquid crystal N,N'-bis(p-methoxybenzylidene)-α,α'-bi-p-toluidine   (BMBT)   and   on   its   isotropic   analogue   N,N'-bis(p-methoxybenzylidene)-4,4'-diaminodiphenylmethane (BMBDM). The results of this study will be discussed in the light of Martire's solution model. Extension of this study to other PAH isomers was precluded because of the scarcity of needed solute vapour pressure and second virial coefficient data. It will be shown that the mechanisms of separation of plate-like molecules in nematic solvents are essentially similar to those proposed for rod-like molecules.

EXPERIMENTAL

Chemicals

The liquid crystal BMBT and its non-mesomorphic analogue BMBDM were synthesized as described in ref. 20. Both products were recrystallized twice from ethanol before use. The BMBT solid–nematic and nematic–isotropic phase transition temperatures were measured by differential scanning calorimetry at 181°C and 337°C, respectively. The solid–isotropic transition temperature for BMBDM occurs at 184°C. The phase transitions of BMBT coated on solid support were slightly lower than those of the bulk material.

The solutes studied were phenanthrene and anthracene. These were chosen as representatives of plate-like polycyclic aromatic hydrocarbons because of the availability of physical data (Table I) necessary for the derivation of thermodynamic quantities from GLC measurements. The solute vapour pressures, P_2^0, were calculated from the Antoine equation and the second virial coefficients, B_{22}, were computed from Kreglewski's equation (p. 47, ref. 21). The constants for these equations are available in ref. 21. Alternatively, B_{22} may be calculated from the equation of corresponding states of McGlashan and Potter[22], using available critical constants[21].

TABLE I

SOLUTE PHYSICAL PARAMETERS

Temperature (°C)	Phenanthrene			Anthracene		
	P_2^0 (mmHg)	$-B_{22}$* ($cm^3\ mol^{-1}$)		P_2^0 (mmHg)	$-B_{22}$* ($cm^3\ mol^{-1}$)	
190	15.86	5650	6738	15.50	5764	6628
200	22.61	5183	6232	22.03	5269	6131
210	31.67	4789	5777	30.76	4850	5685
220	43.60	4410	5368	42.23	4465	5283

* Values in the first column were calculated with the Kreglewski equation, those in the second with the McGlashan and Potter equation.

Preparation of columns

The packing material was prepared by coating BMBT and BMBDM on Chromosorb W HP (100–120 mesh) using the solvent slurry technique. A weighed amount of the liquid phase was dissolved or finely suspended in glass-distilled chloroform and transferred to a chloroform slurry of weighed solid support in a large evaporating dish. The excess of chloroform was initially removed in a rotatory evaporator and the slurry was dried in a drying oven at 60°C with gentle stirring. The dry packing material was resieved to the appropriate mesh specifications to ensure uniformity of particle size. The mass percent of liquid phase on the solid support was determined gravimetrically by ashing duplicate samples. Two columns (1.5 ft. × 0.25 in. copper tubing) were packed with each phase. The packed columns were conditioned at 230°C with a gentle flow of helium for about 3 h. Details of the column composition are given in Table II.

TABLE II

COMPOSITION OF COLUMNS

Column	Liquid phase	Mass of packing (g)	% liquid phase	Mass of liquid phase (g)
1	BMBDM	2.4659	11.96 ± 0.12	0.2949 ± 0.0029
2	BMBDM	2.5135	10.50 ± 0.11	0.2638 ± 0.0026
3	BMBT	2.7092	14.96 ± 0.15	0.4053 ± 0.0041
4	BMBT	2.4692	15.68 ± 0.16	0.3871 ± 0.0039

Apparatus and procedure

A Perkin-Elmer Sigma 1 gas chromatograph equipped with a dual-column, forced air oven, two flame ionization detectors, electronic carrier gas flow controllers and a Sigma 10 data station was employed. The column inlet pressure was measured with an auxiliary instrument pressure gauge accurate to $\pm$ 0.2 p.s.i., and column outlet pressure was read off a barometer. Oven temperatures were independently calibrated with a thermocouple. Temperature control at 200°C was accurate to $\pm$ 1.5°C. Retention data were directly recorded by the on-line data station. An average of three measurements was taken for each solute. The retention time of benzene (10 $\pm$ 2 sec at all temperatures) was used to correct for column dead-volume. At 220°C, the highest column temperature, this value was less than 2% of the retention time of phenanthrene. Minimum sample sizes were delivered as injections of generally less than 0.05 μl of solutions of solutes in benzene. All solute peaks were observed to be symmetrical and independent of sample size, indicating that infinte-dilution conditions have been satisfied. High liquid phase loadings on highly inert Chromosorb W HP were used in order to minimize any contribution to retention from adsorption of solute molecules on the solid support surface. Helium carrier gas flow-rates were read off the digital display, but were also checked at the column outlet with a calibrated soap-film meter.

RESULTS

Specific retention volumes, V_g^0, were obtained for both solutes at four well spaced temperatures in the nematic temperature region of BMBT, and at corresponding temperatures in isotropic BMBDM. The values reported in Table III represent the averages from two separate determinations on two columns each with different liquid phase loadings.

TABLE III

SPECIFIC RETENTION VOLUMES AND INFINITE-DILUTION ACTIVITY COEFFICIENTS

Solute	Liquid phase	$V_g^0 \pm 3\%$				$\gamma_2 \pm 3\%$			
		190°C	*200°C*	*210°C*	*220°C*	*190°C*	*200°C*	*210°C*	*220°C*
Phenanthrene	BMBT	719.0	532.0	397.0	300.6	3.33	3.17	3.03	2.91
Anthracene	BMBT	1013	736.0	539.5	401.7	2.43	2.35	2.31	2.25
Phenanthrene	BMBDM	2706	1923	1405	1045	0.917	0.905	0.885	0.866
Anthracene	BMBDM	2942	2075	1519	1118	0.863	0.861	0.845	0.836

V_g^0 is obtained from primary chromatographic data by the expression[15]

$$V_g^0 = t' \bar{F}_c^0 / g_1 \tag{1}$$

where t' is the corrected solute retention time, $\bar{F}_c^0$ is the volume flow-rate of the carrier gas adjusted to the mean column pressure and 0°C and g_1 is the mass of liquid phase in the column.

The random errors reported for V_g^0 data represent the maximum scatter of the two experimental values from the mean. Authors[10-18] often cite an average random error in V_g^0 data of 1 % or better. The higher uncertainty reported here is perhaps due to a larger than average error in g_1 and to poorer temperature control resulting from the use of the forced air oven at elevated temperatures. Examination of the results (Tables III–VI) clearly show that the important differences in the thermodynamic quantities are much larger than the error margin, allowing unqualified identification of the experimental trends.

The molar enthalpies, ΔH_2^s, and entropies, ΔS_2^s, of solution (Table IV) accompanying the infinte dilution transfer of solute (suscript 2) from its reference state (ideal gaseous mixture of solute plus carrier gas) to the real solution (both gaseous and liquid phases being at the same temperature and pressure) are obtained from the expression[23]

$$\ln V_g^0 = \frac{-\Delta H_2^s}{RT} + \frac{\Delta S_2^s}{R} + \ln\left(\frac{273.2\,R}{M_1}\right) \tag{2}$$

where M_1 is the molar mass of the liquid phase. The correlation coefficients of the linear regression of average $\ln V_g^0$ vs. reciprocal temperature were all in excess of 0.999.

TABLE IV

SOLUTE THERMODYNAMIC DATA

Solute	Liquid phase	$-\Delta H_2^s$ (kJ mol^{-1})	$-\Delta S_2^s$ (J K^{-1} mol^{-1})	H_2^c (kJ mol^{-1})	S_2^c (J K^{-1} mol^{-1})
Phenanthrene	BMBT	55.3 ± 1.7	78.1 ± 2.3	8.57	8.51
Anthracene	BMBT	58.7 ± 1.8	82.6 ± 2.5	4.60	2.59
Phenanthrene	BMBDM	60.2 ± 1.8	78.0 ± 2.3	3.64	8.56
Anthracene	BMBDM	61.1 ± 1.8	79.3 ± 2.4	2.18	5.92

Infinite dilution solute activity coefficients, γ_2^∞, were calculated from V_g^0 data via[15]

$$\ln \gamma_2^\infty = \ln\left(\frac{273.2\,R}{M_1 P_2^0 V_g^0}\right) - \frac{B_{22} P_2^0}{RT} \tag{3}$$

where all the terms are as defined earlier and the B_{22} values are those determined by Kreglewski's equation. The resulting γ_2^∞ values are listed in Table III. The virial coefficients may, alternatively, be calculated with the McGlashan and Potter equation[22], but since the total fugacity correction, $B_{22} P_2^0 / RT$, contributes less than 1.5 % to the corresponding γ_2^∞ values, it is immaterial which approximate B_{22} values are used. It is to be noted that the relative standard deviations in the slopes and intercepts of the $\ln V_g^0$ vs. $1/T$ plots are all less than ± 1 %. However, since the accuracy of the derived ΔH_2^s, ΔS_2^s and γ_2^∞ values primarily depends on the accuracy of the V_g^0 data, the uncertainties in these quantities are essentially not better than those reported for V_g^0.

The thermodynamic relationship between the activity coefficient at infinite dilution and the solution partial molar excess properties is given by

$$\ln \gamma_2^\infty = \frac{G_2^e}{RT} = \frac{H_2^e}{RT} - \frac{S_2^e}{R} \tag{4}$$

where G_2^e, H_2^e and S_2^e are, respectively, the infinite dilution partial molar excess Gibbs energy, enthalpy and entropy of solution. A linear regression analysis of $\ln \gamma_2^\infty$ vs. reciprocal temperature yields H_2^e from the slope and S_2^e from the intercept. Plots of $\ln \gamma_2^\infty$ vs. $1/T$ are shown in Fig. 1, and the resulting H_2^e and S_2^e values are listed in Table IV. All correlation coefficients were in excess of 0.996, but the relative standard deviations in H_2^e and S_2^e are essentially the same as those for ΔH_2^s and ΔS_2^s respectively.
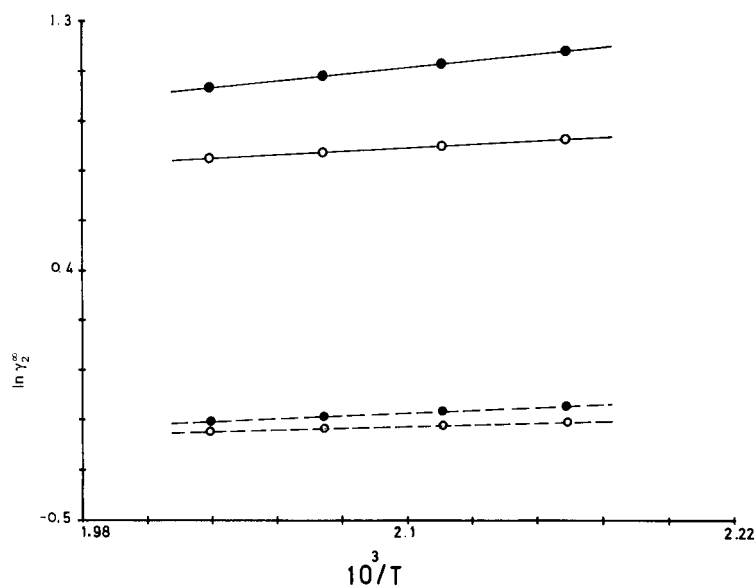
Fig. 1. Plots of $\ln \gamma_2^\infty$ vs. $10^3/T$ for phenanthrene ($\bullet$) and anthracene ($\bigcirc$) in BMBT (———) and BMBDM (– – –).

Furthermore, ΔH_2^s is related to H_2^e through the thermodynamic relation

$$\Delta H_2^s = H_2^e - \Delta H_2^v \tag{5}$$

where ΔH_2^v is the molar enthalpy of vaporization of the pure solute. ΔH_2^v values were calculated for both phenanthrene and anthracene using eqn. 5 with data from Table IV, and the results were compared with literature values (Table V). The excellent agreement reflects the quality of the data.

The mechanisms of retention of polycyclic aromatic hydrocarbons in nematic solvents could be studied via the temperature dependence of the relative retention, α,

TABLE V

COMPARISON OF GLC-DERIVED ENTHALPIES OF VAPORIZATION WITH LITERATURE VALUES

Solute	Liquid phase	ΔH_2^v (experimental) (kJ mol^{-1})	ΔH_2^v (literature)* (kJ mol^{-1})
Phenanthrene	BMBT	63.83	63.43
	BMBDM	63.83	
Anthracene	BMBT	63.27	63.01
	BMBDM	63.27	

* Calculated average value at the four experimental temperatures: 190, 200, 210 and 220°C, with data from ref. 21.

of isomeric solute pairs[15]. The relevent equation for the solute pair phenanthrene (P) and anthracene (A) is simply derived from the basic definition:

$$\alpha(A/P) = (V_g^0)_A/(V_g^0)_P \tag{6}$$

It follows from eqn. 2 that:

$$\ln \alpha (A/P) =$$

$$\frac{-\Delta H_A^s + \Delta H_P^s}{RT} + \frac{\Delta S_A^s - \Delta S_P^s}{R} = \frac{\Delta(\Delta H^s)_{P-A}}{RT} - \frac{\Delta(\Delta S^s)_{P-A}}{R} \tag{7}$$

where $\Delta(\Delta)_{P-A}$ indicates the value for phenanthrene minus that for anthracene. $\Delta(\Delta H^s)_{P-A}$ and $\Delta(\Delta S^s)_{P-A}$ values may be obtained directly from data in Table IV, or from the slopes and intercepts of plots of $\ln \alpha (A/P)$ vs. $1/T$. The two procedures yield identical results in nematic BMBT liquid phase where the $\alpha(A/P)$ values are large. In contrast, the correlation coefficient for the $\alpha(A/P)$ vs. $1/T$ plot in isotropic BMBDM is poor and, therefore, the results presented in Table VI are those calculated from data in Table III.

TABLE VI

RELATIVE RETENTION AND DIFFERENCES IN ENTHALPY AND ENTROPY OF SOLUTION BETWEEN ANTHRACENE AND PHENANTHRENE

Solute pair	Liquid phase	$\alpha(A/P)$				$\Delta(\Delta H^s)_{P-A}$ (kJ mol^{-1})	$\Delta(\Delta S^s)_{P-A}$ (J K^{-1} mol^{-1})
		190°C	200°C	210°C	220°C		
A–P	BMBT	1.408	1.383	1.359	1.336	3.40	4.50
A–P	BMBDM	1.087	1.079	1.081	1.070	0.90	1.30

DISCUSSION

First we consider the factors affecting the selectivity of nematic BMBT towards PAH isomers in the light of a theoretical model initially developed[10] and later modified[19] by Martire and co-workers. According to this model, the main sources of deviation from ideality for solutions of rigid isomers in a nematic solvent are rotational and energetic. Furthermore, these two contributions are mutually dependent as confirmed by a recent extension of the Maier-Saupe theory[24]. According to this theory, solute–nematic solvent interactions depend on the shape of the solute molecule, being stronger for a more rod-like solute which is better accommodated in the ordered nematic domain. As a consequence of this greater orientational order, the more rod-loke solute suffers a greater loss of translational and rotational motion than a bulkier isomer.

When comparing the solution behaviour of a pair of solutes in a common solvent the most appropriate thermodynamic quantities to consider are $\Delta(\Delta H_2^s)$ and $\Delta(\Delta S_2^s)$, as they are a direct measure of the differences in solute–solvent interactions (solute–solute interactions are absent in these terms, and solvent–solvent interactions cancel out with a common solvent). For solutions of phenanthrene and anthracene in BMBT, we find that $\Delta(\Delta H^s)_{P-A}$ represents the difference (phenanthrene minus anthracene) in the strengths of the solute–solvent interactions, while $\Delta(\Delta S^2)_{P-A}$ reflects the compatibility of the solute with the solvent domain.

The results presented in Table VI show that $\Delta(\Delta H^s)_{P-A}$ and $\Delta(\Delta S^s)_{P-A}$ values are all positive and significantly larger on BMBT compared to isotropic BMBDM. A positive $\Delta(\Delta H^s)_{P-A}$ results from a more negative ΔH^s for anthracene, indicating stronger solute–solvent interactions for this isomer. Since intermolecular interactions for both anthracene and phenanthrene with the same nematic solvent are expected to be of the same nature (i.e., same potential energy curve), it can safely be assumed that anthracene–solvent interactions operate at shorter average distances than those of phenanthrene. The positive $\Delta(\Delta S^s)_{P-A}$ values add further credence to this assumption since the greater orientational order imposed on anthracene results in greater loss of its translational and rotational entropy. The fact that anthracene is invariably retained on BMBT longer than phenanthrene indicates that the favourable enthalpic enhancement of solubility outweighs the unfavourable entropic factor. As the temperature is increased, the difference in α(A/P) values on BMBT compared to BMBDM diminishes and α(A/P) values decrease. In BMBDM, $\Delta(\Delta H^s)_{P-A}$ and $\Delta(\Delta S^s)_{P-A}$ are quite small and the α(A/P) values are close to unity. This clearly indicates that it is the orientational order in BMBT that is responsible for the solute shape-dependent selectivity. It is concluded that the mechanisms of separation of phenanthrene/anthracene, and of other PAH isomers, are essentially the same as those proposed for rigid rod-like molecules[15].

Finally, we collate the solution properties of nematic BMBT and isotropic BMBDM at the same experimental temperatures. It has repeatedly been demonstrated that liquid crystals are relatively poorer solvents in their nematic regions compared to their isotropic melts[10–15]. Typically, V_g^0 is smaller and γ_2^∞ is more positive at a given temperature in the nematic state than the corresponding values extrapolated to the same temperature from the isotropic state. However, this type of comparison is not feasible with liquid crystals having high nematic–isotropic transition temperatures.

BMBT columns, for example, exhibit excessive bleeding and liquid phase deterioration at high temperatures, rendering impossible the collection of relevant chromatographic data in the isotropic region at temperatures greater than 337°C. Furthermore, the temperature region of prime importance in practical GLC separations is that immediately above the solid–nematic transition temperature[5]. To circumvent this problem, non-mesomorphic BMBDM was chosen for this type of comparison. It is ideally suited for this purpose since it is analogous to BMBT in chemical structure except for one less methylene group in the central linkage.

Examination of the data in Tables III and IV reveals that for a given solute in BMBT solvent, $\gamma_2^\infty > 1$, $H_2^e > 0$ and $S_2^e > 0$. Positive S_2^e indicate higher solubility, but it is clear that this favourable entropic factor is overwhelmed by the unfavourable positive H_2^e contribution as $\gamma_2^\infty > 1$. This relatively large positive deviation from ideality is attributed to the incompatability of the non-mesomorphic solute in the ordered solvent domain. The bulkier the solute, the more incompatible it is in the nematic solvent. This explains the more positive γ_2^∞, H_2^e and S_2^e values for the phenanthrene isomer in BMBT. In contrast, both solutes exhibit negative deviations from ideality in isotropic BMBDM ($\gamma_2^\infty < 1$) as the excess entropic contribution slightly outweighs the excess enthalpic effect. Isotropic BMBDM is, therefore, a much better solvent for this class of compounds, but nematic BMBT is a far more selective liquid phase for the separation of PAH isomers.

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BINARY STATIONARY-PHASE COLUMNS FOR GAS CHROMATOGRAPHY OF BARBITURATES

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SUMMARY

A general procedure is presented which allows the prediction of gas–liquid chromatographic behaviour and the separation of complex barbiturate mixtures. Using E 301 and FFAP, binary stationary-phase columns were obtained that separated a complex mixture of barbiturates selected on the basis of the their frequency. The barbiturates were analysed in the free form using nitrogen saturated with formic acid vapour as the carrier gas at room temperature.

INTRODUCTION

Barbiturates have been determined by ultraviolet spectrophotometry at different pH values[1,2] but this method, although sensitive and specific, is limited because is does not allow differentiation between individual barbituric acids. As has been reported by several workers[3–8], gas chromatography offers many advantages, *viz.*, sensitivity, speed of analysis and the separation and determination of constituents of mixtures.

It has been noted that the column caracteristics for the gas chromatography of barbiturates are critical. A high liquid loading gives long analysis times, whereas with a low liquid loading high adsorption of barbiturates is observed, which tend to tail badly and highly inert support materials must be used for their analysis. Several workers have used phosphoric acid or organic acids of high molecular weight to reduce tailing[9,10]. This technique yields gas chromatographic columns that are sufficiently inert to barbiturates but of limited stability because of acid decomposition or elution.

Also, not all possible barbiturates can be separated on a single column, although several papers have been published on the use of stationary phases of varying polarity[6], or combinations of polar and non-polar stationary phases[5].

This paper describes the gas chromatography of barbiturates by column dynamic de-activation obtained using a carrier gas saturated with formic acid at room temperature. It also reports the separation, using binary stationary phases, of interesting pairs of barbiturates in a complex mixture that traditionally is separated on two columns of different polarity.

BINARY STATIONARY-PHASE COLUMNS

It has been shown by Purnell and co-workers[11-13] that the simple equation

$$K_R = \varphi_A K_{R(A)}^0 + \varphi_B K_{R(B)}^0 \qquad (1)$$

describes the gas–liquid chromatographic retention behaviour of solutes with a varied number of binary stationary phases, where K_R is the liquid–gas solute partition coefficient for a stationary phase composed of A of volume fraction φ_A and B of volume fraction φ_B, and $K_{R(A)}^0$ and $K_{R(B)}^0$ are the corresponding solute partition coefficients with pure stationary phases A and B. Eqn. 1 is very important in gas–liquid chromatography, as it can be used to predict the optimal composition of A and B that will separate any given mixture.

Eqn. 1 can be written in the form

$$K_R = K_{R(B)}^0 + [K_{R(A)}^0 - K_{R(B)}^0] \, \varphi_A \qquad (2)$$

If no blending occurs between stationary phases A and B, then K_R is a linear function of φ_A. Given that $K_R = V_g \varrho$, where V_g and ϱ are the specific retention volume and the stationary phase density, respectively, and

$$\varphi_A = \frac{m_A \varrho_B^0}{\varrho_A^0 + m_A \, (\varrho_B^0 - \varrho_A^0)}$$

where m_A, ϱ_A^0 and ϱ_B^0 are the weight fraction and the densities of pure A and B, respectively, then $\varphi_A \approx m_A$ if $\varrho_A^0 \approx \varrho_B^0 \approx \varrho$. This approximation broadly applies to high-molecular-weight polymeric silicone oils or gums and so the weight fraction, rather than the volume fraction, can be used. After substitution in eqn. 2, the following equation is obtained:

$$V_g = V_g^0 \, (B) + [V_g^0 \, (A) - V_g^0 \, (B)] \, m_A \qquad (3)$$

The value V_{g_i} of the ith mixture component for any m_A fraction can be obtained from eqn. 3 or, better, from a graph of V_g versus m_A knowing only $V_{g_i}^0$ for the pure stationary phases. Thus, if we require to separate a mixture, a stationary-phase pair A–B is selected such that using stationary phase B it is possible to separate those components not separated by A and vice versa, and the optimal fraction m_A is obtained from a window diagram. This diagram is obtained from graphs of $\alpha_{i/j}$ versus m_A ($\alpha_{i/j} = V_{g_i}/V_{g_j}$). The largest minimal α value for any m_A fraction is obtained. A window diagram can be approximated to an open polygonal of which the absolute maximum coincides with optimal m_A.

The minimal number of theoretical plates required for baseline separate at the best m_A value can be calculated from the resolution equation. For mixtures of unknown composition and complexity, the above-described procedure can be also computerized.

EXPERIMENTAL

Dynamic deactivation of the gas chromatographic system was obtained using a trap with formic acid placed in the gas stream immediately before the injector and used at room temperature. The carrier gas was not bubbled through the formic acid.

The barbiturates were obtained from pharmaceutical products. The formic acid used as tailing reducer was 98 % pure, containing 2 % of water.

The binary stationary-phase columns were prepared with mechanical mixtures of the appropriate amounts of support + selected stationary phase A (FFAP) and support + selected stationary phase B (E 301). Table I lists the barbiturates of the selected model mixture examined. The components were selected on the basis of their frequency.

RESULTS AND DISCUSSION

Fig. 1 shows the shape of the amobarbital peak obtained without (peak A) and with (peak B) formic acid in the carrier gas as tailing reducer. Formic acid greatly reduces the adsorption of barbiturates on silanized or unsilanized Chromosorb. Indeed, from measurements carried out with the barbiturates in the mixture there is a linear response from 10^{-6} to 10^{-9} g injected. Further, with carrier gas saturated with formic acid it is possible to analyse directly barbiturates as their alkaline salts in water or methanol solutions.

A large number of stationary phases were examined but without obtaining the complete separation of all of the compounds of the mixture. With stationary phases of low polarity such as Apiezon L or silicone gum, having methyl or phenyl groups, it is possible to separate allobarbital–butethal and amobarbital–pentobarbital but not
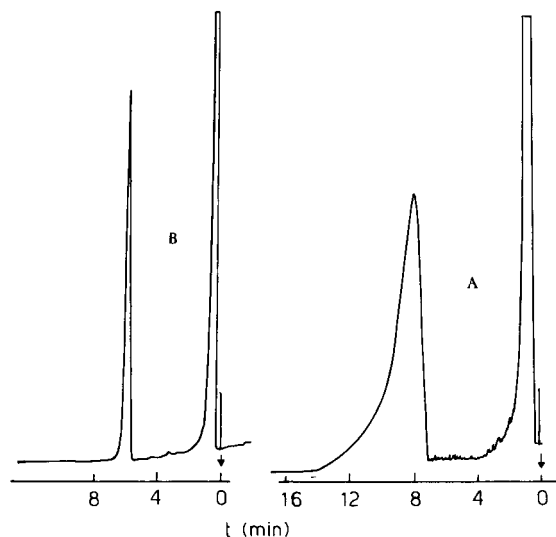
Fig. 1. Shape of amobarbital peak obtained (A) without and (B) with formic acid in the carrier gas. Column temperature, 200°C: carrier gas, nitrogen: detector, flame ionization.

TABLE 1

BARBITURATES EXAMINED
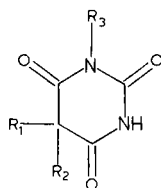
No.	Compound	R_1	R_2	R_3
1	Barbital	Ethyl	Ethyl	Hydrogen
2	Allobarbital	Allyl	Allyl	Hydrogen
3	Butethal	Ethyl	Butyl	Hydrogen
4	Amobarbital	Ethyl	3-Methylbutyl	Hydrogen
5	Pentobarbital	Ethyl	1-Methylbutyl	Hydrogen
6	Secobarbital	Allyl	1-Methylbutyl	Hydrogen
7	Hexobarbital	Methyl	1-Cyclohexenyl	Methyl
8	Phenobarbital	Ethyl	Phenyl	Hydrogen
9	Heptabarbital	Ethyl	1-Cycloheptenyl	Hydrogen
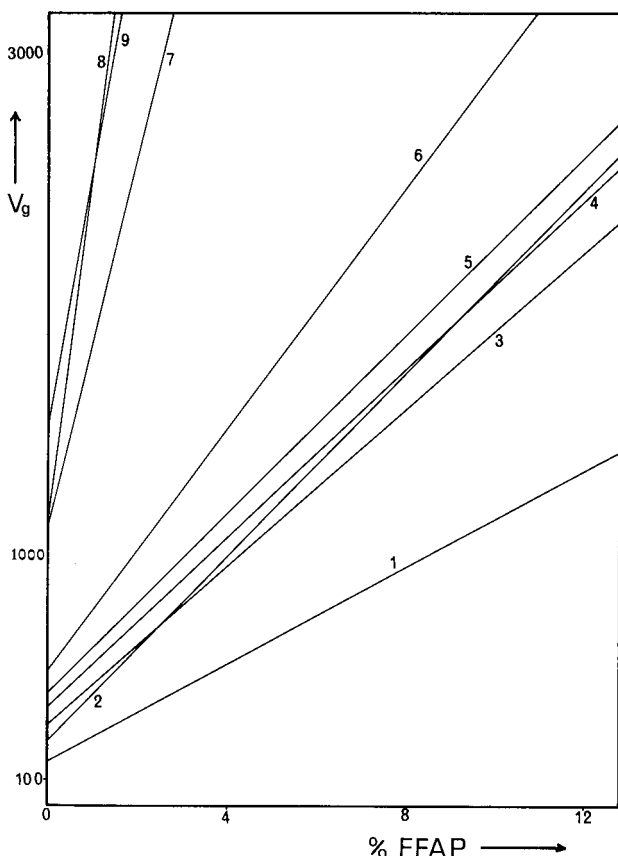
Fig. 2. V_g *versus* concentration of FFAP in E 301 for the barbiturates listed in Table I. Column temperature, 200°C; carrier gas, nitrogen saturated with formic acid. Apolar column: 3 % (w/w) E 301 on Chromosorb W (80–100 mesh). Polar column: 1 % (w/w) FFAP on the same support.

hexobarbital–phenobarbital. With polar stationary phases containing groups such as RCN, ROH, RCOOR′, ROR′ and RCOOH it is possible to separate the last pair but not the others. To separate all of the barbiturates in the mixture a binary stationary phase column was prepared containing E 301 (silicone grease) and FFAP (prepared from Carbowax 20M and a derivative of terephthalic acid) (Carlo Erba, Milan, Italy).
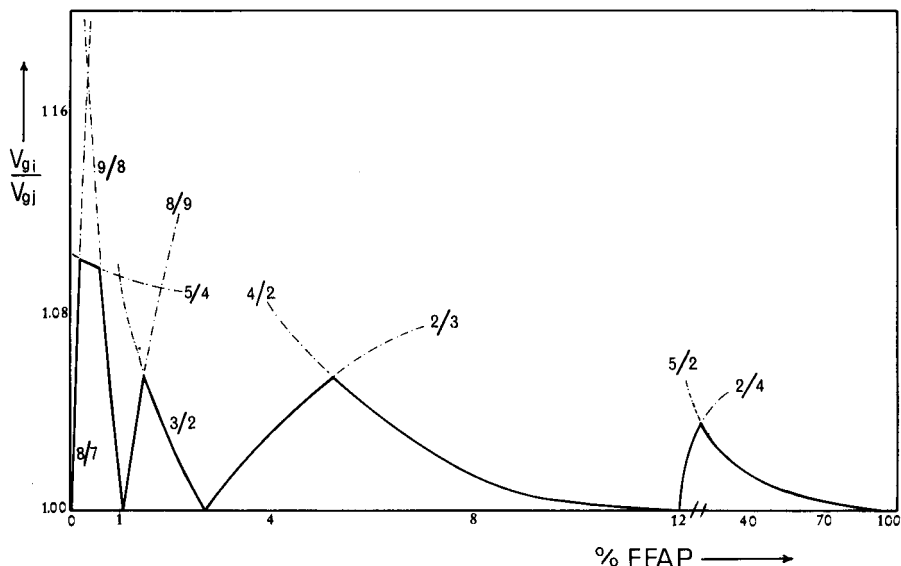
Fig. 3. Window diagram for the barbiturates listed in Table I. The best α value is predicted to lie at 0.2% FFAP in E 301.
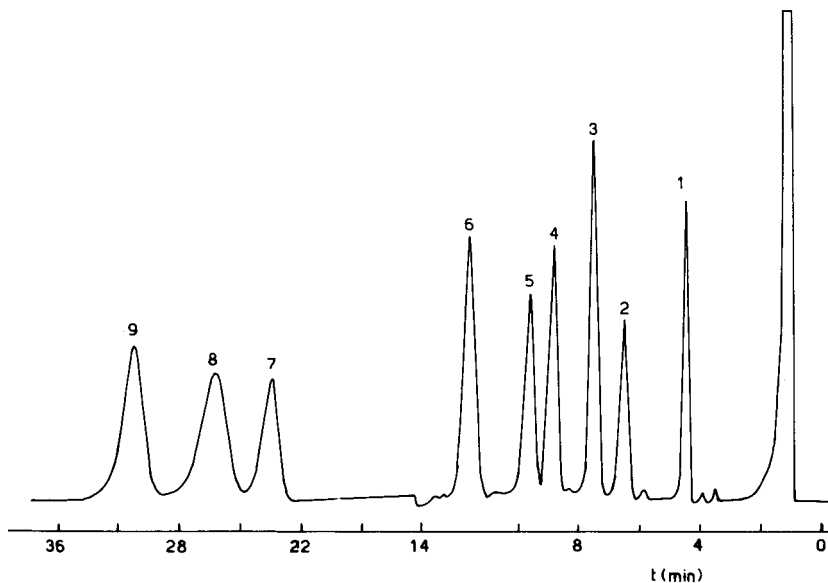
Fig. 4. Chromatogram of the barbiturates listed in Table I at $m_A = 0.2$% FFAP in E 301. Column, 3 m × 0.3 cm I.D.; packing, E 301 (3%, w/w) and FFAP (1%, w/w) on Chromosorb W (80–100 mesh), mechanically mixed; column temperature, 200°C; carrier gas, nitrogen saturated with formic acid vapour.

The apolar column was prepared from 3 % (w/w) E 301 on chromosorb W (80–100 mesh). The polar column was prepared from 1 % (w/w) FFAP with the same support.

Fig. 2 shows graphs of V_g versus FFAP concentration for each of the barbiturates in the mixture. Only the range of FFAP concentrations of interest, 0–12 %, is reported. The points where the lines cross each other corresponds to $\alpha_{i/j} = 1$.

Fig. 3 shows the window diagram for the selected mixture. The largest minimal α value is obtained for the pairs phenobarbital–hexobarbital and amobarbital–pentobarbital and corresponds to 0.2 % FFAP in E 301. With this α value, for a baseline separation of the reported barbiturates 4500 theoretical plates are required, which was obtained with a column 3 m long.

The gas chromatogram in Fig. 4 shows that a complete separation of all of the barbiturates in the mixture is achieved with such a column.

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SORPTION OF AMINO COMPOUNDS ON A NON-POLAR STATIONARY PHASE AND AT THE PHASE BOUNDARIES

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SUMMARY

The sorption of mono-, di- and trialkylamines, aniline and its homologues, cyclohexylamine and 5- and 6-membered nitrogen-containing heterocyclic compounds has been studied by gas chromatography using helium as the carrier gas, Apiezon M as the stationary phase and Celite 545 as the solid support. Dissolution in the liquid stationary phase is the main process responsible for the retention of amino compounds in the system studied. The contributions of interfacial adsorption account for 3–25 % (or almost 30 % in the case of polyfunctional alkylamines) of the total retention volume. For C_3–C_8 n- and isomonoalkylamines, polyfunctional alkylamines, C_4–C_6 dialkylamines, cyclohexylamine and nitrogeneous heterocycles, adsorption at the interfaces is related predominantly to adsorption on the surface of the solid support, whereas with higher monoalkylamines, C_8–C_{14} dialkylamines, trialkylamines and arylamines adsorption at the gas–liquid interface prevails.

INTRODUCTION

Adsorption of compounds in gas–liquid chromatography is a complex process involving (with complete coating of the support surface with the stationary phase) adsorption of sorbate molecules at the gas–liquid interface, dissolution in the bulk of the liquid stationary phase and adsorption at the liquid–solid interface.

The relative contributions of the above processes to retention volumes depend on the nature of the sorbate, the stationary phase and the support. The influence of interfacial adsorption on the separation of non-polar substances on non-polar stationary phases is negligible, whereas the retention of polar compounds on non-polar stationary phases is considerably affected by adsorption on the solid support[1–6]. However, there is much controversy concerning the role of the adsorption of polar compounds at the gas–non-polar liquid interface. It has been established[7,8] that alcohols adsorb insignificantly on the surface of hydrocarbon liquid phases whereas in other studies[9,10] a considerable contribution of this type of adsorption to the retention volumes was reported. According to Conder[11], this discrepancy may be due to variation in the adsorption conditions, *i.e.*, the contribution of adsorption of small

polar molecules at the gas–liquid interface is insignificant at certain final sorbate concentrations, but it tends to increase considerably upon infinite dilution.

The influence of interfacial sorption on the retention of amines has not been studied so far, except for the work of Crowne et al.[12], who measured adsorption coefficients for aniline and dimethylaniline at the interface between the gas and tritolyl phosphate containing 2,4,7-trinitrofluorenone. He took no account of adsorption of arylamine on the solid support. The present investigation was performed in order to evaluate the contributions of solution and adsorption at the gas (helium)–liquid (Apiezon M) and liquid–solid support (Celite 545) interfaces to the retention volumes of mono-, di- and trialkylamines, arylamines, cyclohexylamine and 5- and 6-membered nitrogen-containing heterocycles.

For the description of the total process we used the three-term equation proposed by Berezkin et al.[3]:

$$V_N^S = K_L V_L^S + K_{GL} S_L^S + K_L K_S S_S$$

where V_N is the total specific retention volume per amount of column packing that corresponds to gram of solid support, K_L, K_{GL} and K_S are coefficients of the partition of the sorbate in the bulk liquid phase and of adsorption at the gas–liquid and liquid–solid interfaces, respectively, V_L and S_L are the volume and surface area of the liquid phase per gram of support, respectively, and S_S is the specific surface area of the solid support.

EXPERIMENTAL

Apparatus and columns

The experiments were carried out at 150, 180 and 200°C on a Griffin 2B gas chromatograph equipped with a thermal conductivity detector (the accuracy of temperature regulation in thermostat oven was $\pm$ 0.1°C). Glass columns (3.3 m $\times$ 0.4 cm I.D.) with 7.5, 15 and 20 % of Apiezon M on Celite 545 (44–60 mesh) were used. The flow-rate of the carrier gas was 25 cm^3/min, the inlet pressure was 75,000–90,000 N/m^2 the outlet pressure was 22,500–35,000 N/m^2 and the sample volume 0.2–0.5 μl. The retention times were measured with a two-hand chronometer providing $\pm$ 0.1 sec accuracy. The specific area of sorbents was determined by the low-temperature nitrogen desorption method[13].

Chemicals and calculation methods

The gas chromatographic properties of the following classes of nitrogen-containing compounds were evaluated: C_2–C_{16} n- and iso-mono-, di- and trialkylamines, aniline and its N-alkyl and N,N-dialkyl derivatives, o-, m- and p-toluidines, cyclohexylamine and heterocyclics of the pyrrolidine, pyrrole, piperidine, pyridine, morpholine and 1,4-diazine series.

Specific retention volumes of amines per gram of solid support, V_N^S, were measured. Sorption coefficients and the relative contributions of solution and interfacial adsorption to V_N^S were calculated using the "reference point" method developed by Berezkin and co-workers[4,14].

RESULTS AND DISCUSSION

Partition coefficients of aliphatic amines in Apiezon M (K_L) and the appropriate adsorption coefficients at the gas–liquid (K_{GL}) and liquid–solid interfaces (K_S), as well as the relative contributions of individual types of adsorption to V_N^S determined at 180°C, are summarized in Table I. The corresponding values for cyclic amino compounds are given in Table II. The values of K_L, K_{GL} and K_S for alkyl- and arylamines and nitrogen-containing heterocycles measured at 150 and 200°C can be found in refs. 15–17. The same studies report the absolute values of the contributions to V_N^S of the individual sorption processes. All of the sorption coefficients tend to decrease with increasing column temperature.

Solution of amino compounds in the stationary phase is the predominant type of sorption. The relative contributions of this process to V_N^S is 70–97 %, the lowest

TABLE I

SORPTION COEFFICIENTS OF ALIPHATIC AMINES IN APIEZON M AND AT THE PHASE BOUNDARIES AND CONTRIBUTIONS OF THE PROCESS OF SOLUTION AND INTERFACIAL SORPTION TO THE RETENTION VOLUMES AT 180°C

Amine	K_L	K_{GL}	K_S	$\dfrac{K_L V_L^S}{V_N^S} \cdot 100$	$\dfrac{K_{GL} S_L^S}{V_N^S} \cdot 100$	$\dfrac{K_L K_S S_S}{V_N^S} \cdot 100$
Propylamine	2.97	$2.27 \cdot 10^{-7}$	$4.48 \cdot 10^{-6}$	81.13	0.12	18.75
Butylamine	5.38	$2.36 \cdot 10^{-6}$	$3.60 \cdot 10^{-6}$	83.72	0.70	15.58
Amylamine	9.97	$7.87 \cdot 10^{-6}$	$3.17 \cdot 10^{-6}$	84.84	1.27	13.89
Heptylamine	28.28	$4.06 \cdot 10^{-5}$	$1.94 \cdot 10^{-6}$	88.72	2.41	8.87
Octylamine	48.06	$6.40 \cdot 10^{-5}$	$1.84 \cdot 10^{-6}$	89.25	2.25	8.50
Isopropylamine	1.72	$1.84 \cdot 10^{-6}$	$4.30 \cdot 10^{-6}$	80.50	1.62	17.88
Isobutylamine	4.15	$1.92 \cdot 10^{-6}$	$3.45 \cdot 10^{-6}$	84.28	0.64	14.98
Isoamylamine	8.60	$1.92 \cdot 10^{-6}$	$1.51 \cdot 10^{-6}$	92.41	0.39	7.20
sec.-Butylamine	3.70	$4.46 \cdot 10^{-5}$	$2.63 \cdot 10^{-7}$	80.50	18.40	1.10
tert.-Butylamine	2.35	$1.81 \cdot 10^{-5}$	$6.67 \cdot 10^{-8}$	86.97	12.72	0.31
Diethylamine	4.01	$3.18 \cdot 10^{-7}$	$2.59 \cdot 10^{-6}$	88.10	0.13	11.77
Dipropylamine	11.34	$2.94 \cdot 10^{-5}$	$1.52 \cdot 10^{-6}$	88.68	4.36	6.98
Dibutylamine	32.07	$1.63 \cdot 10^{-4}$	$4.69 \cdot 10^{-7}$	89.25	8.59	2.16
Diamylamine	87.77	$4.56 \cdot 10^{-4}$	$7.14 \cdot 10^{-8}$	90.73	8.93	0.34
Dihexylamine	190.89	$2.20 \cdot 10^{-3}$	$4.60 \cdot 10^{-8}$	81.89	17.92	0.19
Diheptylamine	551.43	$6.62 \cdot 10^{-3}$	$7.27 \cdot 10^{-9}$	81.45	18.52	0.03
Diisopropylamine	6.06	$4.05 \cdot 10^{-5}$	$1.01 \cdot 10^{-7}$	88.36	11.18	0.46
Diisoamylamine	57.44	$3.43 \cdot 10^{-4}$	$2.66 \cdot 10^{-8}$	89.72	10.16	0.12
Triethylamine	7.41	$6.20 \cdot 10^{-5}$	$2.86 \cdot 10^{-8}$	86.20	13.67	0.13
Tripropylamine	27.19	$1.75 \cdot 10^{-4}$	$2.05 \cdot 10^{-8}$	89.02	10.88	0.10
Tributylamine	99.50	$4.70 \cdot 10^{-4}$	$1.69 \cdot 10^{-8}$	91.71	8.21	0.08
Triamylamine	365.79	$1.45 \cdot 10^{-3}$	$4.44 \cdot 10^{-9}$	93.00	7.00	0.01
Ethylenediamine	5.17	$4.79 \cdot 10^{-6}$	$7.85 \cdot 10^{-6}$	70.28	1.24	28.48
1,1,4,4-Tetramethylethylene diamine	13.06	$8.00 \cdot 10^{-5}$	$4.46 \cdot 10^{-7}$	87.79	10.19	2.02
1,1,4,4-Tetraethylethylene diamine	54.29	$3.56 \cdot 10^{-4}$	$4.93 \cdot 10^{-8}$	88.75	11.03	0.22
1,2-Propylenediamine	6.29	$3.06 \cdot 10^{-5}$	$6.51 \cdot 10^{-6}$	70.04	6.45	23.51
Diethylenetriamine	36.50	$1.80 \cdot 10^{-4}$	$3.46 \cdot 10^{-6}$	78.59	7.36	14.05

TABLE II

SORPTION COEFFICIENTS OF CYCLIC AMINES IN APIEZON M AND AT THE PHASE BOUNDARIES AND CONTRIBUTIONS OF THE PROCESS OF SOLUTION AND INTERFACIAL SORPTION TO THE RETENTION VOLUMES AT 180°C

Amine	K_L	K_{GL}	K_S	$\dfrac{K_L V_L^S}{V_N^S} \cdot 100$	$\dfrac{K_{GL} S_L^S}{V_N^S} \cdot 100$	$\dfrac{K_L K_S S_S}{V_N^S} \cdot 100$
Aniline	35.01	$2.69 \cdot 10^{-4}$	$6.45 \cdot 10^{-7}$	86.18	12.55	1.27
o-Toluidine	58.24	$5.26 \cdot 10^{-4}$	$3.10 \cdot 10^{-8}$	85.26	14.60	0.14
m-Toluidine	57.97	$5.35 \cdot 10^{-4}$	$3.02 \cdot 10^{-8}$	85.01	14.86	0.13
p-Toluidine	55.33	$4.93 \cdot 10^{-4}$	$3.60 \cdot 10^{-7}$	84.21	14.23	1.56
N-Methylaniline	57.95	$4.88 \cdot 10^{-4}$	$6.71 \cdot 10^{-7}$	85.36	13.62	1.02
N-Ethylaniline	78.10	$6.38 \cdot 10^{-4}$	$6.48 \cdot 10^{-7}$	85.80	13.28	0.92
N,N-Dimethylaniline	70.31	$6.11 \cdot 10^{-4}$	$5.93 \cdot 10^{-8}$	85.78	14.13	0.09
N,N-Diethylaniline	120.70	$1.06 \cdot 10^{-3}$	$1.06 \cdot 10^{-9}$	85.67	14.32	0.01
Cyclohexylamine	19.72	$1.00 \cdot 10^{-7}$	$4.75 \cdot 10^{-6}$	80.32	0.01	19.67
Pyrrolidine	8.49	$1.67 \cdot 10^{-6}$	$2.86 \cdot 10^{-6}$	86.88	0.32	12.80
Pyrrole	8.71	$2.66 \cdot 10^{-6}$	$2.71 \cdot 10^{-6}$	87.30	0.49	12.21
1-Methylpyrrole	9.98	$2.13 \cdot 10^{-6}$	$2.55 \cdot 10^{-6}$	88.04	0.35	11.60
2,5-Dimethylpyrrole	23.84	$4.99 \cdot 10^{-6}$	$2.57 \cdot 10^{-6}$	87.98	0.35	11.67
Piperidine	13.95	$2.64 \cdot 10^{-6}$	$2.26 \cdot 10^{-6}$	89.27	0.32	10.41
1-Methylpiperidine	14.99	$3.51 \cdot 10^{-6}$	$1.60 \cdot 10^{-6}$	92.02	0.41	7.57
1-Ethylpiperidine	23.05	$4.96 \cdot 10^{-6}$	$1.42 \cdot 10^{-6}$	92.83	0.38	6.79
Pyridine	11.66	$2.71 \cdot 10^{-6}$	$2.47 \cdot 10^{-6}$	88.34	0.38	11.27
2-Methylpyridine	17.72	$4.13 \cdot 10^{-6}$	$1.63 \cdot 10^{-6}$	91.86	0.41	7.73
3-Methylpyridine	22.84	$4.93 \cdot 10^{-6}$	$2.02 \cdot 10^{-6}$	90.20	0.37	9.41
4-Methylpyridine	23.07	$4.13 \cdot 10^{-6}$	$1.91 \cdot 10^{-6}$	90.77	0.31	8.92
2,3-Dimethylpyridine	36.10	$7.60 \cdot 10^{-6}$	$1.54 \cdot 10^{-6}$	92.28	0.37	7.35
2,4-Dimethylpyridine	33.37	$7.71 \cdot 10^{-6}$	$1.53 \cdot 10^{-6}$	92.33	0.40	7.27
2,5-Dimethylpyridine	33.31	$7.28 \cdot 10^{-6}$	$1.44 \cdot 10^{-6}$	92.74	0.39	6.87
2,6-Dimethylpyridine	24.93	$4.84 \cdot 10^{-6}$	$1.39 \cdot 10^{-6}$	92.99	0.34	6.67
2,4,6-Trimethylpyridine	47.20	$2.99 \cdot 10^{-5}$	$4.98 \cdot 10^{-7}$	96.37	1.16	2.47
Piperazine	19.54	$1.15 \cdot 10^{-7}$	$4.30 \cdot 10^{-6}$	81.85	0.01	18.14
1-Methylpiperazine	19.93	$2.68 \cdot 10^{-7}$	$3.64 \cdot 10^{-6}$	84.19	0.02	15.79
1-Ethylpiperazine	31.36	$5.90 \cdot 10^{-7}$	$3.51 \cdot 10^{-6}$	84.63	0.03	15.34
1,4-Diethylpiperazine	53.27	$2.49 \cdot 10^{-7}$	$1.99 \cdot 10^{-6}$	90.70	0.01	9.30
1-(2-Aminoethyl)-piperazine	79.59	$1.61 \cdot 10^{-5}$	$5.61 \cdot 10^{-6}$	77.65	0.30	22.05
Pyrazine	9.46	$3.80 \cdot 10^{-7}$	$3.37 \cdot 10^{-6}$	85.15	0.07	14.78
Methylpyrazine	16.39	$1.44 \cdot 10^{-6}$	$2.56 \cdot 10^{-6}$	88.37	0.15	11.48
Pyrimidine	9.96	$2.96 \cdot 10^{-6}$	$3.94 \cdot 10^{-6}$	82.96	0.38	16.58
Triethylenediamine	50.54	$6.31 \cdot 10^{-7}$	$2.78 \cdot 10^{-6}$	87.46	0.02	12.52
Morpholine	13.88	$3.01 \cdot 10^{-7}$	$2.81 \cdot 10^{-6}$	87.30	0.04	12.66

values (70–80 % V_N^S) being found for polyfunctional aliphatic amines, cyclohexyl-amine and 1-(2-aminoethyl)piperazine (at 150°C also for piperazine[17]); the highest values (90–97 %) are characteristic of C_9–C_{15} trialkylamines, alkylpyridines and al-kylpiperidines. With increase in column temperature the relative contributions of dissolution to V_N^S increase[15–17]. Comparing the values of the relative contributions of dissolution of aliphatic amines to V_N^S over a wide range of molecular masses and

plotting the values of these contributions *versus* the number of carbon atoms in amine molecules, a curve is obtained with a maximum that corresponds to amines containing hydrocarbon chains with 11–12 carbon atoms (Fig. 1). This phenomenon is related to better solubility as a result of increased dispersion forces of intermolecular attraction with increasing number of carbon atoms and to enhanced sorption at the gas–liquid interface due to the surface activity of higher alkylamines.
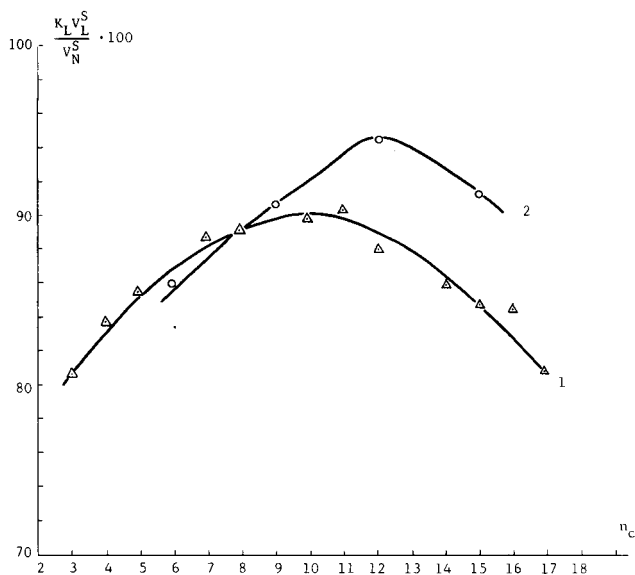
Fig. 1. Dependence of the relative contributions to V_N^S on the number of carbon atoms in the molecule for monoalkylamines (1) and trialkylamines (2).

To evaluate the effects of the physico-chemical properties and structures of amino compounds on the dissolution process the correlation analysis of K_L values was performed. The parameters used included the number of carbon atoms in the molecule, n_C, the number of tertiary carbons in the molecule, $n_{C(t)}$, the number of radicals at nitrogen atoms n_R, dipole moments, μ, and the sum of Palm's steric constants, $\sum E_S^o$. The following regression equations were obtained:

$$K_L = b_0 + b_1 n_C \tag{1}$$

$$K_L = b_0 + b_1 n_C + b_4 \mu + b_5 \sum E_S^o \tag{2}$$

$$K_L = b_0 + b_1 n_C + b_2 n_{C(t)} \tag{3}$$

$$K_L = b_0 + b_1 n_C + b_2 n_{C(t)} + b_3 n_R \tag{4}$$

$$K_L = b_0 + b_1 n_C + b_5 \sum E_S^o \tag{5}$$

$$K_L = b_0 + b_1 n_C + b_3 n_R + b_5 \sum E_S^o \tag{6}$$

TABLE III

COEFFICIENTS OF REGRESSION EQUATIONS DESCRIBING THE PROCESSES OF AMINO COM
POUNDS ADSORPTION

Correlated values	Classes of amines	Equation type	b_0	b_1	b_2
K_L	Alkylamines	1	-105.49	1.44	–
	Alkylamines	2	-107.88	1.53	–
	Alkylamines	3	-115.01	1.51	9.81
	Alkylamines	4	-108.72	1.91	3.37
	Arylamines	6	-271.69	47.77	–
	Pyridine bases	5	-105.48	20.67	–
	Other N-heterocycles	5	-15.49	9.37	–
K_{GL}	Alkylamines	3	$-6.56 \cdot 10^{-4}$	$1.50 \cdot 10^{-4}$	$8.24 \cdot 10^{-5}$
	Alkylamines	4	$-5.15 \cdot 10^{-4}$	$1.90 \cdot 10^{-4}$	$2.56 \cdot 10^{-4}$
	Arylamines	6	$-2.63 \cdot 10^{-3}$	$5.69 \cdot 10^{-4}$	–
	Pyridine bases	5	$-6.02 \cdot 10^{-4}$	$1.20 \cdot 10^{-4}$	–
	Other N-heterocycles	5	$-3.53 \cdot 10^{-6}$	$2.04 \cdot 10^{-6}$	–
K_S	Alkylamines	7	$8.88 \cdot 10^{-6}$	–	–
	Alkylamines	8	$-3.48 \cdot 10^{-5}$	–	–
	Alkylamines	9	$2.63 \cdot 10^{-5}$	–	–
	Alkylamines	10	$-1.40 \cdot 10^{-5}$	–	–
	Arylamines	10*	$-4.43 \cdot 10^{-6}$	–	–
	Pyridine bases	7	$2.24 \cdot 10^{-6}$	–	–

* The pK_b values were used instead of ionization potential to obtain factor b_6.

The coefficients found by the method of least squares and correlation coefficients, r, are given in Table III. As shown, alkylamines (only monoamines were tested) exhibit a satisfactory correlation between K_L and n_C values (eqn. 1). Introduction into the regression equation of terms including μ and $\sum E_S^0$ values fails to improve the correlation, and consequently these factors do not affect K_L values. On the other hand, introduction into eqn. 1 of terms characterizing the branching of hydrocarbon chains, *i.e.*, $n_{C(t)}$ and n_R (eqns. 3 and 4), significantly increases the r values. Thus, the results of correlation analysis indicate the predominance of dispersion interaction between the stationary phase and alkylamine molecules. The ln K_L values appear to be linearly correlated with n_C (Fig. 2). An increase in K_L values is also observed in the series of cyclic compounds in which the size of the molecule (number of alkyl substituents and chain length) is increased (see Table II). Unlike alkylamines, the K_L values of cyclic amino compounds are influenced by the environment of the nitrogen atoms. The K_L values found for heterocyclic bases are satisfactorily described by eqn. 5 containing steric constants in addition to n_C, whereas the K_L values of arylamines fit eqn. 6, containing n_R in addition to the above factors (Table III). The relationship between K_L and steric factors (the presence of alkyl groups in α-positions to the N atom in alkylpyridines) and the effects of replacement of hydrogen atoms at the nitrogen atoms with hydrocarbon radicals (in arylamines) suggest a specific intermolecular interaction upon solution.

b_3	b_4	b_5	b_6	b_7	b_8	r
—	—	—	—	—	—	0.880
—	2.92	8.26	—	—	—	0.881
—	—	—	—	—	—	0.885
-24.18	—	—	—	—	—	0.904
53.91	—	-76.40	—	—	—	0.995
—	—	33.53	—	—	—	0.978
—	—	-9.96	—	—	—	0.936
—	—	—	—	—	—	0.806
$-2.05 \cdot 10^{-4}$	—	—	—	—	—	0.858
$-2.00 \cdot 10^{-4}$	—	$6.72 \cdot 10^{-5}$	—	—	—	0.984
—	—	$8.35 \cdot 10^{-5}$	—	—	—	0.914
—	—	$-2.09 \cdot 10^{-6}$	—	—	—	0.660
—	$-1.48 \cdot 10^{-6}$	$4.69 \cdot 10^{-6}$	—	—	—	0.720
—	$-4.88 \cdot 10^{-7}$	$-1.87 \cdot 10^{-6}$	$4.57 \cdot 10^{-6}$	—	—	0.877
—	$-7.88 \cdot 10^{-6}$	$3.58 \cdot 10^{-6}$	$-1.95 \cdot 10^{-6}$	$4.17 \cdot 10^{-6}$	—	0.921
—	$3.61 \cdot 10^{-7}$	$-3.54 \cdot 10^{-6}$	$7.49 \cdot 10^{-7}$	$1.34 \cdot 10^{-6}$	$2.66 \cdot 10^{-5}$	0.977
—	$1.94 \cdot 10^{-6}$	$-2.06 \cdot 10^{-8}$	$-2.16 \cdot 10^{-8}$	$6.41 \cdot 10^{-8}$	$1.11 \cdot 10^{-5}$	0.852
—	$-3.47 \cdot 10^{-7}$	$2.80 \cdot 10^{-6}$	—	—	—	0.943

Adsorption at the gas–non-polar liquid interface relies largely on the nature of amino compounds. The K_{GL} values of alkylamines show a wide variation over the range 10^{-3}–10^{-7} (Table I). A sharp increase in K_{GL} values is observed in homologous series with increasing molecular size (e.g., elongation of the hydrocarbon chain in monoalkylamines from C_{10} to C_{18} leads to a 1000-fold increase in K_{GL}[18], whereas in dialkylamines the increase in K_{GL} at elongation of the hydrocarbon chain from C_4 to C_{14} is 10,000-fold). The K_{GL} values show a linear correlation with n_C[18] (Table III). Increased K_{GL} values are also obtained for branched aliphatic radicals (Table III). The contribution of this type of sorption to V_N^S ranges from 0.1 % (for propyl- and diethylamine) and 2.25 % (octylamine) to 18–26 % (for octadecylamine[18] and dihexylamine). The observed dependence of alkylamine adsorption on n_C can be explained by increased surface activity, as the combination of polar amino groups with long hydrocarbon chains in the molecules of higher alkylamines stimulates the accumulation of these molecules on the surface of the non-polar liquid.

According to adsorption at the gas–liquid interface, cyclic amino compounds can be arranged into two groups. The K_{GL} values for aromatic amines are of the order of 10^{-3}–10^{-4} (Table II), the relative contributions of the process to V_N^S amounting to 14–16 %. The high surface activity of arylamines is due to the aromatic nature of the ring. Cyclohexylamine is characterized by very low K_{GL} values and a negligible contribution of the process to V_N^S (Table II). The effect of the phenyl ring is equivalent to
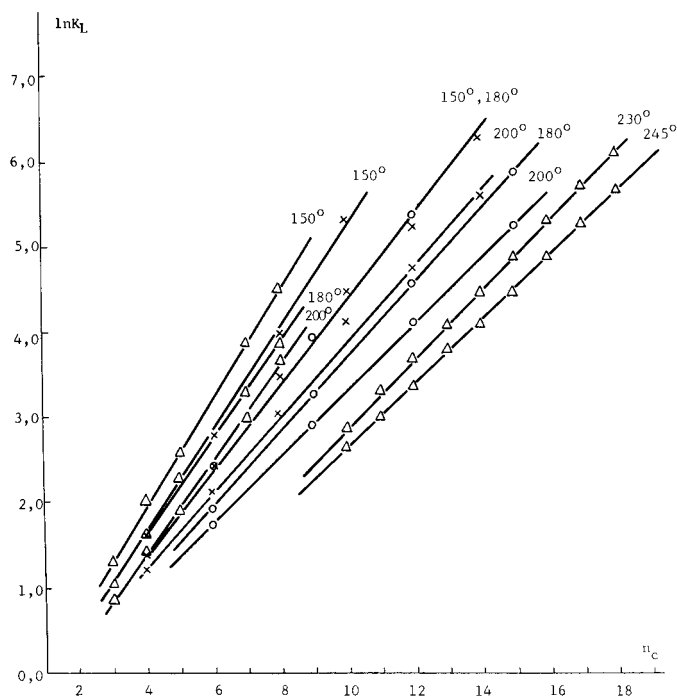
Fig. 2. Dependence of the logarithm of the partition coefficient on the number of carbon atoms in the molecule for *n*-alkylamines in Apiezon M. △, Monoalkylamines; X, dialkylamines; ○, trialkylamines.

elongation of the alkyl chain by 9–10 carbon atoms. This effect is less pronounced in the pyridine series, the contributions of their sorption on the Apiezon M surface being considerable only at $150°C$[17]. For other nitrogen-containing heterocyclic bases adsorption at the gas–non-polar liquid interface is not very typical. Small and compact heterocyclic molecule possess a very low surface activity. Their K_{GL} values are comparable to those of lower alkylamines, the minimum values being found with symmetrical 1,4-diazines. The K_{GL} values of heterocyclic bases show the same correlations with n_C, n_R and $\sum E_S^o$ as the K_L values (Table III).

Adsorption on the solid support is mainly characteristic of comparatively small molecules of alkylamines (10–20 % of V_N^S for C_3–C_{12} monoalkylamines and diethylamine), heterocyclic bases and cyclohexylamine (also 10–20 % of V_N^S). This process is manifested to the greatest extent with aliphatic diamines and 1,4-diazines (15–30 % of V_N^S). The K_S values are comparable to K_{GL} except for 1,4-diazines, which have K_S values 1–2 orders of magnitude higher than K_{GL} (Table II). Adsorption on the solid support is weakened under the influence of factors that reduce the base properties of amines or shield the unshared electron pair on the nitrogen atom (delocalization of the unshared electron pair, steric hindrance preventing the interactions via the nitrogen atom, *e.g.*, in the case of replacement of hydrogen atoms with alkyl radicals at the nitrogen or α-carbon atom). This indicates that at adsorption on the liquid–solid interface dominates the specific interaction between amines and the active sites of the surface, involving the unshared electron pair on the nitrogen atom (the mobile hydrogen atoms at the nitrogen are also implicated to some extent). This suggestion is

confirmed by the results of correlation analysis demonstrating a relationship between K_S values and dipole moments, the number of hydrogen atoms on the nitrogen atom, n_H, ionization potentials, IP (for arylamines pK_b values were used instead of ionization potentials), and a steric constants (Table III, eqns. 7–10):

$$K_S = b_0 + b_4\mu + b_5 \sum E_S^o \tag{7}$$

$$K_S = b_0 + b_4\mu + b_5 \sum E_S^o + b_6 IP \tag{8}$$

$$K_S = b_0 + b_4\mu + b_5 \sum E_S^o + b_6 IP + b_7 n_H \tag{9}$$

$$K_S = b_0 + b_4\mu + b_5 \sum E_S^o + b_6 IP + b_7 n_H + b_8 1/n_C \tag{10}$$

The increase in molecular size in most instances leads to a decrease in K_S values (Tables I and II), the latter being correlated with the reciprocal of the number of carbon atoms. Introduction of this factor into eqn. 9 leads to an increase in the correlation coefficients from 0.921 to 0.977 for alkylamines (Table III) and from 0.581 to 0.852 for arylamines.

Consequently, interfacial adsorption appears to be an important feature in the course of gas chromatographic analysis of amines using a non-polar sorbent. For small alkylamine molecules, cyclohexylamine and nitrogen-containing heterocycles, adsorption occurs mainly on the solid support, whereas for arylamines and higher alkylamines it occurs at the gas–liquid interface. The high values of the correlation coefficients relating K_S and K_{GL} with physico-chemical parameters of the molecules allow the degree of interfacial adsorption to be predicted.

CONCLUSION

Retention of mono-, di- and trialkylamines with normal and branched carbon chains, aromatic amines and nitrogen-containing saturated and aromatic heterocyclic compounds by a non-polar stationary phase is predominantly determined by their dissolution, the contributions amounting to 80–97 % of V_N^S. A certain decrease in the contribution of solution to the retention process (70 % V_N^S) is observed with diamines containing a primary amino group at the α-carbon. These substances are characterized by a significantly higher contribution of adsorption on solid supports.

The contribution of adsorption on the support to amine retention tends to decrease with increase in molecular mass (as much as by two orders of magnitude). The contribution of adsorption at the gas–liquid interface shows a wide variation (0.12–18 %) in the alkylamine series and depends to a much greater extent on the structural properties of amines (normal or branched chain, amino group located on a primary, secondary or tertiary carbon atom, etc.) than the contribution of dissolution. On the other hand, this contribution for aromatic amines is much greater (13–15 %) than the contribution of adsorption on the solid support (0.01–1.6 %) and shows little variation with respect to the structural peculiarities of the molecule. The contribution of adsorption at the gas–liquid interface appears to be insignificant for all of the nitrogen heterocycles studied, varying from 0.01 to 0.49 % (except for 2,4,6-trimethylpyridine).

With increase in temperature the relative contribution of solution to retention increases, together with a simultaneous decrease in the contribution of adsorption on the solid support. Correlation analysis on the relationship between adsorption coefficients on the one hand and structural properties and physico-chemical parameters on the other suggest that in the course of solution of amines in the stationary phase the prevailing type of interaction between the stationary phase and amines is the dispersion interaction. However, when the hydrogen atom in the N–H group is replaced with an alkyl radical or when alkyl groups are situated close to the amino groups, a considerable influence of the specific intermolecular attraction forces on the retention occurs. A similar situation is found for the process of amino compound adsorption at the gas–liquid interface. For the adsorption of amines on the solid support, the specific interaction of the unshared electron pair on the nitrogen atom in amines with the active sites on the surface predominates.

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GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF ALDOSES AS O-METHOXIME, O-2-METHYL-2-PROPOXIME AND O-n-BUTOXIME PERTRIFLUOROACETYL DERIVATIVES ON OV-225 WITH METHYLPROPANE AS IONIZATION AGENT

I. PENTOSES

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SUMMARY   .

Pentoses have been identified by gas chromatography–mass spectrometry using chemical ionization by methylpropane of the O-methoxime, O-2-methyl-2-propoxime and O-n-butoxime pertrifluoroacetyl derivatives, separated on a glass capillary column wall coated with OV-225. Each pentose gave two peaks, the *syn* (Z) and the *anti* (E) alkoxime. The *syn* derivatives of O-2-methyl-2-propoximes give shorter retention times than the corresponding derivatives of O-methoximes. The fragmentation patterns are discussed. M + 1 gives the most intense peak.

INTRODUCTION

Several volatile and thermally stable carbohydrate derivatives have been proposed for gas chromatography and gas chromatography–mass spectrometry (GC–MS), *e.g.*, permethyl[1,2], trimethylsilyl[3–7], peracetyl[8,9], pertrifluoroacetyl[10–12] and isopropylidene[13,14]. Carbonyl groups can be left underivatized, reduced to a hydroxyl group or derivatized to an oxime. The last appears preferable since reduction entails a loss of information and underivatized carbohydrates can produce up to four peaks each. Oximes always gave two peaks, the *syn*(Z) and *anti*(E) isomers (with the exception of symmetrical dihydroxyacetone and 3-pentuloses).

Chemical ionization (CI) MS has been preferred since CI spectra are more readily interpreted, giving fewer fragment ions and more diagnostic hints for the elucidation of molecular structure. It avoids certain drawbacks of conventional electron impact MS as low intensity of high mass ions, absence of the molecular ion and multiplicity of fragmentation pathways.

EXPERIMENTAL

Apparatus

A quadrupole gas chromatograph–mass spectrometer MAT 44S (MAT,

Bremen, G.F.R.) with a 50-m capillary column, wall coated with OV-225 (WGA, Griesheim, G.F.R.), was used.

Materials

D-Arabinose, p.a. was obtained from Serva (Heidelberg, G.F.R.), D-ribose, purum from C. Roth (Karlsruhe, G.F.R.), D-lyxose and D-xylose, puriss. from Fluka (Buchs, Switzerland), ethyl acetate and sodium acetate, p.a. from E. Merck (Darmstadt, G.F.R.), O-methylhydroxylamine hydrochloride from Merck-Schuchardt (Hohenbrunn, G.F.R.), O-2-methyl-2-propylhydroxylamine hydrochloride from Fluka, O-*n*-butylhydroxylamine hydrochloride from Applied Science Europe (Oud-Beijerland, The Netherlands) and trifluoroacetic anhydride (TFAA), *ca.* 99 % from Sigma (München, G.F.R.).

Derivatization

In a 1-ml vial, a solution of 3 mg O-methylhydroxylamine hydrochloride, 5 mg O-2-methyl-2-propylhydroxylamine hydrochloride or 5 mg O-*n*-butylhydroxylamine hydrochloride respectively and 6 mg sodium acetate in 0.1 ml water was added to about 1 mg of a pentose. The mixture was heated at 60°C for 1 h. Water was then evaporated in an air flow at 60°C, 0.1 ml methanol was added and then evaporated. This procedure affords a crystalline precipitate. The last traces of water were removed as an azeotrope by adding 0.1 ml benzene and again evaporating to dryness. The vial was closed immediately with a PTFE-coated septum and 0.03 ml TFAA and 0.015 ml ethyl acetate were injected. After 12 h in a refrigerator or after 2 h at room temperature, the derivatives were ready for injection. The samples are stable at 0°C for several months.

Conditions for GC–MS

Temperature program: 120°C for 2 min, increased at 5°/min, to 180°C then held for 10 min. Carrier gas (helium) flow-rate: 1.5 ml/min. Splitting ratio: 1/10. Sample volume: 1 μl. Pressures: in CI box, 390 μbar; of the forepump, 37 μbar. Electron energy: 150 eV. Emission current: 0.7 mA. Voltage of the secondary electron multiplier: 1800 V. Mass spectrum scanned from *m/e* 200 to 800; scan duration 2 sec. Temperatures: injection port, 250°C; GC separator, 220°C; GC line of sight, 220°C; source, 220°C.

RESULTS AND DISCUSSION

Table I shows the retention times of the O-methoxime, O-2-methyl-2-propoxime and O-*n*-butoxime pertrifluoroacetyl derivatives of the pentoses. As expected, the methoximes show the lowest and the butoximes the highest retention times. It is expected that 2-methyl-2-propoximes always should have longer retention times than the corresponding methoximes. However, three of the four first (*Z*) isomer peaks of the methoximes emerge after the corresponding 2-methyl-2-propoxime analogue. This extreme effect of the bulkiness of a substituent on volatility (outweighing that of the three CH$_2$ groups) is interpreted as a result of the special selectivity of the OV-225 stationary phase towards shape differences of isomeric sugars.

The predominant ion at 10^{-3} bar of methylpropane is C$_4$H$_9^+$ (M = 57)[15]. For

TABLE I

RETENTION TIMES OF O-ALKOXIME PERTRIFLUOROACETATES OF PENTOSES ON OV-225

Carbohydrate	Peak	t_R (min)		
		Methoxime	2-Methyl-2-propoxime	Butoxime
D-Ribose	1	10.88	11.15	13.63
	2	12.07	12.85	15.37
D-Arabinose	1	11.28	11.27	13.95
	2	13.10	13.83	16.82
D-Xylose	1	12.75	12.60	15.45
	2	13.92	14.18	17.33
D-Lyxose	1	12.50	12.35	15.12
	2	13.32	13.98	16.90

all the O-alkoxime pertrifluoroacetyl pentose derivatives, the ion $m/e = M + 1$ (M + 57 − 56) has the highest intensity (Tables II-IV). The O-methoxime and O-n-butoxime derivatives produce fragment ions with higher masses, resulting from the addition of $C_3H_7^+$ (M + 43) and $C_4H_9^+$ (M + 57). The O-2-methyl-2-propoxime derivatives also add to these voluminous substituents (Fig. 1), but the ions are unstable and always lose C_4H_8, giving the masses M + 1 (M + 57 − 56) and M − 13 (M + 43 − 56). Another feature of the O-2-methyl-2-propoxime derivatives is the appearance of the masses M + 1 − 56 and M − 56. The masses M − 113 (F_3C–COO·) and M − 114 (F_3C–COOH) are also observed.

Fig. 2 shows a chromatogram of all the pentoses as their O-n-butoxime pertrifluoroacetyl derivatives obtained by using selected ion monitoring ($m/e = 606 = M + 1$). Thus, especially in complex mixtures of natural products, selected ion monitoring represents an useful detection method for the diverse carbohydrate species.

TABLE II

MASS SPECTRAL DATA OF O-METHOXIME PERTRIFLUOROACETYL DERIVATIVES OF PENTOSES

m/e	Assignment	Relative intensity (%)							
		D-Ribose		D-Arabinose		D-Xylose		D-Lyxose	
		1	2	1	2	1	2	1	2
620	M + 57	5	1	5	1	5	2	5	1
606	M + 43	8	8	8	10	10	12	10	8
565	M + 1 (^{13}C)	16	16	16	17	17	16	16	17
564	M + 1	100	100	100	100	100	100	100	100
563	M	15	50	15	51	53	49	50	48
450	M + 1 − 114	2	16	5	22	3	31	5	22
338	M + 1 − 2 × 113	15	11	16	19	17	17	17	13
222	M + 1 − 3 × 113	1	3	2	2	2	5	2	3

TABLE III

MASS SPECTRAL DATA OF O-2-METHYL-2-PROPOXIME PERTRIFLUOROACETYL DERIVATIVES OF PENTOSES

m/e	Assignment	Relative intensity (%)							
		D-Ribose		D-Arabinose		D-Xylose		D-Lyxose	
		1	2	1	2	1	2	1	2
607	M + 1 (^{13}C)	20	20	19	20	20	20	19	19
606	M + 1	100	100	100	100	100	100	100	100
605	M	21	21	20	22	21	20	19	24
592	M + 43 − 56	11	7	10	5	8	5	7	5
551	M + 1 − 56 (^{13}C)	7	3	2	3	2	3	3	3
550	M + 1 − 56	52	20	15	22	15	23	14	21
549	M − 56	9	3	2	3	3	4	3	3
492	M + 1 − 114	9	15	5	24	8	25	7	22
436	M + 1 − 56 − 114	7	5	3	8	8	8	5	7
380	M + 1 − 2 × 113	16	18	10	27	15	28	16	29
324	M + 1 − 56 − 2 × 113	2	2	1	2	2	2	2	2
266	M + 1 − 2 × 113 − 114	2	2	1	2	2	2	1	2
210	M + 1 − 56 − 2 × 113 − 114	5	8	2	10	3	11	3	14

TABLE IV

MASS SPECTRAL DATA OF O-n-BUTOXIME PERTRIFLUOROACETYL DERIVATIVES OF PENTOSES

m/e	Assignment	Relative intensity (%)							
		D-Ribose		D-Arabinose		D-Xylose		D-Lyxose	
		1	2	1	2	1	2	1	2
662	M + 57	3	1	5	1	3	1	3	1
648	M + 43	3	3	6	3	5	3	4	3
607	M + 1 (^{13}C)	20	20	19	20	19	19	20	20
606	M + 1	100	100	100	100	100	100	100	100
605	M	9	10	13	13	9	9	8	8
492	M + 1 – 114	27	8	8	11	11	11	5	8
380	M + 1 – 2 × 113	16	15	18	13	16	12	23	20
322	M + 1 – 56 – 2 × 114	1	1	1	1	1	1	1	1
266	M + 1 – 2 × 113 – 114	2	2	2	2	2	2	2	2
210	M + 1 – 56 – 2 × 113 – 114	2	1	1	1	1	1	1	1

Fig. 1. Ions with masses greater than M.
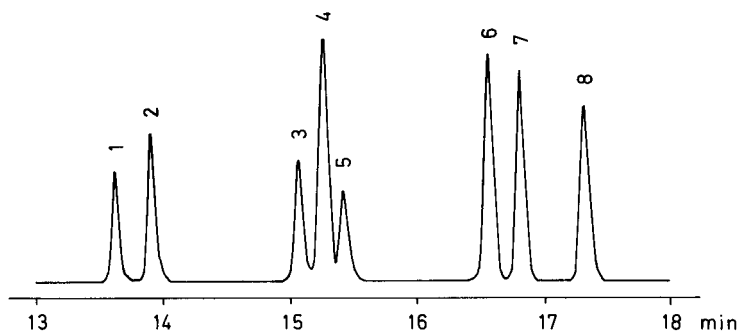
Fig. 2. Chromatogram of O-n-butoxime pertrifluoroacetates of pentoses using selected ion monitoring (m/e = 606). For this analysis, 5 μl of each of the four original derivative solutions were mixed by injecting through a septum into a vial containing 55 μl TFAA and 25 μl ethyl acetate; 1 μl of this mixture was used. Peaks: 1,4 = ribose; 2,6 = arabinose; 3,7 = lyxose; 5,8 = xylose.

ACKNOWLEDGEMENTS

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CHROM. 14,390

GAS CHROMATOGRAPHY–MASS SPECTROMETRY OF ALDOSES AS O-METHOXIME, O-2-METHYL-2-PROPOXIME AND O-n-BUTOXIME PERTRIFLUOROACETYL DERIVATIVES ON OV-225 WITH METHYLPROPANE AS IONIZATION AGENT

II. HEXOSES

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SUMMARY

All aldohexoses as their trifluoroacetylated O-methoximes, O-2-methyl-2-propoximes and O-n-butoximes have been separated by gas chromatography on a glass capillary column, wall coated with OV-225, and identified by chemical ionization mass spectrometry with methylpropane. The 2-methyl-2-propoximes have similar retention times to the corresponding methoximes, thus emerging much earlier than isomeric n-butoximes.

Fragmentation patterns of the *syn* and *anti* isomers are reported, affording always $m/e = M+1$ as the most intense ion peak, except for the *syn* isomer of the glucose n-alkoximes. Glucose gave a different pattern lacking in other hexoses and involving a loss of $F_3C-COO-CH_2$ (M = 127) and of F_3C-COO (M = 113). All spectra contain few masses and are easily interpreted.

INTRODUCTION

The analysis of carbohydrates by gas chromatography (GC) or high-performance liquid chromatography may difficult with samples containing many different carbohydrates along with other compounds. Mass spectrometric (MS) selected ion monitoring may add an additional dimension in such cases. Thus, carbohydrates with different molecular masses or fragmentation patterns can be detected even where they are not distinguishable from other compounds by conventional detectors.

Chemical ionization proves useful here because it affords clear-cut fragmentation patterns. In the preceding paper I reported the characterization of pentoses[1]. Here data are presented on the GC–MS properties of aldohexoses.

EXPERIMENTAL

Apparatus

A quadrupole gas chromatograph–mass spectrometer MAT 44S (MAT,

Bremen, G.F.R.) equipped with a 50-m glass capillary column, wall coated with OV-225 (WGA, Griesheim, G.F.R.), was used.

Materials

The hexoses D-allose, D-altrose, D-gulose and D-idose were products of Sigma (St. Louis, MO, U.S.A.), D-glucose, D-mannose and D-galactose of E. Merck (Darmstadt, G.F.R.) and D-talose of Serva (Heidelberg, G.F.R.). Ethyl acetate and sodium acetate p.a. were purchased from E. Merck. O-Methylhydroxylamine hydrochloride was obtained from Merck-Schuchardt (Hohenbrunn, G.F.R.), O-2-methyl-2-propylhydroxylamine hydrochloride from Fluka (Buchs, Switzerland) and O-*n*-butylhydroxylamine hydrochloride from Applied Science Europe (Oud-Beijerland, The Netherlands). Trifluoroacetic anhydride, *ca.* 99 %, was a product of Sigma.

Derivatization was performed as described previously[1].

Conditions for GC–MS were as described for pentose O-alkoxime pertrifluoroacetates[1].

RESULTS AND DISCUSSION

Table I shows the retention times, t_R, of the derivatized hexoses. As in the case of pentoses[1], hexose 2-methyl-2-propoximes have approximately the same t_R values as the corresponding methoximes. The first peak of each *syn–anti* isomer pair generally appears somewhat earlier, and the second peak somewhat later, than the corresponding methoxime peak (Fig. 1). The *n*-butoximes exhibit more normal t_R values, emerging about 3 min later than the methoxime and 2-methyl-2-propoxime derivatives. This effect is due to the shape selectivity of OV-225.

TABLE I

RETENTION TIMES OF THE O-ALKOXIME PERTRIFLUOROACETATES OF HEXOSES ON OV-225

Carbohydrate	Peak	t_R (min)		
		Methoxime	2-Methyl-2-propoxime	n-Butoxime
D-Allose	1	12.73	12.57	15.15
	2	14.08	14.48	17.38
D-Altrose	1	13.37	13.18	15.82
	2	14.68	15.13	18.33
D-Glucose	1	14.55	14.22	17.35
	2	15.62	15.78	19.55
D-Mannose	1	13.83	13.87	16.58
	2	14.95	15.45	18.63
D-Gulose	1	14.27	14.07	16.77
	2	15.67	15.70	19.50
D-Idose	1	14.85	14.75	17.73
	2	16.60	16.78	20.77
D-Galactose	1	14.30	14.10	17.32
	2	16.50	16.95	20.82
D-Talose	1	14.05	14.18	16.88
	2	15.18	15.88	19.17
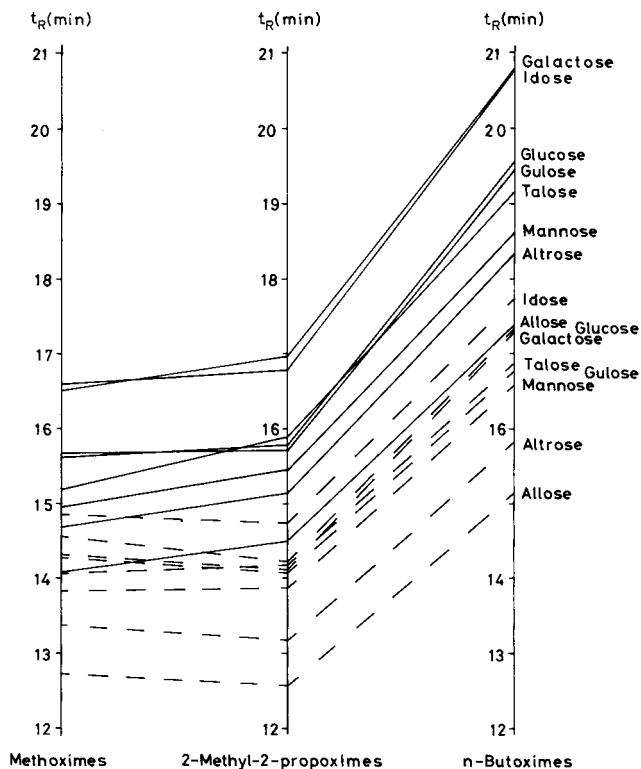
Fig. 1. Retention times of hexose O-*n*-butoxime pertrifluoroacetates: – – –, *syn* isomer; ————, *anti* isomer.

A chromatogram of all the hexoses as their O-*n*-butoxime pertrifluoroacetates obtained using selected ion monitoring ($m/e = 732 = M + 1$) is presented in Fig. 2. The mass spectra (Tables II–IV) show that $m/e = M + 1$ is always the most intense peak except for the first isomer of glucose, derivatized with O-methylhydroxylamine
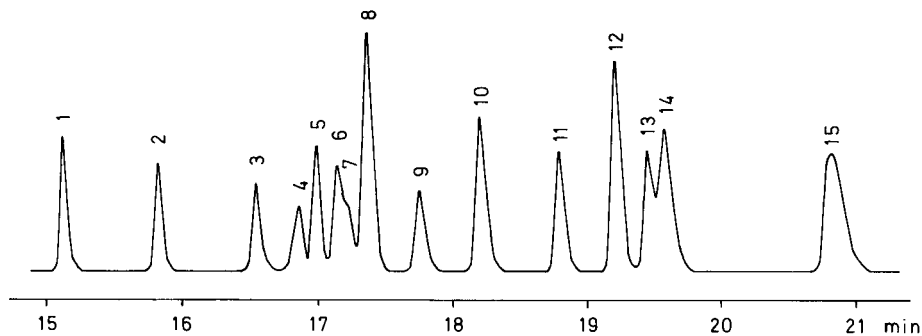
Fig. 2. Chromatogram of O-*n*-butoxime pertrifluoroacetyl derivatives of hexoses using selected ion monitoring ($m/e = 732$). For this analysis, 5 μl of each of the eight original derivative solutions were mixed by injecting through a septum into a vial containing 40 μl trifluoroacetic anhydride and 20 μl ethyl acetate; 1 μl of this mixture was injected. Peaks: 1, 8 = allose; 2, 10 = altrose; 3, 11 = mannose; 4, 13 = gulose; 5, 12 = talose; 6, 15 = galactose; 7, 14 = glucose; 9, 15 = idose. The components of peak 15 were overlapped.

TABLE II
MASS SPECTRAL DATA OF O-METHOXIME PERTRIFLUOROACETATES OF HEXOSES

m/e	Assignment	Relative intensity (%)															
		D-Allose		D-Altrose		D-Glucose		D-Mannose		D-Gulose		D-Idose		D-Galactose		D-Talose	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
746	M + 57	3	1	4	1	2	1	3	1	4	1	5	1	5	1	2	1
732	M + 43	11	7	9	9	5	7	10	8	22	13	15	11	10	12	6	6
691	M + 1 (^{13}C)	20	19	19	19	1	20	19	19	20	20	20	20	20	19	20	19
690	M + 1	100	100	100	100	7	100	100	100	100	100	100	100	100	100	100	100
689	M	52	49	48	52	3	47	50	49	60	55	48	48	59	46	58	53
576	M + 1 − 114	5	14	11	22	1	1	5	20	6	16	9	21	2	32	2	11
464	M + 1 − 2 × 113	14	9	18	17	7	16	14	12	20	14	15	15	16	21	5	11
462	M + 1 − 2 × 114									25							
451	M + 1 − 113 − 127 (^{13}C)					14											
450	M + 1 − 113 − 127	7				100											
449	M − 113 − 127					46											
350	M + 1 − 2 × 113 − 114	2	3	4	3	2	3	3	3	7	5	4	5	4	5		3
348	M + 1 − 3 × 114	1	3		6	1		2	2	16	4		5	2	5		
336	M − 2 × 113 − 127					16											
222	M − 2 × 113 − 114 − 127					54											

TABLE III

MASS SPECTRAL DATA OF O-n-BUTOXIME PERTRIFLUOROACETATES OF HEXOSES

m/e	Assignment	Relative intensity (%)															
		D-Allose		D-Altrose		D-Glucose		D-Mannose		D-Gulose		D-Idose		D-Galactose		D-Talose	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
788	M + 57	3	1	3	1	1	1	3	1	3	1	2	1	3	1	2	1
774	M + 43	4	2	3	3	1	2	3	2	4	3	3	3	5	3	3	2
733	M + 1(^{13}C)	23	24	24	23	5	24	23	23	24	24	24	24	23	23	24	24
732	M + 1	100	100	100	100	20	100	100	100	100	100	100	100	100	100	100	100
731	M	13	14	12	12	20	10	14	13	16	14	15	18	16	13	8	8
618	M + 1 − 114	7	7	12	10	3	8	5	11	7	14	6	9	7	13	7	10
562	M + 1 − 57 − 113	1	1	1	1		1	1	1	1	1	2	1	1	1	1	1
548	M − 56 − 127					8											
506	M + 1 − 2 × 113	16	9	14	12	8	10	18	13	14	10	16	12	14	13	15	12
493	M + 1 − 113 − 127 (^{13}C)					20											
492	M + 1 − 113 − 127					100											
491	M − 113 − 127					8											
449	M + 1 − 57 − 2 × 113	1	1	2	1	1	1	1	1	1	1	1	1	1	1	2	2
392	M + 1 − 2 × 113 − 114	4	2	3	3	2	3	5	4	5	4	5	4	4	4	4	4
378	M − 2 × 113 − 127					5											

TABLE IV

MASS SPECTRAL DATA OF O-2-METHYL-2-PROPOXIME PERTRIFLUOROACETATES OF HEXOSES

m/e	Assignment	D-Allose		D-Altrose		D-Glucose		D-Mannose		D-Gulose		D-Idose		D-Galactose		D-Talose	
		Relative intensity (%)															
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
733	M + 1 (^{13}C)	24	25	24	25	24	25	24	24	25	25	25	24	25	24	25	25
732	M + 1	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
731	M	18	20	18	21	18	20	17	21	19	21	18	19	21	21	18	19
718	M + 43 − 56	10	5	8	7	7	7	5	7	11	8	9	6	10	6	9	5
677	M + 1 − 56 (^{13}C)	13	3	9	8	7	6	5	12	8	7	6	6	5	6	8	5
676	M + 1 − 56	64	18	50	40	33	33	29	53	39	36	30	34	24	34	42	26
675	M − 56	11	3	29	7	18	5	16	9	21	7	16	6	12	6	7	3
618	M + 1 − 114	13	18	14	40	7	32	6	29	9	31	9	24	6	29	9	19
562	M + 1 − 56 − 114	9	3	11	11	4	8	5	12	5	11	6	6	4	7	7	3
506	M + 1 − 2 × 113	20	11	29	39	15	36	16	48	23	38	18	29	12	34	14	16
450	M + 1 − 56 − 2 × 113	4	2	9	7	4	7	5	12	9	9	4	5	3	6	1	2
433	M + 1 − 73 − 2 × 113	5	5	4	8		8	3	10	2	5	4	6	1	6	5	5
392	M + 1 − 2 × 113 − 114			8	11	3	12	6	19	8	16	7	11	3	11	7	7
380	M + 1 − 2 × 113 − 126					7											
336	M + 1 − 56 − 2 × 113 − 114	8	5	8	18		20	5	5	3	15	7	15	1	16	9	9
324	M + 1 − 56 − 2 × 113 − 126					3											

hydrochloride or O-n-butylhydroxylamine hydrochloride. In these derivatives of glucose, C–C bond clearage occurs with loss of F_3C–COO (M = 113) and F_3C–COO–CH_2 (M = 127).

The O-methoxime and O-n-butoxime pertrifluoroacetates of hexoses can add to $C_4H_9^+$ (M = 57), the predominant ion of methylpropane at 10^{-3} bar, and to $C_3H_7^+$ (M = 43), cf., Fig. 3. However, since the corresponding addition products of the O-2-methyl-2-propoxime pertrifluoroacetates are very unstable, only m/e = M + 1 (M + 57 − 56; C_4H_8, M = 56) and m/e = M − 13 (M + 43 − 56) are observed. Besides these fragments, loss of trifluoroacetic acid (M = 114) and O–C_4H_9 (M = 73) occurs.
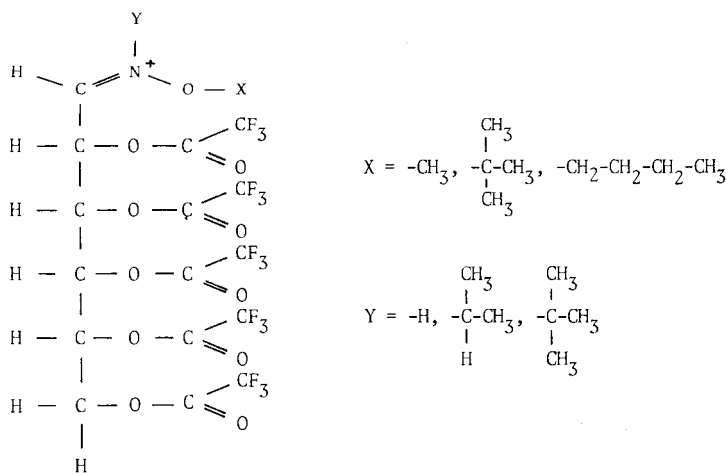
Fig. 3. Ions with masses greater than M.

The unique fragmentation pattern of the *syn* isomers of glucose n-alkoximes (cf., Tables II and III) is very significant in terms of the unique biochemical rôle of glucose. It may even allow specific detection of glucose in the presence of all the other hexoses at M + 1 − 113 − 127 since only D-allose affords minute amounts of this glucose-specific fragment.

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CHROM. 14,420

GAS–LIQUID CHROMATOGRAPHY ON OV-225 OF TETROSES AND ALDOPENTOSES AS THEIR O-METHOXIME AND O-n-BUTOXIME PERTRIFLUOROACETYL DERIVATIVES AND OF C_3–C_6 ALDITOL PERTRIFLUOROACETATES

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SUMMARY

The separation of tetroses and aldopentoses as the pertrifluoroacetates of acyclic O-methoximes or O-n-butoximes on silicon OV-225 using packed or capillary columns is reported. The trifluoroacetates are formed at lower temperatures than acetates, require lower separation temperatures and do not cause silicon deposits in the flame-ionization detector. OV-225 gives a relatively large response to structural differences of isomeric sugars. Besides the pentoses, OV-225 allows a fast separation of C_3–C_6 alditols as pertrifluoroacetates.

INTRODUCTION

Monosaccharides have been separated by gas chromatography (GC) as volatile permethyl[1,2], trimethylsilyl[3–5], peracetyl[6,7], isopropylidene[8] and pertrifluoroacetyl[9–12] derivatives. Since direct derivatization produces mixtures of α- and β-pyranose and furanose derivatives respectively, acyclic derivatives have been introduced. The simplest method, reduction to alditols, has been widely used but implies a loss of information, since a given alditol can originate from different ketoses and aldoses. Derivatization to oximes and alkoximes avoids this drawback. Each aldose gives two peaks, the *syn (Z)* and *anti (E)* isomers. Whereas this redundancy increases the risk of overlapping, it may also save information where one isomer peak is convoluted.

We propose a new combination, the separation of alkoxime pertrifluoroacetates on OV-225. This method combines the use of well defined derivatives, the redundancy introduced by *cis-trans* isomers, the ease of trifluoroacylation and the special selectivity of OV-225 towards differences in the shapes of isomeric carbohydrate derivatives. It proved useful in the separation of the complex monosaccharide mixture arising from the autocatalytic formaldehyde condensation[13,14]. Here we report the separation of all tetroses and aldopentoses and of C_3–C_6 alditol pertrifluoroacetates.

EXPERIMENTAL

Apparatus

For column gas chromatography a Becker-Packard gas chromatograph was equipped with on-column injection, FID and glass columns (2 and 4 m × 3 mm I.D.) filled with 1 % OV-225 on Chromosorb W HP (80–100 mesh).

For capillary gas chromatography, a Hewlett-Packard 5830 A gas chromatograph was equipped with a flame-ionization detector and a 50-m capillary column wall-coated with OV-225 (WGA, Griesheim, G.F.R.). The split liner was filled about 2 cm with 3 % OV-225 on Chromosorb W HP (80–100 mesh), fixed on both sides by quartz wool.

Materials

The carbohydrates glycolic aldehyde, D-erythrose, D-erythrulose and D-arabinose were obtained from Serva (Heidelberg, G.F.R.), D-ribose from C. Roth (Karlsruhe, G.F.R.), and D-lyxose and D-xylose from Fluka (Buchs, Switzerland). Threose was a gift of Dr. Morgenlie, Agricultural University, Department of Chemistry, N-1432 Åas-NLH, Norway. Ethyl acetate and sodium acetate *pro analysi* were obtained from E. Merck (Darmstadt, G.F.R.), O-methylhydroxylamine hydrochloride from Merck-Schuchardt (Hohenbrunn, G.F.R.), O-*n*-butylhydroxylamine hydrochloride from Applied Science Europe (Oud-Beijerland, The Netherlands) and trifluoroacetic anhydride, *ca.* 99 % from Sigma (München, G.F.R.). A mixture of erythrose, threose and erythrulose was obtained in 10 min by incubation of a solution of glycolic aldehyde at pH 11 at room temperature.

Derivatization

To about 1 mg of the carbohydrate in an 1-ml vial were added a solution of 3 mg O-methylhydroxylamine hydrochloride resp. 5 mg O-*n*-butylhydroxylamine hydrochloride and 6 mg sodium acetate in 0.1 ml water. The mixture was held at 60°C for 1 h. Water was then removed by evaporation in an air flow at 60°C. A 0.1-ml volume of methanol was added and evaporated as above, producing a crystalline precipitate. The last traces of water were removed as an azeotrope by adding 0.1 ml of benzene and again evaporating to dryness. The vial was closed immediately with a PTFE-coated septum. To the dry oximes (or 1 mg alditol) were added 0.03 ml trifluoroacetic anhydride (TFAA) and 0.015 ml ethyl acetate using a 50-μl syringe. After 12 h in a refrigerator or 2 h at room temperature, the derivatives were ready for injection.

Condensation of formaldehyde and reduction of carbohydrates

0.016 M Formaldehyde, $1.6 \cdot 10^{-3}$ M glycolic aldehyde and 0.036 M calcium acetate in 0.087 M NaOH were held for 1 min at 70°C. The reaction was started by adding the NaOH and stopped by neutralization. The carbohydrates were reduced by NaBH$_4$[9] and the alditols derivatized as above.

RESULTS AND DISCUSSION

The retention times of the derivatives of the tetroses and the aldopentoses,

TABLE I

RETENTION TIMES (t_R IN min) OF THE O-METHOXIME AND O-*n*-BUTOXIME PERTRIFLU-
OROACETATES OF TETROSES AND ALDOPENTOSES

50-m Capillary column; conditions as in Fig. 1.

Carbohydrate	O-Methoxime pertrifluoroacetates		O-n-Butoxime pertrifluoroacetates	
D-Erythrose	20,28	21.13	23.38	25.61
Threose	20.74	22.42	23.79	26.98
D-Erythrulose	21.84	22.66	25.34	26.88*
D-Ribose	22.83	24.22	26.48	28.82
D-Arabinose	23.23	25.48	26.85*	30.61
D-Lyxose	24.71	25.67	28.52	30.95
D-Xylose	25.03	26.41	29.01	31.67

* Overlapping of peaks.

separated by capillary gas chromatography, are presented in Table I. A capillary gas chromatogram of O-methoxime petrifluoroacetyl derivatives of aldopentoses is shown in Fig. 1; one of the O-*n*-butoxime pertrifluoroacetates is in Fig. 2. In both series the sequence of pentoses is ribose, arabinose, lyxose and xylose for both the *syn* and the *anti* isomers. The pentose derivative with all hydroxyl groups on one side in Fischer's projection, ribose, emerges first, that with alternating hydroxyl groups on the right and on the left side, xylose, appearing last.
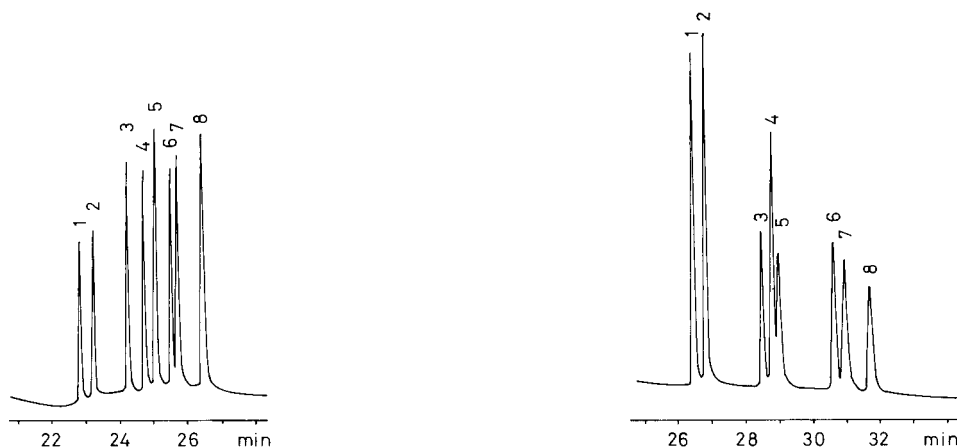
Fig. 1. Gas chromatogram of O-methoxime pertrifluoroacetyl derivatives of aldopentoses. Temperatures: column, 70°C for 2 min, then 70–180°C at 5°/min, finally 180°C; injection and detector. 250°C. Gas flow-rates: N_2 carrier gas, 1.5 ml/min; H_2, 20 ml/min; air, 200 ml/min. Sample volume; 1 µl. Splitting ratio: 1/15. For this analysis, 5 µl of each of the four original derivative solutions were mixed by injecting through a septum into a vial containing 55 µl TFAA and 25 µl ethyl acetate; 1 µl of this mixture was injected. Peaks: 1,3 = ribose; 2,6 = arabinose; 4,7 = lyxose; 5,8 = xylose.

Fig. 2. Gas chromatogram of O-*n*-butoxime pertrifluoroacetyl derivatives of aldopentoses. Preparation of derivatives and GC conditions as in Fig. 1. Peaks: 1,4 = ribose; 2,6 = arabinose; 3,7 = lyxose; 5,8 = xylose.
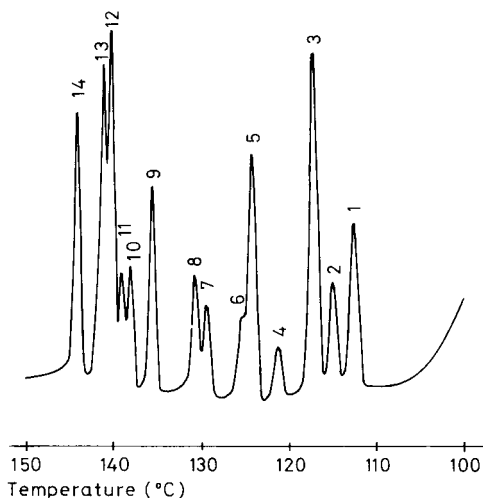
Fig. 3. Gas chromatographic separation of O-methoxime pertrifluoroacetates of tetroses and aldopentoses on a 2-m packed column. Temperature program: 2 min at 100°C, then increased at 10°/min. Peaks: 1,3 = erythrose; 2,5 = threose; 4,6 = erythrulose; 7,9 = ribose; 8,12 = arabinose; 10,13 = lyxose; 11,14 = xylose.

Owing to the redundancy of isomers, also in chromatograms obtained on 2-m and 4-m packed columns all pentoses can easily be identified using 5°/min or 10°/min temperature programs, in spite of partial overlapping (Fig. 3). 4-m columns are also sufficient for the separation of pertrifluoroacetates of alditols. All alditols from C_3 to C_6 are separated, with the exception of iditol and galactitol, which remain at least partially overlapped with the glucitol peak (Fig. 4).

We prefer sodium acetate and ethyl acetate[10] for oximation, thus avoiding a pyridine solvent peak which would interfere with the low-molecular-weight sugars.
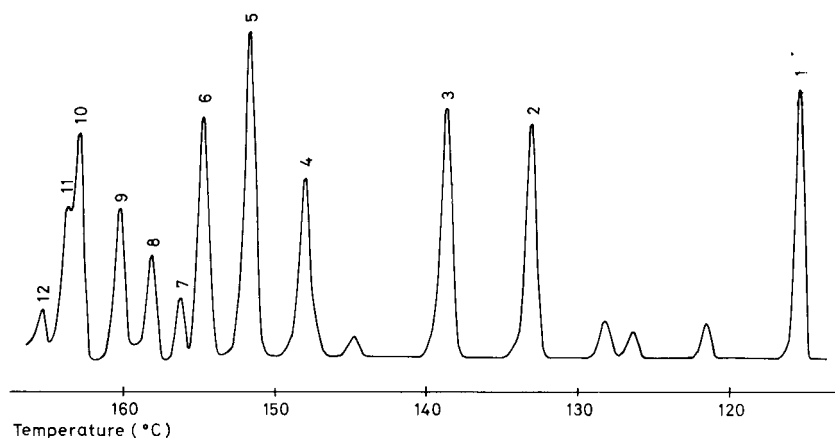
Fig. 4. Gas chromatogram of alditol pertrifluoroacetates from a formaldehyde condensation on a 4-m packed column. Temperature program: 3 min at 100°C, then increased at 5°/min to 180°C. Peaks: 1 = glycerol; 2 = erythritol; 3 = threitol; 4 = ribitol; 5 = arabinitol; 6 = xylitol; 7 = allitol; 8 = mannitol; 9 = altritol; 10 = glucitol; 11 = iditol; 12 = galactitol. Other peaks belong to unknown products.

This allows us to apply trifluoroacylation which occurs at room temperature and much faster than the reaction with acetic anhydride. Trifluoroacetyl (TFA) derivatives separate at much lower temperatures than acetates: pentose O-methoximes emerge at $< 145°C$ from a 2-m column and the C_3–C_6 alditol derivatives appear between 120 and 170°C, allowing one to perform consecutive runs (including recooling) each in about 15 min.

So far, OV-225 has been applied only casually in GC of sugars[15]. It exhibits an excellent selectivity towards the shape of isomeric sugar derivatives. Even on a 2-m column at 10°/min a distance of 5.5 peak half-widths (of 6 sec) between erythritol and threitol, and 13 half-widths between the slowest pentitol (xylitol) and the hexitol (galactitol) respectively are obtained. On the capillary column the region of pentose derivatives extends over as much as 50 half-widths.

In the case of O-*n*-butoximes we observe even an overlapping of subsequent isomer series (see Table I): the second peak of erythrulose has nearly the same retention time as the first peak of arabinose, followed by the second peak of threose.

There are interesting differences between the retention times of the O-methoxime and the corresponding O-*n*-butoxime derivatives, a field that deserves further exploration since in temperature-programmed GC, retention times are linear functions of the functional groups (and interactions thereof) of both the stationary phase and the solute[16,17]. Therefore a variation in substitution of the derivatizing agent theoretically should entail variations of retention times similar to those resulting from variations in the constitution of the stationary phase. Such a strategy would have the advantage of being much easier to apply than the use of different columns.

There are minor drawbacks in our method. TFA derivatives may decompose on metal surfaces and therefore require all-glass equipment, and the maximum temperature of OV-225 makes it unsuitable for the separation of oligosaccharides.

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CHROM. 14,439

EFFECT OF CONCENTRATION OF IMMOBILIZED INHIBITOR ON THE BIOSPECIFIC CHROMATOGRAPHY OF PEPSINS

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SUMMARY

A specific sorbent for porcine pepsin containing 0.85 μmol of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$ per gram of dry carrier (hydroxyalkyl methacrylate copolymer) sorbed 29.4 mg of pepsin per gram of dry sorbent, which means that 99% of immobilized inhibitor molecules participated in the specific complex formation with the isolated enzyme. With increasing amount of bound inhibitor this fraction decreased sharply (only 26% for 4.5 μmol). A specific sorbent with a content of 155 μmol/g appeared to be unsuitable for the affinity chromatography of pepsin (possibility of formation of multiple non-specific bonds between isolated enzyme and specific sorbent). The sorption of chicken and human pepsin was found to be lower thant that of porcine pepsin. The cause is seen in differences between the equilibrium constants of the individual enzyme–immobilized inhibitor complexes. The amount of sorbed chicken pepsin increased after reaction with *o*-nitrobenzenesulphenyl chloride. Using experimentally determined curves representing the dependence of the amount of sorbed enzyme on the content of immobilized inhibitor, it is possible to estimate the order of magnitude of the equilibrium constant of the respective specific complex.

INTRODUCTION

Owing to the complementarity of binding sites, the biospecific bond is characterized by much greater strength than that observed with non-specific bonding. If, however, the affinity sorbent contains the affinity ligand in a concentration which makes possible protein binding by means of a greater number of non-specific bonds, non-specific sorption of inert proteins may occur, or binding of a compound capable of biospecific interaction with the immobilized affinity ligand in an incorrect orientation may also take place[1]. Non-specific bonding may be caused by electrostatic or hydrophobic interactions, or by a combination of the two. Multiple non-specific bonds may then become stronger than a single complementary biospecific interaction between enzyme and inhibitor covalently bound to the carrier. Combined with the biospecific complementary bond, non-specific multiple bonds increase the bond strength in a specific complex. As a consequence, the same enzyme may be eluted in

several fractions[2], or there may be difficulties in the elution of the enzyme from the specific sorbent[3].

In order to restrict non-specific sorption and at the same time to guarantee the highest possible utilization of the immobilized affinant, a specific sorbent with a low concentration of the affinity ligand must be applied. At such a concentration, multiple non-specific bonds on the affinity sorbent cannot become operative, and molecules are preferentially bound biospecifically, by means of complementary binding sites. This, of course, is possible only in those instances where there is no steric hindrance to the formation of a biospecific complex. For most solid carriers, including hydroxylalkyl methacrylate gels, an uneven surface must be assumed. The unevenness of the surface after binding of low-molecular-weight inhibitors through a spacer is reflected in the different accessibility of the immobilized inhibitor[4]. Differences between readily accessible, less accessible and sterically hindered affinity ligands are the greater, the denser is the occupation of the solid carrier with the immobilized inhibitor[1]. These steric hindrances explain not only the low saturation of immobilized inhibitor molecules with the isolated enzyme[5,6], but also the heterogeneity in their affinity[7].

To provide experimental evidence for the effect of the density of immobilized inhibitor on the course of the affinity chromatography of proteolytic enzymes, we prepared specific sorbents for carboxylic proteinases[8,9] containing various amounts of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$ and determined the amounts of sorbed porcine, chicken and human pepsin depending on the concentration of immobilized inhibitor.

EXPERIMENTAL

Materials

Separon H1000 hydroxylalkyl methacrylate gel modified with epichlorohydrin (exclusion molecular weight 1,000,000, specific surface area *ca.* 30 m^2/g, particle size 100–200 μm, epoxide group content 800 μmol/g), Separon H300 modified with epichlorohydrin (exclusion molecular weight 300,000, specific surface area *ca.* 90 m^2/g, particle size 125–200 μm, epoxide group content 600 μmol/g) and the corresponding unmodified Separon H1000 and H300 were obtained by courtesy of Dr. J. Čoupek (Laboratory Instruments, Prague, Czechoslovakia). ε-Aminocaproyl-L-Phe-D-Phe-OCH$_3$ was synthesized and bound on Separon H1000-E by employing methods described earlier[10]. Porcine pepsin (proteolytic activity 14 units/min · mg) was produced by Léčiva (Pharmaceuticals) (Dolní Měcholupy, Czechoslovakia). Chicken pepsin (proteolytic activity 15.2 units/min · mg) was obtained by courtesy of Dr. V. Kostka and Dr. M. Baudyš (Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). Human gastric juice (proteolytic activity 1.5 units/min · ml) was provided by courtesy of Dr. L. Korbová and Dr. Z. Kučerová (Faculty of Medicine, Charles University, Prague, Czechoslovakia).

Methods

The amount of peptides bound to Separon H1000 and H300 and the proteolytic activity were determined by methods described earlier[10].

Preparation of affinity sorbents

(a) ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (250 mg; *cf.*,

ref. 10) was dissolved in the necessary amount of dimethylformamide, and triethyl-amine (76 μl) and Separon H1000 modified with epichlorohydrin (4 g, epoxide group content 800 μmol/g) were added. The mixture was shaken for 48 h, filtered, the sorbent was washed with dimethylformamide, water, 1 M hydrochloric acid and water until the acid reaction disappeared, and then with ethanol and diethyl ether. The product was further washed with 6 M guanidinium chloride solution and water, dried for analysis to constant weight at 105°C and transferred for affinity chromato-graphy into the respective buffer. At the original tripeptide concentrations in solution of 0.02, 0.04, 0.12 and 0.25 mol/l the dried product contained 0.85, 1.2, 2.5 and 4.5 μmol/g of affinity ligand, respectively.

(b) ε-Aminocaproyl-L-phenylalanyl-D-phenylalanine methyl ester (11.1 g) was dissolved in a small amount of methanol and the solution was made up to 75 ml with Britton–Robinson buffer (pH 11). This solution (30 ml) was shaken with 5 g of dry gel (Separon H300, containing 600 μmol/g of epoxide groups), the suspension was fil-tered and the gel was washed with water, 6 M guanidine hydrochloride solution and water again. Further treatment was as in (a). The content of bound inhibitor was 155 μmol/g of dry carrier.

Chromatography of porcine pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon columns with (A) a low and (B) a high concentration of immobilized inhibitor

A solution of pepsin (1 g per 200 ml) in 0.1 M acetate buffer (pH 4.5) was applied continuously to a column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon [content of immobilized inhibitor: (A) 0.85 μmol/g of dry carrier and (B) 155 μmol/g of dry carrier] equilibrated with 0.1 M sodium acetate (pH 4.5) until the eluate had the same activity as the applied pepsin solution. The column was washed with the equilibration buffer, and pepsin was desorbed with 0.1 M acetate buffer containing 1 M sodium chloride. The chromatographic runs are shown in Fig.1.

Chromatography of porcine, chicken, sulphenylated chicken and human pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon columns with the concentrations of immo-bilized inhibitor of 0.85, 1.2, 2.5 and 4.5 μmol/g, respectively

The chromatograms were run by employing the procedure described in the preceding section. With human pepsin, 200 ml of filtered gastric juice diluted 1:1 with the 0.1 M acetate buffer after adjustment of the pH to 4.5 was applied to the columns each time. The amount of desorbed pepsin was determined on the basis of absorbance at 278 nm and of the proteolytic activity of the combined active fractions. The amounts thus determined were in good agreement with the amounts of the individual pepsins isolated from the combined fractions after their dialysis and lyophilization.

Chromatography of porcine, chicken and human pepsin on unmodified Separon

A 50-mg amount of porcine pepsin or 20 mg of chicken pepsin dissolved in 20 ml of 0.1 M acetate buffer (pH 4.5) or 40 ml of diluted (1:1) gastric juice (pH 4.5) were applied to a column (9 × 0.8 cm) of unmodified Separon H1000; the chromatograms were run under the same conditions as described in the preceding section.
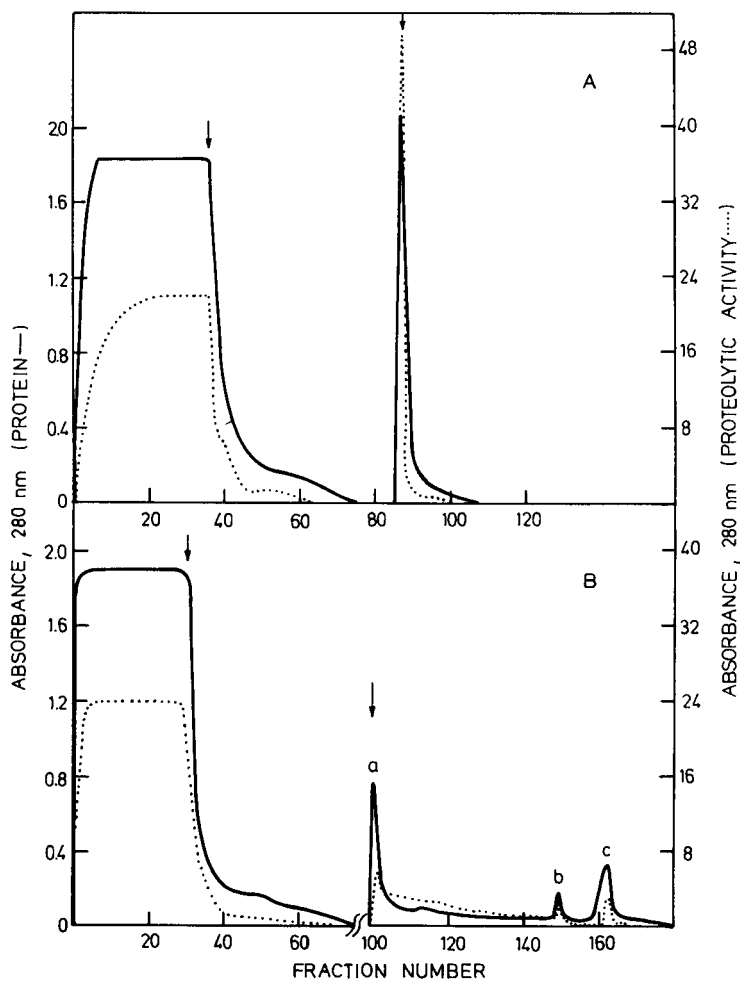
Fig. 1. Affinity chromatography of porcine pepsin on ε-aminocaproyl-L-Phe-D-Phe-OCH₃-Separon columns with (A) low and (B) high concentrations of the immobilized inhibitor. The solution of crude porcine pepsin was applied continuously (see text) on to the affinity columns (5 ml) equilibrated with 0.1 M sodium acetate (pH 4.5). At the position marked by the first arrow equilibrated buffer was applied to the columns to remove unbound pepsin and non-specifically adsorbed proteins. At the second arrow, 0.1 M sodium acetate containing 1 M sodium chloride (pH 4.5) was applied. Fractions (5 ml) were taken at 4-min intervals. The inhibitor concentration of affinity sorbents were (A) 0.85 and (B) 155 μmol/g of dry support). Solid line, protein; broken line, proteolytic activity. a, b and c, fractions of pepsin of the same specific proteolytic activity.

Chromatography of porcine pepsin in a mixture with serum albumin on ε-aminocaproyl-L-Phe-D-Phe-OCH₃-Separon

A mixed sample of 50 mg of porcine pepsin and 100 mg of human serum albumin dissolved in 20 ml of 0.1 M acetate buffer (pH 4.5) was applied to a column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH₃-Separon (content of immobilized inhibitor 4.5 μmol/g of dry sorbent), and the chromatogram was run under conditions of biospecific chromatography described in Fig. 1. A 50-mg amount of por-

cine pepsin was chromatographed under analogous conditions. The chromatograms of pepsin (A) in the absence and (B) in the presence of human serum albumin are shown in Fig. 2.
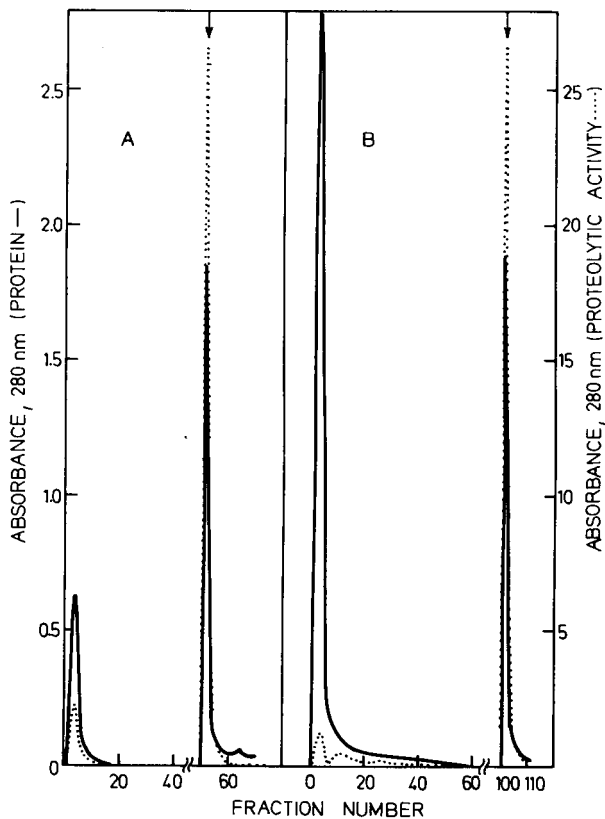
Fig. 2. Chromatography of porcine pepsin (A) in the absence and (B) in the presence of human serum albumin. 50 mg of porcine pepsin (A) in the presence of 100 mg of human serum albumin (B) dissolved in 20 ml of 0.1 M acetate buffer (pH 4.5) were applied to the column (9 × 0.8 cm) of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon (inhibitor content 4.5 μmol/g of dry carrier) equilibrated with 0.1 M sodium acetate (pH 4.5). After washing the column with the equilibration buffer, 0.1 M acetate buffer containing 1 M sodium chloride (pH 4.5) was applied at the position marked with an arrow. Fractions (6 ml) were taken at 5-min intervals. Solid line, protein; broken line, proteolytic activity.

Chicken pepsin was modified with o-nitrobenzenesulphenyl chloride as described by Becker et al.[11].

RESULTS AND DISCUSSION

In order to show the importance of the low concentration of the immobilized inhibitor (i.e., the amount of inhibitor bound per gram of dry solid carrier) in specific sorbents of carboxylic proteases, porcine pepsin was chromatographed on columns of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon containing a low (Fig. 1A) and a high (Fig. 1B) concentration of bound inhibitor. At a low concentration of inhibitor,

pepsin was eluted from the column by the increased ionic strength of the elution buffer in a single sharp peak (Fig. 1A). In contrast, on the column of affinity sorbent containing the immobilized inhibitor at a concentration of 155 μmol/g of dry carrier, several peaks of pepsin exhibiting the same specific proteolytic activity were eluted (cf., Fig. 1B). Such a different behaviour of the enzyme on affinity sorbents with low and high contents of immobilized inhibitor can be attributed to the formation of multiple non-specific bonds of molecules of the enzyme and inert proteins.

Under the same conditions as those in Fig. 1, chromatograms were run of porcine, chicken and human pepsins on columns of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon with concentrations of immobilized inhibitor of 0.85, 1.2, 2.5 and 4.5 μmol/g of dry sorbent, respectively. Under these conditions, the pepsin fractions were desorbed in a single sharp peak. Fig. 3A shows the dependence of the amount of eluted porcine, chicken and human pepsin on the concentration of immobilized ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$ in the individual affinity sorbents. Fig. 3B illustrates the proportion of molecules of immobilized inhibitor involved in specific bonding with pepsin, again as a function of the concentration of immobilized inhibitor. Comparison of the curves obtained for the individual pepsins reveals that ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon is a very good sorbent for porcine pepsin. A specific sorbent containing 0.85 μmol of inhibitor per gram of dry carrier sorbed 29.4 mg of porcine pepsin per gram of dry sorbent. Using the molecular weight of pepsin (35,000) and the amount of sorbed protein, it can be calculated that 99 % of immobilized inhibitor participated in the specific complex formation. With increasing content of bound inhibitor there was a sharp decrease in the portion of immobilized inhibitor molecules involved in the specific complex with pepsin. On the sorbent containing 4.5 μmol/g of inhibitor, only 26 % of the total amount of inhibitor molecules attached took part in the sorption of porcine pepsin.

Dunn and Chaiken[12] described the use of affinity chromatography for the determination of the equilibrium constants of the enzyme complex, both with the free (K_I) and with the immobilized inhibitor (K_L). K_L was calculated using the concentration of immobilized affinity ligand determined on the basis of the so-called "working capacity". When studying the affinity constants of trypsin and chymotrypsin with immobilized inhibitors, benzamidine and Z-Gly-D-Phe[13–15], we found that the concentration determined from the working capacity was much lower than that of the inhibitor determined from the amino acid analysis of hydrolyzates of sorbents. The results in Fig. 3 show that, e.g., a suitable sorbent for investigation of the specific interaction by means of affinity chromatography carried out with porcine pepsin would be a sorbent with the lowest concentration of bound inhibitor, because only with this sorbent is the concentration of immobilized inhibitor determined from the working capacity the same as the concentration determined by the amino acid analysis of the acid hydrolysate of the respective sorbent. Our earlier results[14,15] will be revised in this respect.

In principle, the molecular weights of chicken and human pepsin do not differ from that of porcine pepsin, which allows us to expect similar steric hindrances. From this standpoint, the lower sorption of these two pepsins may be assigned, in the first place, to the lower complementarity of immobilized inhibitor for binding sites of chicken and human pepsin. This is in good agreement with the already reported[11,16] specificity of porcine and chicken pepsin. Z-His-L-Phe-D-Phe-C$_2$H$_5$ is an efficient
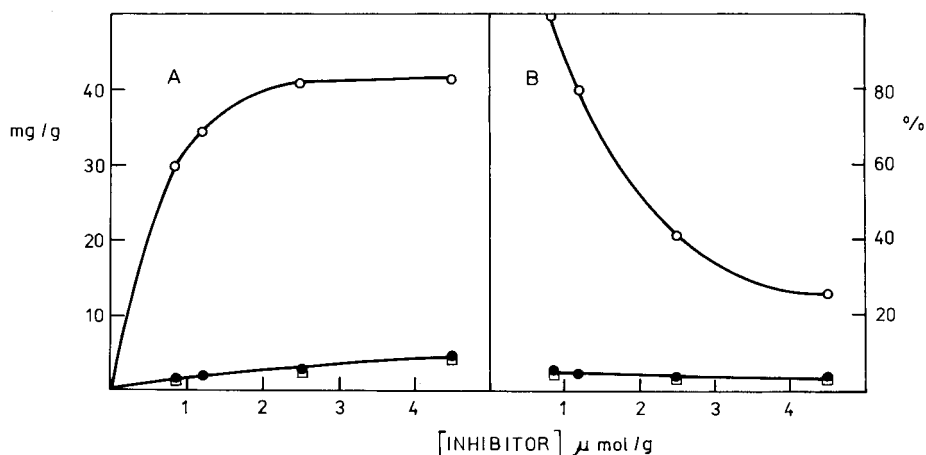
Fig. 3. (A) Capacity of immobilized inhibitor sorbent (ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon) in milligrams of pepsin per gram of dry sorbent and (B) proportion of immobilized inhibitor molecules involved in specific complex formation (%) with respect to immobilized inhibitor concentration (μmol of inhibitor per gram of dry sorbent). O, Porcine pepsin; ●, chicken pepsin; □, human pepsin.

inhibitor of porcine pepsin (K_1 = 0.27 mM), the same as Z-His-L-Phe-L-Phe-C$_2$H$_5$ (K_m = 0.18 mM) or Z-His-L-Phe-L-Phe-OCH$_3$ (K_m = 0.33 mM) are its good substrates[16]. Becker et al.[11] studied the specificity of chicken pepsin. The value of the ratio of the catalytic and Michaelis-Menten constants, k_{cat}/K_m, for the substrate Z-His-L-Phe-L-Phe-OC$_2$H$_5$ for native enzyme could not be determined, because it was too low (< 0.1 M^{-1} sec^{-1}). If, however, the authors[11] modified chicken pepsin with o-nitrobenzenesulphenyl chloride, k_{cat}/K_m for the same substrate increased to 40 M^{-1} sec^{-1}. The modification of chicken pepsin with o-nitrobenzenesulphenyl chloride changes the conformation of the active site and renders it more suitable for binding small peptidic substrates.

The chromatography of chicken pepsin modified with o-nitrobenzenesulphenyl chloride on ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon revealed a 4-fold increase in the amount of sorbed modified pepsin. Thus, according to the results obtained, the cause of differences in the amounts of various pepsins sorbed depending on the concentration of immobilized inhibitors can be sought in differences in the equilibrium constants of enzyme–immobilized inhibitor complexes. Fig. 4B shows such experimentally determined curves recalculated to the same concentrations as those used by Graves and Wu[17] in the theoretical derivation of analogous dependences (Fig. 4A). There is a good fit between Fig. 4A and B, which justifies the assumption that the shape of experimentally determined curves makes possible an estimate of the order of magnitude of the equilibrium constant of the specific complex. The different sorption of acetylcholinesterases from various sources as a function of the concentration of the immobilized affinity ligand in N-methylacridinium-Sepharose has also been described by Sekar et al.[18], who similarly see a possible explanation of the differences in the different specificities of individual acetylcholinesterases.

Application of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon with a low content of immobilized inhibitor in high-pressure liquid affinity chromatography (HPLAC) or in the large-scale isolation of pepsin is based on the observation that the
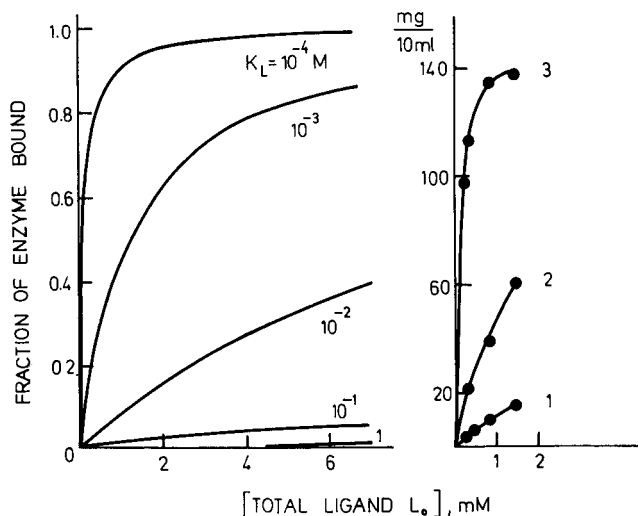
Fig. 4. Comparison of the theoretical relationship between the amount of the sorbed enzyme, concentration of the immobilized affinity ligand and the equilibrium constant of the enzyme–ligand complex, K_L[17] with the experimentally obtained values for chicken pepsin (1), chicken pepsin modified with o-nitrobenzenesulphenyl chloride (2) and porcine pepsin (3).

sorption of an enzyme on a column of a specific sorbent is independent of the enzyme concentration in the applied sample, as has already been demonstrated in a preceding paper[10]. Fig. 2 shows that the amount and activity of isolated pepsin are also independent of the presence of excess of inert protein in the applied sample. In the application of a mixture of pepsin with twice the amount of serum albumin on the column of ε-aminocaproyl-L-Phe-D-Phe-OCH$_3$–Separon, all of the serum albumin was eluted in the first peak with the equilibration buffer. Fractions of desorbed pepsin obtained in the absence (Fig. 2A) and in the presence of serum albumin (Fig. 2B) contained the same amounts of protein and exhibited the same proteolytic activity.

In conclusion, it can be said that biospecific chromatography of the enzyme requires a low content of immobilized inhibitor, in order to prevent the formation of multiple non-specific bonds and to achieve the highest possible utilization of immobilized affinity ligands. Such sorbents are then suitable not only for the efficient isolation and analytical determination of enzymes, both in the classical and in the HPLAC arrangement, but also for the investigation of the respective biospecific interactions.

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CHROM. 14,466

SEPARATION OF METHYLATED ALDITOL ACETATES BY GLASS CAPILLARY GAS CHROMATOGRAPHY AND THEIR IDENTIFICATION BY COMPUTER

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SUMMARY

A gas chromatographic procedure has been developed for the separation and identification of more than 50 partially methylated alditol acetates during a single run. Separation has been achieved using an on-column injection technique with a glass-capillary column wall-coated with SP-1000. Retention coefficients, based on two widely separated compounds as standards, are calculated using a program written for a computing integrator. These coefficients are stable under the chromatographic conditions employed, and enable accurate identification of methylated alditol acetates found during methylation analysis of polysaccharides.

INTRODUCTION

A technique of major importance in structural investigations of polysaccharides and oligosaccharides is that of methylation analysis in which the mixture of methylated sugars obtained after hydrolysis of a methylated polysaccharide is derivatised and separated by gas chromatography (GC). To eliminate complications arising from anomeric forms, the derivatives most commonly employed are the alditol acetates. Such derivatives have the additional advantage that they give readily identifiable fragments when examined by mass spectrometry, enabling their structure to be determined.

GC separation of alditol acetates was first achieved with the phase ECNSS-M[1]. Use of this phase was extended to the separation of partially methylated alditol acetates[2], and for several years it was considered uniquely suitable because of the separations achieved. However, its low thermal stability and the consequent limited column life led to a search for alternatives, especially for GC–mass spectrometry studies[3–6]. The introduction of glass capillary columns resulted in more efficient separations of partially methylated alditol acetates, and various attempts have been made to achieve separation of all the methylated sugars commonly found in natural polysaccharides[7–9]. More recently, the availability of fused-silica, wall-coated glass capillary columns, which offer less active surfaces and significant mechanical advantages, has led to further improvements in chromatographic separations, though published studies to date have dealt mainly with non-methylated sugars[10,11].

Polysaccharides from plant cell walls contain a considerable number of sugars, the most common being glucose, galactose, mannose, xylose, arabinose, rhamnose and fucose. In the course of studies on changes in plant polysaccharide structures arising from various degradative processes, it became necessary to examine products containing mixtures of polysaccharides and oligosaccharides using methylation analysis. Since each sugar can theoretically produce many different methylated derivatives, it was necessary to develop a chromatographic procedure which would separate, if possible in a single run, the methylated sugars liable to be found in such polysaccharides.

Test mixtures containing a range of methylated derivatives of each sugar have been prepared by partial methylation of the corresponding glycosides. After derivatisation and separation, the identity of each component of the mixture was established by mass spectrometry. Where possible the test mixtures were supplemented by methylated sugars which had been synthesised or which originated from polysaccharides of established structure.

For separation of such complex mixtures in a single chromatographic run, only wall-coated glass capillary columns were capable of giving the necessary resolution. Of the stationary phases tested, SP-1000 was the most effective, enabling more than 50 methylated derivatives to be separated under suitable chromatographic conditions. It would be desirable if identification could be based on GC evidence without routine recourse to mass spectrometry.

A major problem in GC is the reproducibility of relative retentions. Numerous attempts have been made to overcome this problem. Kováts[12] introduced a system, using the homologous series of *n*-alkanes as standards, which has been widely used and works well for non-polar solutes on non-polar phases. Various efforts have been made to extend the usefulness of Kováts indices to more polar solutes and phases[13–15]. All these methods utilise more than one standard, usually members of a homologous series. None is particularly applicable to partially methylated sugars, especially when dealing with highly complex natural mixtures. Lindberg[16], working with methylated sugars, makes reference to the use of two standards to improve reproducibility of retention data though he quotes their results relative to a single standard.

It can be confusing to refer to retention data obtained by interpolation using two standards as relative retentions. We have therefore used the term *retention coefficient* for data which we obtained using two widely separated internal standards. These values for a particular component are much more stable than relative retention values based on a single standard. They are less dependent on temperature and other chromatographic conditions. We have obtained retention coefficients for more than 60 commonly encountered partially methylated sugars on SP-1000 and, using a programmable computing integrator, have been able to identify automatically large numbers of unknown peaks in methylated biological samples. The method described here is applicable to data obtained from any capillary column which gives adequate resolution and sufficiently symmetrical peaks for accurate computer identification.

EXPERIMENTAL

Gas chromatography

Separations were carried out with a Carlo-Erba Fractovap 4160 gas chromato-

graph fitted with a flame ionization detector and a non-vaporizing septumless on-column injector of the Grob type[17] with adjustable temperature profile. The column oven was cooled to 60°C during injection and then raised rapidly to 206°C and maintained at this temperature. The detector temperature was 250°C and the carrier gas (helium) pressure 60 Pa. Glass capillary columns (20 m × 0.3 mm) wall-coated with SP-1000 were obtained from Erba Science (UK), Swindon, Great Britain.

Recording and calculation of chromatographic data was carried out with a Spectra-Physics SP-4100 computing integrator. BASIC programmes were written enabling retention coefficients to be calculated based on two standards, and peaks to be identified and named.

Mass spectrometry

Mass spectra were obtained from a VG Micromass 16 mass spectrometer connected to a VG 2025 data collection and processing system. The ionization potential was 70 eV and the source temperature 200°C.

Standards and test mixtures

Mixtures of partially methylated derivatives of each sugar were obtained by partial Purdie methylation[18,19] of the corresponding methyl glycosides prepared as previously described[20]. Periods of methylation were 120 min for rhamnose, 90 min for the hexoses and 35 min for xylose and arabinose. After derivatisation and separation by GC, the compounds were identified by mass spectrometry. Other mixtures were obtained by methylation analysis of commercial samples of xylan, arabinan and galactan.

Authentic samples of 2, 3, and 6-O-methylgalactose were kindly donated by Dr. R. Begbie. Samples of 2- and 3-O-methylglucose, 3,4-di-O-methylglucose, 2,4- and 2,6-di-O-methylgalactose and 2,3,6-tri -O-methylglucose were from the collection of the late Dr. D. J. Bell. A sample of 2,3-di-O-methylglucose was prepared from glycogen, and 2- and 3-O-methyl-, 2,4- and 3,6-di-O-methyl-, and 2,4,6- and 3,4,6-tri-O-methylmannose were obtained from original ovomucoid glycopeptides and glycopeptides derived from them. Quebrachitol (monomethyl-L-inositol) was obtained from Calbiochem, Bishops Stortford, Great Britain.

Methylation analysis of polysaccharides

Samples of soluble carbohydrate polymers from the rumen fluid of sheep, and of milled barley straw were methylated by the method of Stellner *et al.*[21], hydrolysed by a formolysis/hydrolysis procedure[16], and the liberated methylated sugars converted to the corresponding alditol acetates. The mixtures were subjected to GC using the procedure described above.

RESULTS AND DISCUSSION

As the first step in the development of a standard separation procedure we examined the efficiency of glass capillary columns coated with a number of stationary phases most of which had been used previously, mainly in packed columns, for separating methylated alditol acetates. These included OV-330, OV-225[3], OV-17[22], OV-1[23] and SP-1000[7]. Of these phases, SP-1000 gave the most satisfactory separa-

tions of our mixtures, with no tailing or asymmetry of the peaks. Better separation of the 2- and 3-O-methylxylitol derivatives was obtained with OV-1 (resolution $\approx$ 0.6), but this phase was not quite as efficient for the separation of some of the other compounds, and gave less symmetrical peaks with non-linear isotherms, making peak identification less accurate. It has, however, proved to be the most satisfactory phase for the separation and determination of methylated aminosugar derivatives[24].

The first step towards the identification of peaks obtained using packed columns is the relation of the peak retention time to that of a standard. Such relative retention values are, however, affected by column temperature and by the composition and condition of the column packings, especially for packings containing mixed stationary phases. A comparison of relative retentions obtained by different laboratories will often show considerable variation. When capillary columns are used, the problem of obtaining reproducible retention times and relative retentions is greatly increased because of difficulties in coating and because many different methods of preparing and deactivating the columns are employed.

The method of transferring samples which has been shown to give the most reproducible quantitative results is that of on-column injection[24,25]. In this procedure, the chromatographic run is started with the column oven cool or cold, and after injection the temperature is rapidly raised to the operating value[17]. Under such conditions, however, there is no accurate zero time which is implicity required for the ratio method of identification described above.

This problem can be overcome by using as standards two compounds whose peaks are widely separated in the chromatogram. The two standards are assigned retention coefficients (ρ) usually based on relative retention values either previously determined or taken from the literature. Assuming a linear relationship between retention times and retention coefficients, it is then possible to derive retention coefficients for any compound by interpolation or extrapolation from the standards. The equation used is

$$\rho_x = \frac{t_{Rx}(\rho_2 - \rho_1) + \rho_1 t_{R2} - \rho_2 t_{R1}}{t_{R2} - t_{R1}}$$

where ρ_x = retention coefficient of compound x
        t_{Rx} = retention time of compound x
        ρ_1, = retention coefficient of first standard
        ρ_2 = retention coefficient of second standard
        t_{R1} = retention time of first standard
        t_{R2} = retention time of second standard

The equation is derived from that for a straight line ($y = mx + c$). The retention times can be measured from any convenient starting-point, as it is only the difference between the retention times of the two standards which is important in the calculation. The retention coefficients so calculated need no correction for the gas hold-up volume of the column. In practice the retention coefficients do vary slightly with temperature, though this is only noticeable with a large temperature change. They are also affected to a slight extent by the column being used, according to its source and method of manufacture. For greatest accuracy it is therefore preferable

that new values for retention coefficients should be determined for new columns before general use.

With our equipment and procedures, retention times varied between runs within $\pm$ 0.3 % and tended to decrease in the course of a day, probably due to small changes in carrier gas pressure. This did not affect retention coefficients, but because of this variability it is necessary to use internal standards in every sample. The standard peaks are located by a computer program which compares the retention time of each peak with the retention time of the standard found in the previous run, and selects the largest peak within a set "window" around this retention time. The size of window used —a percentage of the retention time— is double that used for the identification of peaks and is usually 2 %. In difficult situations this procedure can be bypassed and appropriate values for the standard peaks inserted manually. The program uses the found internal standards to calculate the retention coefficients of each peak. Over a period of months the calculated values were stable within about 0.15 % relative standard deviation.

Controlled, incomplete methylation of sugars can be used to produce a range of partially methylated compounds, though usually not all those theoretically possible. After separation by GC and identification of the individual sugars by mass

TABLE I

RETENTION COEFFICIENTS $(\rho)^{\star}$ OF PARTIALLY METHYLATED ALDITOL ACETATES

Location of methyl group	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
Unmethylated	1.892	2.081	2.623	3.023	6.630	7.419	6.430
2	1.403	1.462	1.815	1.988	4.793	5.176	5.023
3	1.547		1.936	1.988	5.488	6.185	5.399
4	1.470			$\star\star$	$\star\star\star$	$\star\star\star$	
6	–		–	–	3.529	4.096	
2,3	0.945	1.027	1.125	1.208	3.218	3.620	3.417
2,4	1.000	1.076	1.205	1.154	3.691		3.417
2,5			0.977				
2,6	–	–	–	–	2.603	2.736	2.707
3,4	0.888		1.171	$\S$	3.276		
3,5			0.935		$\S\S$		
3,6	–	–	–	–	2.907	3.037	2.893
4,6	–	–	–	–	2.603		2.893
2,3,4	0.540	0.649	0.671	0.686	1.807	2.379	1.931
2,3,5			0.564				
2,3,6	–	–	–	–	1.765	1.842	1.889
2,4,6	–	–	–	–	1.803	1.912	1.680
3,4,6	–	–	–	–	1.621		
2,3,4,6	–	–	–	–	1.000	1.143	1.000

* Standard 1 was the 2,3,4,6-tetra-O-methyl-D-glucitol derivative ($\rho = 1.000$) and Standard 2 was the monomethyl-L-inositol (quebrachitol) derivative ($\rho = 4.300$).

** This compound yields an alditol acetate identical to that obtained from 2-O-methylxylose. By using sodium borodeuteride for reduction the two compounds can be distinguished by mass spectrometry.

*** Yields identical product to corresponding 3-O-methylhexose (see footnote **).

§ Yields identical product to corresponding 2,3-di-O-methylxylose (see footnote **).

§§ Yields identical product to corresponding 2,4-di-O-methylmannose (see footnote **).

spectrometry, the mixtures can be used as a source of reference compounds for the assembly of retention coefficients for a particular column. We have also assembled additional data from authentic derivatives obtained by synthesis or from methylation analysis of polysaccharides of known structure. Using these sources, it has been possible to produce a table containing retention coefficients for more than 60 partially methylated sugars, including most of those commonly encountered during methylation analysis of polysaccharides from plant and other sources (Table I). As with almost all other types of column, it has not proved possible to separate the 2,3,4,6 tetra-O-methyl-glucitol and mannitol derivatives. These cannot be distinguished by

TABLE II

METHYLATION ANALYSIS REPORT FOR COMPUTER PROGRAM APPLIED TO DATA FROM FIG. 1

Peak number	Area (%)	Retention coefficient (ρ)*	Identification**		
			1st	2nd	3rd
1	4.3	0.540	2,3,4-Rha***		
2	1.0	0.649	2,3,4-Fuc		
3	0.4	0.673	2,3,4-Ara		
4	0.6	0.681	2,3,4-Xyl		
5	3.0	0.885	3,4-Rha		
6	2.1	0.940	3,5-Ara	2,3-Rha	
7	16.5	1.000	2,3,4,6-Glc §	2,3,4,6-Man §	2,4-Rha
8	3.3	1.028	2,3-Fuc		
9	4.3	1.075	2,4-Fuc		
10	0.5	1.125	2,3-Ara		
11	5.0	1.139	2,3,4,6-Gal		
12	2.2	1.200	2,4-Ara	2,3-Xyl	
13	0.8	1.397	2-Rha		
14	3.4	1.460	2-Fuc	4-Rha	
15	0.5	1.542	3-Rha		
16	2.4	1.625	3,4,6-Man		
17	4.8	1.677	2,4,6-Glc		
18	1.7	1.750	2,3,6-Man		
19	1.9	1.804	2,4,6-Man	2-Ara	
20	3.1	1.829	2,3,6-Gal	2-Ara	
21	14.2	1.891	Rha	2,3,6-Glc	
22	5.2	1.931	2,3,4-Glc	3-Ara	
23	1.0	2.322			
24	2.0	2.359	2,3,4-Gal		
25	0.6	2.586	4,6-Man	2,6-Man	
26	3.4	2.709	2,6-Glc §	2,6-Gal §	
27	0.5	2.830			
28	1.3	3.393	2,3-Glc	2,4-Glc	
29	0.8	4.074	6-Gal		
30		4.300	Quebrachitol (standard)		

* Based on the standards 2,3,4,6-Glc ($\rho = 1.000$) and quebrachitol ($\rho = 4.300$).
** Identifications confirmed by mass spectrometry are shown in italics.
*** 2,3,4-Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methylrhamnitol etc.
§ These pairs of compounds cannot be distinguished by mass spectrometry.

TABLE III

METHYLATION ANALYSIS REPORT FOR COMPUTER PROGRAM APPLIED TO DATA
FROM FIG. 2

Peak number	Area (%)	Retention coefficient (ρ)*	Identification**		
			1st	2nd	3rd
1	2.6	0.569	*2,3,5-Ara****		
2	0.1	0.597			
3	0.1	0.667	2,3,4-Ara		
4	0.2	0.688	*2,3,4-Xyl*		
5	0.5	0.754			
6	1.1	0.851			
7	0.1	0.887	3,4-Rha		
8	0.3	0.937	3,5-Rha	2,3-Rha	
9	0.5	0.976	*2,5-Ara*		
10	1.4	1.000	*2,3,4,6-Glc*	*2,3,4,6-Man*	2,4-Rha
11	0.3	1.030	2,3-Fuc		
12	0.1	1.075	2,4-Fuc		
13	0.5˙	1.125	2,3-Ara		
14	1.1	1.143	*2,3,4,6-Gal*	2,4-Xyl	
15	23.8	1.218	*2,3-Xyl*		
16	0.2	1.536	3-Rha		
17	1.0	1.677	2,4,6-Glc		
18	0.3	1.756	2,3,6-Man		
19 §	50.4	1.929	3-Ara	2,4,6-Gal	
20	6.8	1.984	*2-Xyl*	*3-Xyl*	
21	1.3	2.687	*2,6-Glc*		
22 §§	0.9	2.863			
23	0.4	3.000	Xyl		
24	2.3	3.397	*2,3-Glc*	2,4-Glc	
25	0.9	3.844			
26		4.300	Quebrachitol (standard)		

   * Based on the standards 2,3,4,6-Glc ($\rho = 1.000$) and quebrachitol ($\rho = 4.300$).
  ** Identifications confirmed by mass spectrometry are shown in italics.
 *** 2,3,5-Ara = 1,4-di-O-acetyl-2,3,5-tri-O-methylarabinitol etc.
   § Peak 19 has been identified as 2,3,6-Glc, but, due to deliberate overloading, was not identified correctly in this run.
  §§ This peak was identified as a 3,6-hexose by mass spectrometry.

electron impact mass spectrometry, but the few other compounds which co-chromatograph can readily be distinguished by such means. A recent paper describes the separation of these components using a Silar-10C column, though the separation of some other derivatives was inferior to that on our SP-1000 column[9]. Our program uses the table of retention coefficients established for the column to identify each peak in a chromatogram. The identification window is again set as a percentage of the retention coefficient (usually 1 %) and possible identifications are given in the printed output ranked in order of closeness of fit (see Tables II and III)*.

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   * Copies of the BASIC computer program for use with the Spectra-Physics SP-4100 computing integrator may be obtained from the authors.

Application of the procedure is illustrated by two examples showing separations obtained during methylation analysis and the identifications obtained using the computer program. The first example (Fig. 1) shows the methylated sugars obtained from the soluble oligosaccharide complexes found in the rumen liquor of sheep fed on dried grass. Table II shows the report for this run, together with the results of GC–mass spectrometric analysis which confirms almost all the identifications made by GC alone, and enables positive assignment of most of the peaks with multiple identification. Fig. 2 and Table III show the chromatogram and report for a methylated sample of milled barley straw. In this case the column has been heavily loaded to
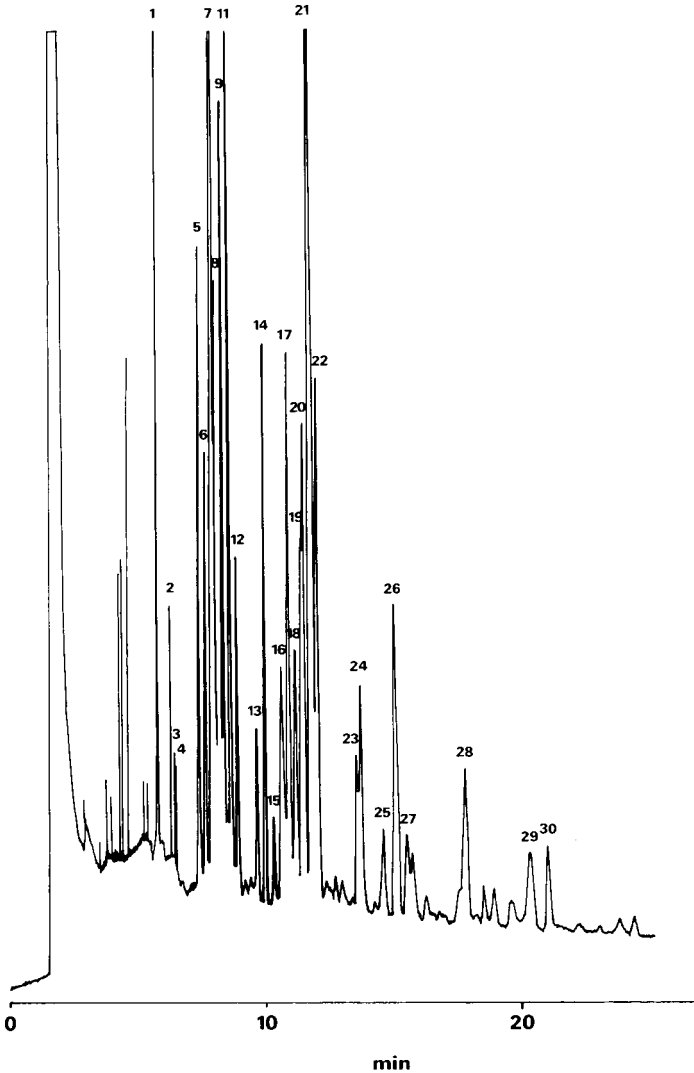

Fig. 1. Gas chromatogram showing separation of the partially methylated alditol acetates from the soluble oligosaccharide fraction from sheep rumen liquor. For details of conditions see text. Peak numbers are given in Table II.

allow identification of those components present in small amounts. This has resulted in an overload of the flame ionization detector amplifier and an incorrect retention time for the largest peak which from other runs is known to be 2,3,6-tri-O-methylglucitol. In both examples the standards used for calculation of retention coefficients were the 2,3,4,6-tetra-O-methylglucitol and mono-methyl-L-inositol (quebrachitol) derivatives. The identification window was 1 %, though in fact most of the peaks were identified even with a window of 0.5 %.

While simpler mixtures of methylated sugars may be resolved satisfactorily using other columns and less complex identification techniques, we consider that the chromatographic procedure described, allied to a programed computing integrator,
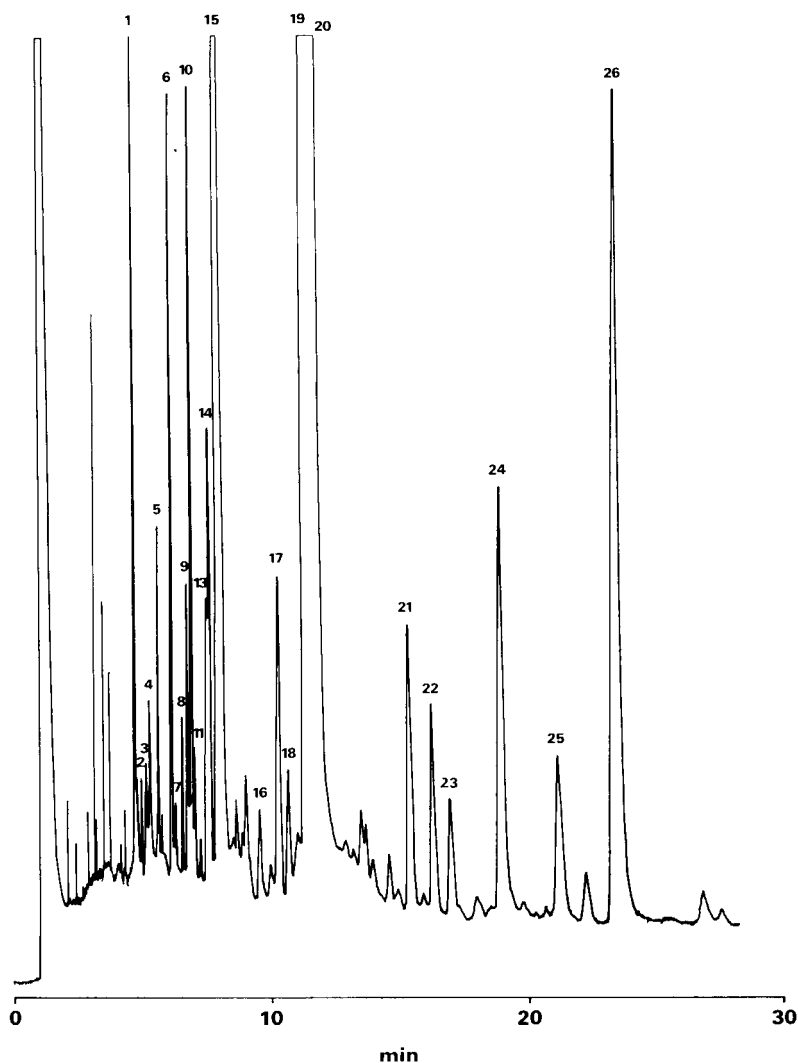

Fig. 2. Gas chromatogram showing separation of the partially methylated alditol acetates from barley straw. For details of conditions see text. Peak numbers are given in Table III.

provides a satisfactory means of obtaining with a single column the identification and approximate quantitation of most of the methylated sugars liable to be encountered in the course of applying methylation analysis to carbohydrate polymers from plant and other sources.

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CAPILLARY GAS CHROMATOGRAPHY OF METAL–PORPHYRIN COMPLEXES

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SUMMARY

The first reported (conventional) gas chromatographic results for transition metal porphyrin complexes are presented. They may be the first metal complexes containing a closed macrocyclic ring to be gas chromatographed. Kovats retention indices are in the range of 5200–5600, necessitating the use of short capillary columns. For Aetioporphyrin I, complete separation of Cu, Ni, VO, Co, Pd and Pt complexes is possible, but with octaethylporphyrin the nickel and vanadyl complexes are incompletely resolved.

INTRODUCTION

Some years ago, Corwin and co-workers[1–3] described the hyperpressure gas chromatographic (GC) technique for analysis of a range of metallo- and free-base porphyrins. Whilst the chromatographic behaviour (efficiency and resolution) of solutes subjected to this technique was not particularly good, the fact that porphyrins could be supercritically chromatographed was significant since they were previously believed to be not suited to GC analysis. Even in the light of Corwin's work, such compounds were still considered "non-chromatographable" using conventional GC appratus (the hyperpressure or supercritical technique relies upon a "solvent" carrier gas operated at high pressure, thereby increasing a solute's solubility in the mobile gas phase). This unsuitability of the porphyrins and metalloporphyrins was attributed to their low vapour pressure, the constraints placed upon the then conventional GC technique being insurmountable. This was predominantly due to adsorption upon the solid support within the packed columns employed. Since these early reports, however, no re-evaluation of the GC of free and metalloporphyrins has appeared in spite of recent advances in GC technology. Not surprisingly, therefore, White *et al.*[4] placed little importance on the supercritical technique as holding value for further studies.

An alternative approach to porphyrin GC is that initiated by Boylan and Calvin[5], involving the preparation of volatile bis(trimethylsiloxy)silicon(IV) derivatives of alkyl porphyrins. These compounds were gas chromatographed using conventional packed column procedures; however, their behaviour was believed to be

somewhat unfavourable until their analysis on flexible fused-silica capillary columns was realised[6]. It is reasonable, therefore, that if the facile determination of silicon derivatives has been made possible with highly deactivated flexible silica columns, then other metalloporphyrins, including those studied by Corwin, should be likewise reinvestigated. Further, it was felt that since many metalloporphyrins possess quantifiable vapour pressure and can be readily sublimed at temperatures of around 200 C under diffusion-pump vacuum, the only limitation to their GC analysis would be the temperature at which these analyses should be carried out.

Abundant precedence for the chromatography of metal chelates exists in the literature, and some reviews[7,8] illustrate the extent to which this area of analysis has been expanded. Recently studied chelates include the dithiocarbamates[9] and dithiophosphates[10], though tetradentate ligands, with chelation through nitrogen and oxygen or sulphur, have received considerable attention[7]. The latter are not, however, closed ring systems of the type represented by porphyrins. In this paper we describe the succesful gas chromatographic analysis of a range of metalloporphyrins.

EXPERIMENTAL

Gas chromatography

The gas chromatograph employed in this work was a Carlo Erba FTV 4160 instrument incorporating Grob-type split/splitless and on-column injectors. Flame ionisation detection was used. The same instrument was interfaced with an AEI MS30 magnetic-sector mass spectrometer to confirm that chelates were chromatographed unchanged.

A Hewlett-Packard 6 m × 0.3 mm I.D. flexible fused-silica capillary column, OV-1 coated and siloxane deactivated, was used for the results presented; however, satisfactory chelate elution was also obtained on a Chrompack CP Sil 5 coated glass column (10 m × 0.3 mm I.D., on-column injection).

Usual chromatographic conditions were: detector and injector (latter for when split/splitless mode used) at 315°C, on-column injection (with secondary cooling) and oven at 60°C. Hydrogen carrier at 0.2 kg cm^{-2} inlet pressure producing an average carrier flow velocity of *ca.* 100 cm sec^{-1} was usually used, with temperature programming for splitless and on-column injection modes of either heating from 60 C to 300°C at 7°C min^{-1}, or heating from 60 C to 240 C (in approximately 3 min) followed by programming to 300°C at 7°C min^{-1}.

Metalloporphyrin preparations

Free-base porphyrins, 1,3,5,7-tetramethyl-, 2,4,6,8-tetraethyl porphyrin (Aetio[1], 1) and 1,2,3,4,5,6,7,8-octaethyl porphyrin (OEP, 2) were obtained from Dr. J.

1, 2   3
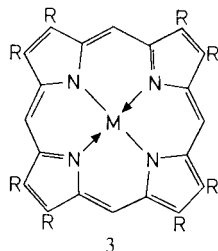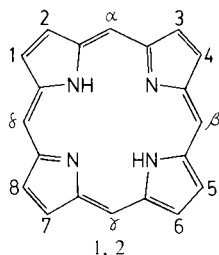
1   Aetio[1]; 1 = 3 = 5 = 7 = CH$_3$; 2 = 4 = 6 = 8 = C$_2$H$_5$; α–δ = H
2   OEP; 1–8 = C$_2$H$_5$; α–δ = H
3   M(II) porphyrin

G. Erdman and Porphyrin Products (Logan, UT, U.S.A.), respectively. They were used as starting materials for preparation of the various metal(II), vanadyl and titanyl porphyrins listed in Table I. Structure 3 represents that of a typical metal(II) porphyrin; vanadyl and titanyl possess an axial oxide ligand with the $M=O$ bond perpendicular to the plane defined by the four nitrogen atoms. Standard methods were used for the bulk of the preparations[11,12]. The procedure of Fournari et al.[13] was used to prepare the titanyl derivative.

TABLE I

GAS CHROMATOGRAPHIC RETENTION DATA FOR METALLOPORPHYRINS, COLUMN AND CONDITIONS AS GIVEN IN THE EXPERIMENTAL SECTION

ND = Not determined.

Metal	Aetio[1] derivative		OEP derivative	
	Relative retention*	Kovats index**	Relative retention*	Kovats index**
Zn(II)	3.4	5220	6.5	5350
Cu(II)	3.6	5240	7.3	5390
Ni(II)	4.8	5290	8.4	5410
V(IV)***	5.7	5320	8.6	5420
Co(II)	6.8	5380	11.2	5490
Pd(II)	10.4	5470	17.0	ND
Ti(IV)***	13.2	5530	–	–
Pt(II)	15.8	5600	–	–

* Given as minutes to peak maximum after elution of n-pentacosane (C_{50}). n-C_{50} elutes at ca. 6 min after reaching 300°C —the isothermal hold region at top of temperature programme— under the conditions employed.

** These values are calculated by interpolation within two n-alkane standards —n-pentacosane and n-hexacosane, and are approximate. They are quoted to nearest 10 index units.

*** V(IV) as vanadyl, $V=O$ and Ti(IV) as titanyl, $Ti=O$.

RESULTS AND DISCUSSION

The range of metalloporphyrins successfully chromatographed is presented in Table I, along with relative retentions, given as elution time after n-pentacosane (n-C_{50}), and approximate Kovats indices. All of these compounds elute at the top of the temperature programme (conditions as in Experimental) after isothermal conditions (300°C) have been established. Under the standard conditions employed to obtain these results, the hydrocarbon reference appears at 6 min after onset of isothermal.

A chromatographic trace of a mixture of metalloporphyrins is given in Fig. 1. Initial success for these chelates was obtained on a 2-m length of the Hewlett-Packard OV-1 capillary column. Poor separations and broad peaks were obtained on this short column (with the chelates eluting at similar times after n-C_{50} as given in Table I, at an isothermal hold temperature of 270°C). A 6-m length of the same material was utilised to give better separations and greater overall efficiency, whilst only requiring a moderate increase in temperature to 300°C, with results as illustrated in Fig. 1.

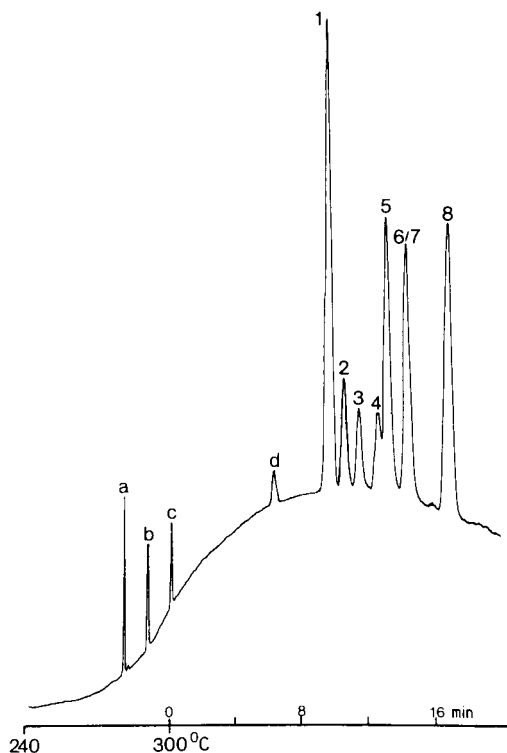At the maximum temperature employed (300°C), a marked increase in the

Fig. 1. Capillary gas chromatogram of a mixture of *n*-alkanes and metalloporphyrins. Flexible silica column (6 m × 0.3 mm I.D.), operated under conditions given in the Experimental section, with isothermal hold at 300°C. Peaks: a = *n*-tetracosane (*n*-C_{40}); b = *n*-dotetracosane (*n*-C_{42}); c = *n*-tetratetracosane (*n*-C_{44}); d = *n*-pentacosane (*n*-C_{50}); 1 = Cu Aetio; 2 = Ni Aetio; 3 = VO Aetio; 4 = Co Aetio; 5 = Cu OEP; 6 = Ni OEP; 7 = VO OEP; 8 = Co OEP.

detector baseline occurred. This was due to phase bleed, as indicated by solvent blank trials, rather than any effects such as compound decomposition. Peak symmetry appears to be quite good; however, some fronting behaviour, indicative of an concave isotherm, may be noticeable at higher concentrations. This effect gives rise to the slight shoulder on the leading edge of peak 1 in Fig. 1. There was no evidence of metal exchange between different metalloporphyrins in the gas phase, although transmetallation of certain porphyrin complexes can occur[12].

Studies on silicon(IV) alkyl porphyrins, which have been reported previously[6], revealed that retention indices for the Aetio and OEP bis(trimethylsiloxy) derivatives were approximately 3315 and 3440, respectively, on a methyl silicone-coated column. Clearly the conditions employed for such studies (25-m column, temperature programming to 260–280°C) would not be suitable for the chelates in Table I which have retention indices of the order of 5200–5600. For the 6-m column, the calculated resolution (R_s) between pairs of compounds (Aetio[l] and OEP) of the same metal species is in the region of $R_s \approx$ 6–7 for Ni, Cu and Co under the non-optimal conditions quoted for Fig. 1 (non-optimal because the carrier gas flow-rate is far in excess of that which would correspond to a minimum on the Van Deemter curve).

The vanadyl pair were resolved to $R_s \approx 5$, and this is a consequence of an apparent reduction in retention volume difference between the Aetio and OEP compounds rather than any adverse chromatographic behaviour (such as band broadening or peak tailing). This may be seen by comparing the positions of peaks 2 and 6 against 3 and 7 for the Ni and VO pairs on Fig. 1. The short 2-m column afforded the nickel pair with a resolution of 2–3, with rather broad peaks resulting, though again peak shapes were symmetrical.

Some of the other metalloporphyrins, most notably the zinc porphyrins, exhibit less favourable chromatography and their elution is characterised by fronting peaks even at low injected concentrations. However, this behaviour may still be an overloading effect due to very low solubility in the stationary phase, possibly related to the lack of planarity of zinc porphyrins[14] (the zinc atom is out of the plan defined by the four nitrogens of the pyrrole groups, although in another crystal structure it is coplanar[15]). Axial ligand attachment is possible for zinc porphyrins[16] but no manifestation of this in peak tailing (through metal–stationary phase adsorptive interaction) is observed.

The relative retention trends of the metalloporphyrin complexes, such as Ni, Pd, and Pt, follow those usually observed for other metal-chelating systems, such as the dithiophosphates[10] and the tetradentate β-thiono enamines[17] and probably reflect a molecular-weight trend influencing volatility. The close similarity of molecular weights of Cu, Co and Ni porphyrins may account for the similar retention volumes of these compounds. However, no definitive work on chromatographic retention mechanisms for a range of metal chelates appears to have been undertaken. Thus, the physicochemical parameters which affect retention of the chelate are still open to speculation. Clearly some interrelation must exist between volatility and solubility (as measured by gas chromatographic retention) and the bonding status of the metal atoms within a series of metal-chelates.

For Zn, Cu, Ni and Co porphyrins, the elution order is the reverse of the molecular-weight order, though it agrees with that for the GC of other metal chelates[17]. The observation of the lower-molecular-weight species eluting later also holds for the VO- and TiO-porphyrins, with the latter having a molecular weight about three mass units less than the vanadyl but with a significantly longer retention. The order of increasing retention for Cu, Ni and VO is also found for other chelating systems[18].

A generalised mechanism for retention behaviour of metalloporphyrins would require consideration of a variety of physical phenomena such as intermolecular attraction and volatility.

Heats of sublimation (ΔH_s) of a range of phthalocyanines have been determined by MacKay[19], and the results correlated with calculated electron distributions around the molecules (both out-of-plane densities on the metals and the peripheral density on the *meso* nitrogens). The resulting trend predicted from distribution considerations was Co > Cu-Ni > Zn > H$_2$, and this agreed with experiment (thus, the cobalt has greater electron density which is manifested in greater intermolecular interaction, and therefore higher ΔH_s value). MacKay felt that the general order of peripheral density (arising from *meso* positions) for the phthalocyanines would correspond to that for porphyrins. MacKay's order for the metallophthalocyanines agrees with that which we have obtained for the GC of metalloporphyrins, except that

the vanadyl, palladium and platinum phthalocyanines have ΔH_s values which do not fit in with the GC trend (these were not included in the considerations of electron distributions in ref. 19). This might imply that sublimation behaviour alone cannot be reliably used to predict relative GC elution orders for the porphyrin–metal chelates.

One trend which does fit much better with our elution order is the stability order of the chelates derived from spectroscopic data. Chelate stability usually refers to stability towards demetallation by various acid treatments[11]. Phillips[20] discussed spectroscopic correlations with complex-stability for a variety of tetrapyrrole ligands, including Aetioporphyrin II (where an increasing shift to lower wavelengths in the visible and ultraviolet spectra indicated increased chelate stability). Relative stabilities decreased through the series Pt > Pd > Ni > Co > Cu > Zn. The position of Co is the only apparent anomaly when this order is compared with chromatographic retentions. Perhaps it is not surprising that spectroscopic data should give a similar order to the electron-density distributions since electronic transitions giving rise to the spectra will be perturbed by electron-density changes. Exactly why such a measure of stability should closely reflect GC behaviour is unclear.

Whilst it would be informative to have thermogravimetric results on the metalloporphyrins, such data are not yet available. It is common practice to compare such behaviour with GC retention[17,18,21].

The limited GC data for metalloporphyrins do not permit us to propose a generalised mechanism for retention at this stage.

The hyperpressure procedure[3] gave a retention order for the Aetioporphyrin II chelates of Cu < Co < Ni < Zn < TiO < Pt < VO < Pd on an Epon 1001 column, and Co < Cu < Zn < Ni < TiO < Pt < Pd < VO on an XE-60 coated column (with dichlorodifluoromethane solvent (carrier) gas). Both these stationary phases are quite polar; Epon 1001 is an epichlorohydrinbisphenol resin (McReynolds index 2319) and XE-60 is a 25 % 2-cyanoethyl silicone rubber (index 1785). Generally such polar phases are not favoured for the GC of metal chelates[7] (with the exception of fluorinated phases for F-containing metal-derivatives). These orders differ greatly from that which we report here, and are at variance with generally observed GC relative retentions. The metal acetylacetonates, also studied by Karayannis and Corwin[22] using hyperpressure GC, likewise gave retention orders not consistent with those usually expected for GC and again, as with the porphyrins, the peak shapes and column efficiencies appear rather unattractive. Evidently, supercritical chromatography must involve different physicochemical interaction mechanisms than those operative in gas-liquid chromatography.

CONCLUSIONS

This paper has demonstrated GC analysis of a wide range of metal–porphyrin complexes. Capillary columns (of either glass or fused silica) provide the necessary low-activity support surface and high temperature stability required for compounds with retention indices in the region of 5200–5600. The self-association phenomenon of these macrocyclic compounds may contribute to such high retention volumes.

Results are reported for C_{32} and C_{36} fully alkylated porphyrins (Aetio[I] and OEP respectively); however, an extension into the "biological" porphyrins, which usually possess acidic side-chains, appears possible after appropriate derivatisation.

The ability of the porphyrin ligand to complex a wide variety of metal species (with successful chromatography of just a few of these reported here) could give this ligand a unique position within the field of metal-chelate GC.

Significant improvements in the chromatographic results would be made if increases in both resolution and overall efficiency could be effected by increasing the length of the column and/or using a carrier flow-rate closer to optimum. Both of these options would necessitate programming to higher temperatures, perhaps in the region of 340°C, and continued cycling of columns to such high temperatures would probably reduce column lifetime. Narrow-bore columns ($\approx$ 50 μm I.D.) and/or the use of very thin-film phase coatings are both possible areas which could be studied, however, the former precludes the use of the on-column injection system and the latter would place an increased importance on deactivation of the glass surface, and also limit the capacity of the GC capillary system. We are currently investigating various ways of improving upon the results presented herein.

ACKNOWLEDGEMENTS

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CHARACTERIZATION OF COMMERCIAL WAXES BY HIGH-TEMPERATURE GAS CHROMATOGRAPHY

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SUMMARY

Sixteen different waxes (including two oils) were characterized by temperature-programmed gas chromatography up to 400°C, using flame ionization detection. Chromatographic patterns were obtained for the untreated samples and for samples treated with diazomethane and acetic anhydride. Included with the patterns are physical characteristics, origins of the waxes, and where available, references to gas–liquid chromatographic work done by others.

INTRODUCTION

Natural waxes, both animal and vegetable, have been used by man for centuries because of their many useful properties. In nature, waxes serve to protect plants against environmental influences such as microorganisms and drying. Waxes are extremely stable substances with high resistance towards both chemical and biological degradation. Because of this, waxes have found extensive use in the protection of food.

Chemically, natural waxes consist mainly of even-numbered straight-chain carboxylic acids and alcohols in the region of *ca.* C_{12}–C_{36}. Hydrocarbons including both odd numbered and unbranched compounds in the same chain-length region are also usually present[1–5]. Although the composition of the fatty acids in waxes is similar to those found in animal fat used for human nutrition, the latter are usually present as glycerides whereas the former are generally esterified with the long-chain alcohols.

The initial analysis and characterization of waxes, particularly natural waxes, were limited to the estimation of acids, esters and a few other constituents. The analysis of hydrolysis products of Japan wax demonstrated that it was a triglyceride wax[6], while sugar cane wax contained a large portion of polyaldehydes[7].

Thin-layer chromatography (TLC) has been used in the past for characterization of waxes and wax constituents[8–10]. Holloway and Challen[11] carried out a systematic TLC study of a number of wax constituents and applied the technique to the characterization of natural waxes from various sources. Their technique can be particularly useful when used complementary with gas chromatography for the identification of waxes of unknown origin.

Several published reports have shown that gas–liquid chromatography (GLC) employing high temperatures could be useful for the analysis of glycerides, waxes and some polymers[12-14]. However, with the exception of the work of Tulloch[13] and Valmalle and Karleskind[14] no extensive characterization of commercial waxes has been reported. Tulloch[12,13] reported GLC patterns only after diazomethane and acetylation, while Valmalle and Karleskind[14] carried out direct analysis using a 2 % JXR column programmed to 380°C.

The purpose of the present work is to obtain GLC "finger-print" patterns on commercial wax samples to permit the ready identification of the waxes used in industrial and, in particular, food products. Because of the nature of the waxes, special GLC conditions are required, particularly the need for a column stationary phase stable up to 400°C. This report contains a comprehensive account of the GLC patterns of a number of waxes in order that they may be identified in unknown samples. This in-depth characterization in most cases includes patterns obtained by: (a) direct injection of the wax without chemical treatment; (b) after treatment with diazomethane; (c) after treatment with diazomethane and acetic anhydride. The direct analysis yields the hydrocarbon and mono-ester patterns while the diazomethane treatment will include the free fatty acids (as methyl esters). Finally, the free alcohols present will appear as acetates after treatment with acetic anhydride. The resulting three chromatograms yield valuable information useful for characterizing the many different commercial waxes.

EXPERIMENTAL

Apparatus

Gas chromatography was carried out using a Varian Model 2700 gas chromatograph with flame ionization detector. Columns were 3 ft. × 1/8 in. I.D. stainless steel, packed with 80–100 mesh, acid-washed and silanized Chromosorb W coated with 1.5 % Dexil 300. The temperature was programmed from 150 to 400°C at 4°C/min. Helium carrier gas flow-rate was 60 ml/min. Detector attenuation was 32 × 10^{-11}. Injection volumes were normally 3–5 μl of a 1 % wax (or oil) solution.

Esterification

Methyl esters of the fatty acids were formed according to the method of Tulloch[9], briefly described as follows. Wax (50 mg) was weighed and dissolved in 5 ml of chloroform. Freshly made diazomethane in ether was then added and the mixture refrigerated overnight. The solution was evaporated to dryness, then dissolved in 5 ml of chloroform for chromatographic analysis.

Acetylation

The solution from above was evaporated to dryness and 1.0 ml of acetic anhydride followed by 1.0 ml of anhydrous pyridine ("Pyridine-Plus"; Pierce, Rockford, IL, U.S.A.) were added to the residue. The contents were mixed and left at room temperature overnight. The solvent was removed by evaporation and the residue dissolved in chloroform for analysis.

RESULTS AND DISCUSSION

Paraffin waxes

Paraffin waxes are prepared under pressure from paraffinic hydrocarbons[15]. They are mixtures of solid saturated aliphatic hydrocarbons with melting points in the range 43–65°C. Their molecular weights vary from 300 to 500 with chain lengths of 20 to 35 carbon atoms. Figs. 1 and 2 shows the patterns obtained from two paraffin waxes (m.p. 53°C, with carbon chain maximum around C_{23}–C_{25}; m.p. 58°C, carbon chain maximum near C_{26}–C_{28}). The figures show that the two waxes have the same hydrocarbon composition but with varying maxima, the lower maxima being found for the lower-melting waxes. A GLC tracing of a 53°C paraffin wax obtained earlier[14] is similar to the one shown in Fig. 1.
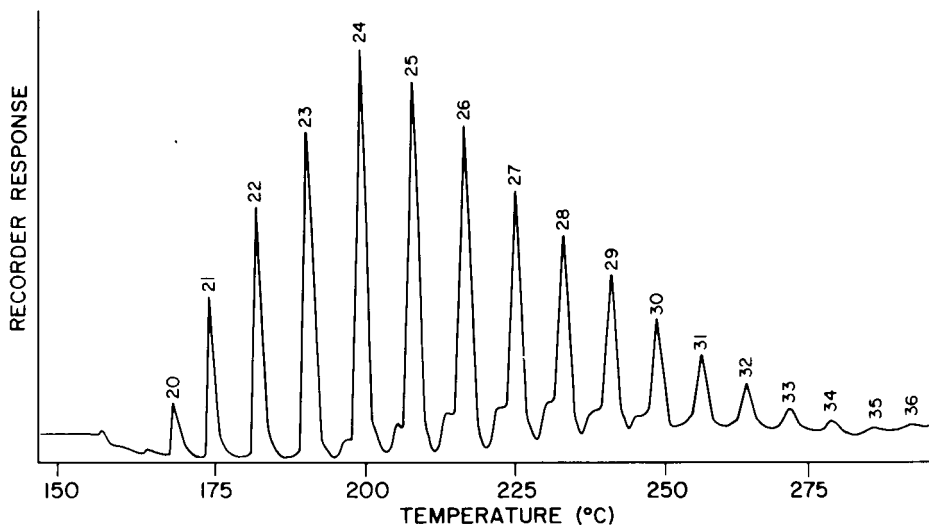
Fig. 1. Paraffin wax, melting point 53°C.

Microcrystalline waxes

After distillation of petroleum waxes, the residues obtained after crystallization are microcrystalline waxes which are solid hydrocarbons. They are made up of saturated hydrocarbons containing 25–50 carbon atoms with a melting point greater than 65°C. Their molecular weight exceeds that of the paraffins (400–700).

Figs. 3 and 4 show the curves obtained for the two microcrystalline waxes (types 1140/10 and 1251/7), showing the sequence of saturated hydrocarbons with a maximum around C_{38}–C_{42} and C_{32}–C_{35} for waxes 1140/10 and 1251/7, respectively. Valmalle and Karleskind[14] reported a similar GLC pattern of this type of wax.

German montan waxes

These are considered fossil vegetable waxes[16] (also considered mineral waxes) obtained by extraction of coal or lignite. The wax is purified by vacuum distillation or by solvent processing.

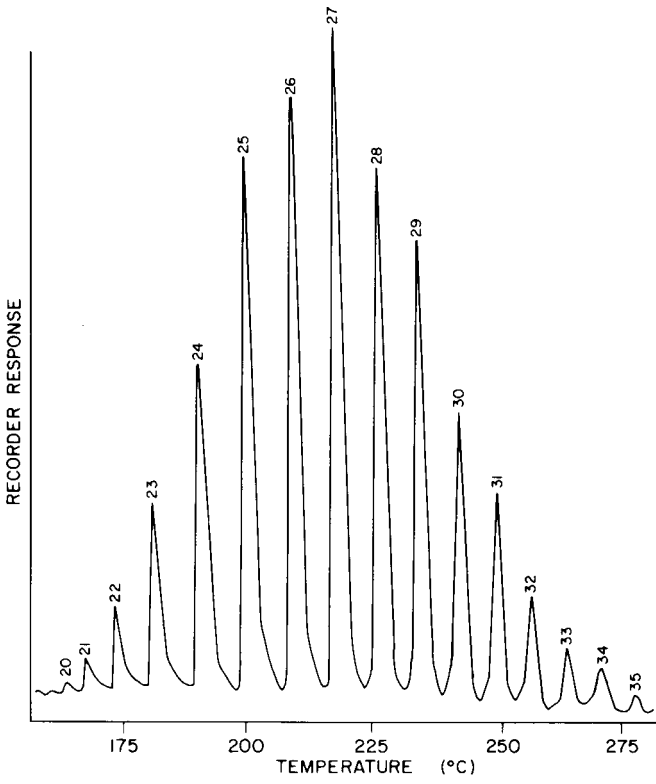It has been reported by Lange and Wildgruber[17] that the main components of
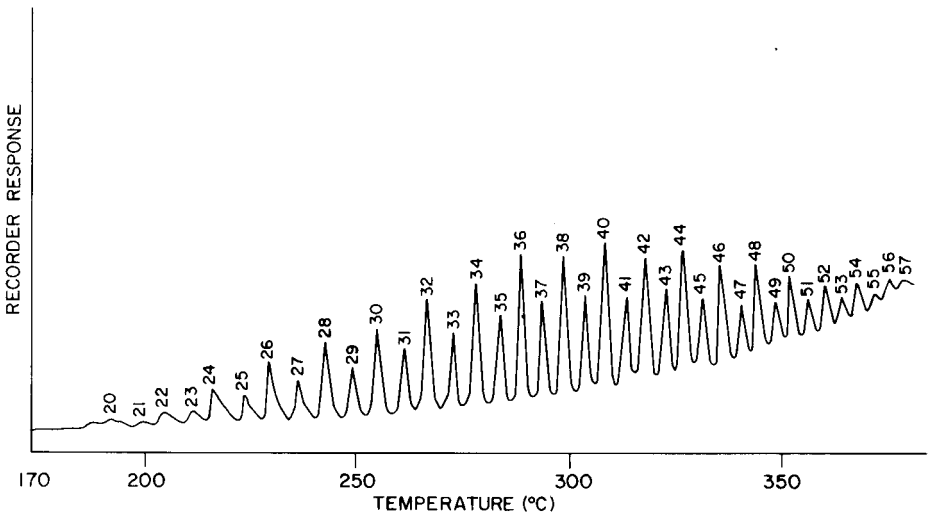
Fig. 2. Paraffin wax, melting point 58°C.

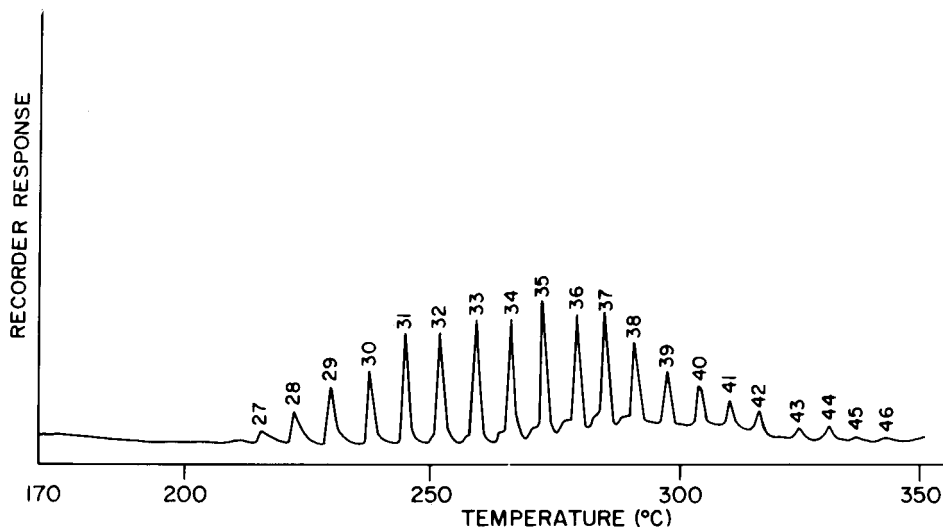Fig. 3. Microcrystalline wax, type 1140/10.

Fig. 4. Microcrystalline wax, type 1251/7.

montan waxes are esters of the C_{28} and C_{30} acids as well as C_{28} and C_{30} alcohols. Valmalle and Karleskind[14] reported the following composition for a sample of the wax: esters, 50%; alcohols, 1–2%; resin and asphalt, 25%; unidentified, 8%.

Fig. 5 shows chromatographic results before and after the treatments outlined in the Experimental section. The untreated wax shows mainly hydrocarbons in the region C_{25}–C_{33}. The peaks in the region C_{50}–C_{62} represent monoesters which remain unaffected after the chemical treatments. The diazomethane and the diazomethane–acetylation treatments show the fatty acids (methyl esters) and alcohols (acetates) contained in the sample. The major acid peaks shown in Fig. 5 are C_{26}, C_{28} and C_{30} acids. Montanic acid is the C_{28} peak, usually the most predominant constituent[17].

Beeswax

This is a digestion product secreted by the common *Apis mellifera* bee. A yellow wax, it can be bleached by various decolorization processes (solar UV radiation, conventional decolorizing earth, active charcoal) without significantly changing its composition.

It has been reported by Tulloch[13] that only 63% of the components in beeswax are volatile after diazomethane and acetylation treatment and it consists of 15% hydrocarbons, 12% acids and 36% long-chain esters.

Fig. 6 shows GLC patterns obtained with yellow beeswax, while Fig. 7 shows the corresponding results for white (decolorized) beeswax. For the latter, the acetylation treatment produces no significant change from the diazomethane results indicating the absence of alcohols in the C_{20}–C_{35} region. It has been reported that the chromatographic patterns of beeswax (white) are rather consistent regardless of geographical origin[14,18]. The yellow beeswax differs from white beeswax in the short-chain fatty acid composition as well as in the presence of some alcohol peaks.
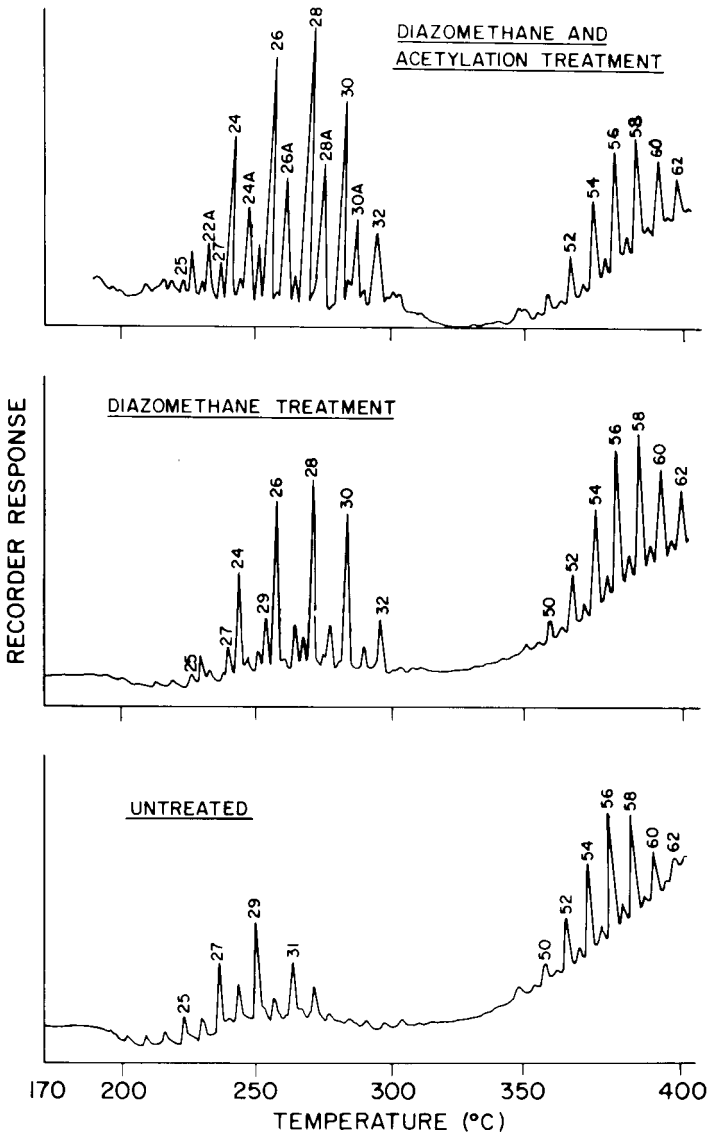
Fig. 5. German montan wax.

Spermacetti substitute

Spermacetti is a whitish hard wax obtained from the head of the sperm whale. It consists of over 95 % esters and some alkanes, fatty acids, and *ca.* 3 % alcohols. Of the esters, chiefly cetyl palmitate (C_{32}) is present in appreciable amounts. Esters of lauric, stearic and myristic acids and esters of higher alcohols are also present.

The substitute received by us is an artificial mixture prepared and sold in place of spermacetti due to its increasing cost and limited availability. From Fig. 8 it can be seen that the spermacetti substitute when injected without any treatment shows a
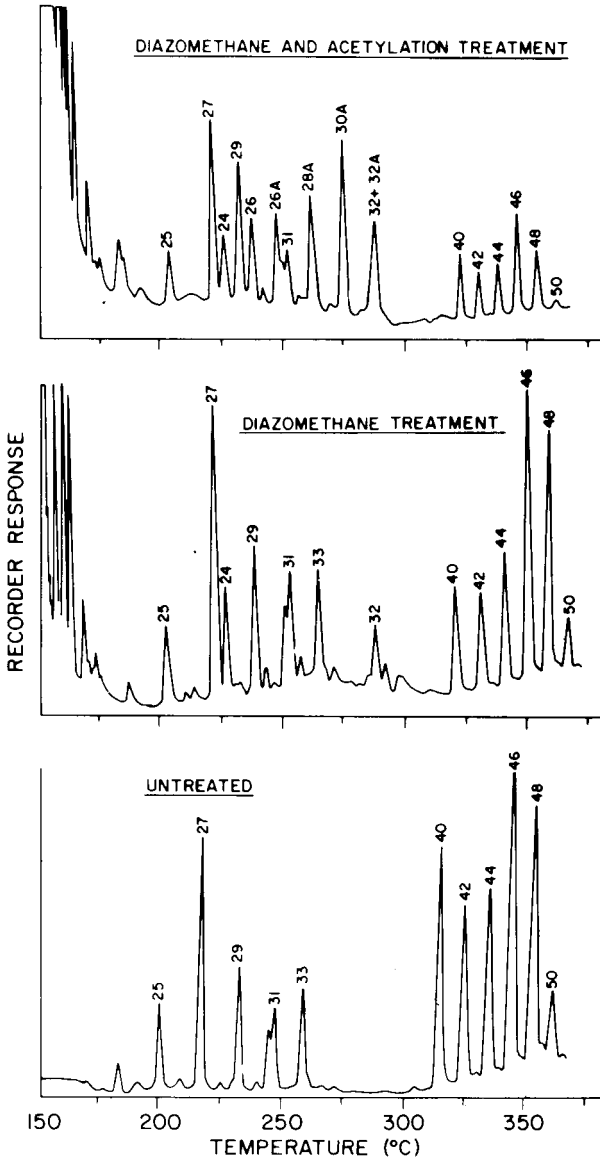
Fig. 6. Yellow beeswax.

maximum at C_{32}, similar to that found by Valmalle and Karleskind[14] and Holloway[19] for the original wax. After treating the substitute with diazomethane, methyl esters of C_{20}, C_{22}, and C_{24} were observed. After diazomethane and acetyation treatments, some alcohol acetate peaks were observed.

Japan wax

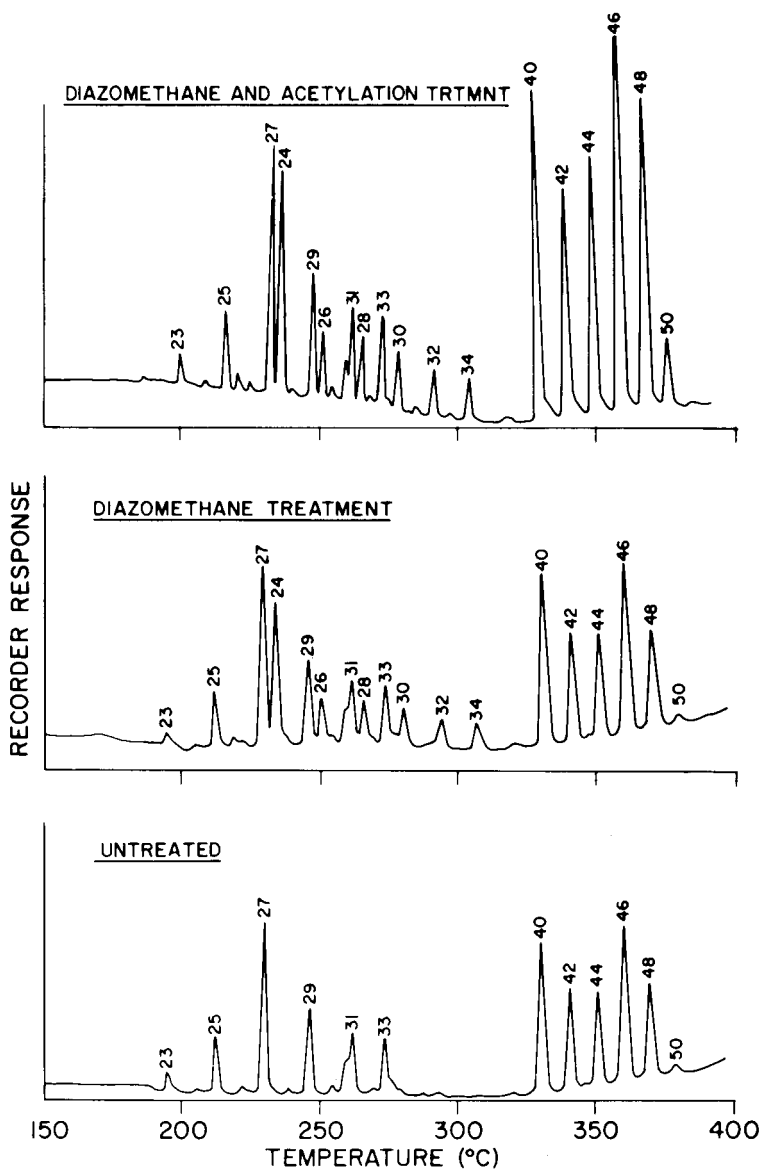This wax is extracted from the protective coating of Sumac kernels[15].

Fig. 7. Decolorized (white) beeswax.

Tsujimoto[6] reported that it is a glyceride wax and gives, on hydrolysis, mainly palmitic acid and 6% long-chain dicarboxylic acids, and on methanolysis gives methyl esters, together with esters of eicosanedioic (0.2%) and docosanedioic (2.6%) acids. Similar percentages of dicarboxylic acids have been reported by Tazaki[20.]

Tulloch[13] reported that the volatile amount of Japan wax was 46% and it was composed of: free acids, 3%; triglycerides, 36%; unidentified, 5%. The same author reported that by GLC (diazomethane and acetylation), three glyceride peaks were
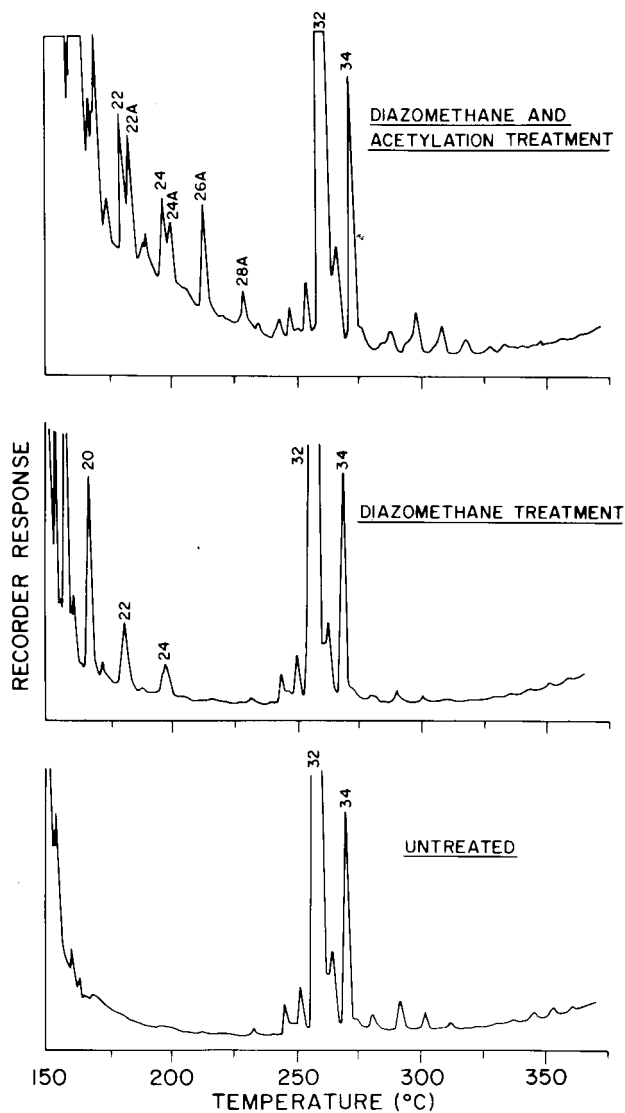
Fig. 8. Spermacetti substitute.

obtained, namely 48G, 50G and 52G, where 48G (tripalmitin) was the major compo-
nent. Valmalle and Karleskind[14] reported the same composition by injecting the wax
without any treatment.

Fig. 9 shows the three chromatograms for Japan wax. The untreated chroma-
togram is somewhat different than that obtained by Valmalle and Karleskind[14] and
Tulloch[13] in that they report 48G (glyceride) as the predominant peak and few con-
stituents below this in carbon-chain length. Fig. 9, untreated, indicates the presence of
traces of hydrocarbons in addition to the glyceride peaks.'The differences here might
be explained by the age of the kernels before extraction of the wax. Tulloch[21] showed

through his work on leaf wax of Selkirk variety spring wheat, that from 44 days to 100 days the hydrocarbons, alcohols and monoesters disappeared to a great extent, giving rise to long-chain esters and diesters with 2,3-unsaturations.
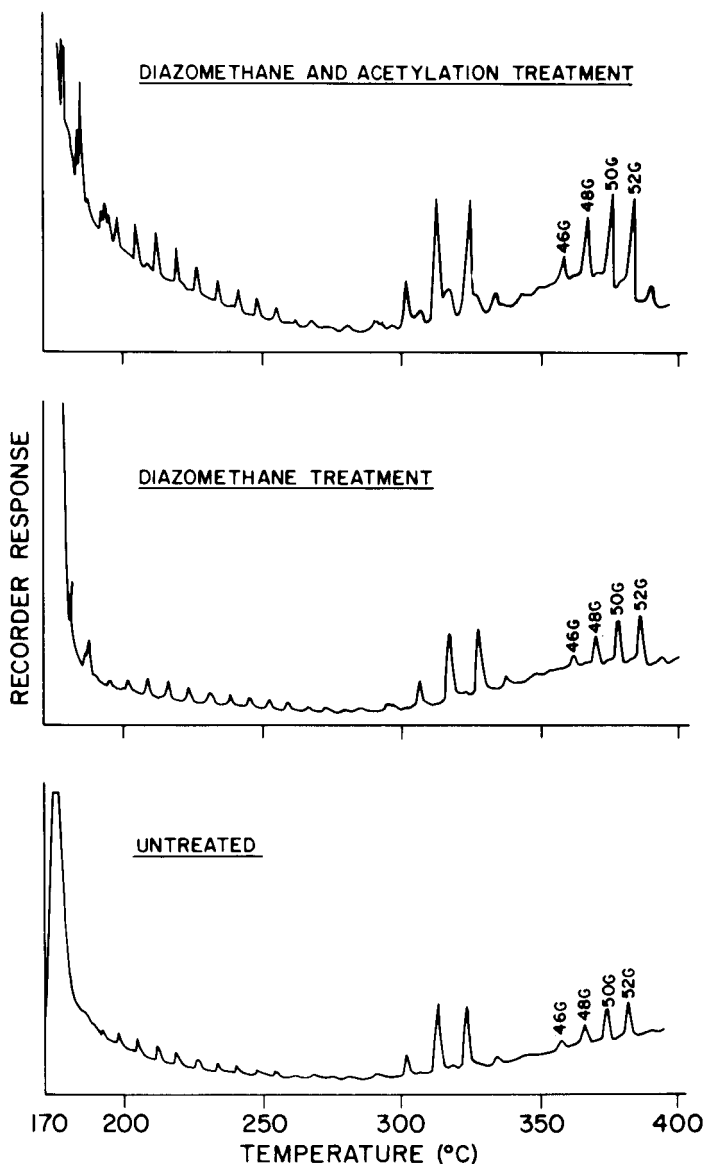

Fig. 9. Japan wax.

Candelilla waxes

These waxes are extracted from various plants, such as *Euphorbia antisiphilitica*, that grow in Southern Texas and Northern Mexico. They are yellow waxes containing *ca.* 40–65 % hydrocarbons, 10 % aliphatic alcohols, 30 % esters and 8–10 %

fatty acids[14]. Tulloch[13] reported that 65 % of the wax is volatile on GLC after di-
azomethane and acetylation treatments. The hydrocarbon content of this wax was
reported to be *ca.* 41–55 % with C_{31} as the principal component, being nearly 80 %
(refs. 22–24).

Fig. 10 compares the results of the chemical treatments. It can be seen that the
components of the wax consists of hydrocarbons, some fatty acids and alcohols all in
the region of C_{23}–C_{34}. These results agree with results reported earlier[13,14,21,25].
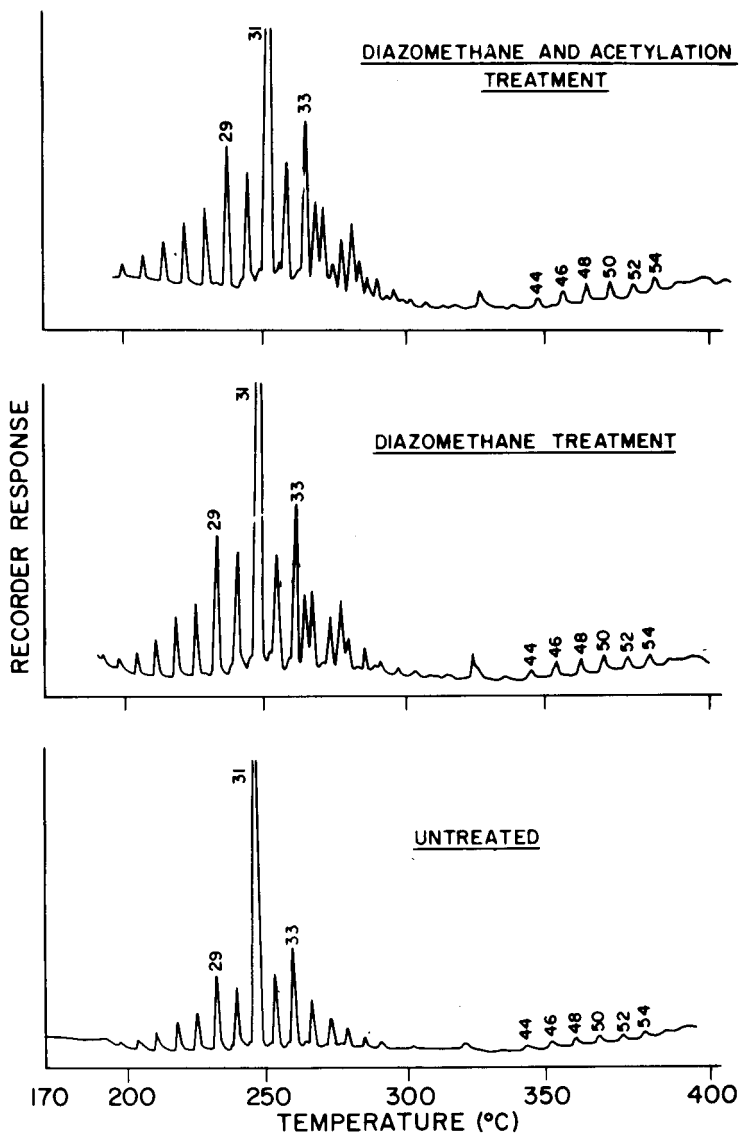

Fig. 10. Candelilla wax.

Ouricury wax

Because of its physical properties, this wax has often been considered a substitute for Carnauba wax. However, the GLC patterns are significantly different. Fig. 11 shows the chromatographic results obtained for ouricury wax.
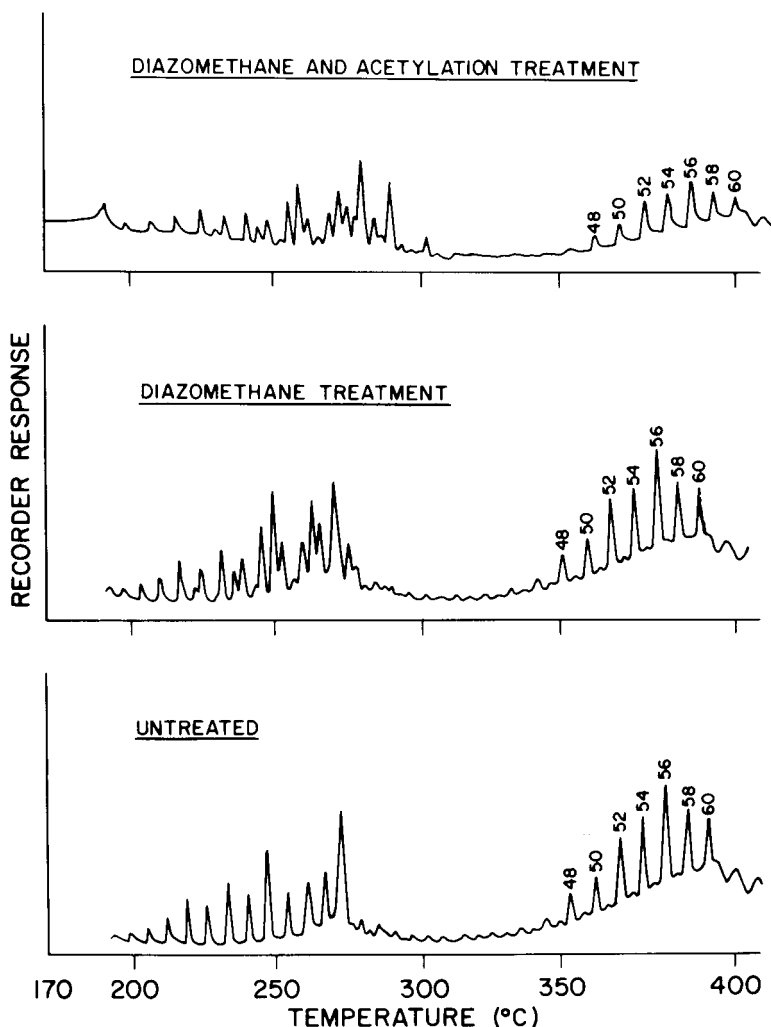

Fig. 11. Ouricury wax.

Carnauba wax

This is one of the most useful and valuable waxes and is extracted from leaves of *Cerifera* palm trees[26]. Carnauba is the highest melting of the commercial vegetable waxes (83–86°C m.p.), a fact which accounts for many of its uses. It is an important component of polishes, and is also used in lipsticks and cosmetic creams, glazing for paper, pills and candies, carbon paper, ink, candles, varnish and enamel[26].

It has been reported by Tulloch[13] that only 47% of the components in Car-

nauba wax are volatile after diazomethane and acetylation treatments, which consist of 11 % alcohols and 36 % esters. According to Valmalle and Karleskind[14], the composition of Carnauba wax is as follows: esters (*ca.* 80 %), alcohols (10–15 %), free acids (3–5 %) and hydrocarbons (2–3 %). Fig. 12 shows the chromatograms obtained for this wax. The untreated wax shows hydrocarbons and monoesters C_{48}–C_{64} with a maximum at C_{56}. No change is observed after diazomethane treatment; however, four alcohols (peaks C_{28}, C_{30}, C_{32}, C_{34} with a maximum around C_{32}) are observed after
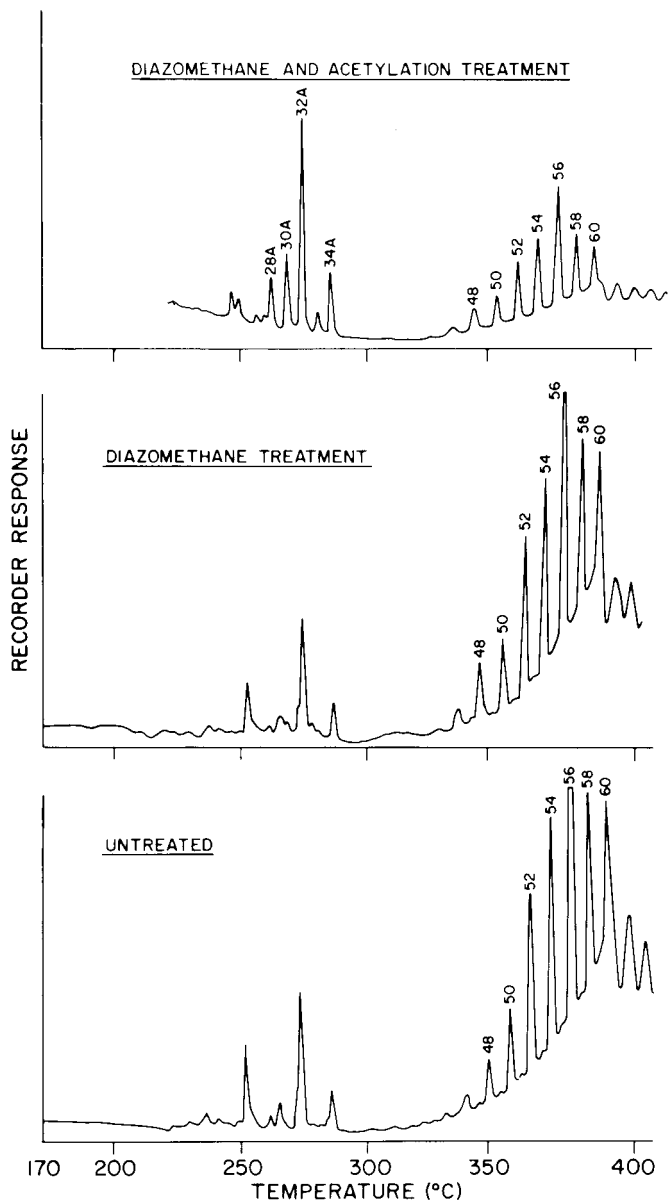

Fig. 12. Carnauba wax.

acetylation (one third or original sample size injected). This is similar to results reported earlier[13,14].

Rice bran wax

Rice bran wax is, as the name suggests, isolated from rice bran oil, which is a commercially important source of edible oil. The purified wax is hard, slightly crystalline and varies in color from tan to light brown. No GLC patterns have been reported up to the present. Fig. 13 shows the results obtained with and without chemical treatment. There is little difference between the untreated and diazomethane extracts. However, acetylation does show at least four alcohols.
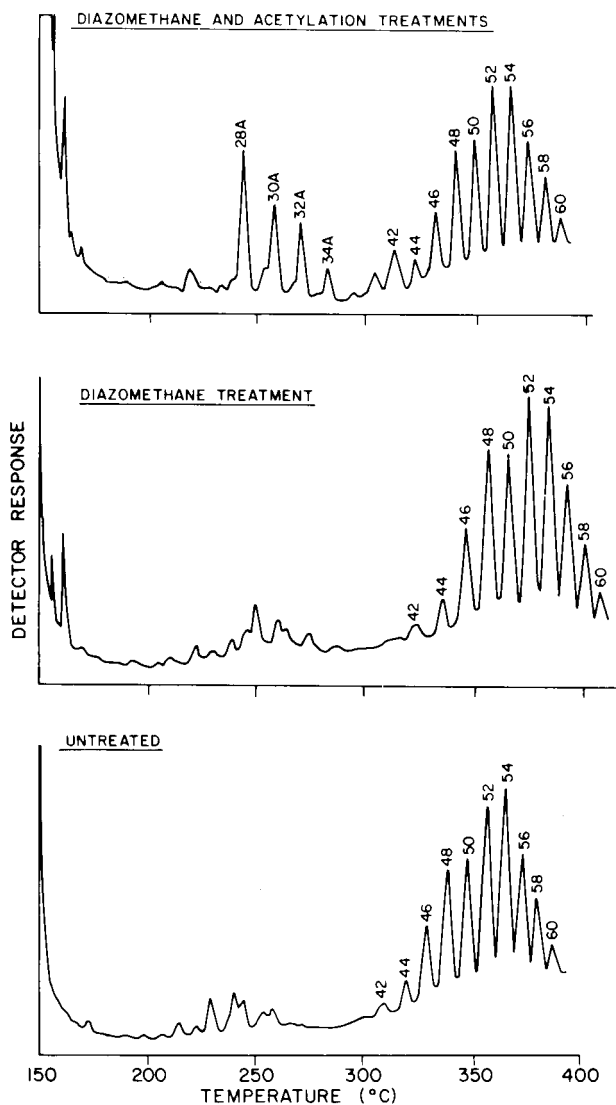

Fig. 13. Rice bran wax.

Shellac wax

Shellac wax is derived from *Tachardia Lacca* and has been studied by several groups by classical means[29–31]. Tulloch[13] has shown that after diazomethane and acetylation the GLC indicated a large free alcohol content. This is also observed in Fig. 14; however, there is little difference between the untreated and diazomethane results.

Mineral and paraffin oils

These oils consist largely of saturated straight-chain (C_{14}–C_{18}) and cyclic hydrocarbons. They are extensively used in baking operations and for fruit and
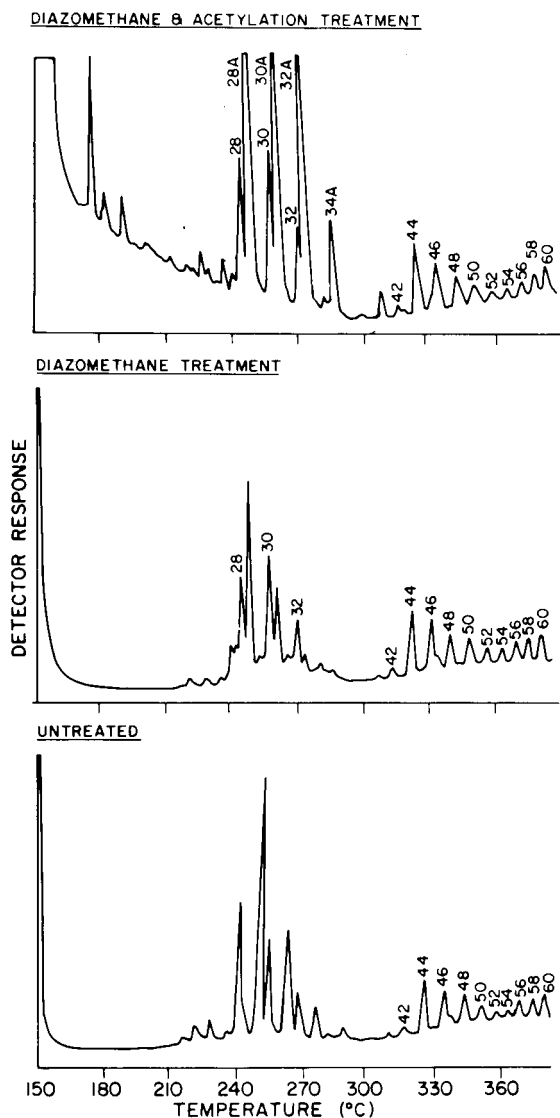

Fig. 14. Shellac wax.

vegetable coatings. Fig. 15 shows GLC chromatograms of the untreated oils analyzed under the same conditions as for the waxes. No discernable peaks were observed for the two. Also, no changes were produced as a result of the chemical treatments.
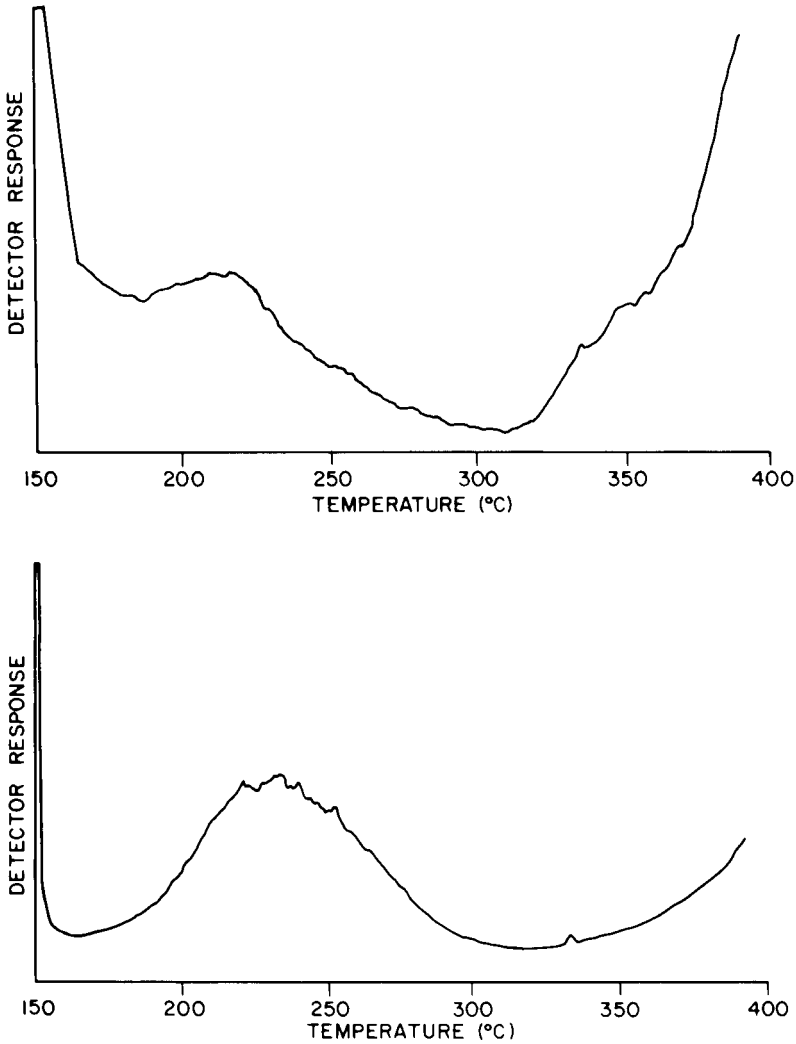

Fig. 15. GLC chromatograms of paraffin oil (top) and mineral oil (bottom).

CONCLUSION

The GLC patterns of fourteen waxes and two oils have been obtained before and after various chemical treatments. These results provide very useful data for the identification of unknown commercial waxes, especially in the food-processing industry where the use of waxes is subject to regulations.

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GAS CHROMATOGRAPHIC ANALYSIS OF CHLOROPHENYLMERCAPTURIC ACID LINDANE METABOLITES

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SUMMARY

An analytical method for phenols has been adapted for the analysis of chlorophenylmercapturic acids in rat urine. Chlorothiophenols were produced from the mercapturic acids by hydrolytic cleavage with sodium hydroxide. Acetate esters of the chlorothiophenols were formed by addition of acetic anhydride to the aqueous alkaline solution. After acylation, the acetate derivatives were extracted into hexane. Forming the acetate esters of the chlorothiophenols prevented their oxidation to disulfides and significantly improved their chromatographic properties. Electron-capture gas chromatographic analysis of the stable acetate esters was performed on a mixed phase column, 4% SE-30 + 6% OV-210. Recoveries of four chlorothiophenols ranged from 82 to 93%. This method required no sample transfer steps; therefore, sample loss and analysis time were minimized.

INTRODUCTION

The formation of mercapturic acids (N-acetyl-cysteine conjugates) is an important detoxification mechanism. The conjugation of glutathione (a tripeptide: glutamic acid, cysteine, glycine) with a xenobiotic is the first step in mercapturic acid biosynthesis. Glutamic acid and glycine are catabolically eliminated from the glutathione conjugate. The resulting cysteine moiety is then acetylated to form the mercapturic acid. A wide variety of potentially harmful electrophilic compounds is detoxified by this mechanism.

When lindane (γ-hexachlorocyclohexane) was administered intraperitoneally to rats, approximately 15% of the dose was recovered in the mercapturic acid fraction[1]. In 1965, Grover and Sims[2] were the first to document the existence of a chlorophenylmercapturic acid as a mammalian metabolite of lindane. They presented chromatographic evidence for the existence of a small amount of 2,4-dichlorophenylmercapturic acid in urine from lindane-treated rats. Koransky *et al.*[3], studying α-hexachlorocyclohexane in rats, identified several isomers of dichlorothiophenol in urine following alkaline hydrolysis. More recently, Kurihara, *et al.*[4] reported the presence of mono- and polychlorophenylmercapturic acids as lindane metabolites.

The chlorophenylmercapturic acids have been analyzed intact and as chloro-

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thiophenols (Fig. 1). Kurihara et al.[4] analyzed intact chlorophenylmercapturic acids in urine. The chlorophenylmercapturic acids were butylated following separation from the netural compounds, free chlorophenols, and conjugated chlorophenols. Butyl esters of the chlorophenylmercapturic acids were then acetylated with trifluoroacetic anhydride and examined by gas chromatography–flame photometric detection. Koransky et al.'s[3] analysis of chlorophenylmercapturic acids was performed on chlorothiophenols. The chlorothiophenols were generated from the chlorophenylmercapturic acids by alkaline hydrolysis, derivatized with diazomethane, and analyzed as the methyl ethers by gas chromatography–electron-capture detection.

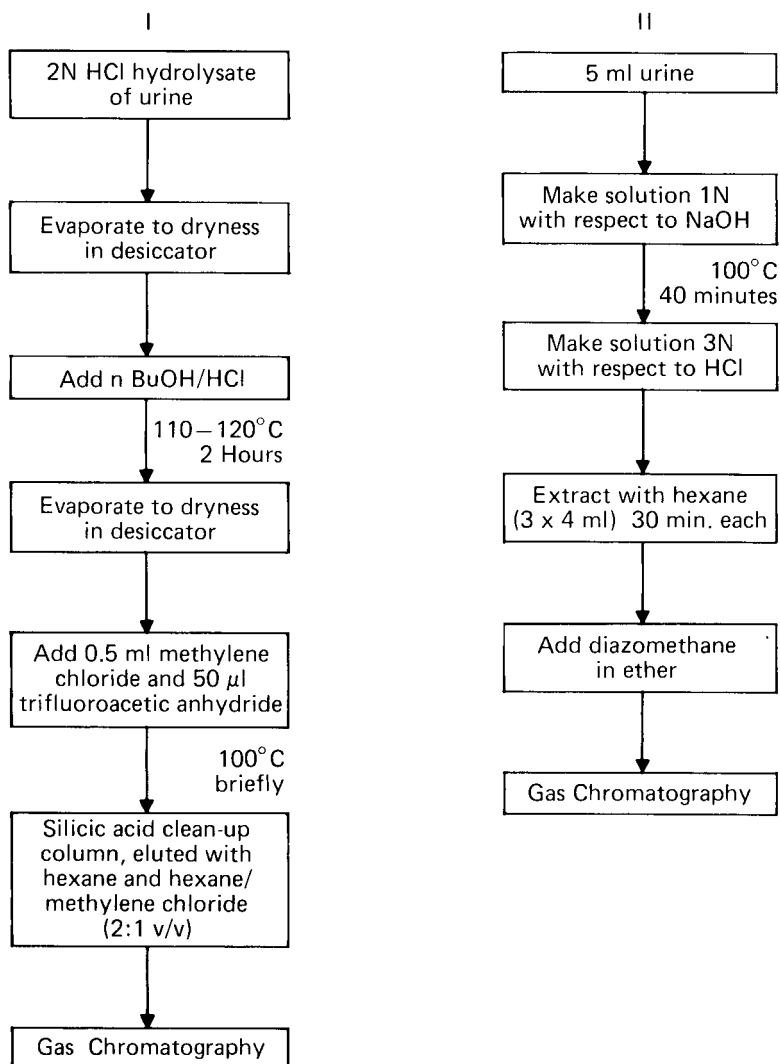An improved analytical method was developed in our laboratory for the de-

I

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┌─────────────────────────┐
│   2N HCl hydrolysate     │
│       of urine           │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│  Evaporate to dryness    │
│     in desiccator        │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│    Add n BuOH/HCl        │
└─────────────────────────┘
            │ 110–120°C
            │ 2 Hours
            ▼
┌─────────────────────────┐
│  Evaporate to dryness    │
│     in desiccator        │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│  Add 0.5 ml methylene    │
│    chloride and 50 μl    │
│  trifluoroacetic anhydride│
└─────────────────────────┘
            │ 100°C
            │ briefly
            ▼
┌─────────────────────────┐
│  Silicic acid clean-up   │
│  column, eluted with     │
│  hexane and hexane/      │
│  methylene chloride      │
│      (2:1 v/v)           │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│   Gas Chromatography     │
└─────────────────────────┘
```

II

```
┌─────────────────────────┐
│       5 ml urine         │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│   Make solution 1N       │
│  with respect to NaOH    │
└─────────────────────────┘
            │ 100°C
            │ 40 minutes
            ▼
┌─────────────────────────┐
│   Make solution 3N       │
│  with respect to HCl     │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│   Extract with hexane    │
│  (3 x 4 ml)  30 min. each│
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│   Add diazomethane       │
│      in ether            │
└─────────────────────────┘
            │
            ▼
┌─────────────────────────┐
│   Gas Chromatography     │
└─────────────────────────┘
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Fig. 1. (I) Analysis of mercapturic acids intact by Kurihara et al.[4]. (II) Analysis of mercapturic acids as thiophenols by Koransky et al.[3]. n BuOH = n-Butanol.

termination of chlorophenylmercapturic acids in urine. The analysis of chlorothiophenols derived from chlorophenylmercapturic acids was preferred because analysis of the intact chlorophenylmercapturic acids was time-consuming and tedious. Analysis of the derivatized chlorothiophenols had two significant advantages compared to the analysis of the underivatized compounds: greatly improved chromatographic properties and increased stability against autooxidation. Due to the extremely hazardous properties of diazomethane, several other derivatizing reagents were examined.

The similarities in the reactivity of phenols and thiophenols suggested that the literature concerning derivatization of phenols also should be reviewed. Various reagents have been used in the derivatization of phenols including: diazomethane[5], diazoethane[6], heptafluorobutyrylimidazole[7], and silanizing reagents[8]. However, all of these reagents required that the phenol be in an organic solvent prior to derivatization. The solvent extraction of phenols from aqueous solution often resulted in sample loss; therefore, a new derivatization procedure was investigated.

Coutts et al.[9] omitted the solvent extraction step and formed the acetate esters of various phenols in an aqueous sodium bicarbonate solution. This method eliminated several steps necessary in most phenol derivatizations including: acidification of the matrix, extraction of the phenol into an organic solvent, drying, and concentration before derivatization. The direct formation of the acetate esters in aqueous alkaline solution aided in the quantitative extraction of the phenols.

The derivatization method of Coutts was very effective for phenols; however, chlorothiophenols required the stronger base sodium hydroxide to provide consistently reproducible yields. It was also necessary to increase the volume of acetic anhydride from 500 μl, used by Coutts, to 5 ml possibly due to the biological matrix in the extract.

The method described in this paper improved on the methods of Kurihara et al. and Koransky et al. in several areas (see Figs. 1 and 2). The most significant improvement was the reduction in analysis time. The approximate times required for the analyses were Kurihara et al.'s method ($>$ 5 h), Koransky et al.'s method ($>$ 2 h), and our method (1.5 h). The method presented here had fewer transfer steps which resulted in higher yields. Utilizing gas chromatography, recoveries obtained by this method ranged from 82–93 % which was significantly higher than 75–85 % reported by Kurihara et al.. The study published by Koransky et al. was primarily based on radiolabelled compounds and gave no gas chromatography recovery data.

EXPERIMENTAL

Apparatus

The gas chromatograph used was a Tracor Model MT-222 equipped with a ^{63}Ni electron-capture detector (ECD) operating in the pulsed linearized mode.

Chromatographic conditions

A silanized glass column (75 cm $\times$ 4 mm I.D.) was packed with 4 % SE-30 + 6 % OV-210 on 80–100 mesh Gas-Chrom Q, obtained from Applied Science Labs. (State College, PA, U.S.A.). The gas chromatographic conditions were as follows: column temperature 130°C (4 min initial hold), then programmed to 185°C (2 min hold) at 6°/min; detector temperature 350°C; injection port temperature 220°C;

transfer block temperature 235°C; carrier gas (argon with 5% methane) flow-rate 85 ml/min.

Reagents

Sodium hydroxide, sodium sulfate, and acetic anhydride were obtained from Fisher Scientific (Raleigh, NC, U.S.A.). Nanograde hexane was purchased from Mallinckrodt (Paris, KY, U.S.A.). The chlorothiophenols used in this study were: 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol, and 2,4,5-trichlorothiophenol with stated purities of 98%, 98%, 97%, and 90%, respectively. All of the chlorothiophenols were obtained from Aldrich (Metuchen, NJ, U.S.A.).

Hydrolysis, derivatization and extraction

Chadwick et al.[10] provided the rat urine which was extracted for the removal of neutrals, free chlorophenols, and conjugated chlorophenols. This urine was obtained from control and lindane-treated rats. The preextracted control urine and reagent blanks were used to determine fortification recoveries.

A 5-ml sample of urine was hydrolyzed with 20 ml of 1.0 N sodium hydroxide in a screw-capped tube for 45 min at 100°C, a slight modification of Parke's[11] procedure. After hydrolysis, 30 ml of distilled water, followed by 5 ml of acetic anhydride, were added to the sample. The resulting mixture was shaken well and derivatized in a warm water bath of approximately 65°C for 35 min. After derivatization, 5 ml of hexane were added, and the sample was shaken vigorously for 1 min. The hexane extract was dried by filtering through anhydrous sodium sulfate and collected in a concentrator tube. The extraction was repeated twice more, combining the extracts and adjusting the volume (Fig. 2).

Preparation of standards

Stock solutions of standards, 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol and 2,4,5-trichlorothiophenol, were prepared at a concentration of 1 mg/ml in acetone and maintained at −15°C. Acetate derivatives of the chlorothiophenols were synthesized using the described derivatization procedure and maintained at −15°C as quantitative standards.

RESULTS AND DISCUSSION

Gas chromatography utilizing temperature programming provided separation of the acetylated chlorothiophenols while maintaining an analysis time of less than 30 min. A representative chromatogram is shown in Fig. 3. When the column was consistently equilibrated between programming runs, less than one percent difference was observed in the acetate retention times. However, there were contaminating substances which coextracted with the chlorothiophenols. These coextracted substances caused a deterioration in peak height reproducibility over a period of time. This problem was corrected by daily replacement of the injection port glass insert with its silanized glass wool plug. It was also necessary to preextract the distilled water used in the hydrolysis procedure with methylene chloride–hexane (1:10), to remove some impurities that appeared as extraneous peaks. For quantification purposes, the height of the chlorothiophenol acetate peaks obtained from sample ex-
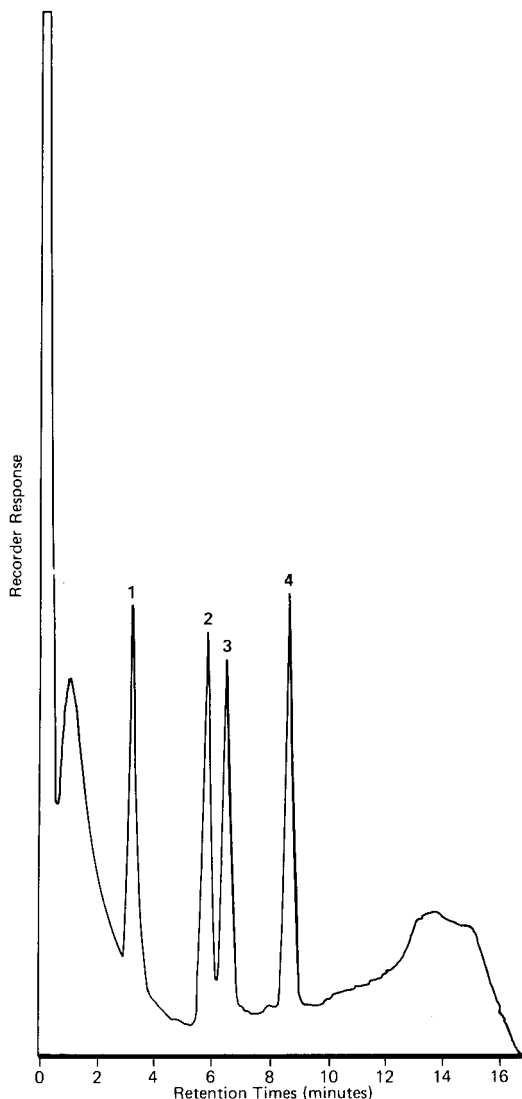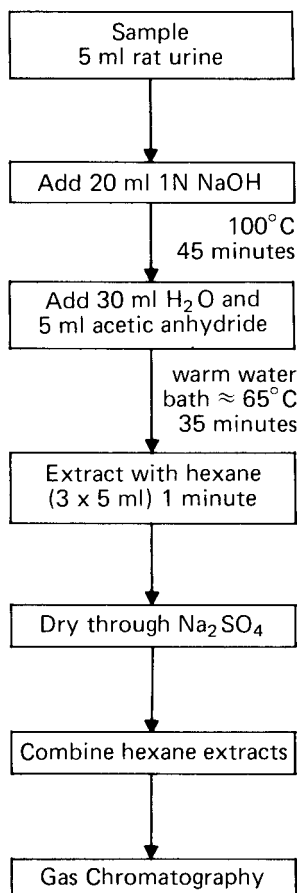
Fig. 2. Hydrolysis, derivatization, and extraction of chlorophenylmercapturic acids.

Fig. 3. Gas chromatographic separation of chlorothiophenol acetate standards. Chromatographic conditions are given in text. Each acetate represents 120 pg. Peaks: 1 = 4-chlorothiophenol acetate; 2 = 2,5-dichlorothiophenol acetate; 3 = 3,4-dichlorothiophenol acetate; 4 = 2,4,5-trichlorothiophenol acetate.

tracts was compared to peak heights obtained with known amounts of chlorothiophenols following acylation.

There were significant differences between recoveries in blanks and in control urines when they were fortified prior to hydrolysis. The recovery of four chlorothiophenols ranged from 4 to 15% lower in blanks than in control urines, indicating that urine deterred loss. In addition, a comparison was made between control urine samples fortified prior to hydrolysis and control urine samples fortified following hydrol-

ysis. We observed a 0–7% loss of chlorothiophenols during the hydrolytic process.

Coutts *et al.* used aqueous sodium bicarbonate as the reaction medium for the acetylation of phenols. However, in modifying this method for derivatization of thiophenols, it was determined that the use of aqueous sodium hydroxide as the reaction medium produced higher acetate yields. Another advantage in using sodium hydroxide was the elimination of the frequent venting necessary with sodium bicarbonate. To determine the optimum derivatizing conditions, normalities of sodium
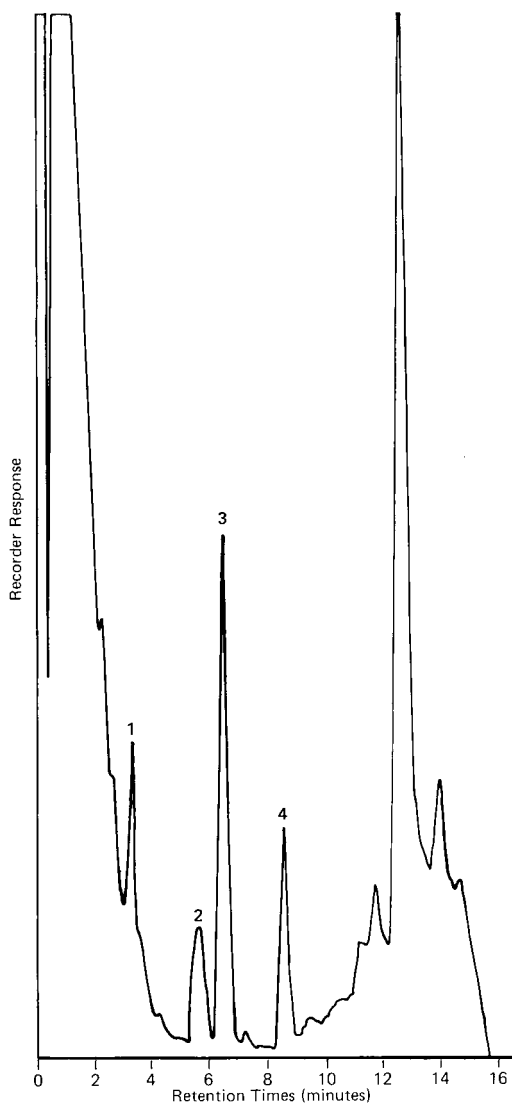
Fig. 4. Representative chromatogram of chlorothiophenols found in rat urine following treatment with lindane. Chromatographic conditions are given in text. Peaks: 1 = 4-chlorothiophenol acetate (84 ppb, 10^9); 2 = 2,5-dichlorothiophenol acetate (49 ppb); 3 = 3,4-dichlorothiophenol acetate (215 ppb); 4 = 2,4,5-trichlorothiophenol acetate (91 ppb).

hydroxide, ranging from 0.1 to 1 N, and volumes of acetic anhydride, ranging from 1 to 10 ml, were examined. The optimum reaction medium providing the most consistent and efficient derivatization proved to be 50 ml of 0.4 N sodium hydroxide and 5 ml of acetic anhydride. A reaction temperature of 65°C had the highest yield of acetate derivatives. Because this method required no sample transfer steps, sample loss was minimized and analysis time reduced.

Extraction of the acetates from the reaction medium was a simple and efficient procedure. However, the acetates were adsorbed by the anhydrous sodium sulfate when insufficient hexane wax used in the extraction or in the rinse. An extraction volume of 5 ml followed by a 3-ml rinse proved adequate to prevent adsorption. Further extraction of the reaction medium with a more polar solvent showed no residual acetates.

The derivatized standards were examined for determination of stability at room temperature and at $-15°C$ with no detectable degradation after three days. After the stability of the standards was ascertained, the four chlorothiophenols were derivatized on four consecutive days and then analyzed with good reproducibility (S.D. 4.3%). Once the reproducibility of the standards was established, fortified control urine samples were analyzed for recovery values. Recoveries of 4-chlorothiophenol, 2,5-dichlorothiophenol, 3,4-dichlorothiophenol, and 2,4,5-trichlorothiophenol from the fortified control urine samples were 84%, 93%, 82%, and 92%, respectively. Due to the high levels of chlorothiophenols found in the hydrolyzed urine, no effort was made to determine the method sensitivity. A representative chromatogram of the chlorothiophenol acetates from a treated rat is shown in Fig. 4.

In summary, mercapturic acid biosynthesis is a vital detoxification mechanism, the full importance of which is still being investigated. Therefore, an improvement in the analysis of mercapturic acids would be advantageous. We have described a simple and rapid procedure for the analysis of chlorophenylmercapturic acids as chlorothiophenol acetates. With this method, we feel that important toxicological information can be obtained.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 2,4- AND 2,6-TOLUENEDIAMINE IN AQUEOUS EXTRACTS

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SUMMARY

A method is presented for the determination of 2,4- and 2,6-toluenediamine (TDA) in aqueous extracts of food-contact boil-in-bag and retortable pouches. The extracts are subjected to a methylene chloride extraction clean-up procedure which effects a 50-fold concentration of the amines. A 500-μl portion of the concentrate is analyzed by high-performance liquid chromatography on a reversed-phase, 5-μm, C_8 column. The mobile phase is 10% acetonitrile in aqueous pH 7.4 phosphate buffer at a flow-rate of 1 ml/min. The lower limit of quantitation is *ca.* 1 ng of TDA on-column or a concentration of *ca.* 40 ng of TDA per liter in the unconcentrated aqueous extracts. Recovery data averaged 89 $\pm$ 4% for standard solutions and spiked extracts over the concentration range 50–2000 ng/l. Results of migration studies are also presented.

INTRODUCTION

Polymeric food-packaging materials contain numerous components that have the potential to migrate out of the polymer matrix and into the food. If any of these components are hazardous to humans, the extent of their migration must be measured and shown to be within acceptable safe limits. Our laboratory wanted to determine whether any 2,4-toluenediamine (TDA) migrates from boilable and retortable plastic food pouches. Toluenediisocyanate (TDI), a component in adhesives used for laminating these products, is readily hydrolyzed to TDA and is the source of its potential migration. Any migration of 2,4-TDA into foods must be closely monitored because animal-feeding studies have indicated that it is carcinogenic[1–3].

Evidence for the migration of aromatic amines at the low parts-per-billion (ppb) (micrograms per liter) level is found in data submitted in manufacturers' petitions for the regulated use of adhesives containing TDI. Earlier studies in our laboratory also suggested such migration. Because the method used to monitor this migration was a non-specific colorimetric technique that responded generally to primary and secondary aromatic amines, we sought a new method which would be both specific and sensitive for quantitating 2,4-TDA at low or sub-ppb concentrations.

Several methods have been reported in the literature that use both gas (GC)

and liquid chromatography (LC) for the analysis of aromatic amines[4-8]. However, none of the reported uses deals with aqueous solutions of TDA at the ppb level, where interactions with "active sites" would be most pronounced. The method reported here employs the technique of ion-suppression high-performance liquid chromatography (HPLC) specifically to minimize these interactions. Consequently, peak tailing is minimized and both sensitivity and resolution are maximized.

We were able to improve sensitivity even more by studying the effect of sample solvent composition on chromatographic peak shape[9]. We achieved minimal peak broadening as the net result while using the exceptionally large injection volume of 500 μl.

The lower limit of reliable quantitation was reduced still further by a methylene chloride extraction procedure which effected a 50-fold concentration and also removed interfering and late-eluting peaks.

EXPERIMENTAL

Equipment

The liquid chromatograph used was a DuPont 850 LC (DuPont, Wilmington, DE, U.S.A.) with fixed-wavelength UV detector, column oven compartment, and a Rheodyne 7125 syringe-loading injector (Rheodyne, Cotati, CA, U.S.A.). LC operating conditions were the following: flow-rate, 1 ml/min; oven temperature, 40°C; UV detector, 254-nm filter, 0.002 a.u.f.s.; injection volume, 500 μl. The UV detector was connected in series to a Spectrum Model 1021A electronic filter/amplifier (Spectrum, Newark, DE, U.S.A.), operated at a frequency cutoff of 0.02 Hz and a gain of either 1X or 2X, and a Hewlett-Packard 7130A strip chart recorder (Hewlett-Packard, Palo Alto, CA, U.S.A.). The reversed-phase column was a 5-μm, Ultrasphere-octyl (Altex, Berkeley, CA, U.S.A.), 250 × 4.6 mm I.D. Other columns were also evaluated: Zorbax ODS, Zorbax TMS (DuPont), 6-μm, 250 × 4.6 mm I.D.; Spherisorb ODS (Spectra-Physics, Santa Clara, CA, U.S.A.), 10-μm, 250 × 2.1 mm I.D.

Methylene chloride extracts were concentrated in a Buchler flash evaporator (Buchler, Fort Lee, NJ, U.S.A.) connected to the house vacuum (*ca.* 20 in. Hg) and a water bath maintained at *ca.* 30°C. Retortable and boil-in-bag pouches were sealed with a Sentinel Model 12AS heat sealer (Packaging Industries, Hyannis, MA, U.S.A.).

Reagents and materials

The acetonitrile used in the mobile phase was HPLC grade (Fisher, Pittsburgh, PA, U.S.A.), and the mono- and disodium phosphate buffers were Fisher reagent grade. Water was prepared by passing the "house" distilled water through a Millipore (Bedford, MA, U.S.A.) Milli-Q purification system. Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) distilled-in-glass, pesticide grade, methylene chloride was used for the sample concentration procedures. The 2,4- and 2,6-TDA, 98% purity, were obtained from Aldrich (Milwaukee, WI, U.S.A.) and used as received.

The retortable pouches used in the extraction studies were obtained from four different manufacturers. The boil-in-bag pouches were all purchased locally.

Standard TDA solutions

Standard solutions of 2,4- and 2,6-TDA were prepared by dissolving known weights of TDA in known volumes of solvent. Typically a stock TDA solution in water was prepared at the parts-per-million (ppm) (milligrams per liter) level, and aliquots were taken and diluted to the desired ppb levels with aqueous pH 7.4 buffer having the following composition: 0.01 M HCl, 0.01 M NaOH, 0.001 M NaH$_2$PO$_4$, 0.003 M Na$_2$HPO$_4$. Both the stock (ppm-level) and the working (ppb-level) solutions were stable for periods of several weeks.

Boil-in-bag and retortable pouch extraction procedure

The aqueous boil-in-bag extracts were obtained by filling the bags with Milli-Q purified water (2.5 ml/in.^{2}) and heat-sealing them (25 p.s.i., 350°F, 2 sec), immersing the bags in a boiling water bath (*ca.* 212°F) for 2 h, and then transferring the contents while hot to suitable glass storage containers. Aqueous retortable pouch extracts were similarly obtained by filling the pouches with Milli-Q water (4 ml/in.^{2}), heat-sealing (25 p.s.i., 350°F, 2 sec), and retorting for 2 h (250°F, 15 p.s.i.). The pouches were allowed to cool and then were stored unopened at ambient temperature until analyzed.

Sample clean-up/concentration procedure

Before LC analysis, the aqueous sample extracts were "washed" and concentrated by the following methylene chloride extraction procedure. A 250-ml portion of aqueous sample extract was transferred to a 500-ml separatory funnel. A 1-ml portion of 0.4 M HCl was added and the mixture was shaken with 100 ml of methylene chloride. The methylene chloride layer, which contained the acidic and neutral extractable components, was drained off and discarded. The aqueous extract was then made basic by the addition of 1.5 ml of 0.4 M NaOH, followed by extraction with two 150-ml portions of methylene chloride. The methylene chloride extracts, which totaled 300 ml, were combined in a 500-ml round-bottom flask. A 4-ml portion of 0.01 M HCl was added as a holding solvent and the methylene chloride was removed under vacuum (*ca.* 20 in. Hg) at 30°C. The 4 ml of sample concentrate was transferred to a 5-ml volumetric flask with a disposable pipet and neutralized by dilution to volume with a solution having the following composition: 0.04 M NaOH, 0.005 M NaH$_2$PO$_4$, 0.015 M Na$_2$HPO$_4$.

RESULTS AND DISCUSSION

Analysis of basic solutes, such as 2,4-TDA, by reversed-phase (RP) LC often is complicated by adsorptive interactions with "uncapped" silanol groups present in the silica-based packing material. These adsorptive interactions contribute to chromatographic peak tailing and cause loss of resolution and sensitivity.

Ion-suppression and ion-pairing are two techniques used in RPLC to minimize the detrimental effects of these interactions[10–12]. The overall mechanisms involved in these two techniques are not fully understood, but both have been used to dramatically improve the chromatography of ionizable solutes.

In ion-suppression HPLC, buffers are added to the mobile phase to obtain a pH which minimizes ionization of the solute. Conversely, in ion-pairing HPLC the

mobile phase pH is adjusted to maximize ionization, and a counter-ion such as an alkyl sulfonic acid is added, which forms a "non-ionic, neutral pair". In both cases, the desired result is attainment of a "neutral" solute, one which is less attracted to the residual silanol groups.

We evaluated both of these techniques for the trace analysis of aqueous solutions of 2,4- and 2,6-TDA. Ion-suppression HPLC produced sharper, more symmetrical peaks than ion-pairing HPLC, enabling greater sensitivity and resolution of TDA. Therefore, ion-suppression HPLC was the technique selected for this work.

Ion-suppression HPLC

We achieved ion-suppression of TDA by buffering the acetonitrile–water mobile phase with mono- and dibasic sodium phosphate. The effect of mobile-phase pH on the HPLC determination of TDA was studied over the pH range 4.5–8.5. For a mobile-phase pH range of *ca.* 6.5 to 8.5, good peak symmetry and stable retention volumes were observed (Fig. 1A). When the mobile-phase pH was lower than *ca.* 6.5, chromatographic peak broadening occurred, resulting in diminished peak heights and a significant reduction in resolving power (Fig. 1B).

MILLILITERS
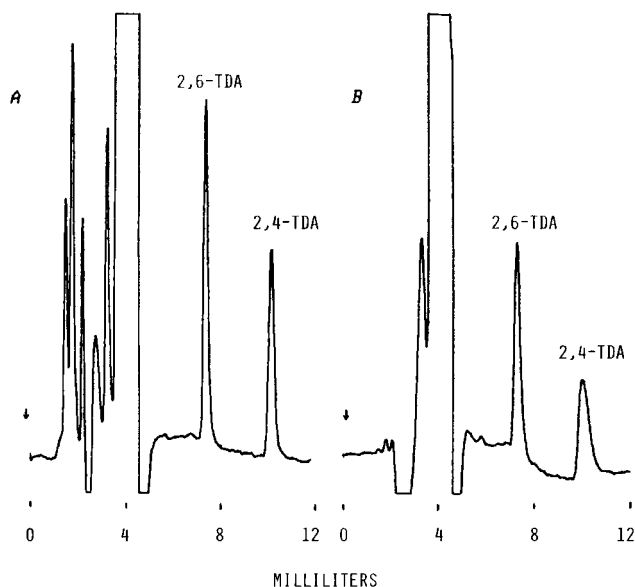
Fig. 1. Mobile-phase pH effect. Conditions: 500-μl injection volume, 1 ml/min, 40°C, 25 ppb of 2,4- and 2,6-TDA in pH 7.4 phosphate buffer, UV detector at 254 nm, 0.002 a.u.f.s. Mobile phase: (A) 10% acetonitrile/pH 7.4; (B) 10% acetonitrile/pH 5.0.

Consequently, we selected a mobile-phase pH of 7.4 (0.001 M NaH$_2$PO$_4$, 0.003 M Na$_2$HPO$_4$) to achieve the ion-suppression of TDA while staying within the pH restrictions of the silica-based column packing material. As a further precaution, we minimized any pH effect on the packing material by flushing the column nightly with 20 ml of water followed by 20 ml of acetonitrile. Columns treated in this manner exhibit useful lifetimes of 5–6 months.

The dependence of TDA retention volume on the acetonitrile concentration in

the pH 7.4 phosphate buffer was also studied (Table I). As would be expected for a C_8 reversed-phase column, the TDA retention volume increased as the acetonitrile concentration was decreased, particularly at acetonitrile concentrations lower than 8%. Low acetonitrile concentrations also resulted in poor column efficiency, as evidenced by the increase in the height equivalent to a theoretical plate (HETP).

TABLE I

VARIATION IN RETENTION VOLUME OF TDA WITH PERCENT ACETONITRILE IN MOBILE PHASE

Conditions: Ultrasphere-octyl, 250 × 4.6 mm I.D. column, 1 ml/min, 40 C, 254 nm, 0.002 a.u.f.s., 500-μl injection of 25 ppb each of 2,4- and 2,6-TDA.

Acetonitrile (%)*	Retention volume (ml)		Peak height (mm)		HETP · 10^{-2} (mm)	
	2,6-TDA	2,4-TDA	2,6-TDA	2,4-TDA	2,6-TDA	2,4-TDA
20	5.1	5.9	150	105	1.5	1.3
15	5.8	7.2	120	75	1.6	1.5
10	7.0	9.8	80	50	1.9	1.7
8	8.0	11.8	70	40	2.1	1.7
5	10.0	16.0	50	25	2.7	1.9

* Percent acetonitrile in pH 7.4 phosphate buffer.

We found that 10% acetonitrile in pH 7.4 phosphate buffer was the mobile phase best suited for the samples of interest in this study. Consistent retention volumes and adequate resolution from surrounding peaks were observed for most samples examined. With this mobile phase, 2,6- and 2,4-TDA retention volumes were *ca.* 6.8 and 9.3 ml, respectively; the corresponding relative standard deviations were ± 0.5 and $\pm 0.4\%$.

Alternative reversed-phase columns

In addition to the Ultrasphere-octyl column, three other reversed-phase columns were evaluated for the HPLC determination of TDA. In these tests the Zorbax ODS and Ultrasphere C_8 columns produced very similar separations. The main difference between them was that the Zorbax ODS column showed slightly more retention of both TDA and the late-eluting components present in most samples analyzed. The second column, a Spherisorb ODS, exhibited limited suitability in actual sample analysis for TDA. Because of its larger particle diameter (10 μm) and smaller column inner diameter (2.1 mm) the number of theoretical plates obtained for this column was insufficient to separate the TDA chromatographic peak from the others surrounding it. With the third column tested, a Zorbax TMS, we were also unable to resolve TDA from interferences present in most sample extracts.

In general, the best separation of 2,4- and 2,6-TDA in aqueous extracts was achieved on the octyl, 5 μm, 250 × 4.6 mm I.D. column. A C_{18} reversed-phase column (5 μm, 250 × 4.6 mm I.D.) would probably be a suitable substitute; however, a slightly longer analysis turnaround time and a marginally broader 2,4-TDA peak might be experienced.

Injection volume

As stated above, TDA was strongly retained on the reversed-phase column when very low acetonitrile concentrations were used in the mobile phase. As a result, it was possible to obtain a "concentration effect" upon injection when distilled water or an aqueous buffer was used as the sample solvent; *i.e.*, TDA partitioned into the stationary phase at the very head of the column and did not begin to elute down the column until the sample solvent was replaced with the mobile phase containing aceto-nitrile. This selection of a sample solvent which was "weaker" than the mobile phase permitted an injection volume of 500 μl, rather than the typical injection volume of 10–50 μl. Thus sensitivity to TDA was maximized with minimal loss of column efficiency (Table II).

TABLE II

VARIATION IN TDA PEAK VOLUME WITH INJECTION VOLUME

Conditions: 10% acetonitrile/pH 7.4, 1 ml/min, 254 nm, 40°C, Ultrasphere-octyl column. Samples: 100 ppb 2,6- and 2,4-TDA in pH 7.4 phosphate buffer.

Injection volume (μl)	Peak volume* (μl)		Theoretical plates** per meter	
	2,4-TDA	2,6-TDA	2,4-TDA	2,6-TDA
20	295	195	62,000	70,000
50	300	205	61,000	66,000
200	315	220	57,000	59,000
500	345	250	51,000	52,000
1000	380	315	47,000	37,000

* Peak volume determined at base by tangent skimming.
** Theoretical plates/meter = [16 (ret. vol./peak vol.)^2] × 4.

This concentration effect is clearly seen in Table II where chromatographic peak volumes are compared with injection volumes. With a 20-μl injection volume, 2,4-TDA eluted *ca.* 9.2 ml after injection with a peak volume that measured *ca.* 295 μl. (Peak volumes were measured at the base by tangent skimming.) With a 500-μl injection volume, 2,4-TDA eluted *ca.* 9.7 ml after injection but the peak volume measured only *ca.* 345 μl, which was *ca.* 30% less than the original injection volume of 500 μl. The net result of this concentration effect was that the number of theoretical plates obtained with the 500-μl injection was roughly 80% of the number obtained with the 20-μl injection.

For this concentration effect to occur, it is imperative that the pH of the sample injected be slightly basic to prevent ionization of TDA; otherwise, much broader chromatographic peaks similar to those observed in Fig. 1B are produced. Therefore, the samples, like the mobile phase, were also buffered to pH 7.4 with mono- and dibasic sodium phosphate.

The routine use of this unusually large injection volume of 500 μl did pose one problem. Both the samples and the standards inevitably contained trace amounts of very fine particulate matter which were not removed by filtration before injection. A

variety of filters were examined, and all either partially retained TDA and/or gave rise to numerous interfering peaks. Consequently, all injected samples and standards were unfiltered and the particulate matter was allowed to accumulate on the column inlet frit (5-μm pore diameter). As a result, the column inlet frit required replacement about once a week. Replacement of the column inlet frit was indicated, not by an increase in the column back-pressure, but by tailing of the chromatographic peaks.

We found that we could further improve chromatographic peak symmetry by removing and repacking the first 2–3 mm of column packing material about once every 2–3 weeks. Evidently some of the particulate matter was small enough to pass through the inlet frit and accumulated on the head of the column.

Detector high-frequency noise

Operation of the UV detector at its maximum sensitivity of 0.002 a.u.f.s. yielded a 20% deflection for a 500-μl injection of 20 ppb 2,4-TDA. However, considerable high-frequency noise occurred at this sensitivity. We effectively removed this high-frequency noise (Figs. 2A and 2B) by connecting the detector output to an electronic noise filter, operated at a frequency cutoff of 0.02 Hz. The electronic filter was also capable of amplifying the detector signal, and a usable baseline was obtained with an amplification factor of 2. Under these conditions (0.002 a.u.f.s., amplification × 2, frequency cutoff 0.02 Hz) a deflection of *ca.* 5% was recorded for a 500-μl injection of 2 ppb 2,4-TDA (Fig. 2B).
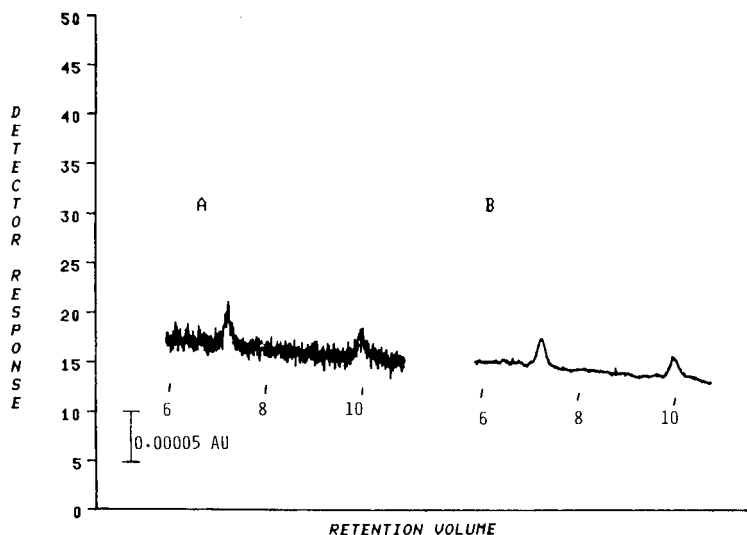
Fig. 2. Effects of detector high-frequency noise filtering. Conditions: 500-μl injection volume, 2 ppb of 2,6- and 2,4-TDA, 10% acetonitrile/pH 7.4, 1 ml/min, 0.002 a.u.f.s., amplification × 2. (A) No high-frequency noise filtering; (B) 0.02-Hz cutoff frequency.

External standard calibration

TDA was quantitated by the external calibration technique; for calibration, 500-μl portions of standard solutions were injected for a series that contained 2,4- and 2,6-TDA in a 2–50 ppb concentration range. The peak maxima were recorded at each

concentration, and a linear regression was calculated. Fig. 3 shows a typical cali-
bration plot having linear coefficients ($y = m x$) of better than 0.988 for 2,6- and 2,4-
TDA with relative standard errors of ± 1 and $\pm 1.7\%$, respectively.

2,4- AND 2,6-TDA LINEAR CALIBRATION
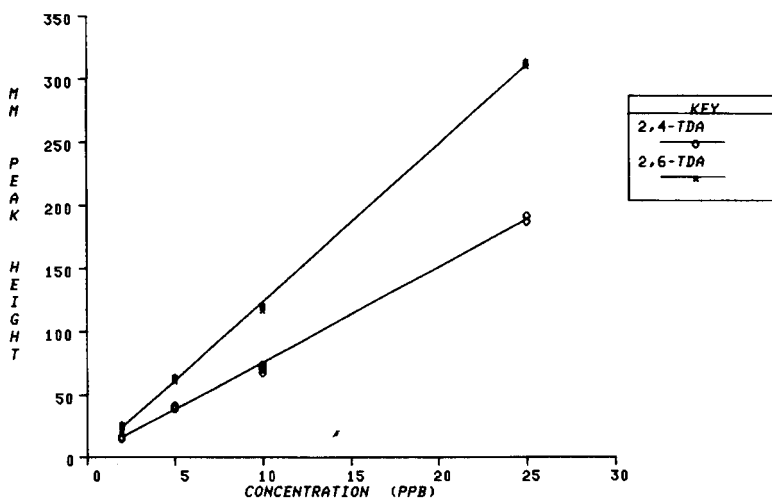
Fig. 3. 2,4- and 2,6-TDA linear calibration plots. LC conditions same as Fig. 2B.

Sample preparation

Aqueous extracts from a variety of boil-in-bag and retortable pouches were
analyzed for 2,4- and 2,6-TDA under the LC conditions outlined above. We obtained
the extracts by filling the boil-in-bag and retortable pouches with water (2.5–4
ml/in.²) and boiling (212°F) or retorting (250°F) for a specified length of time. Pre-
liminary LC analyses of some of the extracts indicated that, if TDA was present, the
concentrations were below the LC quantitation limit (2 ppb) and sample concentra-
tion would be required. In addition to any possible 2,4- and 2,6-TDA, numerous
other components, presumably from both the plastic films and the laminating adhe-
sives, were extracted under these conditions. Therefore a "sample clean-up" was also
needed to remove these potentially interfering and late-eluting components.

Methylene chloride proved to be the ideal solvent for this clean-up and TDA
extraction since partitioning of TDA between methylene chloride and water is very
pH-dependent. Thus, when the samples (250 ml) were acidified (1 ml of 0.4 N HCl) as
the first step, most of the interfering and late-eluting components were selectively
extracted into the methylene chloride layer (100 ml) and all of the TDA was left
behind. After next adding sodium hydroxide to make the sample basic (1.5 ml of 0.4
N NaOH), we achieved an almost quantitative extraction of TDA by re-extracting
with two 150-ml portions of methylene chloride.

The TDA was concentrated by removal of the methylene chloride under
vacuum (20 in. Hg) at 30°C. Evaporating the methylene chloride directly to dryness
and subsequently dissolving the TDA in 5 ml of water gave low and inconsistent

recoveries. Adding 5 ml of water to the methylene chloride before evaporation improved the precision, but TDA recovery was only *ca.* 65%. Acidifying the 5 ml of water with HCl increased TDA recovery to *ca.* 90%.

Because it was necessary to neutralize the final concentration before injection into the LC, we used a 4-ml volume of 0.01 M HCl as the "holding solvent." After the methylene chloride was removed under vacuum, the 4 ml of 0.01 M HCl was quantitatively transferred to a 5-ml volumetric flask. A 1-ml portion of a 0.04 M NaOH solution was added, and the resulting solution was diluted to volume with water. The 0.04 M NaOH solution also consisted of 0.005 M NaH_2PO_4 and 0.015 M Na_2HPO_4, which helped to buffer the sample to the desired pH. The final 5-ml volume represented a 50-fold concentration of the TDA originally present in the 250-ml aqueous sample extract. Using less than 4 ml of 0.01 M HCl as the holding solvent to achieve a greater than 50-fold concentration gave poor TDA recoveries.

TABLE III

RECOVERY OF TDA WITH USE OF METHYLENE CHLORIDE EXTRACTION PROCEDURE

Solution analyzed	Added (ppb)	Found* (ppb)	Average recovery (%)
		2,6-TDA	
Standard	0.05	0.047, 0.052, 0.050	99
	0.10	0.092, 0.085, 0.085	87
	0.25	0.238, 0.233, 0.225	93
	0.50	0.435, 0.450, 0.435	88
	2.0	1.68, 1.73, 1.78	87
Average ± SD			91 ± 3.5
Retortable pouch extract	0.00	1.62, 1.56, 1.54 (1.57)**	
	2.00	3.47, 3.62, 3.59 (3.64)	92
Boil-in-bag extract	0.00	0.09, 0.09, 0.09 (0.09)	
	0.20	0.25, 0.26, 0.26 (0.26)	85
		2,4-TDA	
Standard	0.05	0.041, 0.044, 0.049	89
	0.10	0.078, 0.081, 0.089	83
	0.20	0.190, 0.178, 0.178	91
	0.50	0.455, 0.455, 0.450	90
	1.0	0.88, 0.93, 0.92	91
Average ± SD			88 ± 4.5
Retortable pouch extract	0.00	1.88, 1.86, 1.78 (1.84)	
	2.00	3.71, 3.62, 3.59 (3.64)	90
Boil-in-bag extract	0.00	0.06, 0.07, 0.06 (0.06)	
	0.20	0.22, 0.23, 0.23 (0.23)	84

* As determined by LC analysis.
** Average value is given in parentheses.

Precision and recovery

   We determined the overall precision of the method and the anticipated levels of recovery by performing the methylene chloride concentration procedure, followed by LC analysis, with a series of aqueous standards. Triplicate, 250-ml portions of six standards, containing both 2,4-TDA and 2,6-TDA at concentrations ranging from 50 parts per trillion (ppt) (nanograms per liter) to 2 ppb, were concentrated to 5 ml and then quantitated by LC analysis. The results, presented in Table III, show average recoveries of 88 % for 2,4-TDA and 91 % for 2,6-TDA with relative standard deviations of $\pm 4.5 \%$ and $\pm 3.5 \%$, respectively. Similar levels of precision and recovery were also found for spiked composites of retortable pouch and boil-in-bag extracts (Table III).

   Fig. 4A shows a chromatogram for a 500-μl injection of a 50 ppt aqueous
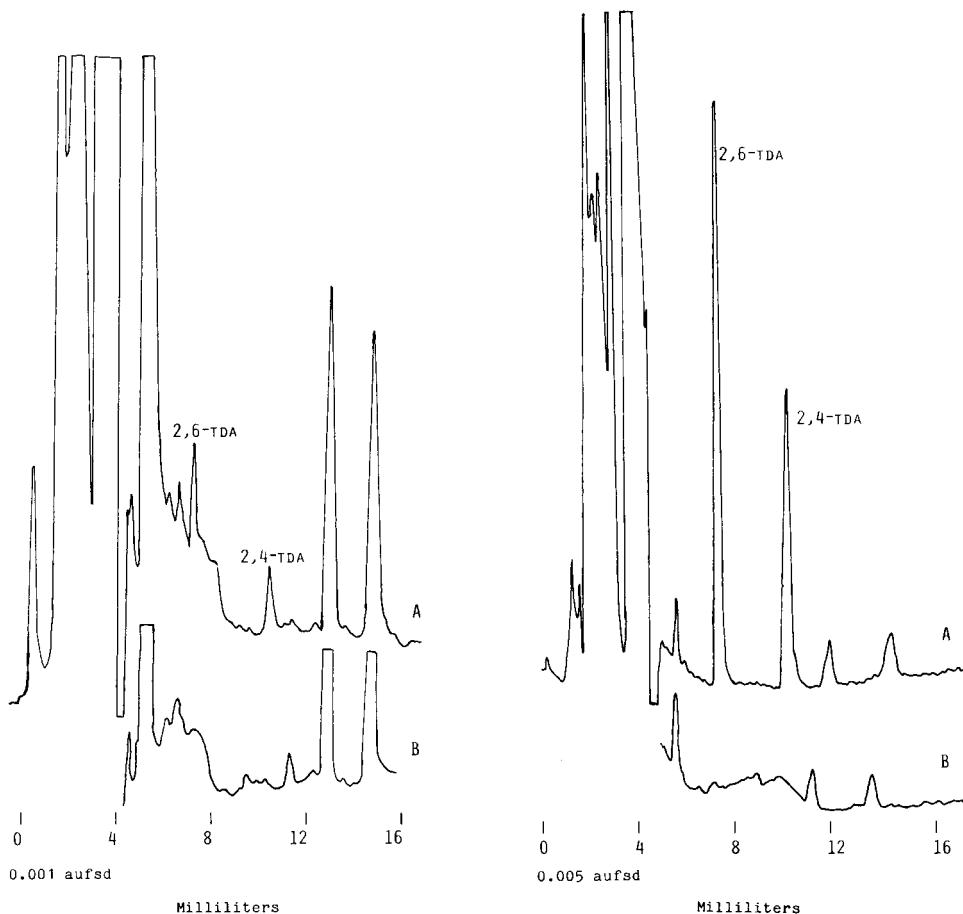
Fig. 4. (A) 500-μl injection of an aqueous 50 ppt 2,4- and 2,6-TDA standard carried through the methylene chloride 50-fold concentration procedure. (B) Distilled water blank carried through methylene chloride procedure. LC conditions same as Fig. 2B.

Fig. 5. (A) 500-μl injection of an aqueous 2 ppb 2,4- and 2,6-TDA standard carried through the methylene chloride 50-fold concentration procedure. (B) Distilled water blank carried through methylene chloride procedure. LC conditions same as Fig. 2B.

standard carried through the methylene chloride concentration procedure; the original aqueous sample extract was 250 ml, and the 500 μl injected was taken from the final 5-ml concentrate. We believe that 50 ppt is the lower limit of reliable quantitation. The calculated concentrations of 2,6- and 2,4-TDA for these peaks are 2.4 and 2.3 ppb, respectively, and indicate corresponding recoveries of 96 and 92%. Fig. 4B shows the chromatogram for a 500-μl injection of a "reagent blank", 250 ml of distilled water carried through the methylene chloride procedure, demonstrating the lack of any significant interferences even at a maximum sensitivity of 0.001 a.u.f.s. Fig. 5A and 5B show chromatograms for 500-μl injections of a 2-ppb aqueous standard of 2,4- and 2,6-TDA and a distilled water blank carried through the methylene chloride 50-fold concentration procedure.

Retortable pouch and boil-in-bag extracts

A variety of retortable pouch and boil-in-bag extracts were analyzed for apparent migration of 2,4- and/or 2,6-TDA. The extracts were obtained according to the conditions described in the Experimental section by extracting with distilled water at either 250 or 212°F for 2 h. Each aqueous extract was concentrated 50-fold by the methylene chloride extraction procedure followed by LC analysis. Apparent levels of 2,4- and 2,6-TDA migration measured in these extracts are listed in Table IV. Typical LC chromatograms for a boil-in-bag and a retortable pouch extract are represented in Figs. 6A and 6B, respectively. The apparent levels of 2,4- and 2,6-TDA are 1.8 and 1.6 ppb, respectively, for the retortable pouch extract and 0.1 and 0.2 ppb, respectively, for the boil-in-bag extract.

CONCLUSIONS

Ion-suppression was found to be a useful technique for the HPLC determi-

TABLE IV

APPARENT 2,4- AND 2,6-TDA MIGRATION FROM RETORTABLE POUCH AND BOIL-IN-BAG EXTRACTS

Manufacturer	Apparent migration (ppb)	
	2,4-TDA	2,6-TDA
	Retortable pouch*	
A	5.0	5.0
B	2.0	4.0
C	1.5	2.5
D	3.0	2.5
	Boil-in-bag**	
E	0.05	0.08
F	0.4	0.6
G	ND***	ND***
H	0.7	0.7
I	0.1	0.2

  * Retorted 2 h at 250°F (4 ml H$_2$O/in.^{2}).
 ** Boiled 2 h at 212°F (2.5 ml H$_2$O/in.^{2}).
*** ND, less than 40 ppt.
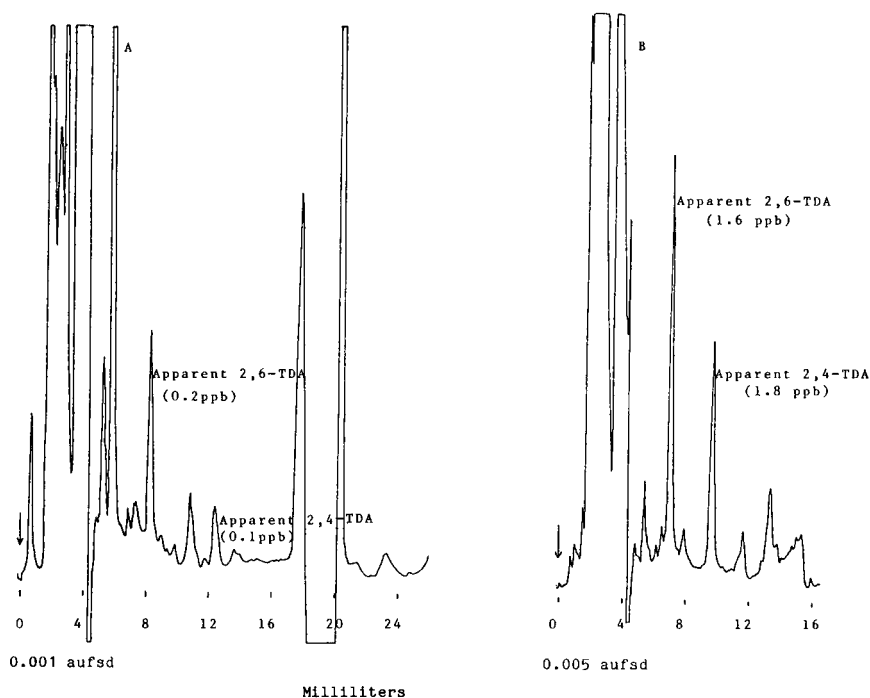
Fig. 6. (A) 500-μl injection of a boil-in-bag extract carried through the methylene chloride concentration procedure. (B) 500-μl injection of a retortable pouch extract carried through the methylene chloride concentration procedure. LC conditions same as Fig. 2B.

nation of 2,4- and 2,6-TDA in aqueous extracts. Quantitation of TDA at the ppt level was made possible by the exceptionally large injection volume of 500 μl. Apparent TDA concentrations ranging from 50 ppt to 5 ppb were measured in a variety of retorted pouch and boil-in-bag extracts. The data were compared with results obtained with a nitrogen-selective gas chromatographic procedure[13]. Analyses by gas chromatography–mass spectrometry afforded data consistent with the presence of TDA in the samples.

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AROMATIC HYDROCARBON METABOLITES IN FISH: AUTOMATED EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION INTO CONJUGATE AND NON-CONJUGATE FRACTIONS

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SUMMARY

An automated extractor–concentrator was used to extract metabolites of naphthalene, 2,6-dimethylnaphthalene, and benzo[a]pyrene from serum, bile and liver homogenate of rainbow trout (*Salmo gairdneri*). The extracts were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. Recoveries of naphthalene and 2,6-dimethylnaphthalene metabolites from all matrices were generally greater than 90%; however, the recoveries of benzo[a]pyrene metabolites from serum ranged from 37–99%. In addition, conjugated metabolites of polycyclic aromatic hydrocarbons (PAHs) were separated from non-conjugated metabolites and parent PAHs by using two diol columns with normal-phase HPLC. The extraction and separation techniques were also applied to isolate metabolites in samples from fish fed 2,6-dimethylnaphthalene.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), which are prominent xenobiotics in the marine environment, have been shown to be toxic to marine life[1–4]. PAHs are readily biotransformed and their metabolic products accumulate in tissues of exposed organisms[5–8]. An important requirement for assessing the effect of PAHs and their metabolites on marine organisms is the identification and quantitation of these compounds in the tissues and fluids of exposed organisms. Sometimes, analytes in biological fluids can be quantitated by injecting the fluid directly into a reversed-phase high-performance liquid chromatography (HPLC) system[9]. Usually, analytes must first be separated from their biological matrices before being determined by the conventional techniques of HPLC, gas chromatography (GC), or gas chromatography–mass spectrometry (GC–MS).

Solvent extraction[1,10–12] is used routinely to extract analytes from biological samples, but this method has its limitations. For example, some extraction techniques do not extract polar analytes efficiently[12]. Also, extracts often contain naturally occurring compounds (*e.g.*, lipids) that interfere with the analyses. Moreover, solvent

extraction can be the most laborious and least precise part of the assay[13]. The precision of HPLC assays with prior solvent extraction is generally poorer than that of assays with direct injection of samples (5–10% S.D. *vs.* 1–3%)[13]. However, direct injection is limited to biological fluids introduced into reversed-phase systems, because non-polar normal-phase solvents can precipitate sample components such as serum proteins and bile salts, which can then block the HPLC injector, tubing or column.

Two totally automated systems, column switching[14] and "FAST-LC"[15], allow for effective separation of analytes from biological fluids and rapid analysis by reversed-phase HPLC; however, tissue samples must be extracted and the extracts injected. Alternatively, Williams and Viola[16] developed a rapid and reproducible method for automated extraction of certain drugs from serum using an extractor–concentrator. Their method appeared promising for our work with PAH metabolites, so we adapted it for use with fish serum, bile and liver homogenate.

In addition, when determining PAH metabolites, separation of samples into conjugate and non-conjugate fractions is frequently desired. Non-conjugated metabolites can be determined directly, but the conjugates are often enzymatically hydrolyzed to aid in their analysis. Consequently, we devised a rapid normal-phase chromatographic method for separating conjugated PAH metabolites from non-conjugated metabolites and parent PAHs.

EXPERIMENTAL*

Chemicals

NPH**, DMN and BaP were obtained from Aldrich (Milwaukee, WI, U.S.A.). The NPH metabolite standards were purchased from Sigma (St. Louis, MO, U.S.A.), and the DMN metabolite standards were prepared in our laboratories[7]. The National Cancer Institute (Bethesda, MD, U.S.A.) provided the BaP metabolite standards. Citric acid monohydrate (U.S.P.) was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Disodium hydrogen phosphate heptahydrate and potassium chloride (both "analyzed-reagent" grade), Ultrex acetic acid, and the HPLC-grade solvents 2-propanol and water were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). The "distilled-in-glass" solvents, acetone, hexane and methanol, were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Fish exposures

Rainbow trout (*Salmo gairdneri*) were maintained in flow-through freshwater

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* Reference to a company or product does not imply endorsement by the U.S. Department of Commerce to the exclusion of others that may be suitable.

** Abbreviations: Naphthalene (NPH) metabolites: 1-naphthyl-β-D-glucuronic acid, naphthyl glucuronide; 1-naphthyl sulfate, naphthyl sulfate; 2-naphthyl-α-D-glucoside, naphthyl glucoside. 2,6-Dimethylnaphthalene (DMN) metabolites: 2,6-dimethyl-3-naphthol, 3-hydroxyDMN; 2,6-dimethyl-3,4-naphthoquinone, DMN 3,4-quinone; *trans*-3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, DMN 3,4-dihydrodiol; and 6-methyl-2-naphthalenemethanol, DMN 2-methanol. Benzo[*a*]pyrene (BaP) metabolites: 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene, BaP 9,10-dihydrodiol; 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene, BaP 7,8-dihydrodiol; 9-hydroxybenzo[*a*]pyrene, 9-hydroxyBaP; 1-hydroxybenzo[*a*]pyrene, 1-hydroxyBaP; 3-hydroxybenzo[*a*]pyrene, 3-hydroxyBaP; benzo[*a*]pyrene 3-sulfate, sodium salt, BaP 3-sulfate; 1-benzo[*a*]pyrenyl-β-D-glucopyranosiduronic acid, BaP 2-glucuronide.

aquaria. Two fish were each force fed 4 mg of DMN and 20 μCi of ^{14}C-labeled DMN (2,6-dimethylnaphthalene-4-^{14}C; 5.9 mCi/mmole, custom synthesized by Wizard Laboratories, Davis, CA, U.S.A.; at least 99 % pure) as described by Collier *et al.*[17]. The DMN was dissolved in 100 μl of ethanol, and the ^{14}C-DMN was dissolved in 50 μl of salmon oil. Two control fish were similarly force fed only the carrier solvents. Twenty-four hours later, the fish were killed and liver, bile and blood samples were collected.

Extraction

Instrumentation. Biological samples were extracted with a Prep I automated extractor–concentrator (DuPont, Wilmington, DE, U.S.A.). Glass microbeads and Type W extraction cartridges packed with a styrene–divinylbenzene copolymer resin were also obtained from DuPont. The extractor–concentrator is a programmable centrifuge, capable of rotating in either direction. During clockwise rotation, the resin cartridge aligns with a waste cup. The prepared biological sample is placed in the cartridge reservoir, and centrifugal force from rotation drives the solution through the bed of resin. The analytes are sorbed by the resin, while water-soluble components elute into the waste cup. When the programmed rotor reverses direction, the outer ring rotates to align the resin cartridge with a sample-recovery cup. An organic solvent then elutes the analytes from the resin into this cup. Detailed operation of this instrument has been described by Williams and Viola[16].

Standard solutions. Solutions of NPH, DMN, BaP and their metabolites were prepared in water by diluting a concentrated methanol solution of each. These water standards were spiked into serum, bile and liver homogenate samples to determine recoveries. The aqueous NPH–DMN stock solution contained (ng/μl): naphthyl sulfate, 15.5; naphthyl glucuronide, 2.35; 1-naphthol, 87.0; NPH, 5.00; DMN dihydrodiol, 17.3; DMN 2-methanol, 3.95; 3-hydroxyDMN, 2.98, DMN quinone, 9.35, and DMN, 4.15. Two dilutions were prepared (40 % and 8 % of stock concentration). The 40 % solution was used to determine recoveries in serum, bile and liver homogenate; the stock solution and the 8 % solution were also used with serum. The BaP standard solution contained (ng/μl): BaP 9,10-dihydrodiol, 1.60; BaP 7,8-dihydrodiol, 1.19; 9-hydroxyBaP, 0.23; 1-hydroxyBaP, 0.18; 3-hydroxyBaP, 0.27; BaP 1-glucuronide, 0.36; BaP 3-sulfate, 0.20 and BaP, 0.205.

Buffers. A citrate–phosphate buffer (0.068 *M* citric acid, 0.064 *M* $Na_2HPO_4 \cdot 7H_2O$, pH 3.8) was prepared for extractions of NPH–DMN reference compounds from serum or liver homogenate. A second citrate–phosphate buffer (1.01 *M* citric acid, 0.2 *M* $Na_2HPO_4 \cdot 7H_2O$, pH 2.5) was prepared for extractions of NPH–DMN reference compounds from bile, for extractions of biologically produced metabolites from serum, bile and liver homogenate and for extractions of BaP reference compounds from serum. An internal standard, 2-naphthyl glucoside (5.75 ng/μl), was added to those portions of the buffer used in sample extraction.

Sample preparation. Serum was obtained from whole blood by centrifugation; bile was used without preparation. For liver, the partially thawed sample was cut into small pieces, 1.5 % (w/v) KCl soln, was added (1:4, w/v), and the mixture was homogenized for 1 min with a Tekmar Tissumizer (Tekmar, Cincinnati, OH, U.S.A.).

To determine recovery efficiencies, serum (0.50 ml), bile (0.10 ml) or liver homogenate (0.50 ml) was mixed with a PAH standard solution (0.50 ml), and the

mixture was added to a cleaned cartridge (see below). Buffer–internal standard (1.00 ml) was added. Samples from rainbow trout fed DMN were prepared as described above, except that the PAH standard solution was omitted.

Cartridge clean-up and extraction. Acid-rinsed glass microbeads (1 g) were added to the cartridge reservoir for extractions of matrices other than serum to prevent the cartridges from plugging. The cartridges were loaded into the instrument, 20 ml of acetone were added to reservoir 1, and the solvent was dispensed and collected. The instrument was reset, reservoir 1 was filled with 10 ml of buffer (without internal standard), and this solution was dispensed and collected. Cleaned cartridges containing the experimental samples (prepared as described above) were loaded into the instrument, and the wash and extraction solvents (see Table I) were added to reservoirs 1 and 2, respectively. The automated program was then executed up to the drying step, and the instrument was stopped and reset to dispense and collect the solvent (Table I) from reservoir 2. This extra extraction step was executed once for samples with spiked reference standards and three times for samples with metabolites produced *in vivo*. After the extraction steps were completed, the total extracts were evaporated at 30°C, under nitrogen, until *ca.* 0.7 ml remained (times of evaporation varied according to the total amounts and types of solvents used for extraction). The entire concentrated extract was transferred to a 1-ml volumetric flask, the volume was adjusted to 1.00 ml, and the solution was analyzed. Twelve samples could be extracted simultaneously, and the total instrument time for the entire procedure was less than 30 min.

TABLE I

WASH AND EXTRACTION SOLVENTS USED FOR AUTOMATED EXTRACTION OF PAH METABOLITES FROM RAINBOW-TROUT SERUM, BILE AND LIVER HOMOGENATE USING THE EXTRACTOR–CONCENTRATOR FOR PROCESSING 12 SAMPLES

Analyte	*Solvents*, ml*				
	Wash (reservoir 1)	*Extraction (reservoir 2)*			
		1	*2*	*3*	*4*
Spiked reference standards	10 W**	10 A***	10 M §	–	–
DMN metabolites formed *in vivo*	10 W**	10 A/M	10 A/M	10 Q	10 Q

* W = water; A = acetone; M = methanol; A/M = acetone–methanol (1:1, v/v); Q = dichloromethane-2-propanol–water (75:25:2, v/v).
** No wash for bile.
*** 20 A for liver homogenate.
§ 20 M for liver homogenate.

High-performance liquid chromatography

Instrumentation

Separations were performed with an 8000B high-performance liquid chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.). An MPF-44A fluorescence spec-

trometer (Perkin-Elmer, Norwalk, CT, U.S.A.) was used for detection and charac-
terization. A UV detector, Spectra-Physics Model 8310, was connected in series with
the fluorescence detector and was used to detect compounds with low fluorescence.
Samples were injected by using a 710A variable-volume automatic sampler (Waters
Assoc., Milford, MA, U.S.A.).

Normal-phase conjugate–non-conjugate separations

Column configuration. Two normal-phase diol columns (Brownlee, Santa
Clara, CA, U.S.A.), a 0.46 × 4-cm OH-GU diol guard column and a 0.46 × 10-cm
OH-MP diol analytical column, were connected to a six-port valve (see Fig. 1). With
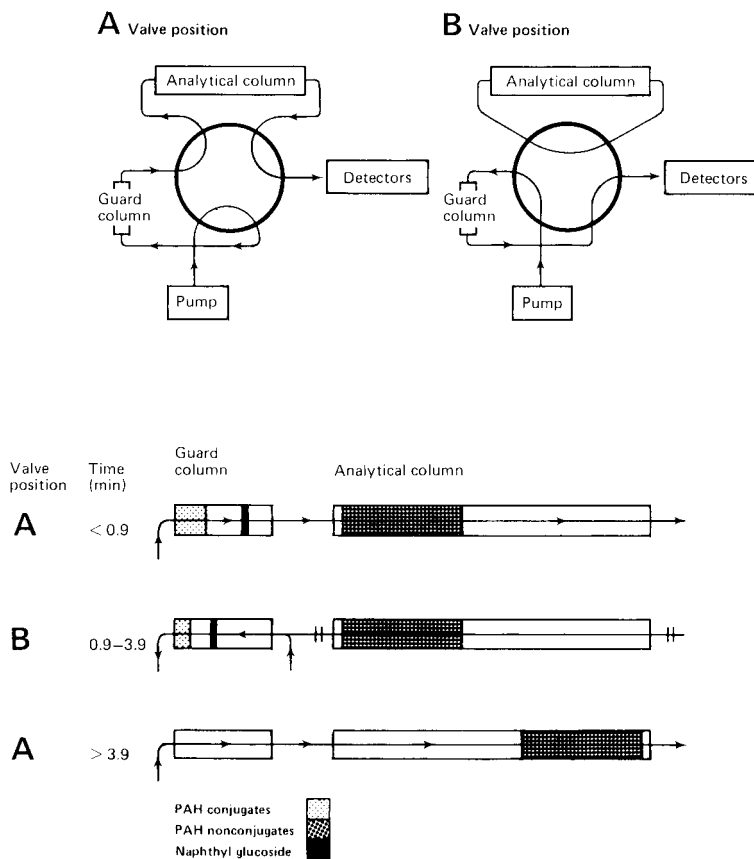
Fig. 1. Top: Connections of diol guard column, diol analytical column, pump and detectors to the six ports
of the two-way valve; the flow-through system is shown for both valve positions A and B. Bottom: Flow
through the two-colomn system as a function of valve position. At injection, with the valve in position A,
eluent flowed through the guard column and the analytical column to the detectors. Conjugated PAH
metabolites moved slowly through the guard column; non-conjugated metabolites and parent PAH moved
quickly through the guard column and on to the analytical column. At 0.9 min, the valve was changed to
position B, trapping the non-conjugated metabolites and parent PAHs on the analytical column and
reversing the flow through the guard column so that the conjugated metabolites were eluted. At about 3.9
min, the valve was returned to position A to elute the non-conjugated metabolites and parent PAHs from
the analytical column.

the valve in position A, the eluent traveled from the pump through the guard and analytical columns to the detectors. When the valve was moved to position B, the eluent traveled from the pump through the guard column in the reverse direction and then to the detectors. The analytical column was isolated with the valve in position B, and materials on this column remained there until the valve was shifted back to position A.

Standard solutions. The stock solutions of NPH–DMN and BaP prepared for the automated extractions were used to calibrate the normal-phase system for conjugate–non-conjugate separations.

Elution conditions. Conjugated and non-conjugated PAH metabolites were separated by using an eluent consisting of hexane–2-propanol (1:1, v/v). The flow-rate was 1 ml/min and the oven temperature was 40°C. The valve position remained at A until 0.9 min after injection, then it was shifted to B; at 3.9 min, the valve position was returned to A.

Fraction collection of serum extracts. Serum extracts from fish fed DMN were separated into two fractions; the first contained conjugated PAH metabolites and the second contained the non-conjugated metabolites and parent PAHs. The conjugated-metabolite fraction was collected while the six-port valve was in position B. The PAH parent compounds and non-conjugated metabolites were collected after the valve position was returned to A (see Fig. 2).

Reversed-phase analyses

Columns. The analytical column was a 0.26 × 25-cm reversed-phase HC-ODS column (Perkin-Elmer). A 0.21 × 5-cm stainless-steel guard column dry-packed with Vydac 37-μm reversed-phase packing (The Separations Group, Hesperia, CA, U.S.A.) was also used.

Gradient-elution separation of NPH–DMN metabolites. Acetic acid–water (0.5:100, v/v; solvent A) and methanol (solvent B) were used in a linear gradient going from 100% solvent A to 100% solvent B in 15 min, then 7 min at 100% B, 3 min to return to 100% A, and 8 min equilibration at 100% A. The flow-rate was 1 ml/min except during equilibration, when it was increased to 2 ml/min. The oven temperature was 50°C. BaP metabolites were separated as reported previously[18].

Quantitations. In chromatograms of analytes recovered from extractions of "spiked" serum, bile and liver-homogenate samples, peak areas were compared with those of chromatographed reference standards. A factor to correct for dilution was applied, and percentage recoveries were calculated. Peak areas of analytes in bile were corrected for contributions from interfering compounds. Responses of the standards were linear in the concentration ranges used.

RESULTS AND DISCUSSION

Automated extractions

Extractions of PAH metabolites from biological samples using the automated extractor–concentrator were rapid and reproducible. Previous automated extraction methods had been limited to biological fluids[14-16], but our adaptation of the extractor–concentrator method allowed extraction of both tissues and fluids. Analytes were separated from the biological matrices by chromatography on resin-packed
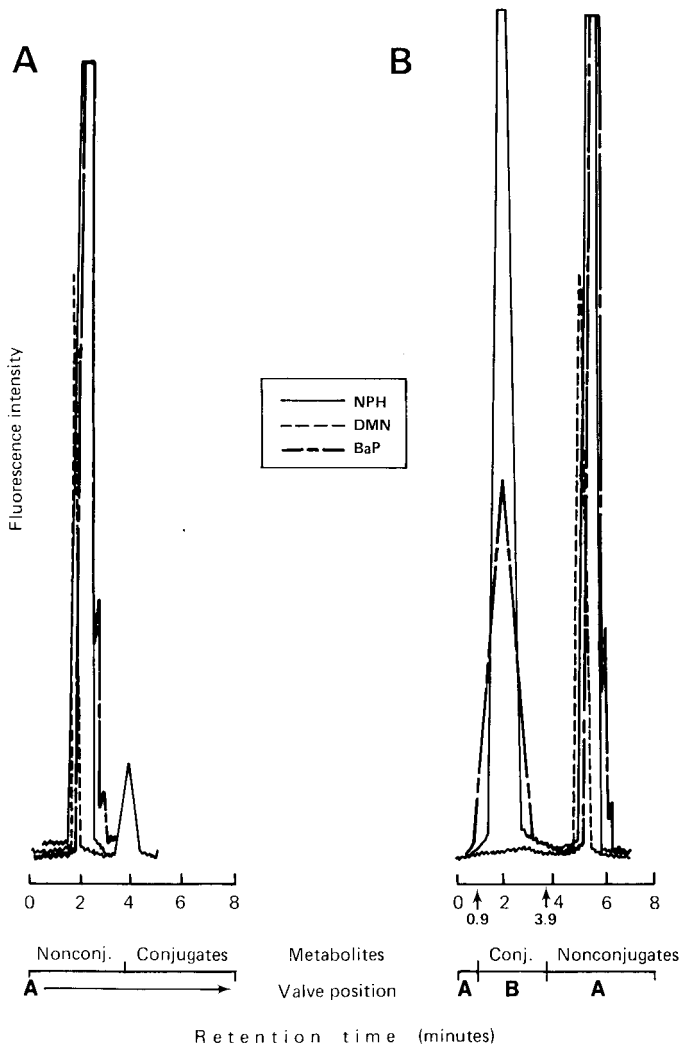
Fig. 2. Normal-phase HPLC chromatograms of NPH, DMN, BaP and their metabolites chromatographed on the configuration of diol columns shown in Fig. 1. The mobile phase was hexane-2-propanol (1:1). A: Valve in position A for the entire chromatogram. Among the conjugated metabolites, only naphthyl glucoside was eluted; the remainder were strongly sorbed to the guard column. B: Valve moved from A to B to A as shown in Fig. 1. Conjugated metabolites were eluted from 0.9–3.9 min and non-conjugates and parent PAHs after 3.9 min.

cartridges. This procedure allowed the processing of 12 samples in 30 min and re-placed laborious conventional extraction schemes[1,11,12].

The extractor–concentrator techniques were adapted to various matrices and analytes by adjusting the operating parameters (*e.g.*, extraction time, centrifigation speed, drying time and drying temperature). In addition, other modifications to the original serum extraction technique[16] were made. For example, glass microbeads

were added to the cartridges to prevent plugging during liver and bile extractions. Also, extraction solvents were chosen to efficiently extract PAH metabolites from the resin. Originally, buffer had been added to the serum samples to aid retention of drugs by the resin in the cartridges[16]. However, in our applications, lowering the pH and increasing the ionic strength (creating a "salting-out" effect) of the buffer were necessary to optimize recoveries of NPH and DMN compounds from bile and of BaP compounds from serum.

Fish serum, bile and liver samples spiked with reference compounds were used to develop the automated extraction techniques, and metabolites of NPH and DMN were recovered efficiently and reproducibly (Table II). However, recoveries of some BaP metabolites from spiked serum were lower than from water (Table III) and were lower than recoveries of DMN and NPH metabolites from either serum or water (Table II). Some of the BaP and its metabolites remained in the water phase and were carried to waste, as confirmed by analysis of the waste-cup contents. By adjusting the pH and ionic strength of the buffer, the recoveries were improved, but the adjustments did not entirely prevent the losses to waste.

Aqueous solutions of the reference compounds were used to avoid quantities of organic solvents that could adversely affect sorption of the analytes by the resin bed, but the concentrations of some reference compounds were not stable in aqueous solution. After overnight storage at 5°C, the concentrations of DMN, BaP and the

TABLE II

RECOVERIES OF NPH, DMN AND THEIR METABOLITES FROM RAINBOW-TROUT SERUM, BILE AND LIVER HOMOGENATE USING AN AUTOMATED EXTRACTOR–CONCENTRATOR

Each recovery ($\pm$ standard deviation) is the mean of three extractions each on two days ($n = 6$). See Experimental for concentrations. NR = not recovered; E = recovery erratic (see Results and Discussion).

Compound	Percentage recovery of spiked compound					
	Aqueous standard	Serum			Bile	Liver homogenate
Percentage of stock	40%	8%	40%*	100% (stock)	40%	40%
Naphthyl glucuronide**	91 ± 3.2	83 ± 3.1	90 ± 3.4	90 ± 4.9	87 ± 3.7	90 ± 2.6
Naphthyl sulfate**	103 ± 2.9	96 ± 4.6	98 ± 4.7	97 ± 1.7	88 ± 3.9	82 ± 2.1
DMN dihydrodiol***	104 ± 2.1	99 ± 6.0	103 ± 2.3	101 ± 5.8	I§	102 ± 3.1
1-Naphthol***	94 ± 3.6	100 ± 2.6	96 ± 6.5	97 ± 5.0	97 ± 4.3	99 ± 4.1
DMN quinone***	97 ± 0.6	NR	NR	NR	NR	NR
DMN 2-methanol**	101 ± 3.6	102 ± 2.6	103 ± 4.3	100 ± 2.6	102 ± 5.5§§	101 ± 3.1
3-Hydroxy DMN**	97 ± 1.0	102 ± 2.9	100 ± 3.2	95 ± 3.8	100 ± 1.8§§	96 ± 3.7
Naphthalene**	56 ± 3.5	106 ± 4.0	103 ± 3.1	90 ± 6.9	83 ± 4.0§§	E
DMN**	65 ± 8.9	E	E	E	91 ± 4.7§§	E
Internal standard (naphthyl glucoside)**	91 ± 6.6	98 ± 4.6	91 ± 3.7	94 ± 5.1	92 ± 5.8	89 ± 2.6

   * ($n = 5$) because a cartridge failed.
  ** Quantitation by fluorescence at ex 290 nm, em 335 nm.
 *** Quantitation by UV at 254 nm.
   § Interfering compounds from bile matrix made quantitation impossible at the concentration used.
  §§ Corrected for contributions to peak areas from bile matrix.

TABLE III

PERCENTAGE RECOVERIES OF BENZO[a]PYRENE AND METABOLITES FROM RAINBOW-TROUT SERUM USING AN AUTOMATED EXTRACTOR–CONCENTRATOR

The compounds were determined by fluorimetry at 430 nm, with excitation at 380 nm. Each recovery ($\pm$ standard deviation) is the mean of three extractions ($n = 3$). See Experimental for concentrations.

Compound	Aqueous standard*	Serum**
BaP 1-glucuronide	83 ± 1.5	66 ± 4.9
BaP 3-sulfate	94 ± 2.3	66 ± 9.5
BaP 9,10-dihydrodiol	94 ± 3.2	98 ± 5.1
BaP 7,8-dihydrodiol	100 ± 5.6	99 ± 6.8
9-HydroxyBaP	73 ± 16.5	49 ± 9.5
1-HydroxyBaP	61 ± 15.3	37 ± 2.6
3-HydroxyBaP	59 ± 7.1	41 ± 2.6
BaP	80 ± 3.6	62 ± 5.9

* Concentrations of some BaP reference compounds are unstable in aqueous solution (see Results and discussion).
** BaP and some of the metabolites appear in the waste cup (see Results and discussion).

hydroxyBaPs decreased 30, 20 and 70–80%, respectively. Consequently, even when freshly prepared solutions were used, recoveries calculated for these compounds were erratic.

To determine if metabolites formed *in vivo* could be extracted as efficiently as spiked metabolites by our techniques, fish were fed radiolabeled DMN. Recoveries of total radioactivity from liver and serum were low when the extraction conditions were identical to those used to extract the spiked samples (Table I, extraction with acetone and with methanol). However, recoveries were excellent (Table IV) when the method was modified to use four extractions with solvents similar to those used for conventional tissue extractions[11] (Table I). When this modified technique was used to extract spiked biological samples, the recoveries were equal to or greater than those reported for two-solvent extractions in Table II.

TABLE IV

RECOVERIES OF TOTAL RADIOACTIVITY FROM [¹⁴C]DMN AND METABOLITES EXTRACTED FROM RAINBOW-TROUT SERUM, BILE AND LIVER USING THE EXTRACTOR-CONCENTRATOR: ANALYSIS OF THE ORGANIC EXTRACTS, WASH SOLUTION, AND CARTRIDGE CONTENTS BY LIQUID SCINTILLATION COUNTING

Each recovery ($\pm$ standard deviation) is the mean of three extractions ($n = 3$).

Matrix	Percentage of radioactivity recovered in:			Total recovery
	Organic extract	Wash	Cartridge*	
Serum	102.9 ± 4.7	1.1 ± 0.2	4.6 ± 1.6	108.6 ± 3.2
Bile	102.8 ± 1.5	1.7 ± 1.1	0.1 ± 0	104.6 ± 2.1
Liver homogenate	94.8 ± 1.7	1.6 ± 0.1	3.6 ± 0.2	100.0 ± 2.0

* Cartridge resin, glass microbeads and liver residue.

Separations of conjugated from non-conjugated PAH metabolites

Conjugated PAH metabolites were readily separated from non-conjugates by normal-phase HPLC on diol columns (Fig. 2). The two-column system allowed for a more rapid analysis than a previous separation on alumina[19], and the analytes were recovered in a small volume of solvent. Naphthyl glucoside, the only conjugated metabolite among the reference standards not retained by the guard column, was
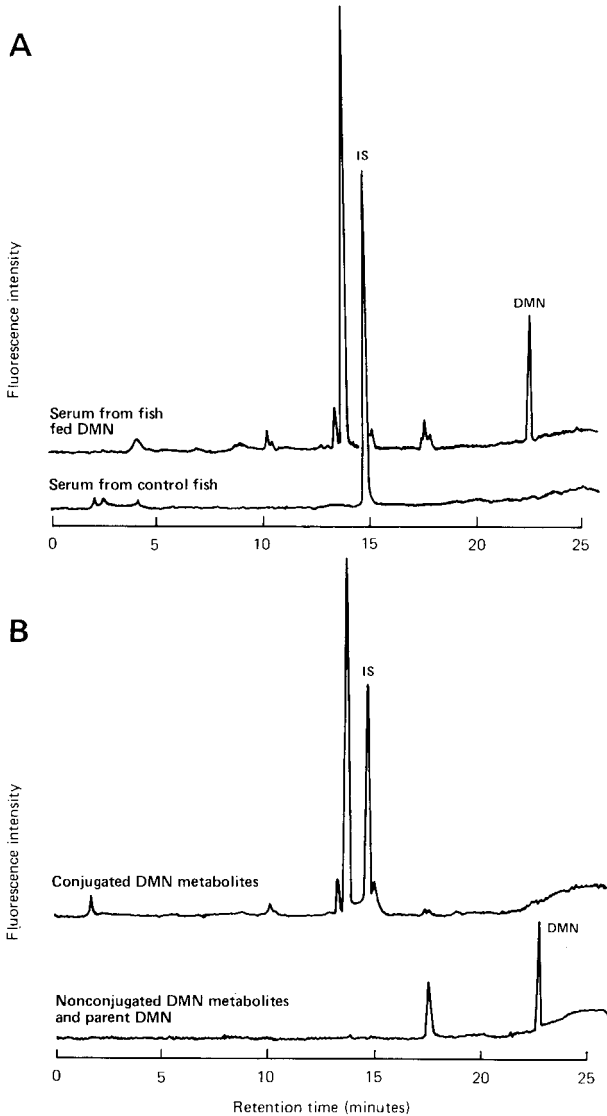
Fig. 3. A: Reversed-phase HPLC–fluorescence chromatograms (excitation at 290 nm; measurement at 335 nm) of serum from fish fed DMN and from control fish. Extracts were prepared with an automated extractor–concentrator. Injection represented extracts from *ca.* 14 mg serum (wet weight). B: Reversed-phase HPLC chromatograms of conjugated and non-conjugated metabolite fractions from extracts of serum of fish fed DMN. The extracts were separated into fractions using the normal-phase HPLC techniques shown in Fig. 1. See Experimental for HPLC conditions.

prevented from eluting with the non-conjugated metabolites by reversing the direction of eluent flow through the guard column after 0.9 min (Fig. 1). The reversal of flow did not appear to degrade the guard column, but, should this occur, it is inexpensive to replace.

This separation technique is an important step in preparing the experimental sample for HPLC analysis of the non-conjugate fraction or for additional procedures to determine the conjugates. For example, enzymatic hydrolyses could be carried out on the conjugate fraction, and the resulting liberated aromatic compounds analyzed by GC or HPLC to identify and quantitate the resulting conjugated metabolites.

Applications to samples from fish fed non-radiolabeled DMN

Serum, bile and liver homogenate from fish fed non-radiolabeled DMN were extracted using the extractor–concentrator, and the extracts were analyzed by reversed-phase HPLC. Extracts of serum (Fig. 3A), bile and liver gave similar HPLC profiles, but the concentrations of metabolites varied; bile had the highest concentration and liver the lowest. From HPLC retention times, most of the metabolites appeared to be conjugates. Similar findings were reported by Gruger et al.[7] for metabolites of DMN in bile of starry flounder (*Platichthys stellatus*) fed radiolabeled DMN.

Separation of the conjugated DMN metabolites in serum extracts from DMN and its non-conjugated metabolites was carried out by the normal-phase HPLC technique. Fractions were collected and concentrated, and the resulting solutions were analyzed by reversed-phase HPLC (Fig. 3B). As predicted from the HPLC retention times, most of the DMN metabolites were found in the conjugate fraction. In future research, we plan to hydrolyze this fraction enzymatically and then identify the liberated aromatic compounds by HPLC or GC–MS.

CONCLUSIONS

NPH and DMN metabolites were extracted efficiently and reproducibly from serum, bile and liver homogenate using the commercially available extractor–concentrator. A simple modification of the extraction procedure was necessary to recover DMN metabolites produced *in vivo* as efficiently as those spiked into biological samples. However, extraction efficiencies for BaP metabolites varied widely, even after modifications in the method were made. Extracts were suitable for reversed-phase HPLC analysis of individual components and for normal-phase HPLC separation into conjugate and non-conjugate fractions. Ultimately, these methods are intended for determination of xenobiotics and their metabolites in aquatic organisms exposed via their natural environment.

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SEPARATION OF FOUR ISOMERS OF LYSERGIC ACID α-HYDROXYETHYLAMIDE BY LIQUID CHROMATOGRAPHY AND THEIR SPECTROSCOPIC IDENTIFICATION

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SUMMARY

A method is described for the separation of four isomers of lysergic acid α-hydroxyethylamide and its decomposition products ergine and erginine using LiChrosorb NH$_2$ as the stationary phase and isocratic elution. The substances under study were determined by ^{13}C and ^{1}H NMR and mass spectroscopy. The relative proportions of individual isomers of lysergic acid α-hydroxyethylamide, ergine and erginine in the fermentation medium are assumed to result from chemical equilibrium reactions. The method is reproducible and suitable for kinetic studies of the isomerization and degradation of lysergic acid α-hydroxyethylamide.

INTRODUCTION

Lysergic acid α-hydroxyethylamide (LAH) is an important substrate of semi-synthetic ergot alkaloids. In fermentation production, LAH[1] decomposes spontaneously[2] and is thus found in a mixture with ergine and erginine[3]. These compounds are also formed on heating ergotamine[4]; other products include C(8) and C(2′) epimers. The same isomerization products were found to be formed on heating other cyclol alkaloids with dilute acid[5–8]. The analysis of these compounds was carried out by Bethke *et al.*[4], who determined ergotamine and the products of its isomerization, hydrolytic and addition reactions by liquid chromatography on a reversed phase. Our aim was to elaborate a separation method for the isomerization and decomposition products of LAH. Because of the considerable instability of the compounds under study it was necessary to prepare reference samples from fermentation broth. The elaboration of the high-performance liquid chromatographic (HPLC) method was based on earlier results[9].

EXPERIMENTAL

Fermentation

The method of cultivation of the saprophytic strain *Claviceps paspali* F 2056 and the conditions of submerged fermentation were described earlier[10].

Alkaloid extraction

On the fourteenth day of fermentation the culture broth was adjusted to pH 9 with aqueous ammonia and extracted with chloroform–isopropanol (4:1); the chloroform layer was separated, dried over sodium sulphate and evaporated to dryness under reduced pressure at a temperature below 15°C. The crude extract was partially dissolved in chloroform, undissolved substances were filtered off and the solvent was evaporated. The crude extract enriched in *iso*-compounds was dissolved in chloroform–methanol (4:1) and used directly for semi-preparative liquid chromatography.

Reagents

Ergine, erginine, ergometrine and ergometrinine standards were obtained from Galena (Opava, Czechoslovakia). The solvents diethyl ether, chloroform, methanol, ethanol and isopropanol (Lachema, Brno, Czechoslovakia) were of analytical reagent grade and were distilled before use. The stationary phase was MicroPak NH_2, particle size 10 μm (Merck, Darmstadt, G.F.R.), in a ready packed column (50 cm × 8 mm I.D.) (Varian Aerograph, Walnut Creek, CA, U.S.A.).

Instruments

Semi-preparative liquid chromatography was carried out on an apparatus consisting of a VCM 300 high-pressure micropump and a variable-wavelength UV detector (both from Development Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia). The substances were eluted isocratically with diethyl ether–ethanol (9:1) as the mobile phase.

Mass spectra were measured on a Varian-MAT 311 instrument under the following conditions: energy of ionizing electrons, 70 eV; ionizing current, 1 mA; ion source temperature, 200°C; direct inlet system operated at 110–180°C. The elemental composition of the ions was determined by the peak-matching technique ($\pm$ 5 ppm; perfluorokerosene standard).

^{1}H and ^{13}C NMR spectra were measured on a JEOL FX-60 spectrometer (59.797 and 15.036 MHz, Fourier transform mode, 25°C) in a mixture of deuteriochloroform and perdeuteriomethanol (4:1). The $CDCl_3$ signal was used as a lock. Chemical shifts were referred to internal tetramethylsilane and were calculated with an accuracy of $\pm$ 0.005 and $\pm$ 0.06 ppm for the digitally obtained address differences.

UV spectra were measured on a Variscan LC instrument immediately during the qualitative determination by a stop-flow method.

RESULTS

Liquid chromatography

Analysis of an alkaloid mixture produced under submerged condition by the fungus *C. paspali* F 2056 revealed eight alkaloids in the extract. The elution times of four of them corresponded to ergine (V), erginine (VI), ergometrine (VII) and ergometrinine (VIII) (Table I, Fig. 1). The other four substances had elution times that did not correspond to any standard at our disposal and had to be isolated by semi-preparative liquid chromatography in order to determine their structures.

Semi-preparative liquid chromatography was carried out under the conditions

TABLE I

RELATIVE CAPACITY FACTORS, $k'_{rel} = k'_i/k'_1$, OF SUBSTANCES i (II–VIII)

Compound i	k'_{rel}
II	0.44
III	1.37
IV	0.53
V	1.28
VI	0.63
VII	1.66
VIII	0.81

given in the legend to Fig. 1. To obtain sufficient amounts of pure standard sub-
stances the chromatographic cycle was repeated 20 times; to prevent possible changes
in the structures of the substances (isomerization, degradation), the individual frac-
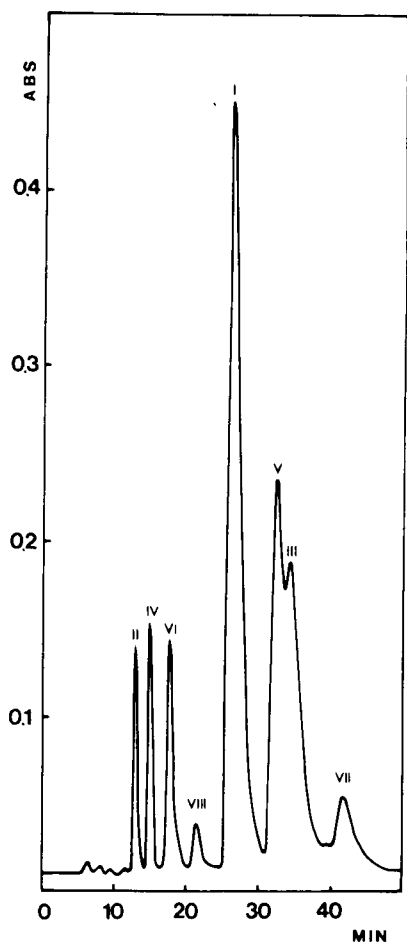
Fig. 1. Chromatogram of alkaloids I–VIII. MicroPak NH_2 (particle size 10 μm), ready-packed column (50
cm × 8 mm I.D.). Mobile phase, diethyl ether–ehtanol (9:1). Flow velocity, 220 ml/h. UV detection: 310
nm, 0.50 A. Injection, 50 μl.

tions were immediately evaporated to dryness after each cycle under reduced pressure at 5–10°C. Analyses of the individual fractions showed that compounds I, II, IV and VI (Fig. 2) were obtained in pure form whereas compounds III and V were mixtures. The resulting standard compounds were used for identification.

$$I \quad R_1 = H \; ; R_2 = CO-NH\cdots\overset{\overset{\displaystyle CH_3}{\big\downarrow}}{\underset{\underset{\displaystyle H}{\big|}}{C}}-OH$$

$$II \quad R_1 = CO-NH\cdots\overset{\overset{\displaystyle CH_3}{\big\downarrow}}{\underset{\underset{\displaystyle H}{\big|}}{C}}-OH; R_2 = H$$

$$III \quad R_1 = H \; ; R_2 = CO-NH\blacktriangleright\overset{\overset{\displaystyle CH_3}{\vdots}}{\underset{\underset{\displaystyle H}{\big|}}{C}}-OH$$

$$IV \quad R_1 = CO-NH\blacktriangleright\overset{\overset{\displaystyle CH_3}{\big|}}{\underset{\underset{\displaystyle H}{\big|}}{C}}-OH; R = H$$

$$V \quad R_1 = H; R_2 = CO-NH_2$$

$$VI \quad R_1 = CO-NH_2; R_2 = H$$

$$VII \quad R_1 = H; R_2 = CO-NH\cdots\overset{\overset{\displaystyle H}{\big\downarrow}}{\underset{\underset{\displaystyle CH_3}{\big|}}{C}}-CH_2OH$$

$$VIII \quad R = CO-NH\cdots\overset{\overset{\displaystyle H}{\big\downarrow}}{\underset{\underset{\displaystyle CH_3}{\big|}}{C}}-CH_2OH; R_2 = H$$
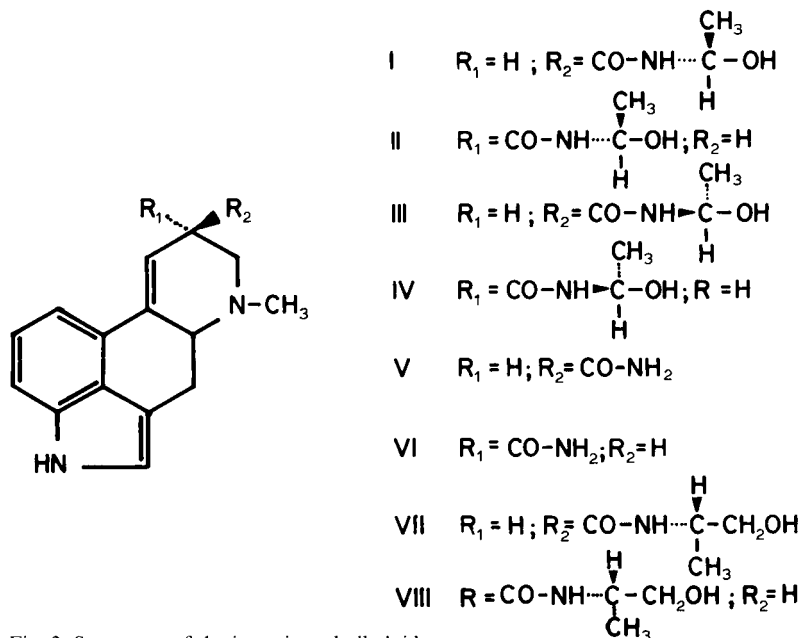
Fig. 2. Structures of the investigated alkaloids.

Identification of substances

All components of the mixture analysed provided suitable UV spectra of $\Delta^{9,10}$-ergolene derivatives. The mass spectra (Table II) always exhibited an ion of m/z 267 $(C_{16}H_{17}N_3O)$ and ions of the fragmentation series typical of ergine (V)[11]. They are therefore substances derived from either ergine or erginine. The observed type of

TABLE II

MASS SPECTRA OF COMPOUNDS I–VI

Compound	m/z (rel. int. in %, composition)
I	295 (14, $C_{18}H_{21}N_3O$), 293 (9, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (37), 224 (49), 223 (60), 221 (100), 207 (86), 196 (46), 192 (43), 180 (69), 167 (46), 154 (66)
II	293 (4, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (16), 224 (26), 223 (30), 221 (56), 207 (54), 196 (36), 192 (20), 180 (46), 167 (26), 154 (40)
III + V	295 (4, $C_{18}H_{21}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (26), 224 (40), 223 (37), 221 (67), 207 (66), 196 (37), 192 (26), 180 (46), 167 (31), 154 (43)
IV	293 (9, $C_{18}H_{19}N_3O$), 267 (100, $C_{16}H_{17}N_3O$), 249 (22), 224 (36), 223 (36), 221 (52), 207 (68), 196 (45), 192 (26), 180 (67), 167 (28), 154 (45)
VI	267 (100, $C_{16}H_{17}N_3O$), 249 (22), 224 (37), 223 (35), 221 (67), 207 (72), 196 (46), 192 (29), 180 (57), 167 (32), 154 (56)

fragmentation and the character of the UV spectra preclude the presence of amides of $\Delta^{8,9}$-ergolenic acid (paspalic acid).

It is possible to distinguish the above derivatives by ^{1}H and ^{13}C NMR spectroscopy. Compounds V and VI differ in the orientation of substitutent on C(8) (Fig. 2); in lysergic acid the substituent is pseudo-equatorial whereas in isolysergic acid is pseudo-axial. The dihedral angle H(8)–C(8)–C(9)–H(9) in the former instance is close to 90°C, causing a small or zero value of $J_{8,9}$; in the latter instance the protons are nearly staggered and the coupling constant may be larger. Using this rule, components II, IV and VI can be classified into an *iso*-series whereas I, III, and V belong to the normal series. The coincidence of the elution time of compound VI with that of an authentic erginine preparation (VI) confirms the correctness of this deduction.

Comparison of the ^{13}C NMR spectra of the pairs ergometrine (VII)–ergometrinine (VIII)[12] and ergine (V)–erginine (VI) (Table III) indicates that the largest differences in chemical shifts are observed on C(7), C(8) and C(9). In the *iso*-series the C(8) always resonates in a lower field and C(7) and C(9) in a higher field than in the normal series. The observed values (Table III) agree with the above classification. The non-stoichiometric ratio of the intensities of some signals in the ^{1}H NMR spectrum of fraction III–V indicates that this is a mixture of substances insufficiently separated by HPLC. Comparison with an authentic standard showed that compound V was ergine.

TABLE III

COMPARISON OF ^{13}C CHEMICAL SHIFTS OF COMPOUNDS I–IV WITH MODEL COMPOUNDS

Position	Ergine*	Erginine*	Ergometrine*	Ergometrinine*	I**	II**	III**	IV**
C(7)	54.7	53.9	55.6	54.0	57.8	54.8	58.0	53.8
C(8)	41.8	43.3	42.8	42.6***	42.6	43.4	42.6	43.4
C(9)	119.9	119.3	120.3	119.2	110.8	110.5	110.7	110.4
C_α	–	–	–	–	71.0	70.6	71.7	71.3
C_β	–	–	–	–	21.4	21.6	21.6	21.4

* In d_6-DMSO.
** In $CDCl_3$ + CD_3OD (4:1).
*** Data from ref. 13, corrected on the basis of spectra measured by the technique in ref. 14.

In the ^{1}H NMR spectra of compounds I, II, IV and mixture III–V (Table IV) the double resonance proved the presence of the $CH_3CH(OH)NH$–moiety. The carbon atoms of this group exhibit resonances at 21 and 70 ppm (off-resonance: quartet and doublet) in ^{13}C NMR spectra (Table III). These findings allow one to interpret the peak with the highest m/z 293 in the mass spectrum as an $M - H_2O$ peak (Table II). The compounds are therefore isomers of α-hydroxyethylamide of the isolysergic and lysergic acids. Compounds II and IV differ in the magnitude of the coupling constant between the NH proton and the secondary alcoholic group of the methine (< 1 Hz and 6.1 Hz); they are therefore epimers on C_α of the side-chain. Compounds I and III form a similar pair. The carbon atom C_α in isomers with higher J_{NH,H_z} value (III and IV) resonates 0.7 ppm downfield of their counterparts I and II. Examples of epimers of lysergic acid dialkylamides hydroxylated in the side-chain

were described by Ishii *et al.*[13]. Although they gave no values for the appropriate coupling constants, the recorded spectrum shows that the epimers also differ in the magnitude of the vicinal constant of the OCH proton. This is probably due to the different population of rotamers caused by different possibilities for hydrogen bond formation. The average conformation observed in the NMR spectra has similar values of chemical shifts for epimer pairs but different magnitudes of the coupling constants.

TABLE IV

COMPARISON OF SELECTED ^{1}H NMR DATA OF COMPOUNDS I–IV AND VI

Spectra measured in a mixture of $CDCl_3$ + CD_3OD (4:1), δ-scale; coupling constants in Hz given in parentheses. Abbreviations: s = singlet; d = doublet; q = quartet.

Compound	$H_{(2)}$	$H_{(9)}$	N–CH_3	$N_{(1)}$–H	CONH–	CHOH α	CH_3 β
I	6.90 s	6.39 s	2.67 s	8.15 s	8.78 s	5.48 q (6.1)	1.40 d (6.1)
II	6.94 s	5.98 d (5.5)	2.61 s	8.17 s	8.68 s	5.41 q (6.1)	1.28 d (6.1)
III	6.94 s	6.45 s	2.67 s	8.39 s	8.82 d (4.9)	5.50 dq (6.1, 4.9)	1.40 d (6.1)
IV	6.92 s	6.56 d (6.1)	2.57 s	7.96 s	8.80 d (6.1)	5.43 dq (6.1, 6.1)	1.30 d (6.1)
VI	6.94 s	6.57 d (4.1)	2.59 s	8.11 s	8.64 s		

Isomerization of LAH

Isomerization of LAH isolated by semi-preparative liquid chromatography was accomplished according to Schlientz *et al.*[5,6]. After alkalization with ammonia solution and extraction with chloroform–isopropanol (4:1), the reaction mixture yielded a sample that was analysed further by liquid chromatography. It contained approximately equal amounts of compounds II, IV and VI (8–10% each) and about double the amounts of I, III and V (about 20% each). The elution times of all substances corresponded to the elution times of compounds obtained from naturally occurring material. The results confirm earlier data[2,3] indicating that ergine and erginine are probably only artifacts arising from LAH.

DISCUSSION

Isomerization of simple lysergic acid derivatives on C(8) is known to proceed readily even under mild conditions. With LAH, the reaction is more complex (as in cyclol alkaloids) as it also includes epimerization on asymmetric carbon atoms of the side-chain. Lysergic and isolysergic acid α-hydroxyethylamides (I, II, III and IV) are hemiacetals derived from acetaldehyde and ergine (V) or erginine (VI). The aldolization reaction is reversible. Mutual transformation of II to IV was observed during a 15-h ^{13}C NMR measurement; the spectrum displayed signals of both C_x atoms. The formation of isomers II, III and IV can likewise be observed during acid-catalysed

isomerization of the parent LAH. The relative proportions of individual isomers of LAH (I, II, III and IV), ergine (V) and erginine (VI) in the cultivation medium during fermentation[10] can be interpreted as the result of chemical equilibrium reactions.

The chromatographic behaviour of compounds I, II, III and IV is determined by the configuration on asymmetric carbon atoms $C(8)$ and C_x. The pseudo-axial position of the side-chain on carbon $C(8)$ may lead to the formation of an intra-molecular hydrogen bond $N(6) \cdots H-N(20)$ which causes a marked decrease in the basicity of alkaloid molecules (II and IV); this is demonstrated by the reduced inter-action with the basic nitrogen atom of the stationary phase. For this reason com-pounds II and IV are less retained on the column. The ^1H and ^{13}C NMR spectra of individual diastereomeric pairs of alkaloids (I and III, II and IV) indicate that these substances differ in the population of rotamers with different possibilities of hydrogen bond formation and with different magnitudes of Van der Waals interactions, which is demonstrated by the different basicities of the molecules and thus in different interactions with the stationary phase.

The developed HPLC method can be used to study the kinetics of isomeriza-tion reactions and degradation reactions of LAH and for the control of the purity of preparations. ·

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SIMULTANEOUS DETERMINATION OF THE CATIONIC AND ANIONIC PARTS IN REPOSITORY PENICILLINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

High-performance liquid chromatographic separations of repository penicillins (hardly soluble salts of organic bases with penicillins) described previously permit only the determination of the penicillin part; the identification or quantitation of the cationic part was possible only by additional analysis. The optimization of the pH value of the mobile phase succeeded in giving a simultaneous determination of the cationic and anionic parts under isocratic reversed-phase conditions.

The elution characteristics of the basic components benzathine, procaine and clemizole depend strongly on the pH of the mobile phase. In mixtures of phosphate buffer and methanol the retention time increases rapidly with increasing pH, whereas the retention time of the penicillin part is only slightly influenced. For the analysis of benzathine penicillins a pH of 5.0–5.5 can be recommended, for procaine penicillin G pH 7.3–7.5 and for clemizole penicillin G pH 2.5–3.0. For all components linear calibration graphs were obtained over the concentration range 0.1–1 mg/ml (injection volume 20 μl). The relative standard deviation, depending on the component and separation system, was 0.5–2%. The separations can be performed within a few minutes at 50°C.

INTRODUCTION

Different methods for the analysis of repository penicillins are described in the various pharmacopoeias. The most important chemical procedure for the quantitative determination of penicillin is still iodimetric titration, which can be traced back to Alcino in 1946[1]. The iodimetric titration is included in the Code of Federal Register (FDA) for all penicillins. A few years ago Karlsberg and Forsman described mercury titration[2], which is included in the draft of the next edition of the European Pharmacopoeia for some penicillins. The cationic component in repository penicillins (benzathine, procaine and clemizole) can be determined be separate chemical analysis. Thus the British Pharmacopoeia describes a titrimetric assay after extraction for

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benzathine[3] as well as for procaine[4]. For procaine different colorimetric methods are also possible[5-7].

Separations of repository penicillins can be performed by thin-layer chromatography (TLC). Fooks and Mattok[8] succeeded in separating free procaine, penicillin G and procaine penicillin G on silica gel plates. The separation system is particularly suitable for tests for identity and purity. Wilson *et al.*[9] described an analogous test method for benzathine penicillin G. As has been demonstrated recently by Nachtmann and co-workers, the high-performance liquid chromatographic (HPLC) determination of penicillin G and V on reversed phases is superior to chemical methods with regard to selectivity and thus to accuracy. The selectivity of HPLC can be explained by the fact that all by-products and degradation products of penicillin are separated[10-12]. For this reason it was obvious to apply HPLC to the analysis of repository penicillins. As the basic cationic part of these compounds also shows UV absorption, it should be possible under appropriate conditions to determine the cationic and anionic component at the same time. The analysis of oral benzathine penicillin V suspensions by HPLC was described by Lebelle *et al.*[13]. According to this method, only the penicillin component can be determined. Tsuji *et al.*[14] succeeded in carrying out a simultaneous determination of procaine and penicillin G. Problems occurred, however, with regard to the reproducibility of separation.

This paper describes the simultaneous determination of the basic and acidic component in benzathine, procaine and clemizole salts of penicillin G and V.

EXPERIMENTAL

The penicillin salts tested, benzathine benzylpenicillin (DBED-G), benzathine phenoxymethylpenicillin (DBED-V), procaine benzylpenicillin (Proc-G) and clemizole benzylpenicillin (Clemizole-G), were manufactured by Biochemie GmbH (Kundl, Austria). All solvents used were of analytical-reagent grade (E. Merck, Darmstadt, G.F.R.). LiChrosorb RP-8 (Merck) was used as the stationary phase for the HPLC separations. This material, particle size 10 μm, was packed in stainless-steel columns (250 × 3.2 mm I.D.) using a slurry technique[15].

Apparatus

For the chromatographic separations an Orlita AE 10-4.4 pump and an Altex 110 A pump in combination with a Rheodyne 7120 injection valve (20 μl) were used. The detection was carried out in the UV region (215–220 nm) with a Schoeffel SF 770 or Perkin-Elmer LC 55 spectrophotometer. The separation column was placed in a thermostated water-bath. All determinations were carried out under isocratic conditions. A Hewlett-Packard 3353 data system was used for integration of the peak areas.

Preparation of samples

The dissolved penicillin salts can be injected into the column without any further preparation. A solution of 1 mg of sample per millilitre of solvent proved suitable for all salts tested. The solvent was composed of phosphate buffer (pH 7.0) (1/15 *M*)–methanol (3:7).

RESULTS AND DISCUSSION

The low solubility of the repository penicillins examined causes some problems. Methanol is certainly an excellent solvent, but it causes rapid degradation of penicillin and the corresponding alkyl-α-D-penicilloic acid is produced[8]. Our own experiments confirmed the instability of penicillin G and V in methanol.

The reaction can be slowed down by adding water to methanol. A compromise could be attained with the mixture of phosphate buffer (pH = 7.0) (1/15 M)–methanol (3:7); 1 mg of the salts examined can be dissolved in 1 ml of this mixture without difficulty.

In order to achieve a simultaneous rapid determination of the cationic (basic) and the anionic (acidic) components of repository penicillins it is necessary to find separation conditions that guarantee optimal resolution of the components. The reversed-phase systems hitherto described for penicillins, mostly utilizing pH values of 6.0–7.0[10–12], do not permit such a separation. Elution of the cationic components of the salts cannot be achieved. For this reason, experiments were carried out in order to reach the desired objective by varying the pH of the mobile phase.

In order to facilitate routine analyses the same type of column (250 × 3.2 mm I.D.) was always used. As earlier experiments carried out by Nachtmann and co-workers have shown, LiChrosorb RP-8 is an excellent stationary phase for the separation of penicillins[10,11] and it is advantageous to carry out the separation at high temperatures. An increase in temperature from 20 to 50°C will shorten the analysis time and reduce the back-pressure of the column if the flow-rate is kept constant. At the same time, the plate number of the column is slightly increased. Therefore, all tests described in this paper were carried out at 50°C. For quantitative determinations an external standard was used. Reproducible elution times for all penicillin salts examined were obtained only after conditioning of the column with the sample. For this purpose it was sufficient to inject the sample 5–10 times prior to analysis. Experiments with different batches of the LiChrosorb RP-8 stationary phase showed differences in the elution characteristics described in below by up to one pH unit.

Benzathine penicillins

The most important types are benzathine benzylpenicillin (DBED-G) and benzathine phenoxymethylpenicillin (DBED-V). In penicillin V p-hydroxypenicillin V is always present as a byproduct. The elution behaviour of these penicillin salts was examined on a C-8 column (Table I). With constant polarity of the mobile phase the elution behaviour of benzathine depends strongly on pH. In the interesting range from pH 4.0 to 5.5 the elution time and the asymmetry of the peak of benzathine increase with increasing pH. By adding 0.1 % of triethylamine to the mobile phase the peak symmetry could be improved considerable but the retention times remained unchanged.

Within the range examined the elution time of penicillin G depends only slightly on pH. The elution characteristics of penicillin V, on the other hand, differ from those of benzathine. The separation selectivity in the range examined is hardly influenced by a change in the ratio of phosphate buffer to methanol. For the determination of DBED-G the separation system at pH 4.0 could be regarded as most favourable with respect to the time of analysis and separation. Under these con-

TABLE 1

ELUTION BEHAVIOUR OF BENZATHINE PENICILLINS

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M)–methanol. Flow-rate: 2.0 ml/min. Temperature: 50°C. Wavelength: 215 nm.

Mobile phase	Retention time (min)			
	Benzathine	Penicillin G	Penicillin V	p-Hydroxypenicillin V
Buffer* (pH = 5.5)–methanol (1:1)	1.8		1.5	0.9
Buffer (pH = 5.5)–methanol (6:4)	2.8		2.6	1.2
Buffer (pH = 5.5)–methanol (7:3)	5.5	3.7	4.3	1.4
Buffer (pH = 5.0)–methanol (7:3)	4.3	3.8	6.7	2.9
Buffer (pH = 4.5)–methanol (7:3)	2.9	4.0	7.0	1.8
Buffer (pH = 4.0)–methanol (7:3)	1.8	4.1		

* Phosphate buffer (1/15 M).
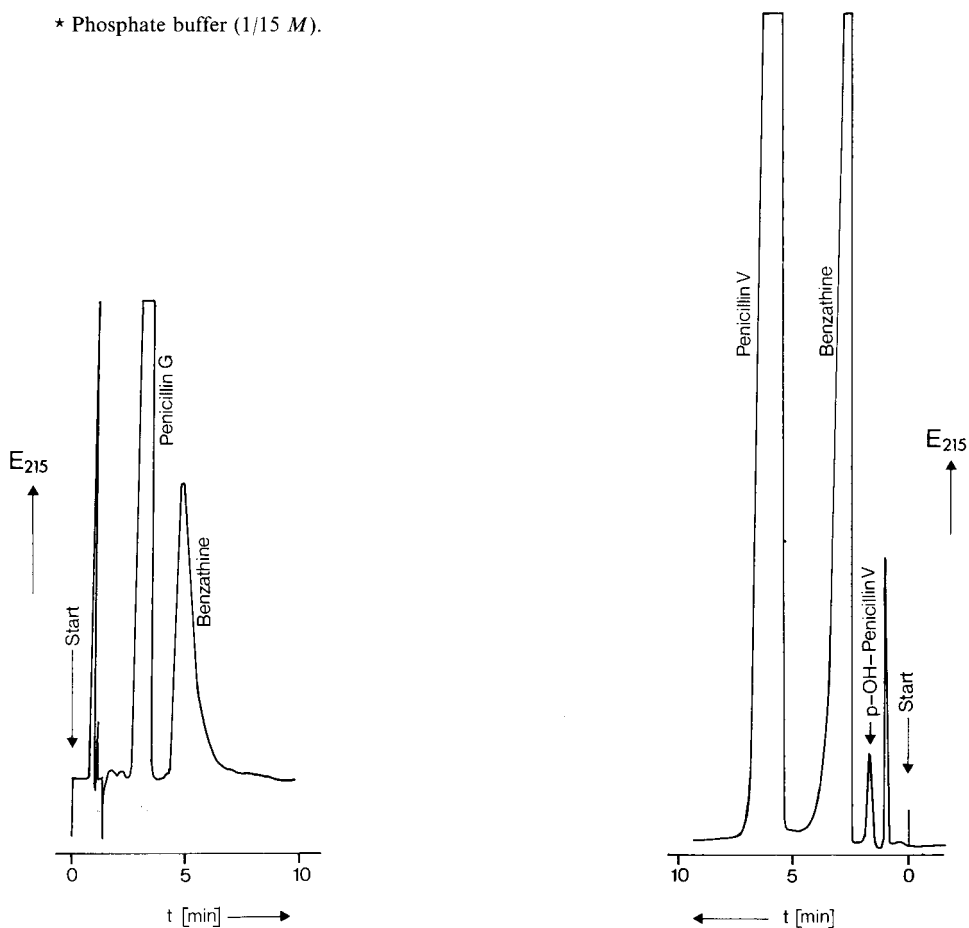
Fig. 1. Determination of benzathine penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M) + 0.1 % triethylamine (pH = 5.5)–methanol (7:3). Flow-rate: 2.4 ml/min. Temperature: 50°C.

Fig. 2. Determination of benzathine penicillin V by HPLC. Stationary Phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M) (pH = 4.5)–methanol (7:3). Flow-rate: 1.8 ml/min. Temperature: 50°C.

ditions, however, any degradation products of penicillin present, such as penicilloic acid, are not separated from the benzathine peak. Assuming that such impurities could be present the chromatography has to be carried out at pH 5.5 (Fig. 1). These separation conditions imply an increase in the time of analysis and, in spite of the admixture of triethylamine, asymmetry of the benzathine peak, but, on the other hand, the selectivity of the separation system is improved. All known degradation products of penicillin are eluted before the peak of penicillin G and thus do not interfere in the analysis.

For DBED-V the separation system at pH 4.5 has to be regarded as the most suitable mobile phase. The p-hydroxy penicillin V occurring as a small impurity is eluted before the two main components and thus a sensitive detection of this by-product is possible. There is a baseline separation for all three eluted peaks. Fig. 2 shows the corresponding chromatogram obtained for a commercial product of DBED-V. A wavelength of 215 nm was chosen for detection as it represents a favourable compromise for all components of interest. For benzathine as well as for the penicillins linear calibration graphs passing through the origin were found for solutions of 0.1–1.0 mg/ml of sample. The method is sufficiently precise for routine tests. In the above range of concentrations the relative standard deviation ($n = 7$) was 0.8 % (conditions as in Fig. 1) and 0.5 % (conditions as in Fig. 2) for benzathine, 1.3 % for penicillin G and 1.7 % for penicillin V.

The following substances are suitable as external standards: benzathine acetate, sodium penicillin G, potassium penicillin V and sodium p-hydroxypenicillin V. The substances used had a purity of greater than 99 %.

The method described was used to examine the stoichiometric composition of the above-mentioned penicillins. Table II shows the results for several batches of DBED-G. When considering Table II one has to take into account that theoretically DBED-G is composed of 68.16 % of penicillin G, 24.50 % of benzathine and 7.34 % of water. An experimental determination of the water content was not carried out. The results show that the stoichiometric composition of the batches examined was confirmed in all instances. The differing absolute contents, however, suggest a varying degree of purity of the samples. Analogous results were obtained for DBED-V.

Procaine penicillin G and clemizole penicillin G

Further important salts of penicillin G with depot character are procaine penicillin G and clemizole penicillin G. As a similar dependence of the elution of the cationic component on the pH of the mobile phase was assumed, a pH profile analogous to that for the benzathine salts was investigated. The results are summarized in Table III. The situation is similar to that with the benzathine salts. The elution times for procaine and clemizole increase with increasing pH, whereas the elution time for penicillin G is only slightly changed. Procaine penicillin G can be analysed in both the acidic and neutral pH ranges. For a phosphate buffer–methanol (7:3) mixture at pH 6.0 procaine is eluted before penicillin G, which is in accordance with the results obtained by Tsuji et al.[14]. In the alkaline range, however, the order of elution is reversed. If detection of the degradation products of penicillin is desired (stability tests) a pH range of 7.3–7.5 is preferable. Under these conditions the degradation products of penicillin elute before penicillin G and can be identified in the chromatogram. Such a determination is shown in Fig. 3. At a wavelength of 220 nm linear

TABLE II

SIMULTANEOUS DETERMINATION OF THE CATIONIC AND ANIONIC COMPONENTS OF BENZATHINE BENZYLPENICILLIN BY HPLC IN DIFFERENT COMMERCIAL PRODUCTS

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (pH = 5.5) (1/15 M)–methanol (7:3). Temperature: 50°C. Wavelength, 215 nm.

Product No.	Content (%)		Stoichiometric ratio
	Penicillin G	Benzathine	
1	68.6	24.3	2.03
2	63.9	22.7	2.02
3	69.3	24.1	2.07
4	67.1	24.0	2.01
5	65.5	23.8	1.98
6	69.2	23.6	2.11
7	66.8	23.1	2.08
8	68.9	24.0	2.06
9	62.5	21.7	2.07
10	69.4	23.8	2.10
11	67.9	24.4	2.00
12	66.1	24.4	1.95

calibration graphs were found for both components in the range 0.05–1 mg/ml of sample. At a concentration of 0.5 mg/ml the relative standard deviation (n = 7) was 1.2 % for penicillin G and 2.0 % for procaine. The low precision for the determination of the procaine portion is due to problems of integration caused by peak tailing.

For clemizole penicillin G the elution power of the mobile phase has to be increased by increasing the concentration of the methanol component. Moreover, the analysis can only be carried out in the acidic pH range from 2.5 to 3.0. Owing to the

TABLE III

ELUTION BEHAVIOUR OF PROCAINE PENICILLIN G AND CLEMIZOLE PENICILLIN G

Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M)–methanol. Flowrate: 1.8 ml/min. Temperature: 50°C. Wavelength: 220 nm.

Mobile phase	Retention time (min)		
	Procaine	Clemizole	Penicillin G
Buffer * (pH = 4.0)–methanol (7:3)	1.1	17.6	4.7
Buffer (pH = 5.0)–methanol (7:3)	1.3	26.0	4.4
Buffer (pH = 6.0)–methanol (7:3)	1.5		3.0
Buffer (pH = 7.0)–methanol (7:3)	4.2		3.7
Buffer (pH = 7.3)–methanol (7:3)	7.1		4.4
Buffer (pH = 7.5)–methanol (7:3)	8.0		4.0
Buffer (pH = 2.5)–methanol (6:4)		4.7	2.2
Buffer (pH = 3.0)–methanol (6:4)		5.2	1.7

  * Phosphate buffer (1/15 M).
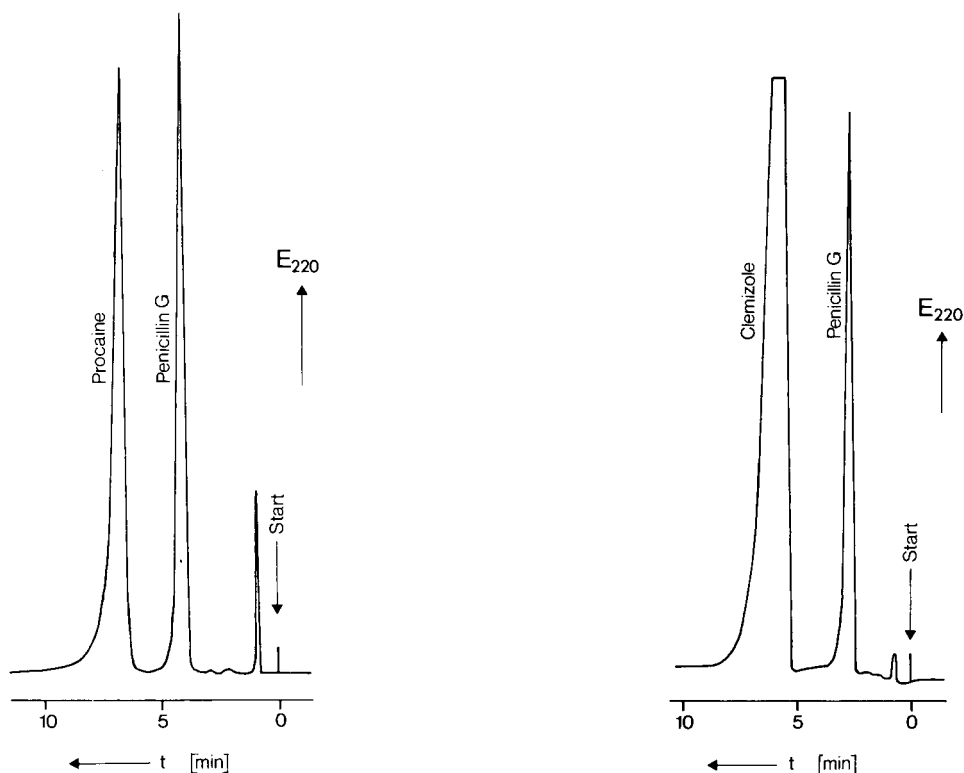
Fig. 3. Determination of procaine penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M) (pH = 7.3)–methanol (7:3). Flow-rate: 1.9 ml/min. Temperature: 50°C.

Fig. 4. Determination of clemizole penicillin G by HPLC. Stationary phase: LiChrosorb RP-8, 10 μm. Mobile phase: phosphate buffer (1/15 M) (pH = 2.5)–methanol (6:4). Flow-rate: 2.1 ml/min. Temperature: 50°C.

rapid chromatographic separation no degratation reaction of penicillin G could be observed. Fig. 4 shows a typical chromatogram for clemizole penicillin G. As with procaine penicillin G, linear calibration graphs were obtained for clemizole and penicillin G at 220 nm in the range of 0.05–1 mg/ml of sample. The relative standard deviation (n = 7) was 0.5% for penicillin G and 0.7% for clemizole. The concentration was 0.5 mg/ml. The fact that the values are superior to those of procaine penicillin G can be explained by the more favourable peak symmetry.

CONCLUSIONS

The HPLC methods described permit the simultaneous quantitative determination of the cationic and anionic components of repository penicillins within a few minutes. They are superiour to all hitherto described procedures for this group of substances. Their application is not limited to the quality control of the pure penicillin salts. The analytical characterization of mixtures of repository penicillins with soluble penicillins or mixtures of different repository penicillins is also possible.

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QUANTITATIVE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMPICILLIN*

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SUMMARY

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method was developed for the potency determination of the trihydrate and anhydrous forms of ampicillin and its sodium salt. An acid phosphate-acetonitrile mobile phase was used with caffeine as an internal standard. Efficiencies exceeding 15,000 plates per meter (or reduced plate height of 13) were obtained with precision normally better than 2 % and a good linear range of detection (0.7–36 µg) at 254 nm. Good statistical correlations were shown when the results obtained from the RP-HPLC method were compared to those from the official methods.

INTRODUCTION

Ampicillin (D(−)-α-aminobenzylpenicillin) (CAS Registry 69-53-4 for the anhydrous, 7177-48-2 for the trihydrate, and 69-52-3 for the sodium salt), derived from 6-aminopenicillanic acid (6-APA), is an antibiotic used therapeutically on a wide scale because of its relatively rapid systemic absorption, its low toxicity, and its biological activity against a broad spectrum of Gram-positive and several Gram-negative pathogens. The assay of ampicillin currently consists of the following official[1] procedures: (a) microbiological agar diffusion, (b) iodine titration, (c) hydroxylamine colorimetry, (d) non-aqueous acid titration, (e) non-aqueous base titration.

The microbiological assay reflects the end use of the drug, but the method is slow and tedious and lacks precision and specificity. The chemical assays require available functional groups in order to react quantitatively. But these assays are limited by the fact that they depend on reactions that may also occur with other components of the drug substance, which interfere. Consequently, the adoption of a concordance test (difference between two non-aqueous titrations) proved relatively successful except in cases when the molecule did not retain its integrity. Clearly a different analytical approach offering greater specificity became a necessity.

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High-performance liquid chromatography (HPLC) offered the best potential for quantitating the intact principle of the drug after separating and differentiating it from undesirable substances such as oligomers, degradation products, and residual contaminants, such as dimethylaniline, which is currently monitored by gas chromatography[2].

Column chromatographic techniques with Sephadex G-25[3,4], ion-exchange[5], and reversed-phase (RP) HPLC[6-8] have been described for the analysis of ampicillin in concurrent polymerization and degradation processes. Bracey[9] used a strong anion-exchange column to isolate and detect ampicillin as a contaminant in nitrofurantoin. A similar type of column was used by Tsuji and Robertson[10] to develop an assay procedure for the antibiotic. Subsequently, qualitative RP-HPLC of ampicillin in dosage forms was promoted for commercial purposes[11] to separate different penicillins[12,13], to determine partition coefficients of penicillins[14], and to analyze antibiotic susceptibility disks[15]. It proved to be an effective method for the analysis of biological materials by direct detection at 225 nm[16] or by post-column derivatization[17], and for separating diastereoisomers[18]. Because none of these references provided a satisfactory analytical method applicable to regulatory use, this study was undertaken to develop a more specific and reliable method for the potency determination of the trihydrate and anhydrous forms of ampicillin and its sodium salt.

EXPERIMENTAL

Apparatus

Investigations were carried out at various times with three different sytems: (1) a S-P Model 3500 liquid chromatograph with a Model 8200 UV detector (Spectra-Physics, Santa Clara, CA, U.S.A.) and a 20-μl loop injector (Valco, Houston, TX, U.S.A.), (2) a Model 995 liquid chromatograph with detector (Tracor, Austin, TX, U.S.A.) and an injector (Rheodyne, Cotati, CA, U.S.A.), (3) a Model 6000A solvent delivery system equipped with a U6K septumless valve injector and a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.). These systems were connected to a strip chart recorder and an electronic integrator such as an H-P 7100 (Hewlett-Packard Co., Palo Alto, CA, U.S.A.) and an Autolab Minigrator (Spectra-Physics), respectively.

Columns

Except for the special Pyrocarbon column[19], the only satisfactory columns were the reversed-phase types such as the octadecyl silane (ODS) bonded silicas like Spherisorb S5-ODS (Phase Separations, Queensferry, Great Britain), Partisil ODS-2 (Whatman, Clifton, NJ, U.S.A.), and Partisil PXS-525 ODS (Whatman), μBondapak C$_{18}$ (Waters) or a well-capped C-2 silica such as Zorbax TMS (DuPont Instruments, Wilmington, DE, U.S.A.) that exhibit some degree of polarity. The columns used for quantitations were Partisil PXS-525 ODS (25 cm × 4.6 mm I.D.) and Zorbax TMS (15 cm × 4.9 mm I.D.). A guard column (10 cm × 4.6 mm I.D.) gravity-packed with an ODS bonded silica of larger size (40–60 μm) was connected to the inlet of the analytical column. Columns were evaluated for performance and suitability as suggested by Bristow and Knox[20] with a program described and modified for the APL programming language to suit our needs[21,22]. The column was conditioned to equilib-

rium by the passage of about 10 to 15 void volumes of eluent, followed by about 75 μl of sample injected in 15-μl portions.

Chemicals and reagents

The ampicillin standard, labeled at 864 μg/mg with 13.52 % moisture, was obtained from the United States Pharmacopeia (USP). Caffeine (USP) was the internal standard. Acetic acid and potassium dihydrogen phosphate were reagent grade. Acetonitrile was "distilled in glass" quality (Burdick & Jackson, Muskegon, MI, U.S.A.). All the water used was purified by the Milli-Q-Reagent-Grade water system (Millipore, Bedford, MA, U.S.A.).

Solutions

Stock solutions. Stock solutions of acetic acid and of potassium dihydrogen phosphate, each 1.0 M, were prepared with water.

Diluent. A 10.0-ml aliquot of 1.0 M phosphate stock solution was combined with 1.0 ml of 1.0 M acetic acid stock solution and diluted to 1.00 liter with water (pH about 4.1). This diluent was used to dissolve all samples as well as the internal standard.

Mobile phase. The eluent was prepared by adding 10.0 ml of 1.0 M phosphate stock solution and 1.0 ml of 1.0 M acetic acid stock solution to about 500 ml of water; then 80 ml of acetonitrile was added and the solution was diluted to 1.00 liter with water while mixing. This solution was degassed by passage through a 0.5-μm filter and ultrasonicated for at least 2 min before use. As an added precaution, helium was allowed to sparge perceptibly through a metal filter into this mobile phase during the analysis to ensure maximum elimination of dissolved air.

Internal standard. Approximately 20 mg of caffeine was dissolved in 100 ml of diluent.

Standard solution. An amount of the working ampicillin standard equivalent to about 25 mg on the dried basis was accurately weighed into a 25-ml volumetric flask. After 1.0 ml of the internal standard was added, the flask was filled to volume with diluent and the solution was mixed. The solution was further shaken and ultrasonicated until it became totally clear. Samples of ampicillins were treated in the identical manner as the standard. It is important that standards and samples be dissolved just before analysis.

Conditions for quantitation

Instrument system 3 as described under *Apparatus* was used for quantitation with operating temperature ambient and flow-rate 1.5 ml/min. The detector sensitivity was controlled by the attenuator of the electronic integrator to produce peak heights greater than 50 % f.s.d. The chart speed was normally set at 2.54 mm/min (0.1 in./min) for assays, but for computation of performance parameters, chromatograms were obtained at 25.4 mm/min to improve the accuracy of manual measurements.

Assay and calculations

Carefully measured 15.0-μl aliquots of standard and sample solutions were injected sequentially into the chromatograph. Quantitation was achieved by normal-

izing the peak height (and/or area) with the internal standard and comparing to that of a reference standard material according to the following equation:

$$[P_x/(C_x \times I_x)/P_s/(C_s \times I_s)] \times 100 = \% \text{ purity}$$

where P is peak height (or area) of ampicillin, C is concentration of solution corrected for moisture content, I is peak height (or area) of the internal standard, and x and s refer to analyte sample and reference standard material, respectively.

    All graphs were plotted and all calculations and data reduction were performed through the APL time-sharing system of the IBM 370/168.

RESULTS AND DISCUSSION

    The sodium salt must be dissolved in the acid phosphate diluent, but good quantitation was obtained for the trihydrate and anhydrous materials by dissolving them in water only. Inasmuch as ampicillin is least prone to hydrolysis in its zwitterionic form, a slightly acid aqueous system with a pH near its isoelectric point will maximize its stability during the course of the analysis, even though its solubility is minimal at that pH[23]. Therefore an ampicillin concentration of about 1 mg/ml was selected, but as noted previously, the samples should be dissolved just before injection.

Calibration
    Varying amounts of loop-injected ampicillin (*i.e.*, same volume of solutions of different concentrations) were plotted against peak heights and corrected for recorder sensitivity for the first calibration. A rectilinear relationship with a coefficient of correlation of 0.9998 and a 0.70 % relative standard error was obtained by regression analysis for a 50-fold dilution of the amount of drug injected (0.7–36 µg); the upper limit of this range is well below the loading capacity of the column. Further examination of 22 values of the calibration factor (peak height per unit concentration) plotted against concentration (different quantities in the same volume) showed a constant value with a coefficient of variation of 4.7 % with the largest deviations occurring at the lower concentrations.

    Another calibration plot based on injections of aliquots of different volumes of the same solution was obtained with the U6K injector. Volumes (2.5–20 µl) injected by syringe and plotted against peak areas (or heights) yielded the straight line shown in Fig. 1. For an injection of 0.4 µg, the extrapolated peak height is about 5 mm, which is more than twice the signal-to-noise ratio at that sensitivity. However, a plot of these volumes against the calibration factor (area/µl) proves that the apparent linearity in Fig. 1 is misleading. The calibration factor, which decreased by about 30 % as volume increased over an 8-fold volume change, is shown in Fig. 2. This discrepancy clearly demonstrates the advisability of using an internal standard and maintaining a constant syringe volume from injection to injection, particularly with non-loop injectors.

System performance
    Fig. 3 is a typical chromatogram of a freshly dissolved sample of ampicillin
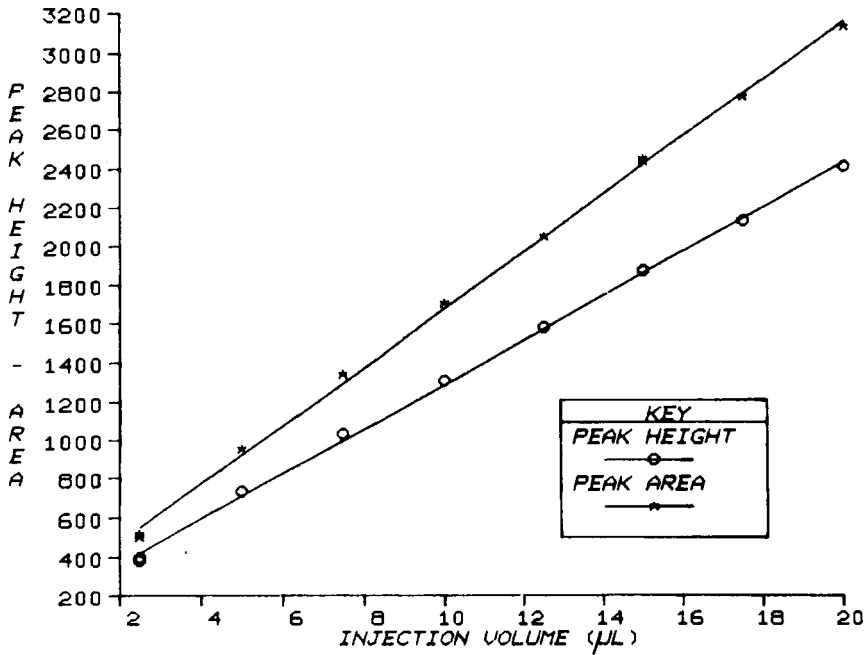
Fig. 1. Calibration of ampicillin syringe and U6K injections.
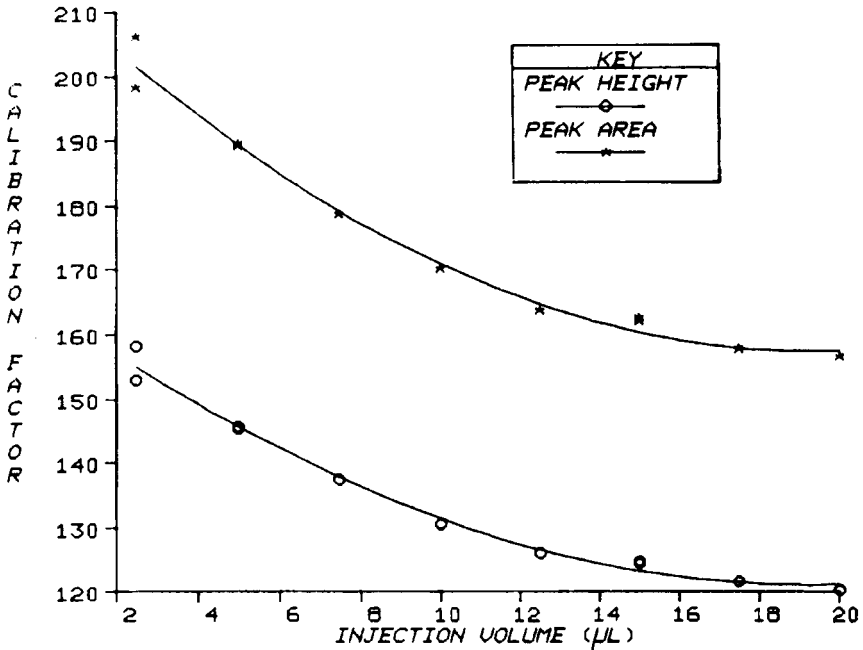
Fig. 2. Constancy of the calibration factor in syringe and U6K injections.
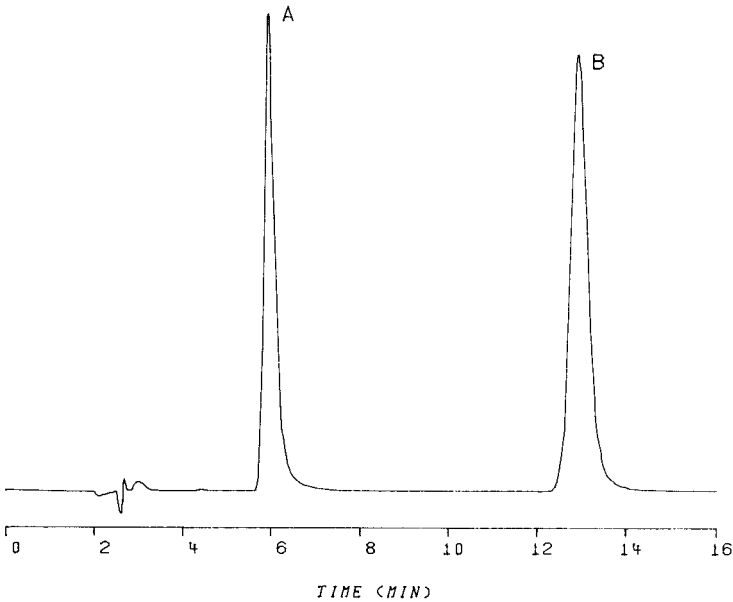
Fig. 3. Chromatogram of freshly dissolved (A) ampicillin and (B) caffeine on Partisil PXS-525 ODS.

used for potency measurement, whereas the chromatogram in Fig. 4 is that of a solution that has incurred a slight degradation just by standing for several days. A chromatogram obtained at higher sensitivity (Fig. 5) shows peaks of minor components.
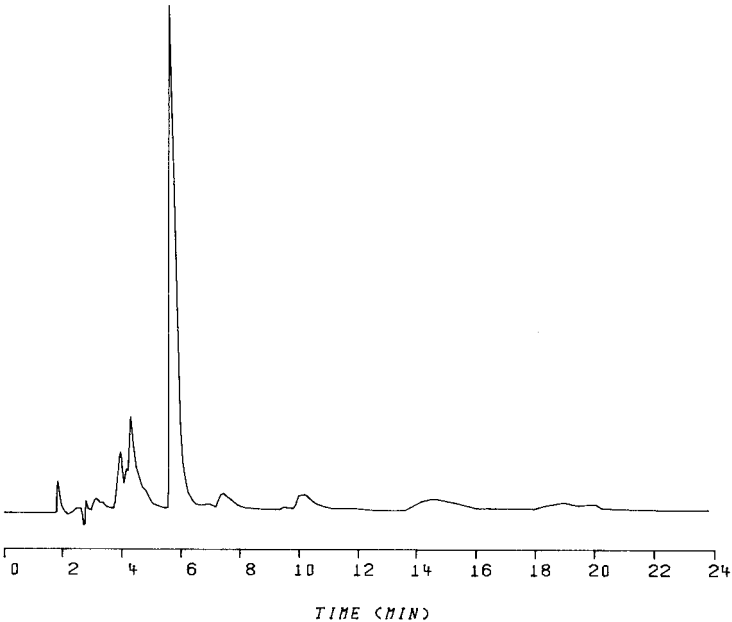
Fig. 4. Chromatogram of slightly degraded ampicillin solution on Partisil PXS-525 ODS.

TIME (MIN)
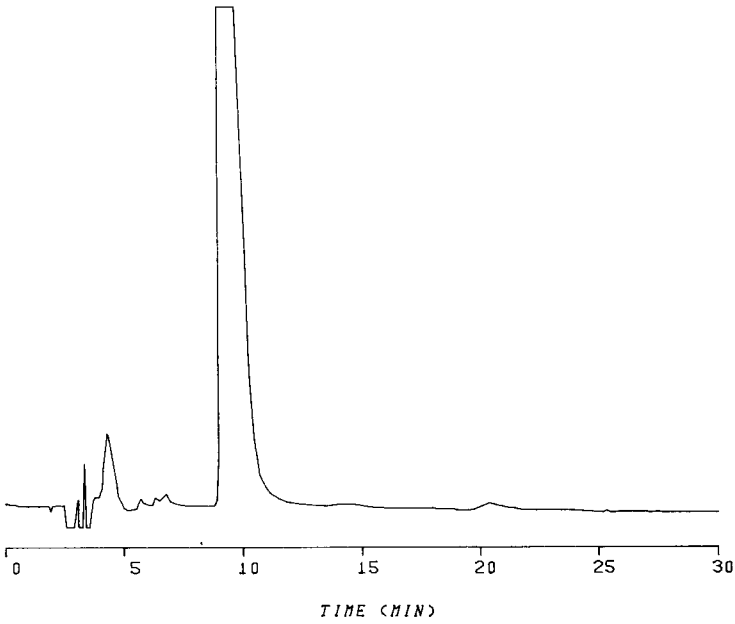
Fig. 5. Chromatogram of freshly dissolved ampicillin at higher sensitivity on Partisil PXS-525 ODS.
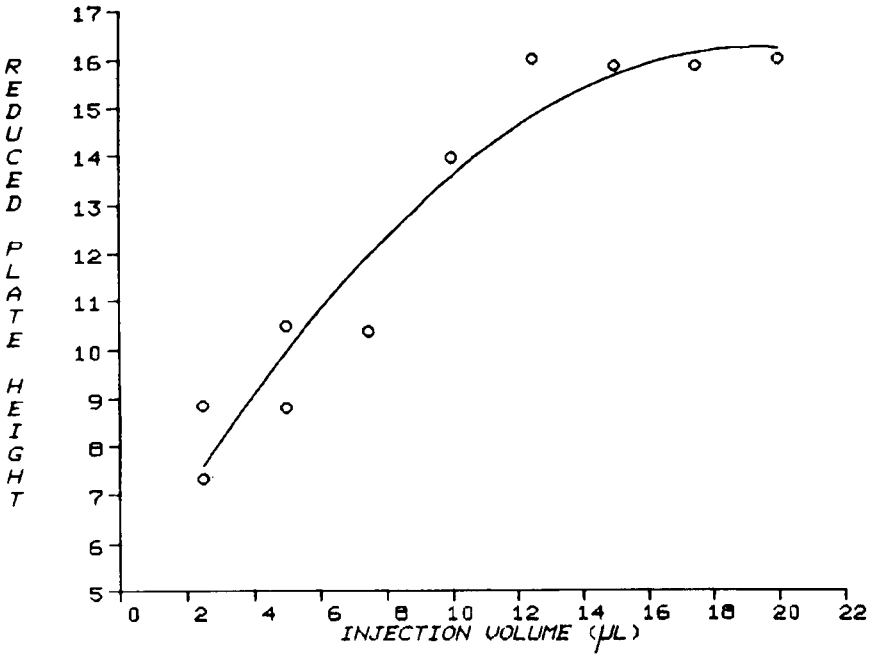
Fig. 6. Effect of sample size on efficiency as reduced plate height, h_r.

A 15-cm Zorbax TMS column typically exhibits efficiencies, n, of 4230 and 11,640 plates or reduced plate heights, h_r, of 9.8 and 3.6, and capacity factors (k) of 0.82 and 1.78 for ampicillin and caffeine, respectively, with a selectivity of 2.16, a retention ratio of 1.5, and a resolution of 8.90 between the two peaks. The shape of the chromatographic peak tends to skew and to shift from the center of gravity with increasing concentration as evidenced by a slight increase in the retention volume and a corresponding increase in the capacity factor. Although greater efficiencies are attained with smaller amounts of injected solutes, (Fig. 6), compromises are necessarily made with respect to quantity injected and flow-rates.

Quantitative analysis

Table I summarizes the results of a number of assays on Partisil PXS-525 ODS of several lots of ampicillin representing a variety of manufacturers. Results were calculated from electronic area integration and from concurrent peak height measurement; Table I gives the combined averages. Values calculated from peak heights correlate well with values calculated from peak areas with a coefficient of variation of 1.1 %. This is a better correlation than that derived from several replicate results obtained from different weights of the same sample.

TABLE I

RESULTS OF AMPICILLIN ASSAYS (OVERALL) ON PARTISIL PXS-525 ODS

S.D. = Standard deviation; C.V. = coefficient of variation.

Sample	Number of assays	Mean potency (%)	Variance (%)	S.D. (%)	
1	4	104.65	0.96	0.98	0.93
2	6	99.95	1.10	1.05	1.05
3	4	96.44	0.11	0.32	0.34
4	5	99.84	0.03	0.16	0.16
5	5	103.37	0.50	0.71	0.69
6	5	102.89	0.51	0.71	0.69
7	4	101.01	0.39	0.62	0.62
8	5	102.62	0.49	0.70	0.69
9	4	99.11	0.08	0.28	0.28
10	9	95.07	1.98	1.41	1.48
11	4	99.72	0.15	0.39	0.39
12	4	103.08	0.01	0.12	0.12
13	5	101.98	0.36	0.60	0.59
14	4	100.53	0.54	0.73	0.73
15	4	100.78	0.19	0.44	0.43
16	4	101.94	0.40	0.63	0.62
17	4	94.97	0.03	0.16	0.17
18	8	95.73	2.11	1.45	1.52
19	4	93.42	1.16	1.08	1.15
20	14	100.27	1.93	1.39	1.39
All	106	99.63	10.10	3.18	3.19
95 % Confidence interval of the mean = 99.02–100.25					
Peak areas　56		99.61	10.88	3.30	3.31
95 % Confidence interval of the mean = 98.73–100.50					
Peak heights　50		99.66	99.43	3.07	3.08
95 % Confidence interval of the mean = 98.78–100.53					

Improved quantitative results were obtained when the system included the guard column.

A correction for loss on drying (LOD) was applied to the initial weighing of all samples, but inaccuracies in this LOD naturally affected the assay results in a like manner.

Good quantitative results, especially with the loop injector, were obtained by direct comparison with an external standard, but the use of an internal standard with a good electronic data system significantly improved precision. The internal standard was used in this analysis solely to enhance the reliability of quantitation. Caffeine was the internal standard selected because it is well resolved from the peaks of interest, is stable, possesses good spectral properties, and is readily available in high purity.

The low absorptivity of the solute accounts for the relative lack of sensitivity at 254 nm, which is indicated by the need to inject upwards of 15 μg into the chromatograph. Nonetheless, this method is amply adequate for pharmaceutical bulk analysis and could easily serve as a stability-indicating method, although the amount injected could be readily reduced 10-fold with a likely increase in efficiency and symmetry. Should greater sensitivity be required, techniques can be devised to magnify the absorptivity of the solute by derivatization of the functional groups with highly chromophoric reagents[17].

Qualitative analysis and impurities

Pyrocarbon packing material is an experimental support prepared by Colin *et al.*[19] by controlled pyrolysis of an organic substance onto a silica substrate. A column packed with this material yielded a chromatogram with good peak separation, but the elution required a mobile phase containing 50 % methanol, indicating a retention mechanism more akin to normal-phase than to reversed-phase separation.

The penicillins appear to be easily separated by reversed-phase chromatography. Table II shows the relative retentions on Spherisorb S5-ODS of several penicillins with the same solvent system as described above for the analysis of ampicillin. Results are also shown for a larger ratio of acetonitrile to phosphate buffer which was used to attain more practical retention times.

Yet with the mobile phase described above, Zorbax ODS, which has a very high carbon content and little or no residual silanol content, could not separate ampicillin from one of its impurities, seen as a shoulder on the main peak.

The identity of the active principle is presumed when the retention of the unknown is identical to that of a reference standard under identical experimental conditions. For confirmation of its identity, the substance under the major peak was collected, freeze-dried, and identified by IR and proton NMR spectroscopy.

Different approaches, such as neutron activation analysis and gas–liquid chromatography of residual dimethylaniline[2], have been attempted in an effort to characterize foreign substances in ampicillin. However, the amount of dimethylaniline found was insufficient to account for the total content of halogens (*i.e.*, mainly chlorides); it is likely that the excess was due to the presence of ampicillin hydrochloride. These past efforts have resulted in reducing the total content of undesirable substances in the drug product. The presence of impurities in these samples has been a subject of intense concern for many years[3–8]. Two reactants in the synthesis and likely contaminants in bulk samples of ampicillin, namely, 6-APA and phenylglycine, were

TABLE II

RETENTION OF PENICILLINS ON SPHERISORB S5-ODS

Compound	k	
	10% Acetonitrile*	35% Acetonitrile
Ampicillin	1.64	NR**
Amoxycillin	0.19	NR**
Potassium penicillin G	6.41	0.76
Procaine penicillin G	—	0.95
Penicillin V	14.7	1.08
Sodium oxacillin	26.2	1.42
Sodium nafcillin	—	2.26

* Essentially same solvent system as described in the HPLC method.
** NR = Not retained.

injected into the chromatographic column and were essentially unretained. Other minor peaks that were observed have not been characterized fully in our laboratories.

Previous studies in other laboratories have clearly demonstrated the presence and character of several contaminants in ampicillin[7,8,10,23,24]. Penicillenic and penicilloic acids with a trace of penicillin G were detected in an alkaline solution of ampicillin[10]. In addition to α-aminobenzyl penicilloic acid, which was not retained on the column, Bundgaard[5-8] detected, identified, and quantitated oligomers which varied in size from dimers to octamers. The formation of these oligomers is promoted in alkaline media through auto-aminolytic degradation, but it should be noted that those
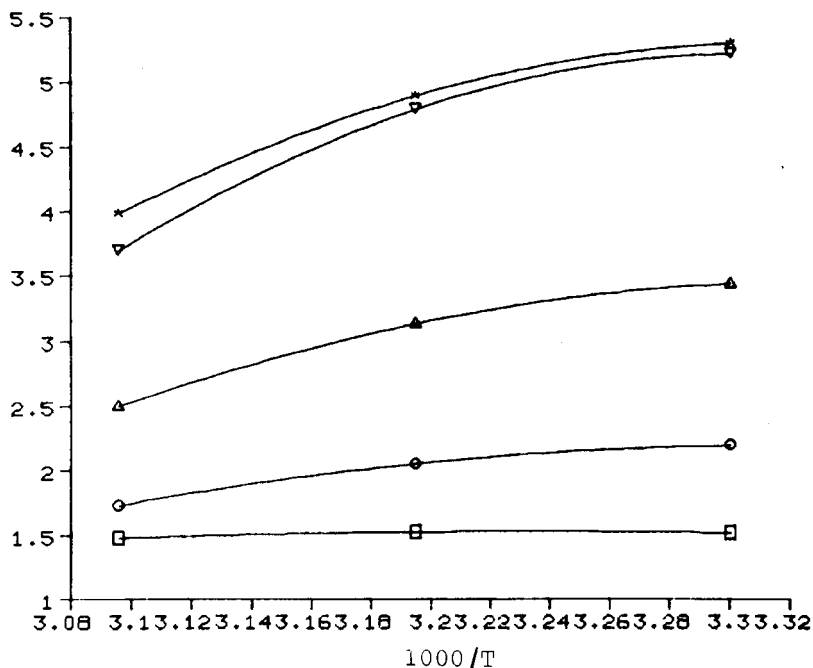
Fig. 7. Effect of temperature, T in °K, on capacity factors, k_1 and k_2, respectively ($\triangle$ and $\triangledown$); selectivity, α ($\bigcirc$); efficiency, n in plates/1000 ($\bigstar$); and pressure drop, P in bars/1000 ($\square$).

solutions for chromatography were prepared in high concentrations at pH levels well above 7 in citrate and borate buffers[7,8], which had been described as deleterious to stability[23]. On the other hand, a strongly acidic solution used in a fluorometric assay yielded a fluorophor, 2-hydroxy-3-phenylpyrazine, as an ampicillin degradation product[24].

Temperature effect

Because the role of temperature has recently attracted more attention as an important parameter in HPLC[19], it was briefly investigated with a Spherisorb S5-ODS column with 12% methanol in 0.5 M sodium dihydrogen phosphate as the mobile phase. A significant decrease in the pressure drop and capacity factor for ampicillin and an impurity peak were observed when the temperature was increased as shown in Fig. 7, but no beneficial effects on efficiency and selectivity were noted.

TABLE III

COMPARISON OF TWO ASSAY METHODS FOR AMPICILLIN

σD = Standard deviation of difference.

Sample	Potency (%)	
	Hydroxylamine	HPLC
1	99.30	104.65
2	103.30	99.95
3	97.60	96.44
4	101.80	99.84
5	100.40	103.37
6	98.50	102.89
7	97.40	101.01
8	102.00	102.62
9	96.10	99.11
10	98.00	95.07
11	98.90	99.72
12	102.60	103.08
13	100.40	101.98
14	100.50	100.53
15	101.00	100.78
16	101.10	101.94
17	99.80	94.97
18	97.00	95.73
19	96.40	93.42
20	100.00	100.27
Number of samples 20		20
Maximum	103.30	104.65
Minimum	96.10	93.42
Range	7.20	11.23
Mean	99.60	99.87
S.D.	2.08	3.18
Median	99.90	100.40
C.V. (%)	2.09	3.18
95% Confidence limit of the mean:		
Lower limit	98.63	98.38
Upper limit	100.58	101.36

$$\sigma D = 2.72$$
$$t = 0.43$$

A statistical difference *is not* detected at the 95% confidence level.

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Low viscosity is inherently crucial in HPLC, but in this system the energy expended to reduce the viscosity of the mobile phase is undesirable because it adversely affects the stability of the analyte. Evidently, it is important to maintain a constant temperature during the analysis for best results.

Validation of results

A comparison of results obtained by HPLC and the official methods by the paired *t*-test indicates that a significant statistical difference exists between the amine titration and each of the other methods, including HPLC. The presence of small amounts of ampicillin hydrochloride could certainly be a contributory interference in the amine titration. On the other hand, the HPLC, acid titration, hydroxylamine titration, and colorimetric assay methods revealed no such difference that could be ascribed to methodology. Statistical data for the hydroxylamine titration and HPLC methods are given in Table III to illustrate this.

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PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THE STABILITY OF NOVOBIOCIN IN MASTITIS PRODUCTS STERILIZED BY ^{60}Co IRRADIATION

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SUMMARY

An isolation procedure and a high-performance liquid chromatographic technique for the quantitative analysis of novobiocin in mastitis products has been developed. The recovery of novobiocin from peanut oil suspensions was 101.2 % with a relative standard deviation of 1 %. The stability of novobiocin to the ^{60}Co irradiation sterilization process was examined. At irradiation dose levels of 1.5 to 4.9 Mrad, the degree of novobiocin degradation was only 3–4.5 %. An increase in the concentrations of two minor indigenous components was observed. However, no new compounds were generated as a result of the sterilization process.

INTRODUCTION

Many drugs, drug preparations and medical devices must be sterilized to ensure a minimum risk of transfer infection. A method of growing acceptance for the sterilization of bovine mastitis products, such as those containing novobiocin [*e.g.*, Albadry® and Albacillin® (Upjohn)], is the post-preparative irradiation of the packaged drug with cobalt-60, an intense source of γ-rays. The question of major concern is the effect this radiation has on the stability of the primary active constituent, novobiocin. Therefore a method of analysis, with a sensitivity of ± 1 %, was required to monitor the effects of this irradiation process on the novobiocin component primarily, and its related minor constituents secondarily.

There are several analytical techniques published in the literature for the detection of novobiocin, either in drug preparations or as residues in biological media. These techniques include microbiology[1-3], paper chromatography[4], thin-layer chromatography[5], gas chromatography[6] and, most recently, high-performance liquid chromatography (HPLC)[7]. The microbiological methods have traditionally been used to assess the activity or potency of drug preparations; these methods are, however, not sufficiently preciese for measuring small differences in drug content, nor are they of any use for measurement of the minor "inactive" components (see Fig. 1). Of the other methods, HPLC is the most appealing in terms of sensitivity, reproducibility, speed and resolving power to separate and measure all of the drug components.

Fig. 1. Structure of novobiocin and analogues.
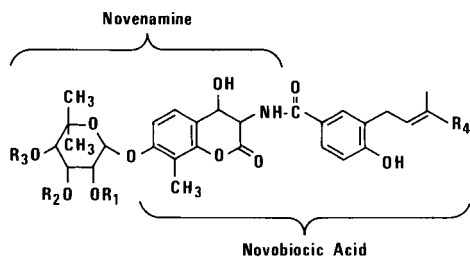
Compound	R_1	R_2	R_3	R_4
Novobiocin	H	$\overset{O}{\overset{\|}{C}}NH_2$	CH_3	CH_3
Isonovobiocin	$\overset{O}{\overset{\|}{C}}NH_2$	H	CH_3	CH_3
Hydroxynovobiocin	H	$\overset{O}{\overset{\|}{C}}NH_2$	CH_3	CH_2OH
Descarbamylnovobiocin	H	H	CH_3	CH_3
Desmethyldescarbamylnovobiocin	H	H	H	CH_3

Dihydronovobiocin = Novobiocin + 2 H (reduced isoprene unit)

The initial efforts to develop an HPLC procedure for novobiocin were based on the procedures of Tsjui and Robertson[7], who used a reverse-phase mode of chromatography, with Zipax HCP as the hydrophobic stationary phase and an aqueous methanol–phosphate buffer as the mobile phase. They reported a relative standard deviation of less than 1 % for the analysis of novobiocin drug; in addition, six minor components were readily separated. However, despite the moderate resolving ability of this method, the novobiocin peak was broad, and the overall elution time of 32 min for all components was considered unacceptably long. Therefore, improvements in the techniques were necessary.

In the last few years, ion-pair chromatography has been one of the more popular topics in HPLC. In particular, reverse-phase ion-pair partition chromatography, referred to as paired-ion chromatography (PIC) by Waters Assoc.[8] or "soap chromatography" by Knox and his co-workers[9,10], is finding application in a variety of areas. This technique, in simplified terms, is based on the chromatographic separation of compounds as ionic species paired with counter-ions, or ionic species of opposite electronic charge, contained in the mobile phase. The separation column is usually a C_{18} or ODS reversed-phase material, but other lipophilic packings have been used. The mobile phase is usually an aqueous methanol or aqueous acetonitrile solution of either 1-heptanesulfonate, which provides the counter-ion species for cationic substrates, or a quaternary amine salt for anionic substrates. The ion-pairs that are in equilibrium with the unpaired ions in the mobile phase are more lipophilic and partition into the organic-like stationary phase. Chromatographic separation is achieved

because each ion-pair has a unique partition coefficient. Kissinger[11] suggests that more subtle processes occur in PIC, but a more detailed discussion is beyond the scope of this paper.

EXPERIMENTAL

Apparatus

Pump. A Varian Model 4200 (Varian, Palo Alto, CA, U.S.A.) was set to deliver mobile phase at 1.0 ml/min.

Column. A Microparticulate Zorbax ODS (DuPont, Wilmington, DE, U.S.A.) column (250 × 4.6 mm) was used, preceded by a Whatman Co:Pell ODS pre-column (50 × 2 mm) (Whatman, Clifton, NJ, U.S.A.).

Detector. The detector used was an Altex Model 150 UV Monitor (Altex, Berkeley, CA, U.S.A.) fitted with a 20-μl analytical flow-cell and a 254-nm wavelength kit operated at 0.2 a.u.f.s.

Injector. The Rheodyne Model 701 sample-loop injector used (Rheodyne, Berkeley, CA, U.S.A.) was fitted with a 10-μl loop.

Recorders. A Varian Model A-25 set at 50 mV and 0.1 in./min was used to monitor the novobiocin and internal standard peaks, and an LIC Model 155 mm (Linear Instrument C., Irvine, CA, U.S.A.) set at 1 mV and 20 cm/h was used to monitor the minor components.

Chemicals and reagents

1-Heptanesulfonic acid sodium salt, was obtained from Eastman Organic Chemicals (Eastman Kodak, Rochester, NY, U.S.A.). Methanol and acetonitrile were "distilled in glass" grade (Burdick and Jackson Labs., Muskegon, MI, U.S.A.).

Reference standard solution preparation

Novobiocin and the various reference compounds were Upjohn Co. reference standards. The novobiocin bulk drug was obtained as the sodium salt from three production lots (693FD, 388FG and 392FG). A series of novobiocin standards for HPLC was prepared from a stock solution of *ca.* 140 mg of sodium novobiocin (120 mg "active" novobiocin) in 20.0 ml of CH_3CN-H_2O (3:1) by dilution to concentrations of 5.0, 4.0, 3.0 and 2.0 mg/ml. These solutions were then diluted 1:1 with a solution (1 mg/ml) of 2,6-dichloro-4-nitroaniline [DCNA (Upjohn)] to yield a series of solutions with novobiocin concentrations of 3.0, 2.5, 2.0, 1.5 and 1.0 mg/ml. The 1.5 and 1.0 mg/ml solutions were diluted 1:1 with CH_3CN-H_2O (3:1) to give 0.75 and 0.50 mg/ml solutions. These solutions served to generate the linear regression curve.

For the internal standard solution, approximately 50 mg of DCNA were weighed into a 50-ml volumetric flask and dissolved in CH_3CN-H_2O (75:25); this gave a DCNA concentration of 1 μg/μl.

HPLC mobile phase

Solutions of 1-heptanesulfonic acid sodium salt, 0.005 *M*, in methanol and in water were prepared separately, degassed under vacuum, then magneticzlly mixed at a ratio of 80:20 (methanol solution to water solution). The mixture was vacuum-filtered through a 4-μm glass-sinter funnel before transfer to the HPLC pump reservoir.

Recovery study

Into each of three 1000-ml separatory funnels was placed 30–32 g of peanut oil, 1500–1700 mg of micronized sodium novobiocin (lot 392FG) —Formulation 1 (to simulate Albadry®)— or 650–750 mg —Formulation 2 (to simulate Albacillin®)— 400 ml of isooctane (distilled in glass) (Burdick and Jackson Labs.) and 400 ml of 75 % acetonitrile (non-spectro-grade distilled in glass) (Burdick and Jackson Labs.) in deionized water. The mixtures were vigorously shaken twice for 20–30 sec at 15–20-min intervals. The lower layer was sampled in triplicate by diluting 5.0 ml with 5.0 ml of $CH_3CN–H_2O$ (75:25), then 10 μl of each sample was injected into the HPLC column. The data are presented in Table I.

TABLE I

RECOVERY OF NOVOBIOCIN FROM PEANUT OIL

Sample	Fortification level, mg	Amount recovered*, mg	Recovery, %
A	1358	1373	101.1
B	1459	1484	101.7
C	1255	1251	99.7
D	598	604	100.9
E	557	567	101.8
F	648	662	102.2
		Mean	101.2 ± 0.9

* Mean of three determinations.

Extraction of drug from plastet samples

Four plastets (a plastic syringe-like device for intramammary injection of drug into the udder) were taken at random from each packet of 12 plastets, weighed as one sample to the nearest 0.01 g, then expressed into a 1000-ml separatory funnel. Then 400 ml of isooctane [saturated with $CH_3CN–H_2O$ (75:25)] and 400 ml of $CH_3CN–H_2O$ (75:25) (saturated with isooctane) were added, and the mixture was vigorously shaken twice for 20 sec each time at 15–20-min intervals. A 10–15 ml portion of the lower layer was drained into a 20-ml screw-cap vial, and duplicate HPLC samples were prepared by diluting 3.0 ml with 3.0 ml of the internal standard solution.

Data calculation

A baseline projection was carefully drawn along the bottom of the chromatogram peaks, and the height of both the DCNA and the novobiocin peaks were measured in mm with a plastic ruler and estimated to the nearest 0.1 mm; the ratio of the peak heights (novobiocin to DCNA) was then calculated. The series of standards produced a standard curve whose slope, ordinate intercept, correlation coefficient and various other parameters were calculated by using linear regression analysis. The drug content in each sample, calculated as mg of novobiocin per g of sample, was calculated from the standard curve data.

RESULTS AND DISCUSSION

     In our initial attempts to study novobiocin by HPLC, we used the commercially available permanently bonded columns ODS, cyano, C-8 and ETH. In no instance was a proper blend of resolution of the minor components listed in Fig. 1 and acceptable novobiocin peak shape observed with typical reversed-phase solvents. However, when the mobile phase was modified with the ion-pairing agent sodium 1-heptanesulfonate, at a conc. of 0.005 M, peak shape and resolution of the minor components greatly improved. A sample chromatogram is shown in Fig. 2. The minor component isonovobiocin was never observed in any chromatogram containing novobiocin because of the overwhelming amount of the latter and their close chromatographic proximity. As far as is known, this was the only minor component whose fate and quantity remained unsolved by this HPLC method.
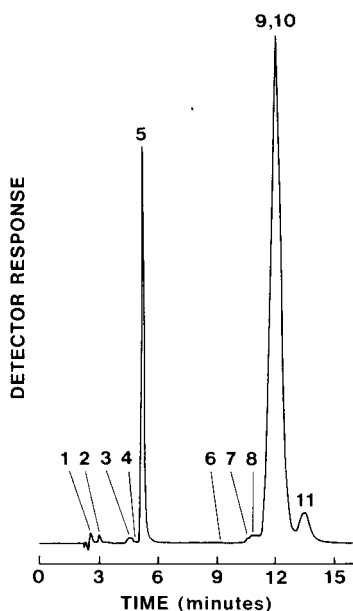
Fig. 2. Chromatogram of novobiocin (lot 388FG); 25μg on column. HPLC conditions: 0.005 M sodium 1-heptanesulfonate in methanol-water (80:20) at 1 ml/min on a Zipax ODS column (250 × 4.6 mm); recorder A-25 at 50 mV and 0.1 in./min. Peaks: 1, novenamine; 2, Unknown 1; 3, Unknown 2; 4, hydroxynovobiocin; 5, DCNA (internal standard); 6, novobiocic acid; 7, desmethyldescarbamylnovobiocin; 8, dihydronovobiocin; 9, novobiocin; 10, isonovobiocin; 11, descarbamylnovobiocin.

     The commercial mastitis products studied in this work were peanut oil suspensions of novobiocin and usually contained a modifier to stabilize this suspension. Therefore, a quantitative extraction of the drug and the drug components from the peanut oil gel suspension was needed. Novobiocin and the various related components are soluble only in highly polar organic solvents, such as acetonitrile. In contrast, the peanut oil is highly lipophilic and will preferentially partition into a non-polar solvent, such as a hydrocarbon. Thus, this isolation could readily be accomplished by a simple partition between CH_3CN-H_2O (75:25) and isooctane.

To test this procedure for efficiency of novobiocin extraction and for HPLC compatibility, a recovery study was run on suspensions of novobiocin in peanut oil. The results, presented in Table I, clearly show that novobiocin was quantitatively extracted. Two levels of fortification were studied at or near the levels in the commercial products; the overall recovery was $101.2 \pm 1.0\%$.

The simple extraction procedure gave quantitative extraction of all the minor constituents present in the bulk drug. Close inspection of the chromatograms in Fig. 3, derived from bulk drug itself and the same bulk drug suspended in peanut oil, then extracted, showed no observable differences. It was also clear that the peanut oil did not cause any interference in the chromatograms.
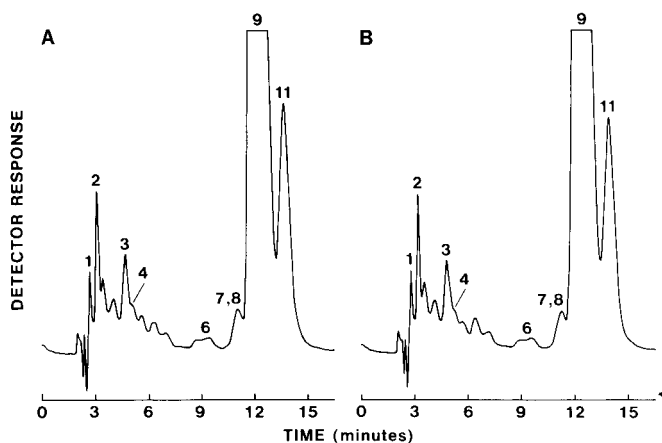
Fig. 3. Chromatograms comparing (A) peanut-oil-suspended novobiocin extracted with CH_3CN-H_2O (75:25) with (B) novobiocin drug standard. Recorder 155 at 1 mV and 40 cm h. Peaks as in Fig. 2.

^{60}Co Irradiated samples

The mastitis products are marketed in a plastic syringe-like device called a plastet. Each plastet contains *ca.* 9 g of the peanut oil suspension of novobiocin and is individually wrapped in plastic and placed in a thin-walled cardboard box (12 plastets per box). Ten of these boxes are packed into a heavier cardboard box, and this box is irradiated with ^{60}Co. The dose levels depend on the length of exposure and the distance from the source, and are generally expressed as a range, since the amount of irradiation which reaches the center of the box is less than that striking the periphery. Detailed descriptions of the process were given by Pope *et al.*[12].

Four sets of samples were irradiated at five dose ranges, and the relative amounts of novobiocin were measured; the results are summarized in Table II. Irradiation at doses of 0.5 to 1.3 Mrad produced a slight decrease of novobiocin of 1 to 1.5%. A slightly higher dose range (1.55 to 1.77 Mrad), however, produced a 3.2% decrease. A jump to the 2.60–4.88 Mrad range generated only a 4.0 tot 4.4% loss. A plot of the percentage of unchanged novobiocin as a function of mean ^{60}Co dose levels (Fig. 4) clearly shows a non-linear bi-phasic plot. Since dose levels above 5 Mrad were not investigated, we shall not conclude that a terminal plateau was reached.

TABLE II

PERCENTAGE DECREASE OF NOVOBIOCIN IN MASTITIS PRODUCTS AS A FUNCTION OF ^{60}Co IRRADIATION*

Dose range	Form. 1**	Form. 1***	Form. 2**	Form. 2***	Mean
0.0	100.0	100.0	100.0	100.0	100.0
0.50–0.57	99.6	98.4	98.9	99.0	99.0
1.05–1.32	99.0	98.4	98.1	98.3	98.5
1.55–1.77	96.2	97.4	97.0	96.5	96.8
2.60–3.13	94.5	96.0	96.9	96.7	96.0
3.94–4.88	94.9	95.7	95.6	96.0	95.6

 * Relative to the 0.0-Mrad sample in each set. Each value is the mean of six determinations.
 ** Sodium novobiocin lot 693 FD.
 *** Sodium novobiocin lot 388FG.

Some changes in the minor components were observed, as seen in Fig. 5, which compares non-irradiated sample with 4.0-Mrad-irradiated sample. There was an increase in the components designated Unknown 1 and Unknown 2 (peaks 2 and 3), respectively. Best estimates, based on peak-height measurements, suggested a 1.5 to 2.5-fold increase in their relative concentrations following irradiation. Perhaps more important, no new compounds were generated by the irradiation process which were not previously present as natural by-products of production or natural degradation. All of the production lots analyzed have contained various amounts of these minor components. Thus, all compounds, including Unknowns 1 and 2, must be defined as indigenous components.
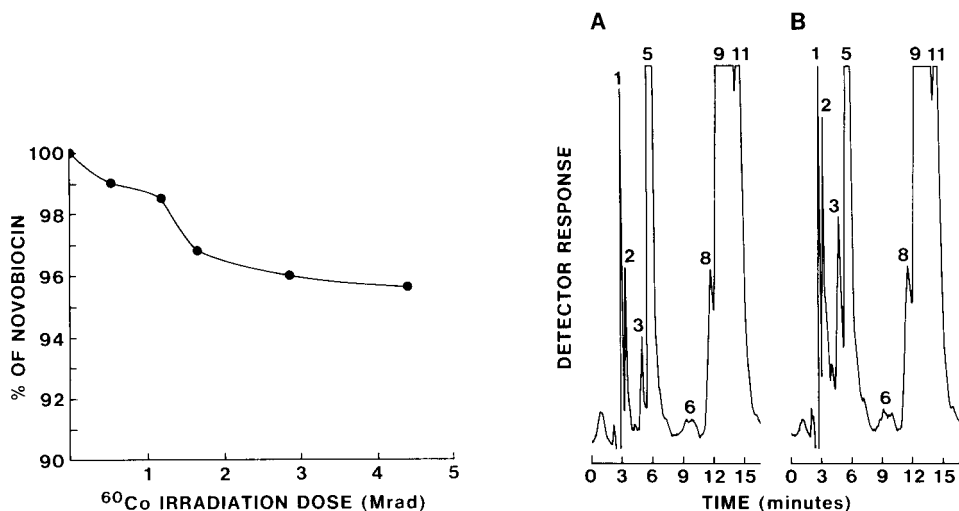
Fig. 4. Dose-response curve of the relative decrease of novobiocin in mastitis products as a function of ^{60}Co-irradiation dose during sterilization.

Fig. 5. Chromatograms comparing the relative intensities of the minor components of novobiocin in mastitis products irradiated with ^{60}Co at an average dose of 0.0 Mrad (A) and 4.0 Mrad (B). Recorder 155 at 1 mV and 20 cm/h. Peaks as in Fig. 2.

One last point should be made. The peanut oil carrier evidently plays only a mediating effect on the irradiation degradation of novobiocin. Irradiation of the raw micronized drug showed an identical degradation pattern and complement of minor components. The decrease in novobiocin was only slightly greater, with a concurrent increase in Unknowns 1 and 2, but again no new components were observed. The results also suggest that the irradiation of the peanut oil does not contribute any observable interference in HPLC.

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Note

Simple programmable controller allowing the timed collection of fractions in high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) is primarily used in conjunction with on-line detection and quantitation. However, for some applications, for example, the separation and analysis of bioactive peptides from tissue extracts, online detection is not always possible because the compounds of interest are either below the limit of detection or are masked by co-eluting material. In such cases, fixed volume fractions of the column effluent can be collected and the substance quantitated using a specific radioimmunoassy (RIA)[1–3] or bioassay[4].

Retention times in reversed-phase HPLC are highly reproducible[1–4] over a wide concentration range. Therefore when the retention time of a standard compound is known, it is possible to collect the portion of column effluent in which it is eluted as a single fraction prior to quantitation.

We describe here a cheap programmable device for controlling a fraction collector which will allow the automatic collection of fractions of varying volume at specified time intervals with a resultant reduction in the number of samples required for assay. The device can be interfaced with an automatic sampler to allow specific fractions to be collected from a series of runs.

MATERIALS AND METHODS

The chromatography was carried out using a Waters 660 gradient programmer controlling two Waters 6000A pumps. The samples were injected from a Waters Wisp 710B autosampler onto a Waters μBondapak C_{18} column. The column effluent was monitored at 206 nm using a LKB Uvicord S detector fitted with an 8-μl flow cell. The fractions were collected on a LKB Ultrorac II 2070 fraction collector controlled via the external input socket by an Acorn series I microprocessor with a R.A.M. 10 chip fitted at the B2 location (Acorn Computers, Cambridge, Great Britain). The microprocessor was programmed to produce a series of up to 100 output pulses, the interval between each pulse being variable from 1 sec to 2 h. The programme was written in machine code and stored on a domestic cassette tape. An interface was constructed from parts obtained from R.S. Components (Fig. 1). Each microprocessor output pulse stepped the fraction collector via this interface and also caused a negative
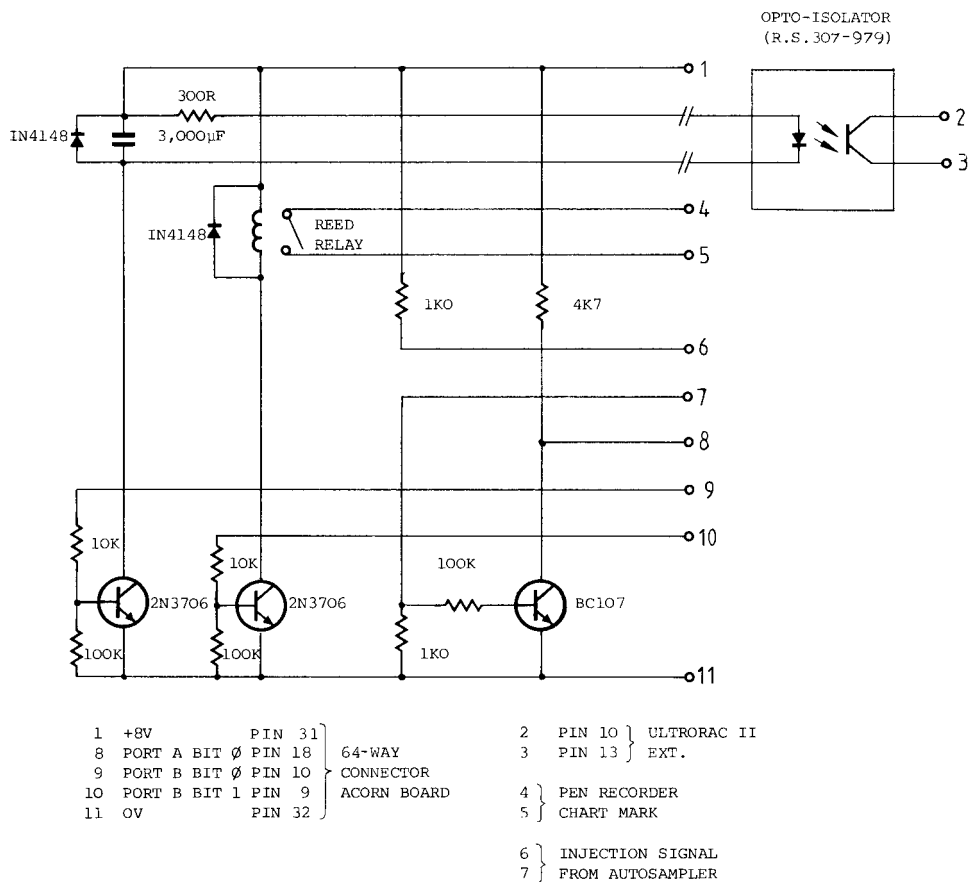
Fig. 1. Circuit diagram of the interface and connections between the autosampler, chart recorder, fraction collector and microprocessor. K = kΩ, R = Ω, 4k7 = 4.7 kΩ, 1KO = 1 kΩ.

(downward) deflection on the chart record. The programme will halt after one run or when linked to an autosampler will recycle for a defined number of runs.

RESULTS

Repeated injections (20 μl) of a standard peptide solution (100 μg/ml) of TRH, LH-RH and substance P were made and from the UV traces the mean elution time for each peptide peak was determined (Table I). After establishing the delay time from detector to collector, the portion of column effluent to be collected was calculated by time using the base width +2 S.D. divided equally about the mid point of the base of the peak (assuming peak symmetry), as the area to be collected for each peak. These times were keyed into the microprocessor. At each step of the fraction collector, a mark was made on the chart. A blank run was then superimposed on the visible standard trace as a means of checking that the collection times fed into the microprocessor correspond with the elution times (Fig. 2).

TABLE I

RETENTION TIMES AND LEVELS OF TRH, LH-RH AND SUBSTANCE P IN THE COLLECTED PEAKS

TRH = thyrotropin releasing hormone; LH-RH = luteinizing hormone releasing hormone.

	Retention time (min)			Peptide levels (pmol)		
	x	S.D.	n	x	S.D.	n
TRH	10.27	0.09	10	1020	26.5	5
LH-RH	14.14	0.10	10	187	9.0	5
Substance P	15.38	0.08	10	177	20.4	5

The reproducibility of the system was assessed by repeated injections of a standard equivalent to levels found in tissue extracts (TRH 1000 pmol, substance P and LH-RH 200 pmol) the collected peaks being assayed using RIA (Table I). Recovery of peptides from the system was calculated using a tritiated peptide ([^{3}H]TRH). 87% of the activity injected on to the column was recovered in one fraction; the mean count of the recovered fraction was 39,577 ± 957 cpm (n = 10) and the initial activity prior to injection was 45,290 cpm.
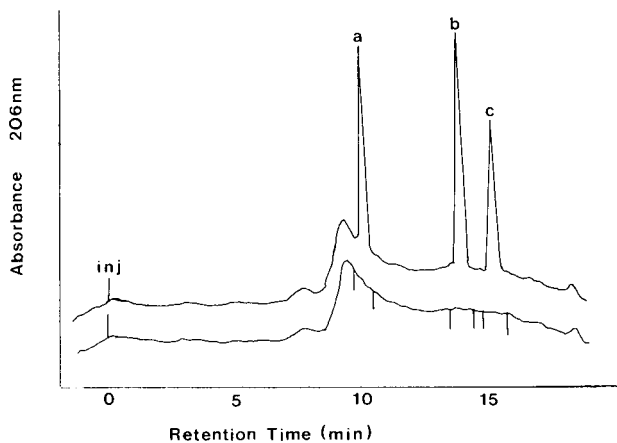
Fig. 2. Separation of TRH, LH-RH and substance P on acetonitrile gradient. Solvent A 0.08% trifluoro-acetic acid (TFA); Solvent B 70% acetonitrile with 0.08% TFA; 20 min. Linear gradient from 5 to 70% B. Peaks: a = TRH; b = LH-RH; c = substance P (all 2.0 µg at 0.5 a.u.f.s.).

DISCUSSION

The system described provides a cheap, simple and highly effective means of recovery of compounds eluting in an HPLC system that are either below the limit of on-line detection or are masked by extraneous peaks that are often present in biological preparations. The system is currently in use in our laboratory for the collection and analysis of peptides from tissue extracts and cerebrospinal fluid. The device should be readily adaptable to other HPLC systems, both analytical or preparative, with only minor modification.

The Acorn microprocessor is flexible enough to allow further programming which could include a base line monitor acting as a level sensing device to enable the collection of visible peaks as well as timed fractions. Finally, the controller could be more permanently programmed by placing a pre-programmed (EPROM) chip into the microprocessor which would obviate the need for a cassette storage system.

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CHROM. 14,428

Note

Contribution to clean-up procedures for serum amino acids

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Most studies dealing with gas chromatographic determinations of amino acids in biological samples involve proteinaceous starting material. Physiological fluids requiring analysis for free amino acid are usually pretreated with a denaturating agent such as picric acid[1,2], sulfosalicyclic acid[3–6], ethanol[7,8] or chloroform[9]. Following removal of the precipitated protein by centrifugation the amino acids are isolated by cation-exchange clean-up. However, variable recoveries of the free plasma amino acids, caused mainly by co-precipitation of the basic amino acids ornithine, lysine and arginine, found by other workers[10–12], led to a search for an alternative procedure.

A simple handy technique, involving dilution of the sample with acetic acid and, thus, elimination of the protein precipitation, was suggested by Pellizzari *et al.*[12], and in combination with cation-exchange clean-up it was used for determination of free amino acids in physiological fluids[12–14]. Even when the results obtained with the acetic acid method proved to be more consistent with those obtained by the amino acid analyzer technique than those obtained by the picrate denaturation[12], the yields of some plasma amino acids were higher with the picrate method, making the acetic acid procedure less attractive[12].

This fact led us to investigate the acetic acid procedure more comprehensively, *i.e.* with respect to the ion-exchange material and the inherent isolation process. It was found that the rate of cross-linking of the ion-exchange polystyrene matrix with the divinylbenzene (DVB) influenced yields of some amino acids markedly, and that by reversing the mechanism of uptake toward the elution a further improvement occurred. Moreover, pieces of polyethylene tubing were found to be a convenient substitute for the ordinary glass columns.

METHOD

Thin-wall polyethylene tubing (3–3.2 mm I.D.), commonly available, was cut into pieces *ca.* 25 mm long, and a plug of glass wool (3–4 mm) was placed in one end. A slurry of 100–200 mesh Dowex 50W-X2 (H$^+$) p.a. resin (Serva, Heidelberg, G.F.R.) in water was sucked in the tubing with help of a syringe to form a 15-mm high column of the wet resin. A peristaltic pump SJ-1211H (Atto, Tokyo, Japan), with a variable flow-rate of 7–700 ml/h, was used to wash the resin bed with 1 ml of 1 *N* hydrochloric acid and 2 ml of distilled water and for the subsequent uptake of the amino acids by suction, as follows.

A 50–100-μl volume of serum (or urine) was placed in a 1.5 ml Silli-Vial (Pierce Eurochemie, Rotterdam, The Netherlands) and mixed with ten times the volume of 25% (v/v) aqueous acetic acid, containing internal standards (25 nmol/ml)[15]. The mixed solution was sucked slowly (ca. 0.5 ml/min) through the activated resin in the plastic column, which was immersed in the liquid by the open end and connected by a piece of glass stem (3.2 mm O.D. and 2 mm I.D.) to the silicone tubing of the peristaltic pump. After the fluid had been sucked off completely, 250–500 μl of water were added and sucked in the resin at a flow-rate increased by two- to three-fold. This step was repeated. For elution of the amino acids it was necessary to place in the lower "suck-in" end of the column a piece of polypropylene tip (a conventional conical tip, used for the push-button pipettes, was cut on both sides to ca. 3.2 and 2 mm O.D., respectively) filled with glass wool. The "suck-off" end of the column was then set on a PTFE luer (8 mm × 3.2 mm O.D.) of a 1 ml syringe (Pressure-Lok®, Pierce Eurochemie No. 19352), which was just filled with 0.7 ml of 2 N aqueous ammonia, and the amino acids were eluted by pushing the syringe plunger down for 15–20 sec. The process is illustrated in Fig. 1.

A) UPTAKE   **B) ELUTION**
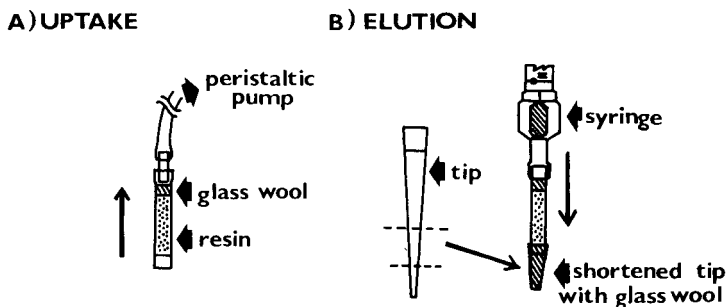
Fig. 1. Schematic illustration of the uptake and elution processer.

RESULTS AND DISCUSSION

Regardless to the kind of sample pretreatment, *i.e.* whether the proteins were precipitated or not, in all the previous procedures[1,2,6–9,12–14] the strongly acidic cation-exchangers of Dowex 50W or Amberlite CG or IR type with higher percentage of DVB-cross-linking (8 or 12%) were used. The higher cross-linkage of the resin provides a doubled resin capacity as compared with the lower one, and this is probably the reason for persistent use of the X8 type resin.

We have tested the Dowex 50W p.a. resin (Serva) with 2–12% of cross-linkage, together with the acetic acid procedure, and found a continuous decrease in uptake of some amino acids as the percentage of cross-linkage increased. When the X8 resin was used instead of the X2 resin, the recoveries for the aromatic (phenylalanine, tyrosine) and long-chain aliphatic (α-aminocaproic acid, being the internal standard in our case) amino acids were lowered to 70–80% and those for arginine and tryptophan to 40–50% only. The yields were even smaller when the X12 resin was employed.

In contrast, the X2 resin improved recoveries for arginine and tryptophan to 70–80% and recoveries of the other protein amino acids were close to 100%. In all these studies the usual procedure was employed, *i.e.* uptake and elution proceeded in

the same direction by forcing the substances through the resin bed under a slightly increased pressure. Under such conditions 0.8–1.0 ml of aqueous ammonia was required to elute amino acids from the resin completely.

The use of reversed flow for the uptake brought a further improvement in the recovery for arginine (more than 90%) whereas that for tryptophan remained unaltered (*ca.* 70%). Recoveries of all protein amino acids proved to be more reproducible under these conditions, and the total amount of the eluant could be reduced to two-thirds of the volume required for the former technique. This method of sample clean-up was used routinely for screening of serum amino acids during thyroid disorders[16]. The amino acids were estimated by means of gas chromatography after their conversion into cyclic derivatives at room temperature[15]. The elaborated technique based on the flow/counter-flow principle might also be useful for other kinds of application.

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CHROM. 14,465

Note

Facile hydrolytic cleavage of N,O-diheptafluorobutyryl derivatives of thyroidal amino acids

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The present United States Pharmacopeia assay method for sodium levothyroxine (T_4Na, sodium salt of 3,3',5,5'-tetraiodothyronine), sodium liothyronine
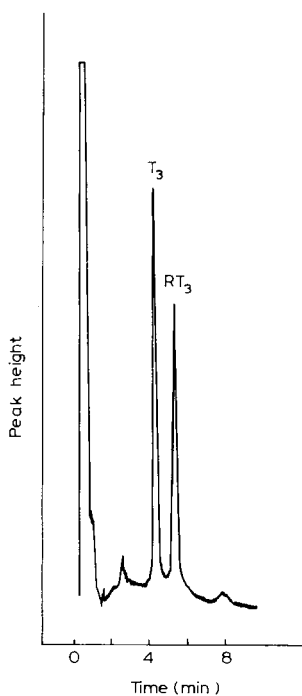
Fig. 1. A Hewlett-Packard 5830A gas-chromatograph, equipped with a Model 18803A Hewlett-Packard electron capture detector (^{63}Ni is the radioactive source) and a Model 18850A Hewlett-Packard GC terminal was used. GLC chromatogram obtained by processing a 5 μg sodium liothyronine tablet. The chromatography was performed on a glass column (4 ft. × 0.4 mm I.D.), packed with 2 % OV-101 on 100–120 mesh Chromosorb W HP. A temperature programming was utilized for the separation of the derivatized T_3 and RT_3, with the initial temperature set at 255°C for 0.5 min and the temperature was increased to 275°C at the rate of 15°/min, to reach 275°C and kept at that temperature for 8 min. The retention times were 5.3 and 7.9 min. respectively for the derivatives of T_3 and RT_3; injection temperature 300°C, oven max 325°C, chart speed 0.5 cm/min. The carrier used was 5 % methane and 95 % argon and the flow-rate was 25 ml/min.

(T_3Na, sodium salt of 3,3′,5-triiodothyronine) tablets and some other thyroid dosage forms[1] is a non-specific, iodometric assay. Recently specific and sensitive high-performance liquid chromatographic (HPLC) methods for analyzing these compounds from tablet formulations were reported[2-5]. However, the reported HPLC procedures were not sensitive enough for conducting either dissolution studies on small dosage forms of T_3Na or T_4Na (smallest dosage form of T_3Na is 5 μg) or to determine plasma levels of these hormones in bioavailability studies.

A highly sensitive gas–liquid chromatographic (GLC) procedure for the determination of T_3 and T_4 from plasma was reported by Petersen and co-workers[6,7], which consists of isolation of the amino acids from plasma and derivatization to the N,O-diheptafluorobutyryl amino acid methyl esters and detection by electron capture. (See Fig. 1 for conditions). We attempted to adapt the above procedure for the analysis of tablets. Basically the procedure consisted of the addition of RT_3 (3,3′,5′-triiodothyronine) as the internal standard, isolation of the amino acids by acidic butanolic extraction[2], followed by derivatization to N,O-diheptafluorobutyryl amino acid methyl ester and GLC determination as described by Petersen et al.[6]. As low as 20 pg injected on the column could be quantitated using this procedure, thus establishing that the method would have the required sensitivity. The resultant peaks had good chromatographic characteristics (Fig. 1). The optimum conditions described for the separation of the peaks were chosen after a preliminary investigation of different columns and chromatography conditions.

The method was reported to exhibit good precision in the analysis of plasma samples[6,7]. However, when we attempted this procedure using pure amino acids, unacceptable coefficients of variation (up to 30 %) resulted. Further kinetic studies revealed that these derivatives are not stable and are sensitive to moisture. The problem was not eliminated even by using freshly distilled dry solvents. The ease of decomposition of the N,O-diheptafluorobutyryl-amino acid methyl ester was T_3 > $T_4 \approx RT_3$.

Similar decomposition phenomena were reported earlier of N,O-ditrifluoroacetyl-T_3 and T_4-O-Me esters by Docter and Hennemann[8]. This is understandable as the trifluoroacetyl group is highly electronegative, thus enhancing the ease of hydrolytic cleavage (Fig. 2). In comparison to the trifluoroacetyl group, the heptafluorobutyryl moiety is more electronegative and is expected to be extremely moisture sensitive. Presently it is not known whether it is the heptafluoro derivative of the amino or phenolic group or both that are cleaved and the reason for the much more facile cleavage of the derivative of T_3 as compared to RT_3 and T_4. It is likely that cleavage occurs more easily at the 4′ (phenolic position), as it is more similar to
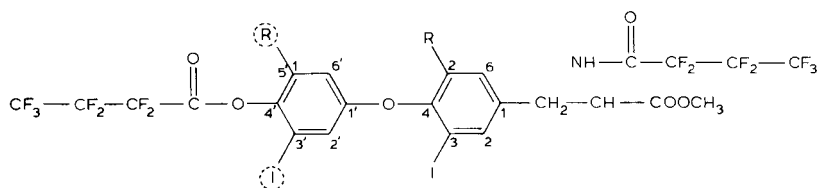
Fig. 2. Chemical structure of basic iodothyronines derivatized as the N,O-diheptafluorobutyryl-amino acid-methyl ester. R_1 = I, R_2 = I is thyroxine (T_4); R_1 = I, R_2 = H is reverse T_3 (RT_3); R_1 = H, R_2 = I is triiodothyronine (T_3 or liothyronine).

an activated ester in structure than at the amide linkage. If indeed the cleavage occured primarily at the phenolic position of the heptafluoro group, it could partially be explained by the presence of steric and hydrophogic interactions. For the derivatized T_3, there is considerable strain due to steric interaction and crowding between the iodine atom and the heptafluoro group. This steric strain would be released by the hydrolytic cleavage of the heptafluoro group. In the case of RT_3 and T_4, the heptafluoro group at the phenolic 4′ position is surrounded by iodine at the 3′ and 5′ positions, with the resultant increase in steric strain. However, hydrophobic interaction between the bulky iodines or between the iodine atoms and the lipophilic heptafluoro moiety is likely. This would result in a hydrophobic interaction, which excludes water, and thus makes hydrolytic cleavage of the heptafluorobutyryl moiety more difficult. This explanation is in agreement with our experimental results that the derivative of T_3 is more prone to decomposition than the derivatives of T_4 and RT_3.

In conclusion, although a procedure was developed for the isolation, derivatization and quantification of thyroidal amino acids using GLC, the procedure was not pursued further due to the relative instability of the derivative N,O-diheptafluorobutyryl amino acid methyl ester. The reason for the instability is explained on the basis of steric interactions and electronegativity.

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Note

Rapid benchtop method of alkaline hydrolysis of proteins

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Tryptophan can easily be quantitated by amino acid analyzer or by specific assays[1]. However, tryptophan analysis of proteins requires a hydrolysis step which can be time-consuming or cause variable losses of tryptophan. Despite promising improvements in acid hydrolysis techniques, significant losses of tryptophan still occur, especially in the presence of carbohydrate[2,3]. Alkaline hydrolysis remains the method of choice when one requires precise determination of tryptophan[4,5].

Spies and Chambers[4] originally noted that increased temperature of hydrolysis dramatically accelerated release of tryptophan. Hare[6] described a technique of rapid acid hydrolysis at 155°C, which eliminated the need for sealing samples in thick walled tubes. I adapted this method for alkaline hydrolysis, using a simple benchtop block heater. Following hydrolysis, tryptophan was quantitated with the sensitive, rapid fluorimetric method of Nakamura and Pisano[1]. Thus, the tryptophan content of a protein can be determined in half a day.

EXPERIMENTAL

Lysozyme was obtained from Boehringer (Mannheim, G.F.R.); tryptophan and the albumins were Sigma products (Sigma, St. Louis, MO, U.S.A.). Glutamine synthetase was prepared by Miller's method[7]. Protein concentrations were determined spectrophotometrically using a molar absorptivity of 37,800 for lysozyme (281 nm, 1 mM HCl), 44,000 for bovine albumin and 35,200 for human albumin (278 nm, 50 mM potassium phosphate, pH 7.0)[8]. Glutamine synthetase concentration was calculated as described[9]. Tryptophan was determined in water with $\varepsilon_{molar} = 5500$ at ·278.9 nm.

The hydrolysis solution was 5 M NaOH (Baker, Phillipsburg, NJ, U.S.A.) with 3% (v/v) thiodiglycol (Pierce, Rockford, IL, U.S.A.). Solutions were made fresh daily, although stability during longer storage was not tested. Thiodiglycol must be included to prevent variable losses of tryptophan as noted by Oelshlegel *et al.*[10] for hydrolysis at 120°C[5].

Hydrolysis vials were Wheaton 1-dram borosilicate glass vials (No. 224882), with a PTFE on silicone cap liner (Pierce No. 12712). They were used without additional treatment or cleaning. Unlined caps were from PGC Scientific (Rockville, MD, U.S.A.). If the usual rubber-lined caps are supplied, this liner must be removed

before inserting the PTFE/silicone liner. (A narrow metal spatula blade permits easy removal of the rubber liner. The caps should then be boiled in water to remove loosely-adhering particles.)

These vials fit into a heating block (Pierce Reacti-Block C) seated in a standard, small, bench-top heater (Lab-Line Multi-Blok Heater No. 2090, with cover). The temperature was set at 155°C. With the cover in place, the temperature usually varied no more than 1°.

Alkaline hydrolysis

Place 25 µl of protein solution (2–200 nmoles in tryptophan) in the hydrolysis vial. Add 500 µl NaOH–thiodiglycol, and flush the vial of air with a stream of argon or nitrogen for 20 sec. Then screw on the cap with the PTFE liner in place. The cap should be snug, but overtightening may crack the neck of the vial. Place the vial in the heating block for the desired time. As shown in the Results section, taking several time points may give more accurate determinations. Times samples may be obtained by using different vials. However, if the amount of protein available is limited, one may sample from a single vial. If a single time point is chosen, 30–45 min is adequate for most proteins. Silicate extraction from the glass increases considerably when hydrolysis time exceeds 45 min.

After cooling, add 500 µl of 25 % acetic acid and then 2.5 ml of 200 mM borate, pH 9.0. (Make by titrating boric acid with NaOH.) Recap and centrifuge the vial for 10 min to precipitate insoluble silicates. Use a 100-µl aliquot of the supernatant for tryptophan determination as described by Nakamura and Pisano[1]. If several time points were obtained, extrapolate to infinite hydrolysis time with a double reciprocal plot (Fig. 2).

RESULTS

Release of tryptophan was followed to assess the rate of hydrolysis of protein. Tryptophan release approaches a maximum at 30–45 min, with release from lysozyme slightly slower than from the other three proteins (Fig. 1). Heating for 90 min or longer leads to a decrease in tryptophan recovery.
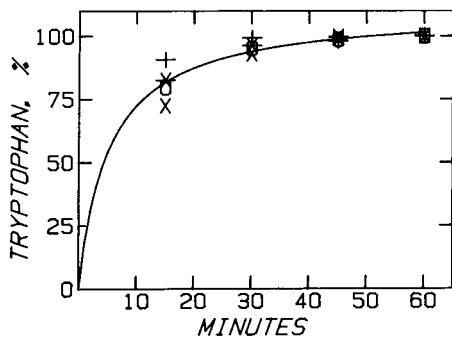
Fig. 1. Release of tryptophan from proteins during alkaline hydrolysis: +, human albumin; *, bovine albumin; ○, glutamine synthetase; X, lysozyme. To permit plotting the results on one graph, the amount of tryptophan released at 60 min was set at 100 %. The curve is the least squares fit to the average of the four proteins. It was fit to a double reciprocal plot, giving

$$\% \text{ Tryptophan} = \frac{110.3 \; x \; (\text{min})}{5.238 \; + \; \text{min}}, r = 0.989$$
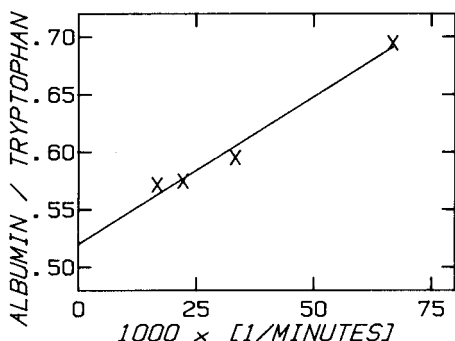
Fig. 2. Release of tryptophan from bovine albumin plotted as a double reciprocal. The fit line is $y = 0.520 + 2.56x$, $r = 0.990$.

The ease of hydrolysis makes it possible to obtain determinations at several times of hydrolysis. Plotting on a double reciprocal plot then permits linear extrapolation to infinite time of hydrolysis (Fig. 2). The results agree with expected values (Table I). If only the 45-min point were used, the average estimated tryptophan content is 10.4 % below the extrapolated value. At 30 min, the value is 14.9 % below the extrapolated value. These factors can be used to adjust single time point determinations.

TABLE I

TRYPTOPHAN CONTENT OF PROTEINS

Protein	Measured*	Expected
Human albumin	0.94	1
Bovine albumin	1.92	2
Glutamine synthetase	1.95	2**
Lysozyme	6.18	6

* By extrapolation with a double reciprocal plot.
** See Discussion.

DISCUSSION

As reported by Spies and Chambers[4] over 30 years ago, alkaline hydrolysis of proteins proceeds much faster at elevated temperatures. As shown in Fig. 1, recovery of tryptophan from four proteins approaches a maximum by 45 min when hydrolysis occurs at 155°C. The use of the block heater permitted hydrolysis to be conveniently conducted on the lab bench using screw-top vials. Coupled with a fluorimetric assay for tryptophan, the method provides a simple, sensitive procedure for assaying the tryptophan content of proteins. The technique also permits recovery of other acid-labile residues, such as phosphotyrosine[11].

The tryptophan content of glutamine synthetase is two residues per subunit. Previously the content had been assayed by ultraviolet spectroscopy, giving a higher

value of three residues per subunit[12]. Determination with second derivative spectroscopy[13] confirmed that two residues is the correct value[14]. An incorrect, high tryptophan content also resulted from spectral analysis of deoxyribonuclease[5].

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Note

Determination of formic acid in aqueous fermentation broth by head-space gas chromatography

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During the anaerobic digestion of complex macro-molecules free fatty acids are formed initially, with subsequent conversion to methane. The types and levels of fatty acid present give an indication of the state of the fermentation. Hence it is valuable to be able to quantify these acids in samples which are usually highly coloured or cloudy.

The C_2–C_6 fatty acids can conveniently be determined by gas chromatography (GC)[1] using a flame ionisation detector but the determination of formic acid is more difficult.

The detection and determination of formic acid have been achieved by using thin-layer chromatography[2,3], column partition chromatography[4], spectrophotometry[5], ion chromatography[6] and GC. Column and thin-layer chromatography, whilst often giving good results are laborious, demanding assiduous sample preparation, condition control and long development time for optimum separation.

The spectrophotometric method put forward by Lang and Lang[5] has been used in a modified form in this laboratory for 3 years but whilst it is quick and sensitive (lower limit of detection 15 ppm), it is not feasible when dealing with highly coloured or cloudy solutions.

Ion exclusion chromatography avoids these particular disadvantages, but the equipment is currently very expensive and it may be difficult to obtain sufficient resolution from other organic acids.

GC may be used but a major drawback exists in that formic acid gives almost no response when using a flame ionisation detector. Free formic acid has been determined gas chromatographically using an automatic titrator[7], a katharometer[8] and an argon detector[9] but these methods are not applicable to the determination of low levels of formic acid in the presence of substantial quantities of other fatty acids.

Formic acid has been derivatised and thus determined in a FID detector but most of the derivative procedures are long and complicated[10–12]. However, Sizova *et al.*[13] put forward a method for the determination of formic and acetic acids and formaldehyde in aqueous media. This has been modified, such that formic acid may be determined in the presence of other fatty acids, down to a lower detection limit of 50 ppm.

EXPERIMENTAL

Chemicals

Ethanol, Synthetic grade was obtained from BP Chemicals, methanol, Ultra grade, toluenesulphonic acid, formic acid and acetic acid, all Analar reagents, from Hopkins & Williams. A 5% (w/v) Toluenesulphonic acid solution was made up in methanol.

Instrumentation

A Pye 104 gas chromatograph, fitted with a heated FID and a wide range amplifier was used. Initially stainless steel columns (1.8 m × 4 mm I.D.) packed with 20% Carbowax 400 on Chromosorb W AW DMCS (80–100 mesh) were utilised, the oven temperature being 80°C. Latterly glass columns (1.5 m × 4 mm I.D.) packed with acetone extracted Porapak Q or QS were utilised. The oven temperature was 100°C and the injection port and detector 200°C and 260°C respectively. The carrier gas was helium, flowing at 30 ml/min.

Derivatisation

The broth samples were spun at a relative centrifugal force of $12,000 \times g$ for 10 min, to provide a supernatant solution for analysis. The reaction was carried out in 1-oz McCartney bottles fitted with neoprene septa inside the metal caps.

A 1.4-ml volume of Ultra grade methanol was pipetted into the bottle; 0.5 ml of 5% toluenesulphonic acid in "Ultra" methanol were added followed by 0.5 ml of the sample supernatant. The bottles were capped, shaken momentarily by hand and heated at 80°C for 30 min in a DRI-BLOCK heater. A beaker, inverted over the bottles produced a draught-free enclosure which was easily removed for sampling. A 0.5-ml volume of the headspace vapour from each bottle was injected into the gas chromatograph using a nylon syringe. The syringe was washed with methanol and dried between injections to minimise carry-over and cross-contamination. The use of Ultra-grade methanol for the analysis is preferred as it gives a lower blank.

Quantitation

Standard solutions of formic acid were prepared over the range of concentrations 0.2–1.0 g/l. These were derivatised and the headspace samples injected as described above. A calibration graph was constructed by plotting the peak height obtained against the formic acid concentration. A typical result is shown in Fig. 3.

Thereafter a standard was included with each batch of samples to check that the calibration was still valid.

RESULTS AND DISCUSSION

The initial paper by Sizova *et al.*[13] described a method whereby formic and acetic acids and formaldehyde were reacted with ethanol in the presence of *p*-toluenesulphonic acid as a catalyst, forming ethyl formate, ethyl acetate and diethoxymethane respectively. When the solution from a mixture of formic acid and formaldehyde treated this way was chromatographed on a Carbowax 400 column it produced the chromatogram shown in Fig. 1. It was found that frequent injections of the acidic solution rapidly destroyed the column.

In order to overcome this problem and noting that the reaction products are volatile, it was decided to try a headspace method of analysis. Use of ethanol as the esterifying alcohol necessitated the use of heated sampling syringes to overcome the problems of condensation. However, the use of methanol as the esterification alcohol minimises this problem since the boiling point of methyl formate is 31.5°C.

The chromatogram given by the use of the headspace method on a standard solution of formic acid at a concentration of 1 g/l is shown in Fig. 2.
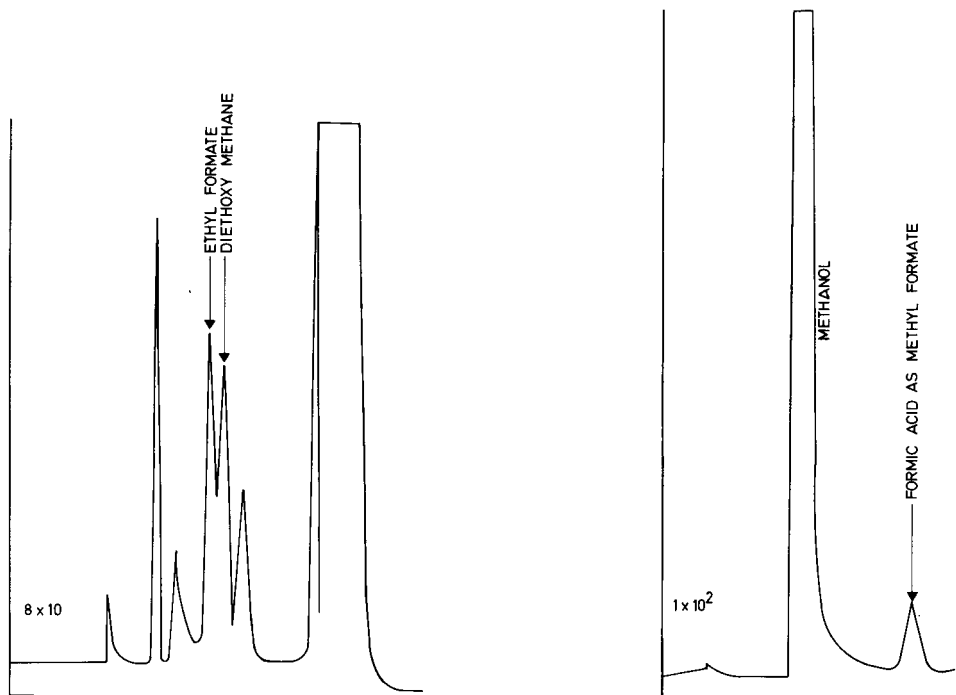
Fig. 1. Chromatogram of formic acid and formaldehyde reacted according to Sizova's method. Column: 1.8 m × 1/4 in., stainless steel, 20% Carbowax 400 on Chromosorb W AW DMCS (80–100 mesh). Temperatures: oven, 80; injector, 200; detector, 260 C. Chart speed: 0.25 in./min. Carrier gas (helium) flow-rate: 30 ml/min.

Fig. 2. Headspace analysis of formic acid at the level of 1 g/l. Column: 6 ft. × 2 mm I.D., Porapak QS (80–100 mesh). Oven temperature: 100 C other details as in Fig. 1.

Control of the temperature during equilibriation of the gas phase, and the salts concentration in the liquid phase are the two primary requirements for consistent headspace analysis. The simple draught shield described, minimises the effect of temperature variation. The high level of p-toluenesulphonic acid fixes the salts level of the final solution such that variations in the salts level of individual broth samples have a negligible effect on the partition of the volatile components.

The calibration graph (Fig. 3) demonstrates good linearity for the method up to 1 g/l indicating a consistent partition of methyl formate into the vapour phase, and good sampling of that phase by the syringe technique described.

The minimum detection level of formate is 0.02 g/l with this sytem. If greater
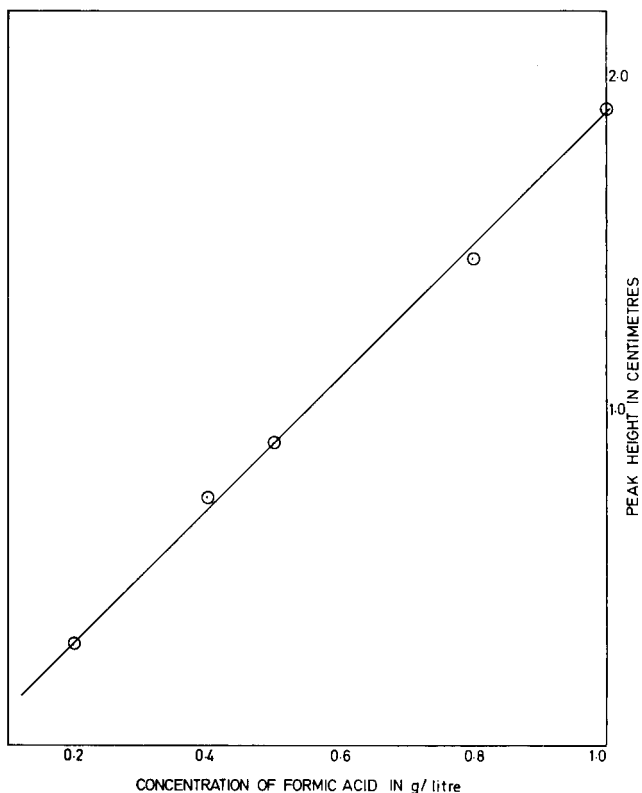
Fig. 3. Calibration graph for the determination of formic acid in aqueous solution.

sensitivity is required a column offering better resolution of the methyl formate would be required.

The standard addition technique should be used if samples are encountered with very variable salt concentrations.

Sizova's original work covered samples within the concentration range 3–15 g/l. Using this headspace method over the range 0.1–1.0 g/l and with standards run with each batch of samples, it has been found that the repeatability between triplicate analysis is of the order of 5 %.

It should be noted that both nylon and glass syringes are compatible with this method, but the ester has a pronounced solvent effect with many other plastics.

This headspace modification of Sizova's method has provided a particularly advantageous technique for the measurement of formic acid in fermenter broths. The sample manipulations are simple, and directly applicable to coloured, heterogeneous broths, total analysis time is 45 min and standard GC apparatus can be used with no ghosting problems as often experienced with the free acid.

The method has been used routinely in this laboratory for 2 years in the analysis of anaerobic effluent digester systems.

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Note

Resolution of RNA by paired-ion reversed-phase high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) has developed into a rapid and efficient method for the resolution and the preparative isolation of a large number of biologically and pharmacologically important molecules. For the most part, analyses of nucleic acids have been limited to purines and pyrimidines[1], nucleosides[2,3], nucleotides[4], and short oligonucleotides[5,6]. Recently, the resolution of large DNA restriction endonuclease digestion fragments by HPLC has been reported using columns containing RPC-5[7] and Kel-F resins[8]. To data, no information has appeared concerning the separation of mRNA size molecules on commercially available columns. We report here preliminary experiments for the partial resolution of tRNA from globin mRNA by paired ion reverse-phase HPLC, using commercially available C_{18} columns in the presence of the tetrabutylammonium counter ion.

EXPERIMENTAL

Yeast transfer RNA (type X) and rabbit globin mRNA were purchased from Sigma and Bethesda Research Labs., respectively. Bacterial RNA was isolated from a culture of *E. coli* strain K-12 in logarithmic growth phase as described previously[9].

Radiolabeling of 1-μg aliquots of RNA with cytidine $3'5'$-$[5'$-$^{32}P]$ bisphosphate (specific activity 2000–3000 Ci per mmol from Amersham) and RNA ligase (P-L Biochemicals) was performed essentially according to the method described by Bruce and Uhlenbeck[10]. After incubation for 16 h at 4°C, the samples were extracted three times each with phenol[11] and diethyl ether.

Chromatography was performed on a Waters Assoc. instrument equipped with two Model 6000 solvent delivery systems, a Model 660 solvent programmer, a Model U6K sample injector, and two μBondapak C_{18} columns (30 × 0.4 cm, each) in series. The pumps were programmed to produce a linear gradient (solvent program number 6) from methanol to water with each solvent containing 5 mM PIC A reagent (tetrabutylammonium phosphate, Waters Assoc.).

Samples containing 5–25 μl (0.1–0.5 μg) of radiolabeled RNA were applied to the columns at a flow-rate of 1 ml/min, with an operating pressure of 55–69 bars. A 40 ml methanol to water gradient was generated immediately after injection, and 1-ml fractions were collected. The amount of radioactivity in each fraction was measured directly by Cerenkov radiation.

Aliquots of 200 μl were removed from fractions to be analyzed by polyacryl-amide gel electrophoresis and dried prior to resuspension in a solution containing 10 mM Na$_2$EDTA, 50% (v/v) formamide, and 0.01% (w/v) bromophenol blue and xylene cyanol FF dyes. The samples were then heated to 68°C for 2 min and layered atop a 3.5% polyacrylamide gel (14 cm × 18 cm × 0.15 cm) containing 7 M urea, using a buffer system described previously[12]. Following electrophoresis for 2 h at 200 V, the gels were dried prior to autoradiography.

RESULTS

Purified tRNA (80 nucleotides) and a population of molecules in which rabbit globin mRNA ($\approx$ 650 nucleotides) predominates were radiolabeled separately with
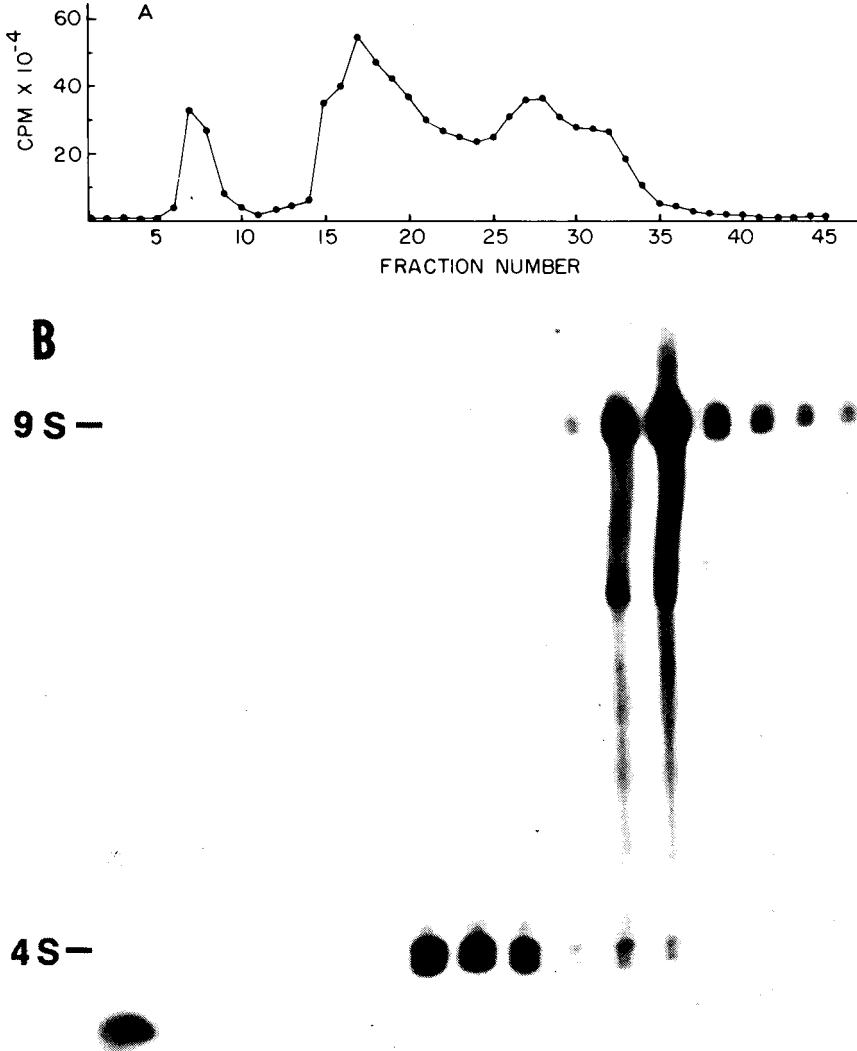
Fig. 1. Elution profile of radiolabeled 4S and 9S RNA following HPLC (panel A) and polyacrylamide gel analysis of fractions 15 through 32 (panel B).

^{32}pCp. Aliquots containing $2 \cdot 10^4$ acid-insoluble cpm from each were pooled and analyzed by HPLC in the presence of tetrabutylammonium phosphate on two C_{18} columns as detailed in the Experimental section. The distribution of radioactivity in the eluted fractions is shown in panel A of Fig. 1, and the electrophoretic mobility of the radioactivity in 200 μl of fractions 15 through 32 is shown in panel B. The peak of radioactivity in the early fractions 6–9 represents authentic unreacted ^{32}pCp as characterized by thin-layer chromatography on polyethyleneimine. The radioactivity which appears in fractions 16–19 is an anomaly. Its amount varies with the lot and "age" of ^{32}pCp used. This material migrates more slowly on polyethyleneimine than authentic ^{32}pCp, suggesting that it is a class of ^{32}P-containing molecules with more than two phosphate groups. Since these molecules are relatively small, they are not

Fig. 2. Elution profile or radiolabeled *E. coli* RNA following HPLC (panel A) and polyacrylamide gel analysis of fractions 17 through 36 (panel B).

retained on 3.5 % polyacrylamide gels under the conditions used here. What is apparent from the autoradiograph is that tRNA molecules elute prior to the larger globin mRNA molecules, predominating in fractions 21 and 26, respectively.

Another demonstration of the ability of this technique to separate RNA molecules on the basis of molecular weight is illustrated in Fig. 2. Here, instead of applying an artificial mixture of molecules from two different sources, RNA was isolated from a culture of *E. coli* growing in logarithmic phase, ^{32}pCp labeled and chromatographed as above. Aliquots containing 200 μl from fractions 17 through 36 (Fig. 2, panel A) were processed for electrophoresis, and the pattern is shown in panel B of Fig. 2. Clearly, a gradient of molecules of increasing molecular weight eluted with increasing time.

DISCUSSION

The probability is low that RNA molecules would interact with non-polar $Si(CH_2)_{17}CH_3$ residues of a C_{18} column. In an attempt to promote this interaction, the tetrabutylammonium counter ion was added to the mobile phase. If the counter ion were to affect the mobility of RNA by providing some non-polar character to these polar molecules, it might be expected that the degree of retention of RNA by the column would be somewhat proportional to the molecular weight, *i.e.*, smaller RNA molecules would elute more rapidly from the C_{18} columns than larger ones. Fig. 3 illustrates this concept.

RNA ALONE
IN MOBILE PHASE

C_{18} COLUMN C_{18} COLUMN

RNA WITH
PAIRED ION (R-N+)*
IN MOBILE PHASE
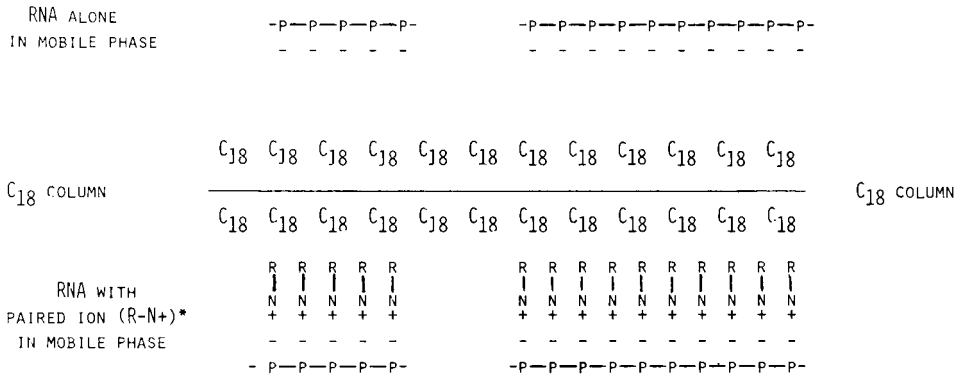
Fig. 3. Idealized view of RNA interaction with C_{18} column matrix in the absence and presence of tetrabutylammonium counter-ion. Above the C_{18} column two RNA molecules are shown; one has five phosphates and the second ten phosphates, each with a negative charge. Below the column these two molecules are shown paired with the counter ion containing the tetrabutyl group (R) which can interact with the C_{18} residues of the column. In this idealized view, the non-polar character contributed by the paired ion is proportional to the number of phosphates, and thus the molecule with five phosphates would be eluted from the column before the longer molecule.

The results presented here demonstrate that RNA molecules of molecular weights greater than tRNA can be fractionated by a simple technique employing commercially available HPLC columns and reagents. At the present state of development, the resolution observed is comparable to sucrose gradient centrifugation. However, the HPLC method offers some immediate advantages. First, comparable resolu-

tion is obtained in 40 min with HPLC as in a minimum of several hours by centrifugation. Second, because both the solvents and paired ion are volatile, subsequent steps, such as ethanol precipitation, are not necessary.

It should be emphasized that the resolution observed in this preliminary report may be increased in future work by manipulating a number of parameters. For example, there are as yet no data on the effects of different solvents, solute concentrations, or shapes of elution gradients. Such work is presently underway. If successful, the utility of this technique in isolating not only individual RNAs, but also individual restriction endonuclease fragments of double stranded DNA may become apparent.

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Note

Fractionation of tRNA on siliconized porous glass coated with trialkyl-methylammonium chloride

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In the course of a study on the recognition mechanism between tRNA and aminoacyl-tRNA synthetase[1], we carried out some studies on tRNA[2-4], but the mechanism was not resolved. Pure tRNA is necessary for investigating the mechanism. For the separation of tRNA, Pearson *et al.*[5] reported a reversed-phase chromatographic technique at a low flow-rate, named RPC-5. Subsequently we developed separation methods using porous glass[6,7]. We report here a method involving high flow-rates for separating tRNA on siliconized porous glass[8] coated with trialkylmethylammonium chloride.

EXPERIMENTAL

Preparation of adsorbent

The porous glass used was CPG-10, 350 Å (100-μm particles) (Electro-Nucleonics, Fairfield, NJ, U.S.A.). After being washed and dried, the glass (1 g) was tightly coated with 200 μl of silicone oil (dimethylpolysiloxane, KF 96; Shinetsu Chemicals, Tokyo, Japan) according to previous work[8]. The siliconized glass (10 g) was shaken for 2 h according to the literature[5] with 20 ml of chloroform containing 0.4 ml of Adogen 464 (a trialkylmethylammonium chloride with the predominant chain length of the alkyl groups being C_8–C_{10}) (Ashland Chemical Co., Columbus, OH, U.S.A.). The slurry of siliconized glass in chloroform was dried in a glass tray. The glass treated with Adogen 464 immediately sank in an aqueous medium even though siliconized porous glass floated on the surface of water[8].

The siliconized glass coated with Adogen 464 was suspended in a solution composed of 0.45 *M* sodium chloride, 0.01 *M* magnesium chloride, 0.01 *M* Tris–hydrochloric acid buffer (pH 7.6) and 1 m*M* β-mercaptoethanol. After being washed with the solution, the glass was packed in a column having a water jacket maintained at 37°C. A sample of tRNA was dissolved at a concentration of 10 mg/ml in the above solution and applied on to the column. After the column had been washed with one column volume of the solution, elution was carried out with a linear gradient of sodium chloride at a flow-rate of 0.6 ml/min · cm².

Transfer RNA

Transfer RNA was prepared from bovine liver as follows. Bovine liver (1 kg)

was minced and mixed in 5 l of 1 M socium chloride–0.02 M Tris–hydrochloric acid buffer (pH 7.5) containing 0.5 mM EDTA, 0.01 M magnesium chloride, 1 % sodium dodecylsulphate and 20 % phenol. The supernatant obtained from the above solution after standing overnight in a cold room was mixed with 2 volumes of ethanol. The precipitate was collected and dissolved in 0.3 M sodium acetate solution. The high-molecular-weight nucleic acid, such as rRNA and DNA, was precipitated with 0.4 part of isopropanol[9]. After the DNA precipitate had been removed, the solution was mixed with 0.6 part of isopropanol relative to the first supernatant and the precipitate was collected by centrifugation at 600 g. Glycogen in the precipitate was removed by treatment with ethylene glycol monomethyl ether[10] and the extract containing tRNA was dialysed. After dialysis, tRNA was incubated in 1 M Tris–hydrochloric acid buffer (pH 9.0) to remove amino acid bound on tRNA and then precipitated by addition of ethanol to 70 %. This tRNA preparation was further purified on Sepharose 6B and fractions eluting at K_{av} 0.25 were collected; 0.98 g of tRNA was obtained from 1.0 kg of bovine liver.

The tRNA (1.1 g) obtained on Sepharose 6B was then dissolved in 100 ml of buffer (pH 4.6) consisting of 0.4 M sodium chloride, 0.01 M magnesium chloride and 0.01 M potassium acetate and chromatographed on a benzoylated DEAE-cellulose column (22.6 × 5.2 cm I.D.) according to the method of Gillam *et al.*[11]. Elution was carried out at 23–26°C with a linear gradient (total volume 4 l) from 0.4 to 1 M sodium chloride in 0.01 M potassium acetate (pH 4.6) and 0.01 M magnesium chloride. The fraction volumes were 50 ml. The chromatographic pattern is shown in Fig. 1.
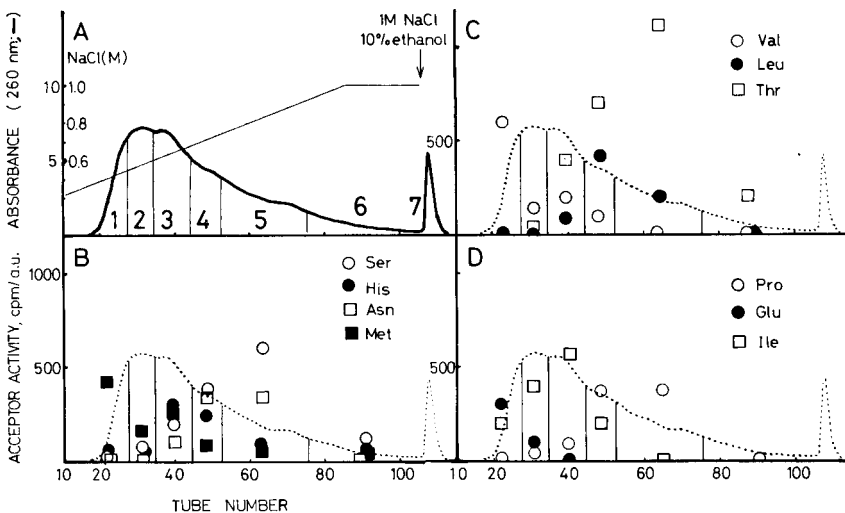
Fig. 1. Elution pattern of tRNA on BD-cellulose. The sample (1.1 g, 16,500 absorbance units) was applied on a column (22.6 × 5.2 cm I.D.) and eluted with a linear gradient (total volume 4 l) from 0.4 to 1.0 M sodium chloride at 23–26°C. (A) Result of measurement of absorbance at 260 nm; identical patterns are shown in (B)–(D) with broken lines. Fraction volumes were 50 ml. (B)–(D), Amino acid acceptor activities of each fraction. The cpm/absorbance unit values indicate the amounts of amino acids bound on tRNA.

Preparation of aminoacyl-tRNA synthetase

The synthetase was prepared from Donryu strain rat liver in a cold room[12]. The supernatant (300 ml) from centrifugation at 105,000 g for 60 min of the homogenate of liver (38 g) in 0.25 M sucrose–0.02 M Tris–hydrochloric acid buffer (pH 7.4) was dialysed against a solution composed of 0.01 M Tris–hydrochloric acid buffer (pH 7.5), 0.01 M magnesium chloride, 0.01 M β-mercaptoethanol and 5 % glycerin, and then loaded on a DEAE-cellulose column (20 × 5 cm I.D.) which had previously been equilibrated with the above buffer. After the column had been washed with the buffer, proteins were eluted with 0.3 M potassium chloride in the buffer. The protein peak measured at 280 nm was dialysed against a solution composed of 0.01 M Tris–hydrochloric acid buffer (pH 7.5), 0.01 M β-mercaptoethanol, 2 mM EDTA and 50 % glycerin. The protein concentration of the dialysate was 28.5 mg/ml by the Lowry method[13]. The dialysate was kept in a freezer at $-20°C$. This dialysate was used as aminoacyl-tRNA synthetase and the activity was generally stable for several months.

Assay of aminoacylation of tRNA was carried out as follows. tRNA was dissolved in a solution composed of 0.2 M Tris–hydrochloric acid buffer (pH 7.6), 0.02 M potassium chloride and 0.02 M magnesium chloride. The tRNA solution (40 μl) was mixed with 50 μl of the same solution described above but containing 5 mM ATP and 40 μM [14C]amino acid (10 μCi per 2 μmol; Radiochemical Centre, Amersham, Great Britain). The aminoacylation reaction was started at 37°C by the addition of 10 μl of enzyme solution to the above tRNA–amino acid solution (90 μl). The incubation was allowed to proceed for 30 min. Before the reaction was stopped, the reaction mixture was layered on to a filter-paper and the paper immersed in 0.2 M hydrochloric acid. After being washed for 20 min with three portions of 0.2 M hydrochloric acid the filter-paper was placed in a solution of diethyl ether–ethanol (1:1) and then dried. The dried filter-paper was counted in common toluene–PPO–POPOP solution with a Searle Analytical liquid scintilation counter. The assay was carried out duplicate. A blank and a control (crude tRNA, 2.4 absorbance units) were usually measured at the same time as the sample. The counting efficiency for [14C]amino acids was about 60 %.

RESULTS AND DISCUSSION

Fig. 2 shows the results of chromatography at 37°C of bovine liver tRNA (the fraction obtained on Sepharose 6B) on a 45 × 0.8 cm I.D. column of siliconized glass coated with Adogen 464. The activity of tRNAs accepting methionine, valine and serine is also shown in Fig. 2. Fraction 1 in Fig. 2 did not contain tRNA^{Val} and tRNA^{Ser}, but contained tRNA^{Met}. tRNA^{Val} and tRNA^{Ser} were contained in fractions 2 and 3, respectively. Thus, tRNA species were separated on the siliconized glass coated with Adogen 464. The order of elution of tRNA species from the glass column is methionine, valine and serine, which is similar to that on an RPC-5 column[5]. However, the concentration of sodium chloride necessary for eluting tRNA on the glass column is higher than the system on an RPC-5 column.

Fig. 3 shows the results of further purification of the partially purified tRNA^{Ser} (fraction 5 in Fig. 1) on a siliconized glass column coated with Adogen 464 (45 × 0.8 cm I.D.). Major tRNA^{Ser} was eluted in fraction 4 in Fig. 3 and minor tRNA^{Ser} in fraction 1. Thus, iso-accepting tRNA^{Ser} species were separated on the siliconized
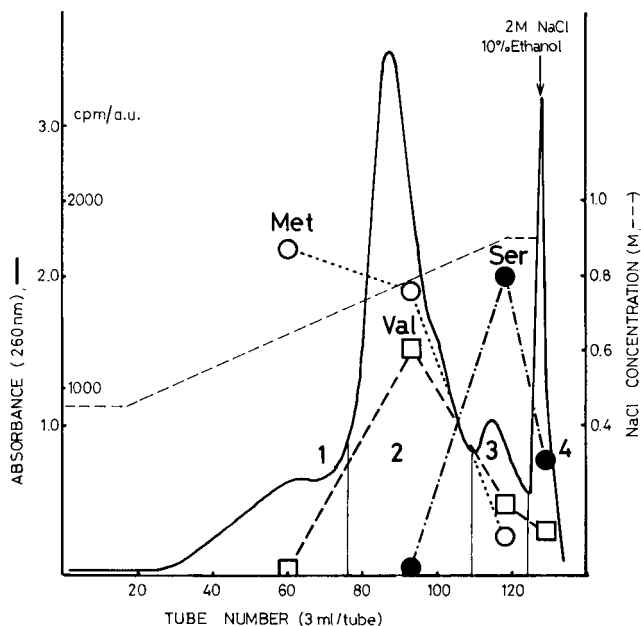
Fig. 2. Elution profile of crude tRNA (20 mg) on siliconized porous glass coated with Adogen 464. Column, 45 × 0.8 cm I.D. Elution was carried out at 37°C with a linear gradient (total volume 300 ml) from 0.45 to 0.9 M sodium chloride in 0.01 M magnesium chloride, 0.01 M Tris-hydrochloric acid buffer (pH 7.6) and 1 mM β-mercaptoethanol at a flow-rate of 0.6 ml/cm^2 · min. Amino acid acceptor activity of tRNA is shown by cpm/absorbance unit as follows; ○ - - - ○, Met; □ - - - □, Val; ● -.-.- ●, Ser.

Adogen 464-coated glass and tRNA^{Ser} was separated from other impurities. It is possible that major tRNA^{Ser} corresponds to serine codons UCX and minor tRNA^{Ser} to AGC and AGU, from the results on RPC-5[14].

Partially purified tRNA^{Ser} (1430 absorbance units, 72 mg; fraction 5 in Fig. 1) was phenoxyacetylated according to the literature[15] and chromatographed on BD-cellulose. Phenoxyacetylated tRNA^{Ser} had an affinity to BD-cellulose and eluted later than other inpurities, as shown in Fig. 4A. The tRNA^{Ser}-rich fraction in Fig. 4A was
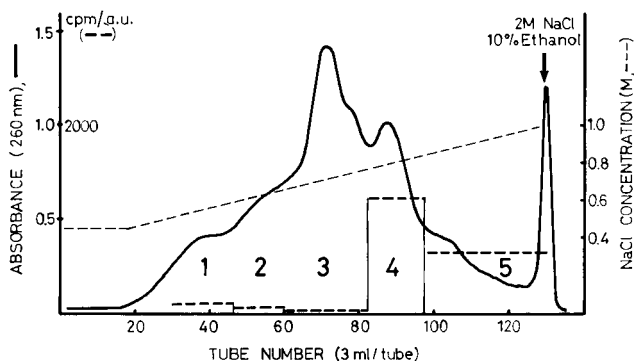
Fig. 3. Purification of tRNA^{Ser} on siliconized porous glass column coated with Adogen 464. The partially purified tRNA^{Ser} (10 mg) used was fraction 5 in Fig. 1. Other conditions identical with those in Fig. 2.
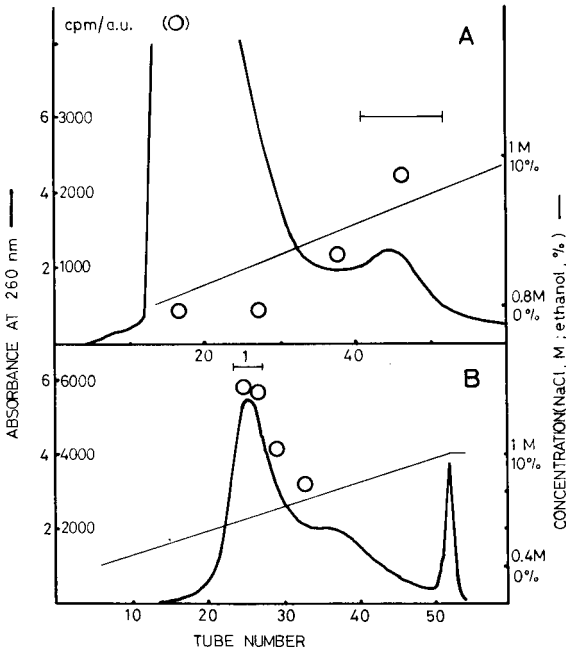
Fig. 4. Purification of tRNA^{Ser} on BD-cellulose. (A) tRNA (1430 absorbance units) was applied on a BD-cellulose column (15 × 1.6 cm I.D.). Total elution volume of a linear gradient from 0.8 to 1.2 M sodium chloride–20 % ethanol was 400 ml. Fraction volumes were 4 ml. (B) Results of re-chromatography of a fraction (tubes 41–51) rich in tRNA^{Ser} in (A) on a BD-cellulose column (10 × 0.4 cm I.D.). Fraction volumes were 1 ml. Fractions in tubes 24–27 in (B) were pooled and used in Fig. 5.
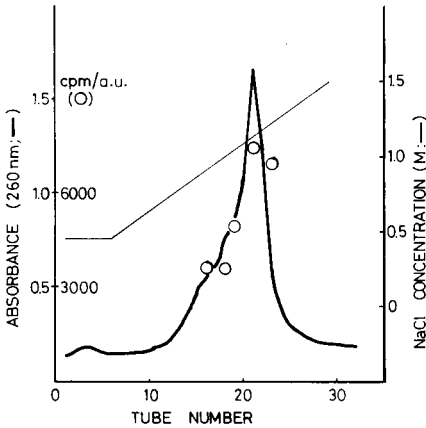
Fig. 5. Isolation of tRNA^{Ser} on the siliconized and Adogen 464-coated porous glass. Sample (62 absorbance units, fraction 1 in Fig. 4B) was dissolved in 0.5 ml of 0.01 M Tris–hydrochloric acid buffer (pH 7.6), 0.45 M sodium chloride, 0.01 M magnesium chloride and β-mercaptoethanol and applied on a 20 × 0.8 cm I.D. column. Elution was carried out at a constant temperature of 37°C with an elution buffer (100 ml) composed of a linear gradient from 0.45 to 1.5 M sodium chloride. Fraction volumes were 4 ml.

deaminoacylated and re-chromatographed on a small BD-cellulose column as shown in Fig. 4B. The fraction in tubes 24–27 in Fig. 4B was further purified on the siliconized Adogen 464-coated porous glass and the chromatographic pattern is shown in Fig. 5. Serine tRNA was eluted in tubes 21–23 and impurities in tubes 12–20 in Fig. 5. Purified tRNA^{Ser} accepts 1.4 nmole of serine per absorbance unit of tRNA.

The recoveries of tRNA in Figs. 2, 3 and 5 were better, averaging 90 %, than that obtained with the Plaskon–Adogen 464 (RPC-5) system. In the work with the RPC-5 column[5], a linear gradient was pumped through the column, because of the small size of the particles of Plaskon. Using the siliconized porous glass coated with Adogen 464, a high flow-rate was obtained under no pressure. The glass was used repeatedly several times and the reproducibility of the chromatographic patterns remained constant. However, the glass floated on an aqueous medium after repeated use, because Adogen 464 was removed from the surface of the siliconized glass. Therefore, it is advisable to re-coat the glass with Adogen 464 after use several times.

CONCLUSION

Chromatography of bovine liver tRNA with use of a sodium chloride gradient and a siliconized porous glass column treated with Adogen 464, eluted tRNA from the column in the order tRNA^{Met}, tRNA^{Val} and tRNA^{Ser}, and the recovery of tRNA from the glass column averaged 90 %. tRNA^{Ser}, partially purified on benzoylated DEAE-cellulose, was isolated on the siliconized Adogen-coated porous glass.

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Note

Large scale purification of phosphatidylcholine from egg yolk phospholipids by column chromatography on hydroxylapatite

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Phosphatidylcholines have been purified from natural sources by column chromatography on silicic acid[1,2] or alumina[3,4], using a mixture of chloroform and methanol. These methods require high capacity columns, tedious elutions and a rechromatography of phosphatidylcholine to remove traces of lysophosphatidylcholine and sphingomyelin. The time and manipulation required can lead to the possible oxidation of unsaturated phosphatidylcholines[5]. Purification of phosphatidylcholine and phosphatidylethanolamine from crude egg phospholipids on radially compressed silica gel columns using a Waters Prep LC-500 has been described[6]. This method requires a large amount of solvent, and instrumentation not readily available in most laboratories.

Hydroxylapatite, $Ca_{10}(PO_4)_6(OH)_2$, a modified form of crystalline calcium phosphate developed by Tiselius *et al.*[7], has been used successfully for the separation of proteins[8], lipoproteins[9], and polynucleotides[10]. Slomiany and Horowitz[11] first reported the use of hydroxylapatite, prepared by the Tiselius method, as an adsorbent during the column chromatographic separation of polar lipids from cattle serum. This method resolved complex lipid mixtures, but it required careful preparation of the adsorbent to achieve good separations and reasonable flow-rates. Recent improvements in the preparation of hydroxylapatite have been reported[12–14]. Commercial products now make possible the use of hydroxylapatite with high crystal integrity and improved flow-rate, load capacity and resolution. This report describes the use of an improved hydroxylapatite for the rapid, large scale purification of phosphatidylcholine from crude egg yolk phospholipids.

This method is advantageous, as a single column and a short period of time is required for purification, thus avoiding the possible degradation of phosphatidylcholine. Extensive preparation of the hydroxylapatite before use is not required and chloroform, a potentially hazardous solvent, is avoided.

MATERIALS

Hydroxylapatite (High Resolution/dry), egg yolk phospholipids and phospholipid standards were obtained from Calbiochem (La Jolla, CA, U.S.A.). Methylene chloride, acetone and methanol were obtained from J. T. Baker (Phillipsburg, NJ,

U.S.A.). Thin-layer chromatographic (TLC) plates coated with silica gel were obtained from E. Merck (Darmstadt, G.F.R.). They were developed in chloroform–methanol–water (65:25:4). Iodine and molybdate spray[15] were used to detect phospholipids on the plates, and the contents of each fraction were identified by direct chromatographic comparison with authentic standards. Phosphate determination were performed by the method of Martin and Doty[16].

EXPERIMENTAL AND RESULTS

Purification of phosphatidylcholine

Hydroxylapatite (300 g) was suspended in methylene chloride–acetone (1:1) and the slurry was poured into a 6 × 50 cm glass column fitted with a coarse fritted disc. The column was equilibrated with 2 l of methylene chloride–acetone (1:1). Crude egg phospholipids (10 g) dissolved in 50 ml of methylene chloride–acetone (1:1) were applied onto the column and carefully rinsed in with 100 ml of the same solvent. The column was eluted at a flow-rate of 30 ml/min, collecting 1-l fractions as indicated in Table I.

DISCUSSION

Rapid, large scale purification of phosphatidylcholine from egg yolk phospholipids can be achieved on hydroxylapatite without prewashing, activation or removal of fines, in sharp contrast to hydroxylapatite prepared by the Tiselius method[11]. Excellent flow-rates are observed with a simple gravity solvent feed system. Hydroxylapatite fines are not observed in any of the fractions.

TABLE I

ELUTION OF EGG PHOSPHOLIPIDS FROM HYDROXYLAPATITE

Acetone–methanol ratio of eluent (v/v)	Total volume (l)	Fraction number
9:1	2	1–2
8:2	1	3
7:3	10	4–13
6.5:3.5	3	14–16
6:4	2	17–18
5:5	2	19–20

Using a 30:1 ratio of hydroxylapatite to sample, pure phosphatidylcholine can be eluted with acetone–methanol (7:3) after brief elution of neutral lipids with acetone–methanol (9:1 and 8:2). Approximately 95 % of the phosphatidylcholine in crude egg phospholipids was recovered without using chloroform, a potentially hazardous solvent used in the traditional purification methods. A single column chromatography provides pure phosphatidylcholine in a short period of time compared to silicic acid or alumina column chromatography.

We have also used this procedure to purify synthetic phosphatidylcholine prepared by the acylation of glycerophosphatidylcholine, and to isolate pure phospha-

tidylcholine from crude soy phospholipids. This procedure provides chromatographically pure phosphatidylcholine for further studies or conversions into intermediates.

Thin-layer chromatograms of each fraction were developed in chloroform–methanol–water (65:25:4) and visualized by iodine vapors or molybdate spray (Fig. 1). TLC indicated that fractions 1–3 contained neutral lipids. Fractions 4–11 contained pure phosphatidylcholine which were combined and the solvents were removed by evaporation. Upon drying, pure phosphatidylcholine (6.3 g) was isolated. The purity and identity was confirmed by comparison using TLC and high-performance liquid chromatography (HPLC)* with authentic standard. Phosphorus analysis based on mean moleculear weight of 772 for phosphatidylcholine showed: theoretical = 4.01 %, found = 3.96 %.
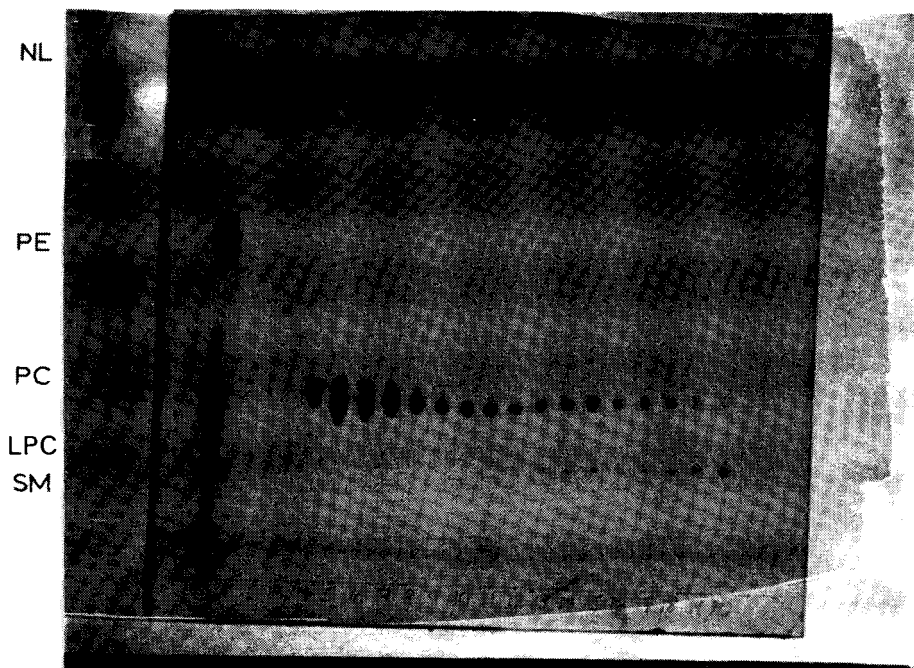
Fig. 1. Thin-layer chromatogram of fractions eluted from the chromatography of egg yolk phospholipids on hydroxylapatite as shown in Table I. The silica gel plate was developed in chloroform–methanol–water (65:25:4) and the spots visualized by exposure to iodine vapors. O = Egg yolk phospholipids; PE = phosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; LPC = lysophosphatidylcholine; NL = neutral lipids.

Evaporation of the solvent from fractions 12–17 (sphingomyelin with a trace of phosphatidylcholine) yielded 0.14 g; fractions 18–20 (sphingomyelin with a trace of lysophosphatidylcholine) yielded 0.20 g.

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* HPLC analysis was performed on a Waters high-pressure liquid chromatographic system using a μPorasil column (Waters Assoc., Milford, MA, U.S.A.). The column was eluted with hexane–isopropanol–water (6:8:1, v/v) at a flow-rate of 1 ml/min and detection at 206 nm.

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CHROM. 14,412

Note

Stevia rebaudiana

II. High-performance liquid chromatographic separation and quantitation of stevioside, rebaudioside A and rebaudioside C[*]

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Stevioside, rebaudioside A, and rebaudioside C (dulcoside B) are the major and the sweetest diterpene glycosides isolated from the leaves of *Stevia rebaudiana* Bert[1]. The potential use of these glycosides in the United States as non-caloric sweetening agents stimulated the authors to devise a simple, efficient, and sensitive procedure for their quantitation in plant material. Several procedures have been published for the assay of *S. rebaudiana* total diterpene glycosides by gas–liquid chromatography of the aglycones[2,3] or by thin-layer chromatographic separation of stevioside, followed by colorimetric estimation[4]. None of these methods was deemed satisfactory. The first successful high-performance liquid chromatographic (HPLC) separation of stevioside and rebaudioside A was reported by Hashimoto and co-workers[5,6] while our study was in progress. A deficiency in one of their procedures will be related herein.

A previous publication by the authors described the HPLC assay of stevioside and rebaudioside A by hydrolysis of the esterified sugars, followed by formation of the chromophoric *p*-bromophenacyl esters[7]. However, it was noted that in our former procedure rebaudiosides D and E produced the same ester derivatives as rebaudioside A and stevioside, respectively. This communication reports the quantitative extraction of diterpene glycosides from *S. rebaudiana*, and a direct, efficient HPLC procedure for the quantitation of underivatized stevioside, rebaudioside A, and rebaudioside C.

EXPERIMENTAL

Plant material

Stevia rebaudiana Bert. (Compositae) was grown from seed; mature plants were authenticated by Mr. Floyd A. Swink of the Morton Arboretum, Lisle, IL, U.S.A. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center, Chicago, IL, U.S.A.

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Apparatus

Liquid chromatographic separations were conducted with a Waters Model 6000A liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Rheodyne Model 7120 syringe-loading sample injector and 100-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) a Waters Model 450 variable wavelength UV spectrophotometer, and a Texas Instruments Servo/Riter II portable recorder (Texas Instruments, Houston, TX, U.S.A.). Separations were carried out on two Waters 30 × 0.78 cm I.D. Protein I-125 columns.

Chemicals

All chemicals and solvents used in this investigation were reagent or certified grade. Solvents for HPLC were redistilled in glass and degassed prior to use.

Stevioside and rebaudioside A were isolated from the leaves of *Stevia rebaudiana* as previously described[7]. Rebaudioside C was obtained from Professor M. Kobayashi, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.

Stevia rebaudiana extraction for HPLC analysis

Dried, powdered (30 mesh) *S. rebaudiana* leaves (0.5 g) were continuously and sequentially extracted in a micro-soxhlet apparatus with chloroform (15 ml) for 3 h (extract A), and with methanol (15 ml) for 5 h (extract B); the plant marc was then refluxed with distilled water (15 ml) for 1 h (extract C). Each extract was evaporated to dryness *in vacuo* and the resulting residues were redissolved in chloroform (extract A, 10 ml) or in 1-propanol–water (49:1) (extract B, 50 ml; extract C, 25 ml) for chromatographic analysis.

For comparative purposes, *S. rebaudiana* was also extracted by the method of Hashimoto *et al.*[5]. Calcium carbonate (0.3 g) and distilled water (6.0 ml) were added to the dried, powdered leaves (1.0 g) and the slurry was mixed well prior to 15 h of maceration. The mixture was heated at 50°C for 4 h, cooled, diluted with acetonitrile (18 ml), filtered, and evaporated to dryness *in vacuo* to yield extract D. The residue was redissolved in 1-propanol–water (49:1) (100 ml) for HPLC analysis.

Thin-layer chromatography (TLC)

Extract A (20 μl), as well as HPLC column eluates of stevioside and rebaudiosides A and C were spotted on precoated silica gel GF_{254} plates (20 × 20 cm, 0.25 mm thick; E. Merck, Darmstadt, G.F.R.) and developed with chloroform–methanol–water (30:20:1). After development, chromatograms were air-dried, sprayed with anisaldehyde–sulfuric acid reagent[8], and heated at 100°C for 5 min to visualize *Stevia* diterpene glycosides.

High-performance liquid chromatography

The operating conditions for HPLC were: ambient temperature; flow-rate of eluting solvent, 1-propanol, 1 ml/min; wavelength of UV detector, 210 nm; recorder chart speed, 3 in./h at 0.04 a.u.f.s. Standard solutions of stevioside, rebaudioside A, and rebaudioside C were injected onto the column and their retention times determined.

Beer's law standard curves were obtained by injecting different quantities of

stevioside (5, 10, 15, 20, 30, 50, 60, 80, and 100 μg per 100 μl injection), rebaudioside A (5, 10, 20, 25, 50, and 100 μg per 100 μl injection), and rebaudioside C (4, 6.25, 12.5, 20, 25, 50, and 100 μg per 100 μl injection) onto the column in triplicate and measuring the resulting peak heights.

RESULTS AND DISCUSSION

Previous attempts by these authors to separate stevioside (SS), rebaudioside A (RA), and rebaudioside C (RC) or their benzoyl, 4-nitrobenzoyl, or 3,5-dinitrobenzoyl derivatives[9] on μPorasil, μBondaPak C_{18}, μBondaPak NH_2, μBondaPak CN, and μStyragel, 500-Å columns (Waters Assoc.) with numerous solvent systems having a UV cut-off below 210 nm were totally unsuccessful. A mixture of SS, RC, and RA was originally separated on a single Protein I-125 column, giving retention times of 19.7, 23.2, and 27.5 min, respectively. However, these compounds were poorly resolved when an aqueous extract of *S. rebaudiana* leaves was applied to the column. Therefore, attempts were made to purify the extract prior to HPLC. Extraction of the plant material with 1-butanol produced a simpler extract, but was found to be nonquantitative for the diterpene glycosides. Extraction of impurities from an aqueous extract with ethyl acetate or chloroform–methanol (9:1) prior to HPLC did not significantly improve resolution. Treatment of the aqueous extract with charcoal, calcium hydroxide, barium hydroxide, or lead acetate did remove impurities from the extract, but it resulted in the partial adsorption and/or precipitation of the diterpene glycosides as well.

Satisfactory resolution of SS, RC, and RA was finally achieved on two protein columns in series, after the plant material was extracted with chloroform (extract A) prior to methanol extraction (extract B). A typical HPLC chromatogram is shown in Fig. 1. TLC of extract A showed that no detectible quantitites of diterpene glycosides
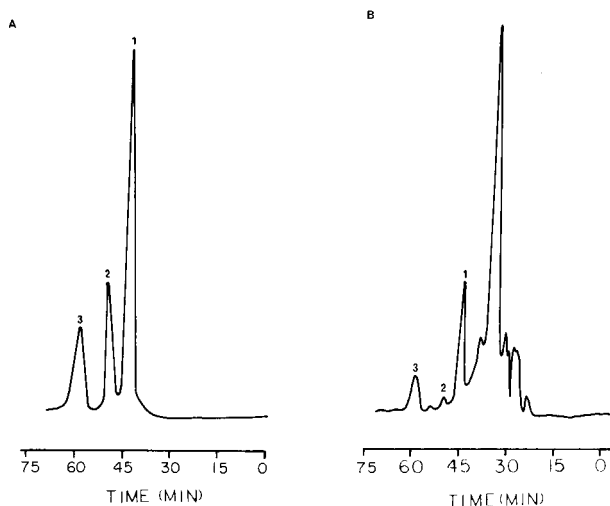
Fig. 1. Separation of stevioside, rebaudioside C, and rebaudioside A. A. Mixture of standards; B, *Stevia rebaudiana* aqueous extract (extract B). Operating conditions: two Protein I-125 columns in series; mobile phase, 1-propanol; flow-rate, 1 ml/min; detector, UV spectrophotometer (210 nm). Peaks: 1 = stevioside (SS); 2 = rebaudioside C (RC); 3 = rebaudioside A (RA).

were removed by this purification step. In addition, an aqueous extract (extract C) of the plant marc after methanol extraction was devoid of diterpene glycosides. Since these glycosides are more soluble in water than in organic solvents, these results indicate that SS, RA, and RC were quantitatively extracted by methanol in this procedure.

Under the HPLC conditions finally used in this study, SS, RC, and RA gave retention times of 43.2, 49.5, and 59.0 min, respectively, and linear detection responses when concentrations of 5–100 μg were employed. Beer's law standard curves gave slopes of 1.68 (SS), 1.22 (RC), and 0.91 (RA); y axis (peak height) intercepts of -1.16 (SS), $+0.52$ (RC), and $+0.35$ (RA); and correlation coefficients of 0.999 (SS, RC, and RA) by linear regression analysis. The minimum detectible quantities of SS, RC, and RA were 1.0, 2.0, and 2.0 μg, respectively. TLC analysis of SS, RC, and RA separated from extract B by this HPLC column showed a single spot for each compound ($R_F = 0.30$, 0.24, and 0.18, respectively) which co-chromatographed with the corresponding standard.

An analysis of extract D showed that the published procedure of Hashimoto et al.[5] removed fewer impurities from the plant. However, comparison with extract B showed that the diterpene glycosides were not quantitatively extracted by their procedure, yielding only $52.78 \pm 0.13\%$ of the extractible SS, $43.32 \pm 0.08\%$ RC, and $48.61 \pm 0.04\%$ RA. These low yields probably result from the small volume of water used for plant extraction.

The data demonstrate that SS, RC, and RA can be quantitatively extracted from S. rebaudiana and rapidly and accurately analyzed by HPLC with the procedures described in this communication. They also demonstrate the versatility of the Protein I-125 column in separating water-soluble compounds other than proteins.

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CHROM. 14,482

Note

Analysis of a model ionic nitrosamine by microbore high-performance liquid chromatography using a thermal energy analyser chemiluminescence detector

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Trace levels of volatile nitrosamines have been reported in a number of different types of foodstuffs and beverages including cured meat[1], cheese[2] and beer[3]. The occurrence of these compounds is of concern because of the carcinogenic properties many of them exhibit in animal feeding studies[4]. The analysis of trace levels of volatile nitrosamines in complex samples of biological origin has been considerably facilitated by the introduction of the nitrosamine-specific thermal energy analyser (TEA) chromatographic detector[5]. The TEA comprises a catalytic pyrolyser which cleaves the nitrosamine N–NO bond and a cold trap for removing organic matter; the nitric oxide is then detected by a chemiluminescent reaction with ozone. Due to the high sensitivity and selectivity of the TEA in comparison with most other gas chromatographic (GC) detectors[6], GC–TEA is now the standard method for trace analysis of volatile nitrosamines[7,8].

The TEA may also be used as a liquid chromatography detector permitting, in principle, the analysis of non-volatile nitrosamines. However, in contrast to volatile nitrosamines the field of non-volatile nitrosamines is still largely unexplored due to the constraints the TEA places on the choice of high-performance liquid chromatographic (HPLC) solvent systems. Inorganic buffers and ion-pair reagents cannot be used with the TEA pyrolyser; additionally, the detector output is highly unstable in the presence of aqueous HPLC mobile phases and only trace levels of water may be used. As a result HPLC–TEA has been used only for the analysis of a limited number of nitrosamine classes, *i.e.* non-polar nitrosamines[9], polar nitrosamines such as nitrosodiethanolamine[10] and only those ionizable nitrosamines (*e.g.* nitrosoamino acids) where the ionization can be suppressed by the HPLC solvent[11]. There are no reports concerning the analysis of ionic nitrosamines such as zwitterions, quaternary nitrogen-compounds and macromolecular peptides and this is principally due to the incompatibility of the TEA with the amount of water introduced into the detector under HPLC conditions required for the chromatography of ionic compounds. However, the recent advent of microbore HPLC with its very low flow-rates may permit the use of these chromatographic conditions with the TEA. To assess the applicability of this technique we have synthesized a model ionic nitrosamine N-nitroso N^1,N^1-dimethylpiperazinium iodide. In this note we report for the first time the TEA analysis of an ionic nitrosamine using microbore HPLC.

EXPERIMENTAL

The analysis was performed by reversed-phase ion-pair chromatography using a Chrompack 50 cm × 1 mm microbore ODS column coupled directly to a 0.5-μl internal-loop Valco valve. The mobile phase of 0.1 M ammonium heptane-sulphonate in methanol–water (70:30) was pumped at 20 μl/min using a Waters 6000A pump modified for microbore flow-rates. The column eluent was mixed with acetone (2 ml/min) and introduced into the TEA pyrolyser operating at 650°C. Two sets of TEA cold traps were used in series (solid carbon dioxide–isopropanol and liquid nitrogen).

N-Nitroso N^1,N^1-dimethylpiperazinium iodide was prepared by reacting N-nitroso N^1-methylpiperazine with methyl iodide in ethanol at 0°C. The results of elemental analysis (found: C, 26.80; H, 5.14; N, 15.49; I, 46.38%. $C_6H_{14}N_3OI$ requires C, 26.58; H, 5.20; N, 15.50; I, 46.81%) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy on the resulting white solid were consistent with the expected structure $[ON \cdot N \cdot (CH_2 \cdot CH_2)_2 \cdot N(CH_3)_2]^+ \ I^-$.

RESULTS AND DISCUSSION

The microbore HPLC–TEA chromatogram of N-nitroso N^1,N^1-dimethylpiperazinium iodide is shown in Fig. 1. Under the reversed-phase ion-pair conditions employed the ionic nitrosamine eluted as a sharp symmetrical peak after 20.5 min (capacity factor, $k' = 1.3$). Due to the very low flow-rate involved the highly

30   20   10   0

MINUTES
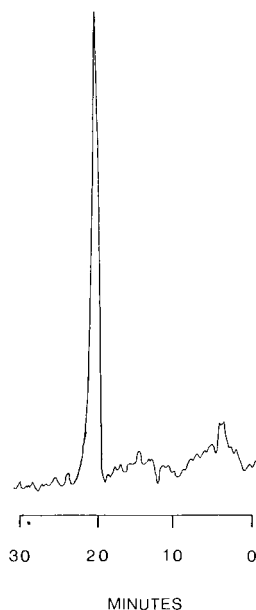
Fig. 1. Reversed-phase ion-pair HPLC–TEA chromatogram of N-nitroso N^1,N^1-dimethylpiperazinium iodide (105 ng); TEA attenuation, × 16.

polar nature of the microbore HPLC mobile phase had little adverse effect on the stability of the detector signal.

As far as we are aware microbore HPLC coupled with the highly sensitive and nitrosamine-specific TEA detector is presently the only chromatographic technique suitable for the trace analysis of ionic nitrosamines. The procedure may be of considerable use for the unexplored field of ionic, zwitterionic and macromolecular nitrosamines in foodstuffs.

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Note

Determination of 2,4,7-trinitro-9-fluorenone in workplace environmental samples using high-performance liquid chromatography

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Recent interest in 2,4,7-trinitro-9-fluorenone began when it was reported that this compound, which is used as part of a charge-transfer complex in several models of business machines, was not only a suspect carcinogen, but was also reported to give positive results in mutagenicity assays[1]. Possible exposure to trinitrofluorenone may occur during maintenance and repair of the business machine, during the manufacture of the photo conductor drum, and during manufacture of trinitrofluorenone. No sampling and analytical method was available to determine the amount of worker exposure to airborne trinitrofluorenone. The equilibrium vapor concentration of this solid at room temperature, based on available vapor pressure data[2], would be only $1.9 \cdot 10^{-3}$ $\mu g/m^3$. Therefore a method for the determination of particulate trinitrofluorenone air concentration was needed. The method presented here uses normal-phase high-performance liquid chromatography (HPLC) with UV detection for the quantitative analysis of trinitrofluorenone in particulate air samples and in bulk spent toner*.

EXPERIMENTAL

Equipment

The liquid chromatograph used consisted of a Waters 6000A reciprocating pump, a Waters Intelligent Sample Processor 710B autosampler, a Waters 440 UV detector equipped with a 280-nm filter, and a Waters Model RCM 100 radial compression module with a Radial-Pak B adsorption cartridge. The detector signal was recorded on a Soltec Model B281 dual-channel strip-chart recorder and integrated by a Hewlett-Packard Model 3354B laboratory automation system. Filters investigated for recovery of trinitrofluorenone were 37 mm polytetrafluoroethylene filters (Millipore, Fluoropore, FH) and glass fiber filters (Gelman A/E).

Reagents

Dilute standards of 2,4,7-trinitro-9-fluorenone (reagent grade, Matheson, Coleman & Bell) were prepared from a concentrated 1-$\mu g/\mu l$ stock solution of the compound in toluene (Burdick & Jackson). The mobile phase solvents were methylene chloride and isooctane (Burdick & Jackson).

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* Business machine toner is a finely powdered pigment and plastic resin. The toner is contaminated with trinitrofluorenone during contact with the photo-conductor drum. The excess toner not used in producing the printed page is discarded as spent toner.

Procedure

All filter samples were placed in 8-ml glass screw-cap test tubes and extracted with 2 ml of toluene. Bulk quantities of spent toner (0.3–1.0 g) were extracted with 10 ml of toluene in 20-ml glass screw-cap vials. The trinitrofluorenone was extracted from the spent toner in bulk or on a filter with the aid of ultrasonic agitation for 5–10 min. Toner particulate was then separated by centrifuging at approximately 1300 g for 20–30 min. A portion of the clear extract was transferred to an autosampler vial and a 100-μl aliquot of the clear extract was then injected into the HPLC system with no further preparation and analyzed under isocratic conditions of isooctane–methylene chloride (20:80) at 2 ml/min and ambient temperature. The chromatograph was calibrated with standard solutions of trinitrofluorenone in toluene.

RESULTS AND DISCUSSION

Although normal-phase chromatography was determined to be the best analytical technique, others were investigated first. Gas chromatography with a nitrogen-selective detector was investigated because of the possibility of enhanced sensitivity. However, the thermal instability of the trinitrofluorenone made this approach unsatisfactory, even when using an all-glass gas chromatographic system. Reversed-phase liquid chromatography and electrochemical detection with various types of C_{18} columns were also tried. However, the trinitrofluorenone eluted too slowly when using the high percentages of water in the mobile phase necessary for operation of the available electrochemical detector. In addition, when an aliquot of the toner extract was injected into the LC system, the system became plugged. This was traced to the precipitation of some of the extracted toner compounds from the sample solvent, tetrahydrofuran, upon dilution of the aliquot with the aqueous mobile phase.

Resolution of trinitrofluorenone from toner components was adequate with normal-phase liquid chromatography when an isooctane–methylene chloride mobile phase was used. The all-organic mobile phase also eliminated the problem of precipitation of the toner components. The trinitrofluorenone, however, exhibited some interesting characteristics with this technique. When the strength of the mobile phase solvents was changed, the elution order of trinitrofluorenone with respect to the toner components changed, as shown in Fig. 1. This permitted more maneuvering of the separation, and good separation of trinitrofluorenone from the other toner components could be obtained. In addition, when working over a wide range of concentrations, the retention time of trinitrofluorenone at a constant mobile phase strength changed with the amount injected. Representative data are shown in Table I. The greater the quantity of trinitrofluorenone injected in the same injection volume, the shorter the retention time tended to be. However, over the recommended range of the method, 12 to 80 ng was injected, and the variation of the retention time during a day's analyses was usually around 1 % relative standard deviation.

Peak area was used for quantitation to minimize effects due to any variation in retention time of the peak of interest. The average slope of nine calibration curves with a range of 120–800 ng/ml was 1035 area counts-ml/ng with a standard deviation of $\pm$ 90. The average y-intercept and its standard deviation for these calibration curves was 1489 $\pm$ 3283 area counts, which is not significantly different from zero. The limit of detection was 20 ng/ml and the limit of quantitation, where the standard deviation was no worse than $\pm$ 12, was 120 ng/ml.
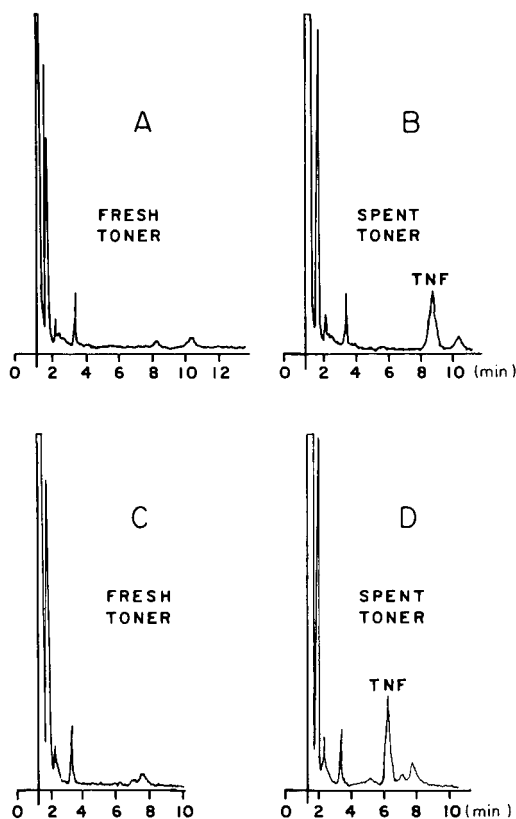
Fig. 1. Chromatograms of the toluene extract of fresh toner (A and C) and spent toner (B and D) showing the change in elution order of trinitrofluorenone (TNF) relative to the other components of the toner with a change in mobile phase strength. For chromatograms A and B the mobile phase was methylene chloride–isooctane (70:30). For chromatograms C and D the mobile phase was methylene chloride–isooctane (80:20).

Since trinitrofluorenone is a solid at room temperature, where it is reported to have a vapor pressure of only $1.1 \cdot 10^{-10}$ Torr[2], a filter sampling medium for particulate is appropriate for the determination of breathing zone and general air concentrations. Glass fiber and polytetrafluoroethylene filters were investigated for recovery of trinitrofluorenone. Six samples for each filter type at two levels, 0.4 and 1.6

TABLE I

CHANGE OF RETENTION TIME WITH AMOUNT OF TRINITROFLUORENONE INJECTED

Amount injected (ng)	Retention time (min)
2	7.16
6	7.02
10	6.82
20	6.75
200	6.55

TABLE II

RECOVERY OF TRINITROFLUORENONE FROM FILTER MEDIA

Type	Loading (μg)	Recovery (average % ± S.D.)
Glass fiber	1.6	82.5 ± 2.9
Glass fiber	0.4	79.7 ± 5.4
Polytetrafluoroethylene	1.6	101.3 ± 1.1
Polytetrafluoroethylene	0.4	93.7 ± 2.4

μg, were prepared. After overnight storage, the samples were analyzed giving results listed in Table II. From these results polytetrafluorethylene filters were selected as more suitable.

Two 2-week stability studies with samples prepared by spiking blank filters with either pure trinitrofluorenone or spent toner were used to further evaluate method recovery (see Table III). From the data collected when trinitrofluorenone only was present stability on polytetrafluoroethylene filters seemed to be adequate for at least 2 weeks at room temperature. However, when trinitrofluorenone was in the presence of toner, it may not have been as stable. Because of the imprecision of the spent toner data and the limited number of data, it was difficult to assess the stability of trinitrofluorenone in this type of sample.

TABLE III

STABILITY OF TRINITROFLUORENONE ON POLYTETRAFLUOROETHYLENE FILTERS

Loading (ng)	Number of samples	Time stored (days)	Average recovery (% ± standard deviation)
400	6	1	86.7 ± 7.6
400	6	7	93.0 ± 3.6
400	6	14	87.7 ± 6.3
417*	6	1	108.2 ± 7.7
417*	6	7	84.2 ± 8.9
417*	6	14	87.8 ± 13.7

* The amount of trinitrofluorenone in spent toner loaded on filters was determined by analyzing five aliquots of the spiking suspension, spent toner in toluene, and had a relative standard deviation of 10.6%.

CONCLUSION

From the work presented, normal-phase liquid chromatography can provide an adequate analysis of trinitrofluorenone in workplace environmental samples. The method is quantitative over the range of 0.48–1.6 $\mu g/m^3$ with an air sample volume of 500 l and exhibits a pooled relative standard deviation of 5.6% over this range. Stability of trinitrofluorenone in samples with toner present may not be as good as samples with only pure trinitrofluorenone, but use of control samples prepared with spent toner would aid in determining the validity of samples stored for any period of time.

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Book Review

Comparisons of fused silica and other glass columns in gas chromatography, by W. G.
Jennings, Alfred Hüthig Verlag, Heidelberg, 1981, VIII + 80 pp., 39 figs.,
price DM 38.00, US$ 19.00, ISBN 3-7785-0729-X.

Initial impressions of this book are highly favourable. The clear, bold print and
distinct figures immediately catch the eye, whilst the convenient, compact size make
the book easy to carry around and refer to. Each chapter and each section within a
chapter are clearly headed and preceeded by a useful summary in darker print. As
fused-silica capillary columns are still relatively new, it is good to see a book extolling
their virtues —and drawing attention to their (fewer) drawbacks.

Chapter 1 is a general introduction to gas chromatography and the parameters
which affect performance. In particular, the theory behind column separation of
components is explained in simple terms —and no equations!— leading on to the
pros and cons of capillary and packed columns.

Having decided that capillaries outperform packed columns in efficiency,
speed, sensitivity, inertness and price, Chapter 2 discusses Materials of construction.
Basically, this can be summarized by, firstly, *why* glass (and not plastic or metal)?
Having answered that (because it is more inert, but not inert enough), one has to
choose *which* glass? The bulk and surface structures of different glasses (soda-lima,
borosilicate, lead and fused silicate/quartz) are well explained and this reveals how
surfaces can have a variety of Lewis acid and silanol active sites. Finally, attention is
drawn to the special problems of drawing capillaries from high purity fused silica.

Once a capillary has been drawn, the surface active sites mentioned above,
must be deactivated. Chapter 3 provides a highly comprehensive survey of both the
theory and practice of glass and fused-silica capillary pretreatment. This must cover
all the common techniques used, including leaching, etching, surface active agents,
salt deposition, deactivation using polymer films, polysiloxanes and silylation, and
relative merits of each in deactivating Lewis acid or silanol sites (and other side
effects) are discussed in detail.

When the column has been appropriately treated, the author proposes that it
should be evaluated for degree of inertness (Chapter 4). This can be done prior to,
and after coating with the final liquid phase (the book is concerned principally with
wall-coated open tubular columns) using a variety of test compounds, and procedures
such as varying temperature, phase "bleed", adsorption of compounds (*e.g.*, tailing
and loss of sample) and compound degradation. This is a particularly useful chapter,
incorporating many hints for chromatographers wanting to evaluate the quality of
their columns. In passing, it would have helped, had the peaks in Fig. 22 been num-
bered, as identification using Table 3 is otherwise impossible.

Chapter 5 —Physical characteristics of the column— should really be renamed
Physical characteristics of the fused silica column, since it devoted to the many

advantages to be gained from a flexible column. One immediately obvious gain is to do away with the need for flame straightening of column ends, which can not only produce active sites, but can enrage operators trying to mount long, fragile straight ends into instruments! Fused-silica columns are very thin, so they are coated with, commonly, a polyimide sheath. The book stresses the need for column ends to be properly cut to avoid projecting sheath fragments, and there are some excellent photographs and practical instructions for perfecting this technique.

Chapter 6 —Cost comparisons of glass and fused silica columns— seems entirely irrelevant. The whole chapter is only two pages, of which one quarter is a summary of the other three quarters. What the author says here —that fused-silica capillaries are cheaper than glass because . . . — could be said in one paragraph at the end of, say, Chapter 2.

The final chapter (7) consists of a diverse selection of Application examples, and is simply an illustration of what can be achieved with fused-silica columns. The message seems to be that capillary gas chromatography is becoming evermore versatile, capable of handling a wider range of high and low molecular weight compounds and the advent of fused-silica columns has given this a healthy boost.

So who is this book aimed at, and what will the reader learn from it? The book is written in the casual style common to American authors, and despite occasional eccentricities of spelling and sentence construction, I found it very easy to read and extremely informative. I anticipate that it will appeal most to people actually using capillary gas chromatography who would like to understand the basic theory and principles —without the mathematics. At the same time, there is a surprising amount of practical advice for such a slim volume.

Brighton (Great Britain)   RICHARD L. PATIENCE

AUTHOR INDEX

Erratum

Journal of
chromatography news section

SIXTIETH BIRTHDAY OF PROFESSOR ISTVÁN HALÁSZ

István Halász began chromatographic research as the assistant of Professor Géza Schay in the Institute of Physical Chemistry at the Technical University of Budapest after receiving his PhD from the University of Szeged in 1949. His habilitation in 1954 was granted on the basis of his work "Vapor Adsorption Methods in the Investigation of Adsorbents and Catalysts". In addition to his research and teaching at the Technical University, István Halász was one of the first scientists who formed the Central Research Institute for Chemistry of the Hungarian Academy of Sciences, and in 1954 he became the Head of the Department of Gas Adsorption and Catalysis.

In 1956, István Halász left Hungary and moved to Frankfurt am Main, G.F.R., where he received a research position at the University in the Institute of Physical Chemistry headed by Professor H. Hartmann. In the Spring of 1958 he received the *venia legendi* for physical chemistry at the same institute and became *Dozent* in 1961 and Professor in 1964. Beside teaching at the University, he had a position in industry as the head of the Gas Laboratory at Scholven Chemie AG from 1957 until 1961. He has been a visiting professor at Northeastern University in Boston, and at the University of Nice in 1970. Since 1970 he has held the Chair for Applied Physical Chemistry at the University of the Saarland in Saarbrücken.

István Halász's early research on gas adsorption led to his involvement in the field of gas chromatography where he has become one of the best known scientists. His pioneering work with capillary columns was followed by numerous contributions encompassing instrumentation, column technology and theory. His work also played a major role in the development of "brush-phases" for use in gas chromatography, among many other wide ranging accomplishments. He clearly recognized the potential of liquid chromatography in the late sixties, and his laboratory has become one of the most prolific and influential research centers in this field. He has been a frequent speaker at international symposia, and a generation of chromatographers has enjoyed his technical presentations delivered not only with perfection but also with his unique wit and often wry humor. As a popular and respected teacher, he has been the *Doktorvater* of graduates in Frankfurt and Saarbrücken, many of them having continued their work in the same field and also become known through their own contributions in the world of chromatography. His work has been recognized by the Chromatography Commemorative Medal of the U.S.S.R. Academy of Sciences in 1978 and the M.S. Tswett Chromatography Award in 1980.

István Halász celebrates his sixtieth birthday on February 28, 1982, and the *Journal of Chromatography* joins his former and present students, friends and colleagues in wishing him good health and much success in his work.

CALENDAR OF FORTHCOMING MEETINGS

April 5–8, 1982
Las Vegas, NV, U.S.A.

17th International Symposium "Advances in Chromatography"
Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. (Complete program published in Vol. 219, No. 3.)

April 14–16, 1982
Amsterdam,
The Netherlands

12th Annual Symposium on the Analytical Chemistry of Pollutants
Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)

April 15–17, 1982 Tokyo, Japan	**18th International Symposium "Advances in Chromatography"** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Central Campus, 4800 Calhoun, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. (Complete program published in Vol. 234, No. 1.)
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umwelt- forschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik 82** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl- Wiechert-Allee 9, 3000 Hannover 61, G.F.R. (Further details published in Vol. 224, No. 3)
May 2–6, 1982 Interlaken, Switzerland	**2nd International Symposium on Instrumental TLC (HPTLC)** Contact: Dr. R.E. Kaiser, Institute for Chromatography, P.O. Box 1141, D-6702 Bad Dürkheim, G.F.R.
May 11–14, 1982 Ghent, Belgium	**4th International Symposium on Quantitative Mass Spectrometry in Life Sciences** Contact: Prof. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, De Pintelaan 135, B-9000 Ghent, Belgium. (Further details published in Vol. 226, No. 2.)
June 1–4, 1982 Goslar (near Hannover), G.F.R.	**3rd International Symposium on Isotachophoresis** Contact: Dr. C.J. Holloway, ITP 82, Abteilung für klinische Biochemie, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, D-3000 Hannover 61, G.F.R. (Further details published in Vol. 219, No. 3)
June 6–12, 1982 Frankfurt, G.F.R.	**European Meeting on Chemical Engineering and ACHEMA Exhibition Congress 1982** Contact: DECHEMA P.O. Box 970146, D-6000 Frankfurt/M 97, G.F.R.
June 7–11, 1982 Philadelphia, PA, U.S.A.	**VI International Symposium on Column Liquid Chromatography** Contact: R.A. Barford, ERRC – SEA, U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118, U.S.A. (Further details published in Vol. 211, No. 3)
June 16 + 17, 1982 Szeged, Hungary	**Symposium on Advances in Thin-Layer and High-Performance Liquid Chromatography** Contact: Dr. Haleem J. Issaq, Frederick Cancer Research Center, P.O. Box B, Frederick, MD 21701, U.S.A.; or Dr. Tibor Devenyi, Institute of Biochemistry, Hungarian Academy of Sciences, Budapest X1, Hungary. (Further details published in Vol. 216.)
June 18–21, 1982 Lund, Sweden	**Flow Analysis II** Contact: Flow Analysis II, c/o The Swedish Chemical Society, Upplands- gatan 6A, 1 tr., S-111 23 Stockholm, Sweden. (Further details published in Vol. 216.)
June 20–23, 1982 Bordighera (near San Remo), Italy	**International Symposium on Chromatography and Mass Spectrometry in Biomedical Sciences** Contact: Dr. Alberto Frigerio, c/o Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62 – 20157 Milan, Italy. Tel.: 35.54.546; Telex: 331268 NEGRI I. (Further details published in Vol. 214, No. 2.)

June 28–30, 1982 East Lansing, MI, U.S.A.	**35th American Chemical Society Annual Summer Symposium** Contact: A.I. Popov, Chemistry Department, Michigan State University, East Lansing, MI 48824, U.S.A.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
July 11–16, 1982 Louvain-la-Neuve, Belgium	**6th IUPAC Conference on Physical Organic Chemistry** Contact: Prof. A, Bruylants, Université Catholique de Louvain, Laboratoire de Chimie Generale et Organique, Batiment Lavoisier 1 Place Louis Pasteur, 1348 Louvain-la-Neuve, Belgium.
July 12–16, 1982 Amherst, MA, U.S.A.	**IUPAC Macromolecular Symposium** Contact: James C.W. Chien, Department of Polymer Science & Engineering, University of Massachusetts, Amherst, MA 01003, U.S.A.
July 19–22, 1982 Prague, Czechoslovakia	**Prague Microsymposium "Selective Sorbents"** Contact: Dr. F. Svec, c/o Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Heyrovského n.2, 162 06 Prague, Czechoslovakia.
Aug. 11–13, 1982 Hameenlinna, Finland	**6th European Symposium on Polymer Spectroscopy (ESOPS 6)** Contact: Professor Johan Lindberg, Department of Wood and Polymer Chemistry, University of Helsinki, Meritullinkatu 1 A, SF 00170 Helsinki 17, Finland.
Aug. 15–21, 1982 Perth, Australia	**12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.
Aug. 22–28, 1982 Vancouver, Canada	**XIth International Carbohydrate Symposium** Contact: Mr. K. Charbonneau, Executive Secretary, XIth International Carbohydrate Symposium, c/o National Research Council of Canada, Ottawa, Ontaria, Canada K1A 0R6. Tel.: (613) 993-9009; Telex: 053-3145.
Aug. 23–27, 1982 Budapest, Hungary	**22nd International Conference on Coordination Chemistry** Contact: Prof. M.T. Beck, Institute of Physical Chemistry, Kossuth Lajos University, Debrecen 10, H–4010, Hungary.
Aug. 29–Sept. 4, 1982 Kyoto, Japan	**5th International Congress of Pesticide Chemistry** Contact: Rikagaku Kenysho (The Institute of Physical and Chemical Research), 2–1 Hirosawa Wako-shi Saitama Pref. 351, Japan.
Aug. 30–Sept. 3, 1982 Vienna, Austria	**9th International Mass Spectrometry Conference** Contact: Interconvention, P.O. Box 105, A–1014 Vienna, Austria. (Further details published in Vol. 206, No. 1)
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.

Sept. 6–9, 1982 Liège, Belgium	**8th European Workshop on Drug Metabolism** Contact: Professor Jacques E. Gielen, Laboratoire de Chimie Médicale, Institut de Pathologie, Université de Liège, Bâtiment B 23, B-4000 Sart Tilman par Liège 1, Belgium. Tel.: (32-41)-56.24.80/81. (Further details published in Vol. 225, No. 2.)
Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 12–17, 1982 Kansas City, MO, U.S.A.	**184th American Chemical Society National Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington DC 20036, U.S.A.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Philadelphia, PA, U.S.A.	**9th National Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Division of Analytical Chemistry, American Chemical Society, Department of Chemistry, Notre Dame, IN 46556, U.S.A.
Oct. 12–14, 1982 Salzburg, Austria	**DIOXIN 82, 3rd International Symposium – Workshop on Chlorinated Dioxins and Related Compounds** Contact: Dr. E. Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland. (Further details published in Vol. 219, No. 3.)
Dec. 6–8, 1982 Parsippany, NJ, U.S.A.	**3rd Biennial Symposium on Advances in Thin-Layer Chromatography** Contact: Dr. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. Tel.: (215) 662-2082. (Further details published in Vol. 235, No.1.)
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
June 4–12, 1983 Cologne, G.F.R.	**29th Congress of the International Union of Pure and Applied Chemistry (IUPAC)** Contact: General Secretariat of the 29th IUPAC Congress, Dr. W. Fritsche, c/o Gesellschaft Deutscher Chemiker, P.O. Box 90 04 40, D-6000 Frankfurt/M 90, G.F.R.
June 27–July 1, 1983 Amsterdam, The Netherlands	**23rd Colloquium Spectroscopium Internationale** Contact: Congress Secretariat, c/o Organisatie Bureau Amsterdam, B.V., 1078 GZ Amsterdam, The Netherlands.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)
Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

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Electrodes of Conductive Metal Oxides: Part A

edited by SERGIO TRASATTI, *Laboratory of Electrochemistry, University of Milan, Italy*

STUDIES IN PHYSICAL AND THEORETICAL CHEMISTRY 11

The discovery by Beer in the second half of the sixties that the performances of anodes made of thermally prepared noble metal oxides were better than those of noble metals provoked something of a technological revolution in the large electrolytic industry. Since then an ever increasing number of fundamental studies have been published but the large amount of data has, until now, not been adequately assimilated.

This two-part work provides a general unifying introduction plus a state-of-the-art review of the physicochemical properties and electrochemical behaviour of conductive oxide electrodes (DSA). The text has been divided into two volumes – Part A dealing mainly with structural and thermodynamic properties and Part B, to be published in due course, dealing with kinetic and electrocatalytic aspects. This division came about due to the large amount of material to be treated and also because, in a rapidly developing field, difficulties arise in collecting all relevant material at one given moment.

The editor approaches the subject from a multidisciplinary angle, for example, the electrochemical behaviour of oxide electrodes is presented and discussed in the context of a variety of physicochemical properties – electronic structure, nonstoichiometry, crystal structure, surface structure, morphology and adsorption properties. For the first time the different groups of oxides are treated together in order to place emphasis on their similarities and differences.

This major reference work is mainly directed to electrochemists and those working on catalysis. It will also be useful to those in the fields of materials science, physical chemistry, surface and colloid chemistry and in areas where oxide surfaces may play a major role as in chromatography and photochemistry

CONTENTS: Chapters. 1. Electronic Band Structure of Oxides with Metallic or Semiconducting Characteristics *(J. M. Honig)*. 2. Chemisorption and Catalysis on Metal Oxides *(A. Cimino and S. Carrà)*.. 3. Oxide Growth and Oxygen Evolution on Noble Metals *(L. D. Burke)*. 4. Electrochemistry of Lead Dioxide *(J. P. Pohl and H. Rickert)*. 5. Properties of Spinel-Type Oxide Electrodes *(M. R. Tarasevich and B. N. Efremov)*. 6. Physicochemical and Electrochemical Properties of Perovskite Oxides *(H. Tamura, Y. Yoneyama and Y. Matsumoto)*. 7. Properties of Conductive Transition Metal Oxides with Rutile-Type Structure *(S. Trasatti and G. Lodi)*.

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