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JOURNAL OF CHROMATOGRAPHY

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HIGH OVEN TEMPERATURE ON-COLUMN INJECTION IN CAPILLARY GAS CHROMATOGRAPHY

I. SAMPLE INTRODUCTION

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(Received January 6th, 1986)

SUMMARY

High oven temperature on-column injection, *i.e.*, on-column injection at column temperatures well above the boiling point of the solvent, is highly attractive for rapid analysis at elevated temperatures. Two concepts are described, involving the use of a temporarily cooled column inlet at least 10 cm long such as is achieved by the extended secondary cooling (Carlo Erba), the movable on-column injector (J & W) and the oven-independently thermostated column inlet (Varian). According to the first concept, a relatively small sample volume (*ca.* 0.5 μl, depending on the length of the temporarily cooled inlet) is injected, the liquid coating the wall of the cooled inlet. The second concept accepts larger sample volumes and uses the cooled inlet as a buffer zone in order to prevent violent solvent evaporation from rejecting sample liquid into the injector. Movements of the sample material within the column inlet are described, from which technical requirements are derived.

INTRODUCTION

The basic concept of on-column injection in capillary gas chromatography (GC) is extremely simple, as the sample is introduced directly into the column inlet, circumventing all the steps involved in extra-column vaporization which are known to present problems. On-column injection is accordingly reliable for quantitative analysis. The basic logic is again simple: losses of sample material (also related to discrimination and high standard deviations) due to injection are ruled out, provided that no sample material returns behind the injection point or is lost backwards out of the column.

A key problem determining the working rules of the technique is related to the volume of the injection zone: the volume in the column inlet is far smaller than the volume of vapour created by evaporation of the sample solvent. As a result, rapid sample evaporation creates an increased pressure in the column inlet, which pushes sample material backwards out of the column. Therefore, it is necessary to provide sufficient time for sample evaporation, allowing the carrier gas to remove the gen-

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erated vapour continuously. This is achieved either by injection at low column temperatures (conventional on-column injection) or by slow introduction of the sample liquid from a cool zone into the heated zone (the kind of techniques to be considered in this paper).

Solvent evaporation at low column temperature

The conventional working rules for on-column injection[1] require the column temperature during injection and solvent evaporation to be below the boiling point of the sample (solvent) at the carrier gas inlet pressure in order to restrict the sample vapour pressure to below the carrier gas inlet pressure. This maximum injection temperature is therefore slightly above the boiling point of the solvent under standard conditions, depending on the inlet pressure applied[2].

On-column injection at low column temperatures proved to be highly reliable: it is reliable for quantitative analysis as it rules out rejection of sample material, but it also ensures that solvent effects optimally reconcentrate the initial bands of volatile solutes. There is no thermal defocusing, the temperature of the column inlet (and its increase) requires no special attention and the technique remains the same whether 0.2-or 200-μl volumes of sample liquid are injected[3]. Hence it contributes to the simplicity of on-column injection.

On the other hand, on-column injection at column temperatures below the boiling point of the solvent often renders analyses awkward. First, the required cooling of the oven is time consuming. If, for example, triglycerides are analysed as a solution in *n*-hexane, the column must be cooled below 70–75°C for injection and solvent evaporation. Subsequently, the oven is heated to above 300°C again for elution of the triglycerides. In addition to being awkward, the cooling step also causes problems concerning the stability of the baseline and reproducibility of absolute retention times. It is therefore understandable that there is much interest in high-temperature on-column injection, even if this means leaving the safe ground of classical conditions and if certain drawbacks must be accepted.

Approaches to high-temperature on-column injection

In 1978 Grob[4] suggested that sample rejection due to violent sample evaporation could be overcome by injecting slowly; assuming instant evaporation of the sample solvent, only such an amount of volatile material should be introduced per unit time that its vapour could be immediately discharged through the column by the carrier gas. However, using conventional on-column injectors, this concurrent solvent evaporation sometimes caused severe losses of high-boiling solute material[1]. Part of these losses were due to sample evaporation inside the tip of the syringe needle reaching into the oven-thermostated, hot column inlet. Another part was caused by sample evaporation on the outer wall of the needle; slow injection causes sample liquid to be drawn backwards into the narrow space between the needle and the column wall by capillary forces. As solvent evaporation proceeds from the rear to the front (the carrier gas picks up vapour from the rear), solvent evaporation occurred primarily between the needle and the column wall, depositing high-boiling solute material on the outer wall of the syringe needle. Some aspects of slow injection at high oven temperatures could be substantially improved by cooling the injection

zone during sample introduction by secondary cooling[5]. Nevertheless, slow injection was abandoned because its reliability was unsatisfactory for the high demands in on-column injection, particularly when using volatile solvents.

At Varian, Yang[6] and Hinshaw and Yang[7] constructed an on-column injector equipped with a device thermostating an inlet section of the column about 12 cm long independently of the oven. This inlet can be kept at low temperature while the oven temperature is far above the boiling point of the solvent. This inlet section can only retain about 0.5 μl of liquid; if larger sample volumes are injected, some liquid flows into the hot column. However, the cool inlet section serves as a buffer, preventing sample material from being rejected as far as into the injector.

The investigations reported in this paper were carried out using a movable on-column injector (J & W, Rancho Cordova, CA, U.S.A.). However, the results and conclusions are expected to be independent of whether the inlet was cooled by the device of Varian (Palo Alto, CA, U.S.A.), by pulling the column inlet out of the GC oven or by using an extended secondary cooling (Carlo Erba, Milan, Italy).

CONCEPTS OF ON-COLUMN INJECTION INTO TEMPORARILY COOLED COLUMN INLETS

Small sample volumes coating cooled column inlet

According to the first of two existing concepts, the sample liquid is coated as a film on the internal wall of the temporarily cooled column inlet. The sample volume injected is kept small enough to prevent sample liquid from flowing into the oven-thermostated column. Solvent evaporation is carried out at a low temperature, and the inlet section is heated to or above the oven temperature only after the solvent has completely evaporated. This technique avoids the generation of large volumes of vapour through rapid heating.

Geeraert *et al.*[8] applied this technique, using a home-made movable on-column injector, to the analysis of triglycerides. Sample volumes between 0.2 of 0.3 μl were injected into the column inlet kept in the atmosphere above the GC oven.

Cool buffer zone preventing rejection from column

The second concept tolerates larger sample volumes and accepts that sample liquid flows out of the cooled inlet into the entrance of the hot, oven-thermostated column. However, liquids cannot really penetrate into a column section at a temperature that far exceeds the boiling point of the sample as the sample liquid is stopped by its own vapour pressure. Therefore, the front of the sample plug remains in the zone where the column temperature changes from below to above the boiling point of the solvent, and solvent evaporation takes place there. After completion of solvent evaporation, the column inlet is heated, allowing the solute material to start the chromatographic separation process.

EXPERIMENTAL

Apparatus

Experiments were carried out using a movable on-column injector from J & W. This injector is fixed to a telescopic system, allowing the injector (and the column inlet) to be moved up and down by 133 mm. The system was mounted on an old gas

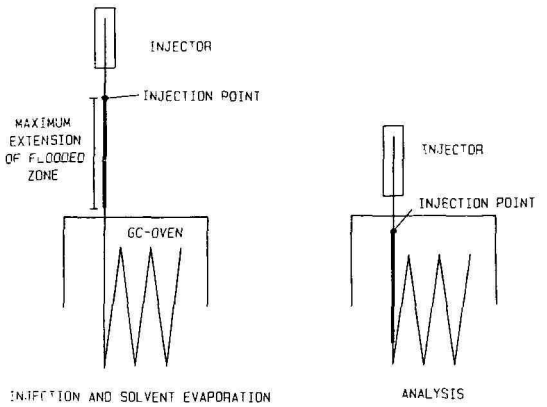

Fig. 1. Working principles of the moving on-column injector used for high-temperature on-column injection. Injection is carried out with the injector in the up position, pulling some 10 cm of the column inlet out of the oven-thermostated zone. The sample floods this cool inlet. After completion of solvent evaporation the injector is moved into the down position, introducing the solute-coated inlet into the oven. The injection point must be positioned such that it is clearly oven-thermostated when the injector is in the down position.

chromatograph (Carlo Erba Model 2150), leaving a gap of 15 mm between the roof of the oven and the base plate of the injector, stuffed with insulation material. This, however, does not fully preclude that a column section just above the base plate of the injector may be warmed by heat irradiated from the mounting bolt or by hot air from the oven blown along the column inlet through the mounting bolt.

Glass capillary columns (8–10 m × 0.31 mm I.D.) coated with PS 255 (a methylsilicone) of 0.4 μm film thickness were used. These columns were equipped with fused-silica inlets of 1–1.5 m × 0.32 mm I.D., either deactivated but uncoated (retention gap) or coated with stationary phase of the same film thickness as the separation column. Connections were prepared by fusion[9].

Description of the system

During chromatographic runs the movable on-column injector is in down position (Fig. 1). In this position the injection point must be located within the GC oven (preferably 10–15 mm below the possibly cooler oven roof) in order to ensure that all sample material deposited on the wall of the column inlet is oven-thermostated during the chromatographic run. This requires adjustment of the length of the syringe needle to the given installation (17 cm in our case).

Movable on-column injectors work as conventional on-column injectors if left permanently in the down position. This is suitable for injections carried out at column temperatures at or below the boiling point of the solvent.

Moving the injector into the up position, a length of 133 mm of the column inlet is pulled out of the GC oven and cooled to a temperature between 25 and 40°C, depending on the oven temperature and the distance from the base plate. However, the useful length of the cooled column inlet section is less than 133 mm; it only spans from the base plate of the injector to the injection point (Fig. 1). In our case it was 100 mm.

Movement of the sample liquid in the column inlet

Movement of the sample liquid in the column inlet was observed visually, either using a fused-silica capillary inlet from which the polyimide coating had been burnt off, or by replacing the top part of the column inlet by a glass capillary with a strongly etched internal surface (giving it a whitish aspect when dry and becoming transparent when coated with liquid).

Movement of solute material was also determined visually, injecting solutions containing 0.1–1% of perylene (a polynuclear aromatic hydrocarbon eluting from standard columns at *ca.* 250°C) and observing the fluorescence of the latter under UV light (366 nm).

RESULTS AND DISCUSSION

Behaviour of the sample liquid in the column inlet

In the first instance the sample liquid injected into the column inlet kept below the boiling point of the solvent behaves as previously described in the context of band broadening in space[10]: the injected sample liquid forms a plug closing the bore of the column. The latter is pushed into the column by the carrier gas, coating the column wall behind itself with a thick film of sample liquid until the plug is exhausted. Mostly, this primary flow of liquid, lasting for a fraction of a second, is followed by a secondary flow along the column wall, which tends to reduce the thick sample layer to a mechanically stable film.

The length of the column inlet flooded by the sample liquid depends on a number of parameters, but as a first approximation it may be assumed that liquids wetting the column (stationary phase) surface flood 20–25 cm of the column inlet per microlitre of injected liquid, 25–30 cm if the inlet is uncoated (retention gap)[11].

In on-column injection into temporarily cooled column inlets, three cases can be distinguished according to the injected sample volume.

(a) According to the first concept described above, with the sample liquid coating the cooled inlet, the length of the flooded zone must not exceed that of the cooled inlet. If the useful length of the cooled inlet is 10 cm, the maximum sample volume is 0.4–0.5 μl if the inlet is coated and 0.3–0.4 μl if it is uncoated.

(b) If slightly larger sample volumes are injected (second concept), some sample liquid flows into the entrance of the oven-thermostated column by the secondary flow of liquid along the column wall. However, rapid solvent evaporation prevents the liquid from entering a column section at a temperature considerably exceeding the boiling point of the solvent. Solvent vapour replaces most of the carrier gas passing through the column, with the consequence that the carrier gas flow in the inlet is at least strongly reduced. This in turn reduces the flow of sample liquid into the hot part of the column. This partly self-regulating system causes the solvent evaporation at the entrance of the oven-thermostated column to be fairly mild. If some liquid does flow too far into the hot column (delayed evaporation), followed by rapid evaporation, the vapour pressure created is discharged by a flow of vapour backwards into the cooled inlet. Such returning vapour does not move far owing to rapid recondensation.

(c) On further increasing the sample volume (under typical conditions and assuming a useful length of the cool inlet of 10 cm, to a volume exceeding about 0.8

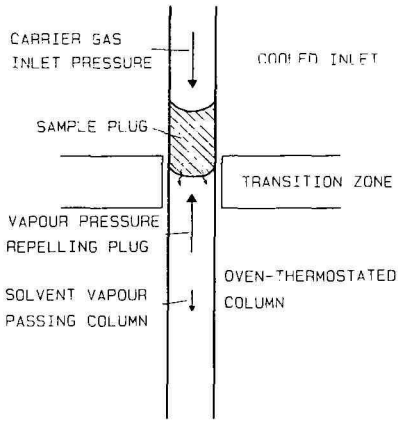

Fig. 2. Solvent evaporation from a stationary sample plug, positioned such that the front of the plug is located at a point where the temperature corresponds to the boiling point of the solvent at the inlet pressure. The plug is kept stationary as the solvent vapour pressure equals the carrier gas inlet pressure.

μl), the sample plug (primary flow) reaches the hot column section and a more violent mechanism becomes active. In contrast to case (b), the column bore is closed by the liquid, preventing easy discharge of excessive sample vapour towards the cooler rear. There is some "bumping", considered in more detail below.

Rejection of sample liquid

Theoretically, a plug of liquid pushed into a hot column should be stopped and driven back to a position such that its front is at a temperature causing the sample (solvent) vapour pressure to be equal to the carrier gas inlet pressure. This should result in a stationary plug as shown in Fig. 2, from the front of which the solvent would evaporate. However, as described in a previous paper[12], in reality the sample plug does not find a stable position in a process as quiet as that described above; owing to delayed evaporation the sample plug enters the hot column much further than it is supposed to (the smooth surface of a stationary phase film efficiently hinders the onset of evaporation). Once evaporation is initiated, evaporation is violent, and the high sample vapour pressure created at the evaporation sites (often the sample plug is split) throws the sample plug(s) back into the cool column inlet. The plugs move a considerable distance until they come to a stop, *i.e.*, until the vapour in front of them has recondensed. A fraction of a second later, the carrier gas inlet pressure exceeds the solvent vapour pressure and drives the (often recombined) plugs back towards the heated zone, and the whole process starts again.

Typical "stopping distances", *i.e.*, distances covered within the cool inlet until the plug of liquid comes to a stop, were found to range between 6 and 11 cm. Liquids that do not wet the surface of the column inlet are driven further back (more than 15 cm) because liquids glide more easily on the unwetted surface but possibly also because recondensation is hindered. The latter observation is of particular importance if on-column injection at elevated column temperatures is used to avoid band broadening in space by samples that do not wet the stationary phase[13].

Effect of prematurely heating the inlet

A delicate point when using temporarily cooled column inlets for high-temperature on-column injection concerns the selection of the moment when the inlet is heated (by lowering the injector into the down position, switching off the secondary cooling or initiating heating of the independently thermostated inlet section). Most producers of temporarily cooled inlets recommend heating this inlet immediately after completion of injection. Corresponding guidelines have been elaborated for slow injection where cooling of the inlet serves for preventing evaporation inside the syringe needle[5]. However, our case is fundamentally different and guidelines must not be transferred blindely.

The sample liquid is coated on the wall of the cooled inlet in order to provide the solvent with the time required for gentle evaporation and transport through the column. Similarly, the concept of considering the cool inlet as a buffer for retaining rejected sample liquid requires that cooling is maintained up to completion of solvent evaporation. Premature heating creates an excessive volume of vapour and a back-flow of the latter. In other words, the benefits of the cooling are nullified.

Experimental observations confirmed this view in general, although presenting a more complex picture. Movements of the liquid were followed visually, injecting volatile solvents into a column at 200°C and lowering the injector into the down position immediately after injection.

On injecting volumes up to 0.3 μl no returning liquid could be observed above the base plate of the injector. Injections of larger volumes, however, created a hump of liquid *ca.* 2–5 mm above the point where the column inlet left the heated zone (mounting bolt in the injector base plate). This standing wave of liquid remained there until solvent evaporation was almost completed. If the injector was lowered slowly, the hump of liquid moved backwards at a corresponding speed, remaining stationary relative to the mounting bolt.

Visual observation of the solvent does not allow one to distinguish the extent to which the solvent returned towards the injector as liquid or as vapour recondensing in the cooler part. This differentiation, however, is crucial, as return of liquid in the liquid phase carries all dissolved solute material along, whereas evaporation and recondensation leaves higher boiling material at the evaporation site.

Movements of the solute material were studied using perylene as a marker. Injections of various volumes and using different solvents were carried out at an oven temperature of 250°C (as required for the isothermal analysis of perylene), again lowering the injector immediately after the injection. Observations were not uniform; in many instances the fluorescence of perylene became clearly visible within the hump of back-flowing liquid. In other instances, however, the hump of returned liquid did not show noticeable fluorescence. However, even in these instances some perylene was located in the transition zone of the inlet located between the oven-thermostated and the cooled sections, *i.e.*, the part situated in the mouting bolt. The extent to which perylene was carried backwards behind the injection point appeared to depend on many factors, among which the speed of lowering the injector was important. A large proportion of perylene returned when the plug of injected liquid did not disappear before the injector was lowered because the vapour pressure created at the front of the plug drove the latter backwards behind the injection point. Considerable back-flow in the liquid phase is also obtained if the stream of back-flowing vapour

drives waves of liquid along the column wall into the rear of the inlet.

In practice, it is not important how much solute material is flushed behind the injection point. As long as such a return cannot be reliably ruled out, the inlet can only be heated when (nearly?) all of the solvent has been evaporated. Currently used guidelines must be changed accordingly.

Sample plug returning to needle tip

The mechanism under certain conditions causing the sample plug to return to the tip of the still inserted syringe needle and its consequences for analyses (discrimination, memory effects) have been described previously[14]. The carrier gas ahead of the freshly injected sample plug acts as a spring; it is compressed by the injected sample liquid (which enters the column at a flow-rate far exceeding that of the carrier gas). The compressed gas slows the movement of the sample plug and, under certain conditions, pushes it back again towards the tip of the still inserted syringe needle (a process that takes place during a fraction of a second). If the returned sample plug touches the needle tip, some liquid is pulled by capillary forces backwards between the needle and the column wall. When the syringe needle is withdrawn, this liquid is pulled backwards further up the column neck, often up to the column entrance. The column neck thus becomes coated with sample liquid, the excess of sample liquid flowing back into the oven-thermostated column. The sample film contains about 30 nl of liquid per centimetre of column length (0.3 mm I.D.). The amount of sample material deposited on the wall of the neck between the column entrance and the injection point depends on the length of the latter, but can easily reach 100–300 nl.

The sample material pulled backwards into the permanently cooled column neck kept inside the injector behaves as sample material, reaching there owing to premature heating of the temporarily cooled inlet section or excessively violent rejection of liquid at the beginning of the heated column (see above). The effects observed in the chromatograms depend on the volatility of the solutes and the retention power in the column inlet, in particular on whether a retention gap is used as a column inlet.

Solutes of high volatility in the cool column inlet return rapidly to the oven-thermostated column and are chromatographed normally together with the bulk of the solute material, forming perfectly shaped peaks representing the total amount of injected solute material. On the other hand, solute material of low volatility remains in the column neck during the whole run. Again, perfectly sharp peaks are obtained, but of insufficient area (discrimination at the high-temperature end of chromatograms!). The lost material is likely to create memory effects; it is washed back into the oven-thermostated column by a following injection bringing liquid into the column neck[14].

Solute material of intermediate volatility returns into the oven-thermostated column with a delay. If this delay is small, the affected solute material is eluted as the tail of a peak. A greater delay causes the tail to become broader, or invisible if the solute material merely lifts the baseline to a marginal extent. Resulting peaks may appear to be of perfect shape. However, the peak areas are insufficient. In contrast to solute materials of even lower volatility, no memory effects are observed as the deposit is removed before the subsequent injection.

Fig. 3a shows an isothermal chromatogram (160°C) of solutes of intermediate

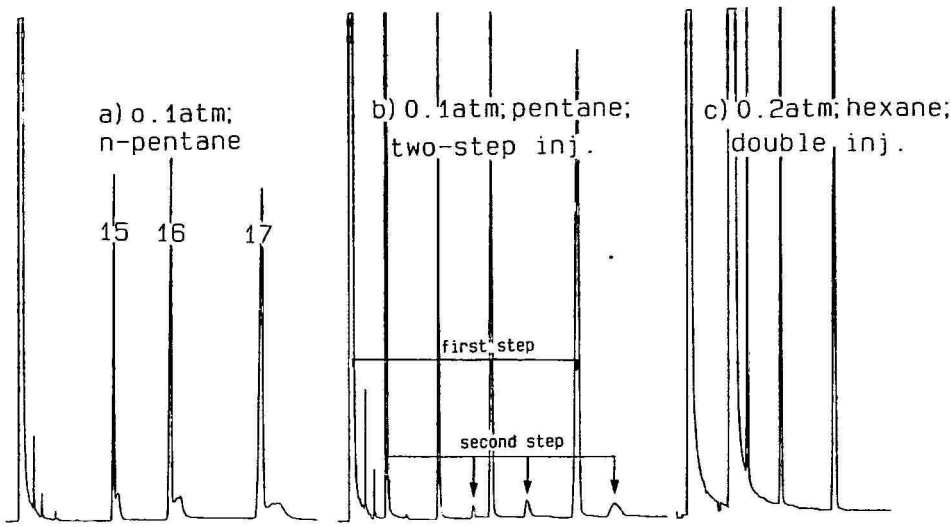

Fig. 3. Certain conditions cause the injected sample liquid to be pulled backwards between the walls of the column and the syringe needle. As the needle is withdrawn, this liquid is pulled further backwards into the column neck. The effects on chromatography depend on volatility of the solutes. Isothermal runs at 160°C; 0.5-μl injections of C_{15}–C_{17} *n*-alkanes (15–17) in *n*-pentane (a,b) and *n*-hexane (c). (a) Normal injection under conditions causing some sample liquid to be pulled into the column neck. Solutes return from there with a delay, forming the broad shoulders eluted after the main peaks. (b) After a normal injection the injector was moved into the up position again and a 5-cm section of column inlet was pulled out of the injector. Lowering the injector caused thermal desorption of solute material from the formerly cooled inlet, resulting in the small peaks eluted after the main peaks with the same distance as the "air" peak of the second "injection" is away from the solvent peak. (c) Normal injection under conditions that do not cause a return of sample liquid behind the injection point, followed by an injection of pure solvent (*n*-hexane) into the column neck to rinse possible deposits of solute material into the oven-thermostated column.

volatility (C_{15}–C_{17} *n*-alkanes) obtained by injection of 0.5 μl of an *n*-pentane solution on to an 8 m × 0.31 mm I.D. capillary column coated with PS-255 up to the column entrance (no retention gap). Injection was carried out with the injector in the up position, and the injector was lowered (the column inlet heated) 10 s after injection. Both the high volatility of the solvent and the relatively low carrier gas flow-rate (about 2 ml/min of H_2) contributed to the fact that the plug of injected sample liquid returned to the tip of the syringe needle. Broad shoulders are observed in the chromatogram, eluted just after the sharp peaks and representing material that entered the oven-thermostated column with a delay due to slow release from the permanently cooled rear part of the column inlet behind the injection point. According to the peak-area ratios, about 30% of the sample was withdrawn into the column neck, *i.e.*, about 0.15 μl of sample liquid, coating a column section about 5 cm long. Return of solute material into the cool column neck was confirmed by a modified injection procedure in which injection was carried out as in (a), lowering the injector 10 s after injection, but the solutes were allowed only 10 s to leave the temporarily cooled inlet by lifting the injector into the up position again after 10 s. In this way the warm inlet section behind the injection point was cooled to ambient temperature also. Then a 5-cm section of the column inlet was pulled out of the injector, accordingly shifting the injection point further into the oven. Lowering the injector into the down position

caused a formerly permanently cool rear column inlet section to become oven-thermostated and desorption of the solute material withdrawn into it.

Two points can be observed in Fig. 3b. First, the broad shoulders observed in chromatogram (a) are absent, which is the result of the rear column inlet having been cooled to a temperature that does not cause desorption of the solutes at a rate that produces a visible deflection of the recorder pen. Second, small, slightly broadened peaks are observed at a distance from their parent peaks corresponding to the time difference between lowering the injector for the first and the second times. These small peaks represent solute material from the cool rear of the inlet, which has only been heated by the second "injection". The areas of these secondary peaks differ from those of the shoulders in chromatogram (a), first because the amount of withdrawn solute material is poorly reproduced, and second because some solute material slowly passed on to the oven-thermostated column without a noticeable rise in the baseline before lowering the injector the second time.

The same evidence as obtained by the above experiment was achieved by subsequent injection of pure solvent into the column head instead of pulling the inlet partially out of the injector. The sample was injected, the inlet heated and cooled again (as in the above experiment), followed by injection of a 1-μl volume of pure *n*-hexane into the column neck just below the column entrance. This solvent rinses possible solute material from the permanently cooled rear part of the inlet into the oven-thermostated column. Such rinsing is highly efficient if an uncoated inlet (retention gap) is used. Rinsing solute material from a coated surface, however, is incomplete.

Fig. 3c shows the result of a double injection carried out under conditions that prevent the return of the sample plug to the tip of the syringe needle; *n*-hexane was used instead of the more volatile *n*-pentane and the carrier gas inlet pressure was doubled. The chromatogram shows the two solvent peaks but no secondary peaks of the solutes, confirming that no solute material was withdrawn far behind the injection point.

Return of the sample liquid to the needle tip was found to be a severe problem, first because such a return occurred frequently, and second because of the length of the column neck that was coated with a large volume of sample liquid. Hence, the effects on quantitative analysis are not negligible.

Frequently, sample liquid was not only pulled backwards up to the column entrance, but out of the column into the needle guide zone and the rotating valve of the injector. As the restriction above the rotating valve consists of a glass tube, the liquid adhering to the needle tip and being smeared within this top section of the injector could easily be observed. Of course, such losses and the resulting contamination of the injector (memory effects) cannot be tolerated.

CONCLUSIONS

It should be emphasized that comments on the movable on-column injector refer to high-temperature on-column injection and not to the use of the injector for conventional on-column injection at lower column temperatures. In fact, the injector was not designed for high-temperature on-column injection, and the latter technique should be considered as an additional possibility offered by injectors that allow tem-

porarily cooling of a column inlet section such as the movable on-column injector.

This paper has dealt only with the process of sample introduction, where several sources of problems, mostly resulting in general or selective losses of solute material, were identified. Part II will deal with possible problems caused by deformed initial solute bands (resulting in deformed peaks or losses in peak area).

On the one hand, high-temperature on-column injection is very convenient for many applications, and on the other, the quantitative results obtained were clearly less reliable than those produced by conventional on-column injection. Sometimes absolute peak areas varied within wide ranges, indicating non-reproducible losses of sample material. In some rare instances memory effects were observed. Finally, the precision and accuracy of the relative peak areas often did not correspond to those obtained by conventional on-column injection. Nevertheless, results were obtained that could not have been produced by vaporizing injection.

ACKNOWLEDGEMENT

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HIGH OVEN TEMPERATURE ON-COLUMN INJECTION IN CAPILLARY GAS CHROMATOGRAPHY

II*. AVOIDANCE OF PEAK DISTORTION

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SUMMARY

High oven temperature on-column injection often produces distorted peaks, particularly when running isothermal chromatograms at the injection temperature. Peak distortion may be due to solute material pulled backwards into the cold column neck, but more often it is due to premature release of solute material from the temporarily cooled column inlet and thermal defocusing of the initial bands during this transition. The sources of peak distortion are described together with the possibilities of avoiding such problems. Summarized guidelines on how to use high oven temperature on-column injection are given.

INTRODUCTION

High oven temperature on-column injection in capillary gas chromatography (GC), *i.e.*, on-column injection at column temperatures well above the boiling point of the solvent, is particularly attractive for rapid isothermal analyses at elevated temperatures. Conventional on-column injection requires cooling of the column below the boiling point of the solvent before injections can be carried out. Cooling and heating of the GC oven for injection considerably prolongs analysis times (frequently to a multiple of the time required for isothermal analyses). Further, such changes in the oven temperature often disturb the baseline (baseline drifts and "ghost" peaks due to impurities cold trapped during the cooling period) and render absolute retention times less reproducible. If analyses involve temperature programming, it is usually no longer very important how far the GC oven must be cooled. Therefore, it is the rapid isothermal analysis at high column temperatures that would profit the most from the possibility of injecting into columns above the boiling point of the solvent.

In Part I[1] we discussed high-temperature on-column injection based on the use of a temporarily cooled column inlet section. Injectors equipped with an extended secondary cooling (Carlo Erba), movable on-column injectors (J & W) and injectors

* For Part I, see ref. 1.

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allowing oven-independent thermostating of the column inlet (Varian) are suitable for this purpose. However, the technique is not simple as there may be losses of sample material backwards leaving the column.

This paper deals with the shape of the initial solute bands created by the two high oven temperature on-column techniques described. The initial band shapes influence the shape of the eluted peaks. However, distorted initial bands may also affect quantitative analysis: under certain conditions solute material is eluted before the main peak in such a way that it remains undetected and, as a consequence, the integrated peak is too small.

High oven temperature on-column injection is again not as simple in these respects as conventional on-column injection because of additional band broadening effects and severe disturbances of solvent effects.

TWO TYPES OF BAND BROADENING

There are two fundamentally different types of band broadening: band broadening in time and in space[2,3].

Band broadening in space

Band broadening in space is due to spreading of the solute material within the column inlet section which is flooded by the sample liquid. The initial bands are characterized in terms of band length, this length corresponding to the length of the flooded zone. Band broadening in space only affects peaks eluted at least 50°C above the injection temperature.

Band broadening in space is virtually non-existent in high oven temperature on-column injection because the sample liquid cannot flow more than about 10 cm into the oven-thermostated column (the sample liquid is rejected by its own vapour pressure). The length of the initial bands, assuming a temporarily cooled inlet 10 cm long, is at most *ca.* 20 cm, which causes peak broadening only in special instances. In fact, on-column injection at column temperatures slightly exceeding the boiling point of the solvent was first proposed for avoiding band broadening in space[4–7].

Band broadening in time

Band broadening in time originates from slow introduction of solute material into the column (*e.g.*, splitless injection) or from slow release of the material from the column inlet. Band widths are expressed in terms of time, whereby initial band widths correspond to the introduction time or the time of release. It is this type of band broadening that frequently causes problems in high oven temperature on-column injection.

TWO CONCEPTS OF HIGH OVEN TEMPERATURE ON-COLUMN INJECTION

In Part I[1] two concepts of high oven temperature on-column injection were distinguished. These concepts are again kept apart because the mechanisms determining the width and shape of the initial solute bands are different.

Sample coating cooled inlet

According to the first concept, the sample liquid is coated on to the wall of

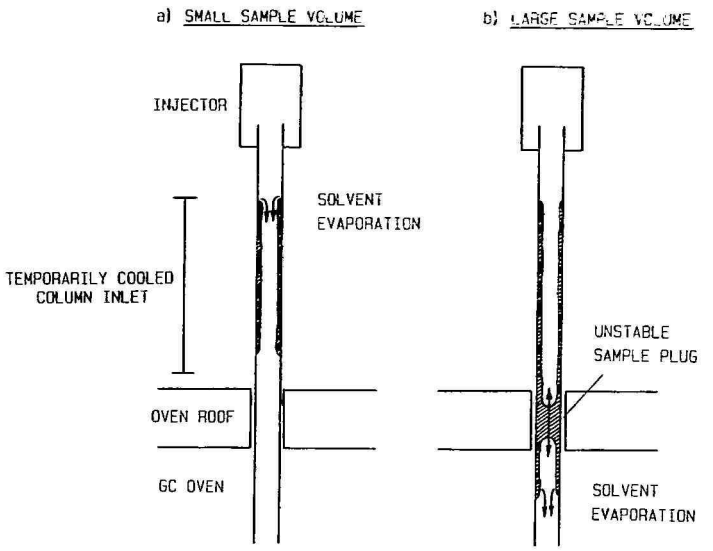

Fig. 1. The two concepts of high oven temperature on-column injection and their implications on solvent effects. (a) A relatively small sample volume (up to about 0.4 μl) is injected, ensuring that the whole sample liquid remains within the temporarily cooled inlet. Solvent evaporation proceeds from the rear to the front of the flooded zone, producing a solvent trapping effect. (b) A larger sample volume is injected, causing some sample liquid to flow into the entrance of the oven-thermostated column. First, the solvent evaporates at the front where the column temperature exceeds the boiling point of the solvent. Subsequently, evaporation continues at the rear of the sample layer within the cooled inlet as in (a).

the column inlet section, which is temporarily cooled below the boiling point of the solvent. The length of the flooded zone must not exceed the length of the cooled inlet as no sample liquid is allowed to flow into the oven-thermostated column (see Fig. 1).

Desorption on heating. According to the concept, the solute material is released from the temporarily cooled inlet on heating of the latter. This, however, presupposes that the solute material remains within the cooled inlet until the latter is heated. Although this is realistic for solutes eluted at high column temperatures, the situation is more complex for volatile solutes; the latter tend slowly to leak out of the cooled inlet and to start chromatography before the inlet is heated. Slow release from the inlet produces broad bands.

Solvent trapping. As solvent evaporation within the cooled inlet proceeds from the rear to the front of the sample layer, there is solvent trapping, a solvent effect[8]. Solvent trapping retains the solute material in the highly retaining solvent envelope up to completion of solvent evaporation. This is particularly important for volatile solutes that escape from the solvent-free, cooled column inlet. It means that solute material starts to leave the cooled inlet at the moment when heating of the inlet is permitted. It follows that heating of the column inlet should occur precisely at the end of solvent evaporation, as earlier heating may cause a backflow and later heating causes distorted initial solute bands.

The kind of distortion of the solute bands obtained by retarded heating of the column inlet is shown schematically in Fig. 2. Solute material prematurely released

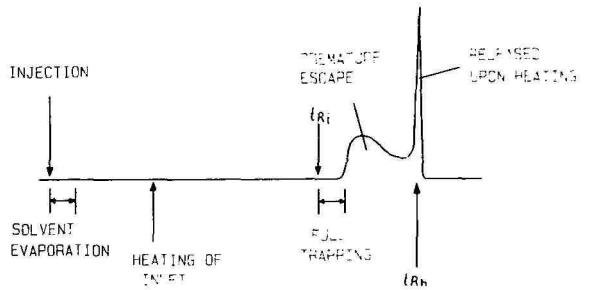

Fig. 2. Typical peak distortion caused by delayed heating of the temporarily cooled column inlet when working according to the concept of coating the whole sample liquid on the cooled inlet. The first solute material elutes with a retention time corresponding to the GC retention time (duration of separation process) plus the solvent evaporation time (t_{R_i}, chromatographic retention time measured from injection). The sharp peak represents material introduced into the oven-thermostated column by heating the inlet. Its retention time corresponds to the chromatographic retention time determined from the moment of heating (t_{R_h}). The ratio of the areas in the pre-peak and the sharp peak depends strongly on the volatility of the solutes in the temporarily cooled inlet (the pre-peak becomes invisible if the retention power of the cooled inlet is sufficient to render premature escape small). Perfectly shaped initial bands (and peaks) are obtained in any case if the inlet is heated exactly at the end of solvent evaporation.

from the cooled column inlet starts to elute with a retention time that exceeds the GC retention time by the solvent evaporation time. Heating of the column inlet causes rapid desorption of solute material that was still located in the cooled inlet. The width of the shoulder representing the prematurely released material corresponds to the delay of heating the inlet. Of course, the shape of a solute band distorted by this pattern depends on the volatility of the solute in the cooled inlet. Also, whether the shape of the initial band is still visible on the finally eluted peak depends on the extent of band broadening during chromatography and on whether cold trapping, the result of increasing column temperature between injection and elution, reconcentrated the band.

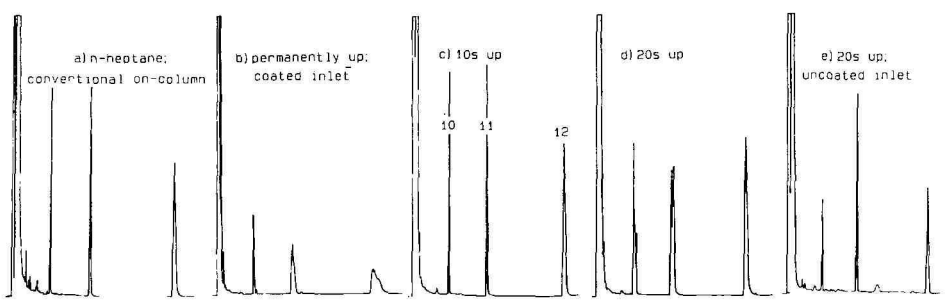

Fig. 3. Peak distortion due to delayed heating of the column inlet. Isothermal runs at 90°C, using a movable on-column injector and an 8 m × 0.32 mm I.D. glass capillary column coated with PS-255 of film thickness 0.4 μm, equipped either with a coated [(a)–(d)] or with an uncoated (e) fused-silica inlet 1 m long. Injection volumes, 0.4 μl; C_{10}–C_{12} *n*-alkanes (10–12) in *n*-heptane (a) or *n*-hexane [(b)–(e)]. (a) Basis of comparison for peak shapes; (b) solute bands broadened owing to slow release from permanently cooled inlet; (c) nearly perfect chromatogram achieved by heating the inlet 10 s after injection; (d) distorted peaks due to delayed heating (20 s after injection); (e) as (d) but using a retention gap, due to the low retention power rapidly releasing solute bands at the end of solvent evaporation (solvent trapping).

Example. Fig. 3 shows chromatograms of isothermal runs at 90°C obtained using the movable on-column injector from J & W as described in Part I[1]. Chromatogram (a) serves as a basis for comparison. It was produced by conventional on-column injection of a 0.4-μl volume of an *n*-heptane (b.p. 100°C) solution containing C_{10}–C_{12} *n*-alkanes. As no temporary cooling of the column inlet was needed, the injector was permanently in the down position. Under these conditions the initial bands are known to be perfectly sharp. Chromatograms (b)–(e) were produced by high oven temperature on-column injection of solutions in *n*-hexane (b.p. 69°C, 0.4-μl volumes). For chromatogram (b) the injector was permanently in the up position, resulting in peaks reflecting the slow release of the solute material from the cooled inlet. The least volatile solute, *n*-C_{12}, is most strongly broadened as its lower volatility caused it to leave the cooled inlet more slowly than the other solutes. Chromatogram (c) was obtained under almost optimal conditions: the injector was lowered 10 s after injection, and no obvious peak distortion is observed. A closer look, however, reveals that the *n*-C_{10} peak is slightly broader than in chromatogram (a) (typically, band broadening in time is first observed for the very sharp peaks). Apparently solvent evaporation took about 9 s, and some *n*-C_{10} left the column inlet about 1 s before heating of the column inlet.

Heating of the column inlet only 20 s after injection caused obvious distortion of the first two solute peaks. The beginning of the peaks corresponds to that in chromatogram (b). However, instead of waiting until the last solute material had left the inlet at low temperature, the remainder was rapidly desorbed by heating, forming sharp initial bands for the latter material.

From the fact that the *n*-C_{12} peak is almost as sharp as in reference chromatogram (a), it may be concluded that for certain applications heating delayed by as much as 11 s does not cause obvious peak broadening. It very much depends on the sharpness of a peak how critical the correct selection of the moment of heating is, because contributions to peak widths are added as squares[9], with the effect that peak broadening becomes small if the initial band widths are reduced to less than 50% of the chromatographic contribution to the peak width.

Chromatogram (e) will be discussed below.

Barrier against back-flow

The second concept of high oven temperature on-column injection is based on the assumption that a volume of sample liquid is injected, causing some liquid to leave the temporarily cooled inlet and to flow into the entrance of the oven-thermostated column. The sample liquid is rejected in the hot zone, flung backwards into the cooled zone (acting as a barrier against back-flow into the injector) and pushed into the entrance of the heated column again by the carrier gas, resulting in a rapid movement backwards and forwards (see Fig. 1).

Solvent evaporation in two steps. In a first step the sample solvent evaporates at the front of the flooded zone. The solvent vapour pressure stops the carrier gas flow into the column, and only solvent vapour passes through the column. There is no solvent trapping during this period of solvent evaporation because there is no condensed solvent ahead of the position where solute material is released from the evaporating solvent. As solvent evaporation is completed in the zone where the column temperature exceeds the boiling point of the solvent, solvent evaporation con-

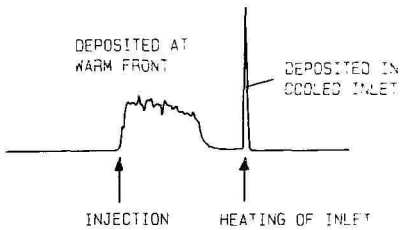

Fig. 4. Initial band shape of a solute injected with a volume of solvent causing some liquid to run into the entrance of the oven-thermostated column, represented as would be recorded by a detector positioned at the beginning of the oven-thermostated column. Some solute material is released from the solvent at the front (Fig. 1), producing an initial band width corresponding to the evaporation time of the solvent that flowed into the hot zone. After completion of evaporation at the front no solute material is released as there is solvent trapping during solvent evaporation in the cooled inlet. Solute material elutes as a sharp band from the cooled inlet provided that the inlet is heated exactly at the end of solvent evaporation. The initial band shape shown may be modified by a kind of cold trapping occurring within the temperature gradient from the cooled inlet to the oven-thermostated column (see Fig. 5).

tinues at the rear of the flooded zone; the carrier gas starts to flow again, carrying a proportion of solvent vapour through the column determined by the solvent vapour pressure within the cooled inlet. As solvent evaporation now proceeds from the rear to the front, there is solvent trapping of the volatile solutes, corresponding to the process taking place when working according to the first concept.

Fig. 4 shows schematically the typical initial band shape of a solute introduced by the second concept of high oven temperature on-column injection. First, there is an irregular release of solute material into the column during solvent evaporation at the entrance of the heated column. Irregularity stems from the fact that on one occasion solute material is carried far into the hot column whereas another time it is deposited more in the rear, cooler zone. The following solvent evaporation within the temporarily cooled inlet occurs below the boiling point of the solvent and therefore solute material is retained by solvent trapping. No solute material enters the oven-thermostated column during this period. Release of the solvent-trapped material corresponds to that described above; in Fig. 4 it is assumed that the inlet is heated at the end of solvent evaporation (otherwise further peak distortion according to that in Fig. 2 would be obtained).

Sharp initial bands? For the first concept there is a generally viable concept for obtaining sharp initial solute bands: heating of the column inlet at the moment solvent evaporation is completed. For the second concept there is no way that is as general and simple because there is no solvent trapping effect hindering solute material from leaking into the oven-thermostated column immediately after injection, *i.e.*, at a moment when heating of the column inlet is not permitted. So far, no sharp peaks can be expected in isothermal runs at the injection temperature when using the second concept.

Focusing within a temperature gradient. Using the movable on-column injector, perfectly shaped peaks of sufficient area were often obtained at high analysis temperatures despite injecting sample volumes that caused some liquid to run into the entrance of the oven-thermostated column. This suggests activity of an additional reconcentration mechanism.

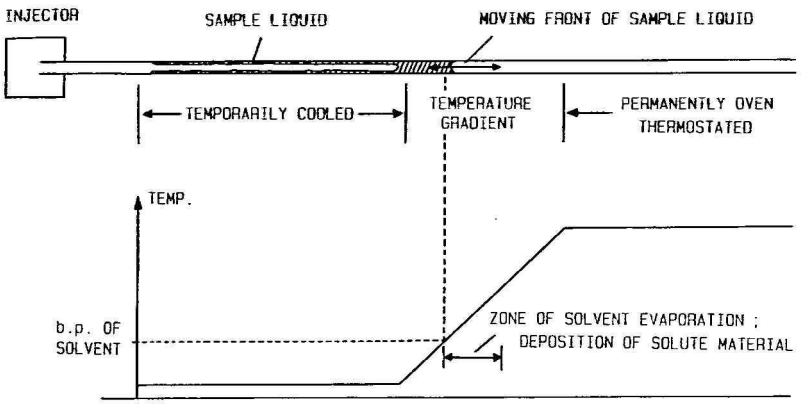

Fig. 5. Solute reconcentration due to cold trapping within the temperature gradient between the temporarily cooled column inlet and the permanently oven-thermostated column (for movable on-column injectors the column section passing through the roof of the GC oven). If solvent evaporation were a smooth process, it would occur at the position where the column temperature corresponds to the boiling point of the solvent. In reality, however, the sample liquid moves beyond this point (cooling of the position and delayed evaporation). Solute material is deposited within the range where solvent evaporation occurs. If the temperature within this range is sufficiently below the analysis temperature, the solute material is cold trapped and is released only on heating the inlet.

The temperature gradient from the cooled to the oven-thermostated column section of the injector used was relatively flat and corresponded to a column length of about 4 cm. If solvent evaporation were a smooth process, the solvent would evaporate at the beginning of the gradient at the point where the column is at a temperature corresponding to the boiling point of the solvent (see Fig. 5). The solute material is released from the solvent envelope and deposited on the column wall at the point of solvent evaporation, at a point that is far too cool to allow the solute material to migrate at a significant speed. It accumulates there and starts chromatography when the inlet is heated. In reality, the often violent solvent evaporation carries solute material considerably beyond the point where the column temperature corresponds to the boiling point of the solvent, *i.e.*, into a zone of higher temperature.

In order to achieve sufficiently efficient cold trapping of solute material, the temperature gradient must fulfil two requirements. First, there must be a sufficient difference between the boiling point of the solvent and the analysis temperature. Cold trapping requires the solute material to be placed at a position that is at least *ca.* 70°C below the analysis temperature. Second, the temperature gradient must be flat and long, accounting for the fact that the liquid moves too far into the hot column: the longer the gradient, the lower is the temperature of the position where the most advanced solute material is deposited. The distance the liquid covers beyond the position at the boiling point of the solvent certainly depends on the slope of the temperature gradient. However, other factors are less temperature dependent: the density of locations initiating solvent evaporation (acting as boiling stones), the stopping distance of a moving plug of liquid and the length of the capillary section necessary for transferring the heat required for solvent evaporation from the oven atmosphere to the liquid (solvent evaporation cools the column considerably).

Reconcentration within a temperature gradient improves with increasing analysis temperature and decreasing boiling point of the sample solvent. It is shown in Fig. 6 that our temperature gradient did not allow reconcentration of solutes eluted at 190°C when using *n*-hexane as solvent. On the other hand, reconcentration was just about complete during analyses of sterols involving an oven temperature of 250°C (*n*-hexane).

There is little chance of finding generally valid guidelines on the conditions that would provide complete reconcentration within a temperature gradient because the latter depends on too many factors (temperature gradient profile, volatility and nature of the solvent, carrier gas flow-rate, surface properties of the column inlet). It appears to be necessary to experiment with each particular instance, special care being required for detecting low pre-peaks, the area of which is usually not integrated. If, for a moment, some sample liquid enters very far into the hot column, some solute material is released into the oven-thermostated column and elutes before the bulk of the solute material. Up to about 20% of the solute material may be eluted prior to the major peak, marginally lifting the baseline, without being noticed. It is therefore recommended to compare peak areas with areas obtained by injecting the same sample volume under conventional conditions (column temperature below the boiling point of the solvent).

Example. Fig. 6 shows two chromatograms illustrating the difference between the initial bands produced by injection of a small sample volume, coating only the cooled inlet, and a larger volume introducing some liquid into the entrance of the oven-thermostated column. The injector was left in the up position for the duration of a run (10 min) in order to show leakage of solute material from the cold trap in the cooled column inlet. The sample contained solutes of intermediate volatility, C_{17}–C_{19} *n*-alkanes in *n*-hexane (of course, the volatility of the solutes strongly influ-

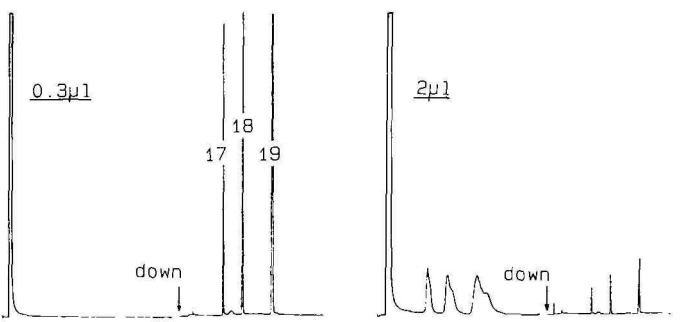

Fig. 6. In practice it is often observed that the injection of small sample volumes creates sharp peaks whereas the injection of larger volumes produces distorted peaks. If small sample volumes are injected, the whole sample liquid remains within the cooled inlet (first concept described in the text). Virtually no solute material leaves the column inlet even if the inlet is cooled longer than required by solvent evaporation, provided that the analysis temperature is above about 180°C (see the first part of the left-hand chromatogram, where the injector was left in the up position and the oven temperature was 190°C). Heating of the inlet ("down") releases sharp bands of solutes (second part of the chromatogram). Injecting larger sample volumes, the sample liquid carries solute material into the warm entrance of the oven-thermostated column from where the latter is immediately released (first part of right-hand chromatogram). Only the small proportion of solute material deposited in the cooled inlet is released on heating the inlet.

ences the outcome of such experiments!). The column was coated up to its entrance; the analysis temperature was 190°C.

Injecting a 0.3-μl volume, no solute peaks were observed as long as the injector was in the up position. Heating of the column inlet produced three perfectly shaped peaks corresponding to the solute material that remained in the cooled inlet after the first 10 min period. From the fact that no peaks or shoulders are eluted during the first part of the run with the inlet still being cooled it is tempting to conclude that no solute material was released during this period. However, integration of the peak areas revealed that about 50% of n-C_{17} is missing, indicating that half of the material had slowly left the cooled inlet before the latter was heated, raising the baseline to a hardly noticeable extent. Losses of n-C_{19} amounted to only 20%, owing to the lower chromatographic migration speed of n-C_{19} compared with n-C_{17}. Of course, one would not leave the column inlet cooled during 10 min after injection. However, the experiment serves as a warning that one cannot rely on the appearance of pre-peaks or shoulders for detecting whether solute material prematurely started chromatography. Disappearance of a peak area in the baseline may cause severe errors in quantitative analysis.

Injection of a 2-μl volume produced broad peaks of large area eluted during the period the column inlet was cooled. The corresponding material had been carried into the temperature gradient zone by the flowing liquid. It did not directly reach the oven-thermostated zone, as shown by the fact that the initial bands became strongly broadened by thermal defocusing. Heating of the inlet produced sharp peaks representing the solute material from the truly cooled inlet. They correspond to the peaks in the left chromatogram, but are smaller because the sample liquid was diluted, accounting for the larger injection volume.

In a realistic case the column inlet would have been heated about at the end of solvent evaporation. Evaporation of a 2-μl volume of n-hexane took about 25 s. The resulting peaks were distorted by broad (25 s) pre-peaks (similar to Fig. 4, although without a valley between the pre-peak and the main peak). Under the conditions used, isothermal analysis yielded sharp peaks only if the sample volume was restricted to about 0.8 μl.

COATED COLUMN INLET OR RETENTION GAP?

In conventional on-column injection (and also in splitless injection[10]), the use of an uncoated column inlet (retention gap) is advantageous for the reconcentration of bands broadened in space[11] (necessary only if sample volumes exceeding 1–2 μl or liquids not wetting the column surface are injected) and for reducing negative effects of sample by-products[12]. In high oven temperature on-column injection a new factor becomes important: the gas chromatographic retention power within the cooled inlet and the temperature gradient zone.

Retention gap at low analysis temperatures

Whether a coated or an uncoated column inlet is preferable depends on the analysis temperature used. In Fig. 3, where the analysis temperature was 90°C, the retention gap provided the best result [chromatogram (e)].

As a rule of thumb, the retention power within a retention gap corresponds to

that of a standard thin film column kept at a temperature about 100°C higher. This means that the retention power within the retention gap section cooled to ambient temperature was below that of the separation column kept at 90°C, and that the solutes left the cooled inlet at a speed exceeding that of their migration through the column. As a result, the initial bands were focused rather than thermally defocused as if using a coated column inlet [comparable chromatograms are (d) and (e)].

At low analysis temperatures the use of a retention gap simplifies high-temperature on-column injection as the moment of heating the column inlet becomes non-critical (in fact, the inlet does not need to be heated at any time!).

Continuing with the rule of thumb mentioned above, the retention power within the cooled retention gap corresponds to that of the oven-thermostated coated column at oven temperatures around 120–140°C. At lower analysis temperatures a retention gap provides some (unnecessary) focusing, whereas at higher analysis temperatures there is thermal defocusing of the solute bands. However, up to about 150°C such defocusing is negligible, and the use of a retention gap is clearly advantageous.

Coated inlets at elevated analysis temperatures

Solute material eluted at high analysis temperatures can be kept within the cooled column inlet, provided that the latter is coated. If there is sufficient retention power in the column inlet to prevent significant escape of solute material from the cooled inlet, the moment of heating is again not critical. As solute materials are

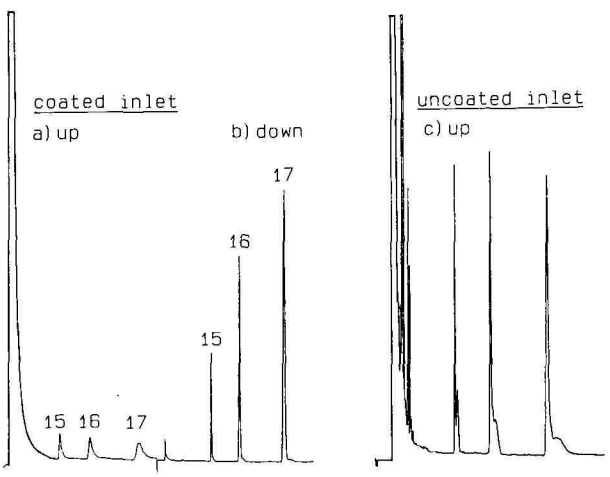

Fig. 7. Basically, high oven temperature on-column injection requires the column inlet to be heated exactly at the end of solvent evaporation. However, if sample volumes are injected that can be coated on the temporarily cooled inlet, it can mostly be arranged that delayed heating does not harm the peaks (which is convenient, as the accurate solvent evaporation time is unknown). Intermediate analysis temperatures (150–190°C) are an exception to this. On the one hand, a coated column inlet does not fully retain the solute material: in (a), where the injector was permanently in the up position, considerable amounts of solute material (C_{15}–C_{17} *n*-alkanes) escaped from the cooled inlet. On the other hand, solutes do not leave a retention gap sufficiently rapidly to form sharp initial bands (c). The injector was again in the up position; in this instance no peaks were detected after heating the inlet (chromatogram not shown). The analysis temperature was 170°C.

released only on heating the inlet, retention times must be measured from the moment of heating the inlet (if a retention gap is used with relatively low analysis temperatures, retention times must be measured from the moment of injection).

In Fig. 6 (left) an example is shown where the escape of solute material from the cool inlet was low enough to cause no significant losses in peak areas if heating of the inlet would have been delayed by, *e.g.*, 10 s. Conditions (analysis temperature 190°C), however, were near the border-line below which solute escape begins to be important.

Fig. 7 shows chromatograms obtained from isothermal runs at 170°C, where leakage from the cooled inlet was no longer negligible. The sample volume was 0.4 μl according to the concept of coating the cooled inlet. A first analysis was again run with the injector remaining in the up position to show elution of solute material from the cooled inlet. The coated column inlet first released a considerable amount of solute material, but retained other material up to the moment the inlet was heated. This is due to the fact that the front of the column inlet kept above the oven roof is considerably warmer than the rear, owing to heat from the oven. It should be noted that again most of the n-C_{15} material is not present in either the first or in the second peak; most of it lies on the baseline between the two peaks. Using a retention gap all solute material left the column inlet before heating (the second part of the analysis, a straight baseline, is not shown). Most of the material was released at the end of solvent evaporation (solvent trapping process) as a sharp band. Some material, presumably deposited in the cooler rear of the sample-coated inlet, entered the oven-thermostated column with a delay and is repesented by the peaks or shoulders eluted after the main peaks.

At analysis temperatures between 150 and 190°C there is no obvious answer as to whether a coated or an uncoated column inlet is preferable. On the one hand, the retention power of the retention gap cooled to ambient temperature is too high for rapid release of the solute material, and on the other, the retention power of the coated column inlet is insufficient to retain the material.

DETERMINATION OF SOLVENT EVAPORATION TIME

The solvent evaporation time is a key parameter in high oven temperature on-column injection, as in many instances the column inlet must be heated at the end of solvent evaporation. Unfortunately, it is impossible to give simple guidelines for the duration of solvent evaporation as the latter depends strongly on many parameters (carrier gas flow-rate, injection volume, volatility of the solvent, vapour volume created per unit volume of liquid). Therefore, the solvent evaporation time must be determined for each particular application.

The current method of heating the column inlet as the solvent starts to elute is certainly not suitable as there is no relationship between the appearance of the solvent peak (approximately corresponding to the dead time of the column) and the solvent evaporation time.

The solvent evaporation time can be roughly calculated from the width of the solvent peak; at high column temperatures the width of the solvent peak is primarily determined by the solvent evaporation time. Contributions from retention in the separation column (phase soaking phenomenon) are small. It is recommended that

pure solvent (of the type and volume of interest for the samples) be injected, leaving the injector permanently in the up position. If highly solvent-sensitive detectors (*e.g.*, flame ionization detectors) are used, the attenuation should be high in order to preclude tailing from contributing to the width of the solvent peak. The peak width is measured at the top.

Estimates of solvent evaporation times obtained by the above method tend to be slightly high. On the one hand, this introduces some safety margin, ruling out premature heating of the inlet. On the other hand, there may still be some distortion of rapidly eluted peaks (as, *e.g.*, in Fig. 3c), which would call for a small adjustment towards earlier heating.

SUMMARIZED GUIDELINES

From the above considerations, the following guidelines for the use of high oven temperature on-column injection can be derived.

Sample coating cooled inlet

(1) Determine the maximum sample volume from the length of the column inlet that is temporarily cooled below the boiling point of the solvent, measured from the injection point. As a rule of thumb, a 0.5-μl volume can be injected on to a 10-cm section of a coated inlet and a 0.4-μl volume on to a retention gap.

(2) Use a retention gap for isothermal and short-range temperature-programmed runs at column temperatures up to about 150°C and coated column inlets at higher temperatures.

(3) Inject by rapidly depressing the plunger (to separate the sample liquid well from the needle tip). The speed of other manipulations is not important.

(4) Heat the column inlet after completion of solvent evaporation. The solvent evaporation time is determined from the width of the solvent peak (see above).

(5) Check the quantitative results by comparison with results obtained using conventional on-column injection, *i.e.*, injecting at column temperatures below the boiling point of the solvent.

Injection of larger sample volumes

Here we refer to the technique of introducing more sample liquid than can be retained on the wall of the temporarily cooled inlet and which uses the cooled inlet as a barrier against a back-flow.

(1) There is no clear upper limit to the sample volume. If the temporarily cooled inlet is at least 10 cm long, up to *ca.* 3 μl of liquid can be safely kept within the inlet. A cooled inlet 13 cm long permits the injection of up to about 10 μl of liquids that wet the inlet surface.

(2) Injection of up to 3-μl volumes should occur rapidly; larger volumes must be injected more slowly (about 1 μl/s).

(3) The column inlet should be coated with immobilized stationary phase.

(4) Initial bands must be reconcentrated by cold trapping, which means that injection must be carried out at least about 70°C below the analysis temperature. At analysis temperatures above *ca.* 240°C this may be unnecessary if there is sufficient reconcentration within the temperature gradient between the cooled and the oven-thermostated parts of the column.

(5) Use high carrier gas (hydrogen) flow-rates and avoid solvents of high volatility such as *n*-pentane or diethyl ether (returning sample plug!).

(6) Check the quantitative results by injections at column temperatures well below the boiling point of the solvent, and check for memory effects due to solute material deposited behind the injection point.

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OPTIMIZATION OF THE PREPARATIVE SEPARATION OF HYDROXYBIPHENYL ISOMERS

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SUMMARY

The separation of hydroxybiphenyl isomers was carried out on silica gel. The choice of the separation conditions was difficult owing to the low solubility of the *para* isomer. Three kinds of the mobile phase were employed, their elution strengths being selected in such a manner that the selectivity factor varied in the range *ca.* 2.6–7.7. The rate of broadening of a band was investigated as a function of mass overloading and solubility of the sample in the mobile phase for similar k' values. The optimal column loadability was established for 99.0% purity, taking into account the column throughput and the volume of the fractions. One operation in a 300 × 50 mm I.D. preparative column yielded 1.25 and 0.63 g of pure *para* and *ortho* isomers, respectively.

INTRODUCTION

Mixtures of *ortho*, *meta* and *para* isomers are well separated on silica gel. The selection of appropriate conditions for preparative separations involves choosing a gel of large specific surface area and a mobile phase that ensures maximum throughput. If the separation is aimed at obtaining small amounts of substances, optimization with respect to the particle size of the packing and the column dimensions is of less significance.

The effect of the selectivity factor, α, and capacity factor, k', on the throughput of the process is already generally known. Among numerous studies, the most useful seem to be those of Gareil and co-workers[1–3], who gave the equation

$$C_v = C_m \exp\left(-\frac{v - V_0 - V_m}{\tau}\right) \quad (1)$$

where C_v is the concentration of the sample for an elution volume v, V_0 is the injection volume, V_m is the column hold-up volume and C_m and τ are model parameters. A limitation to the applicability of this equation to the selection of separation conditions is the poor solubility of the separated compounds in the mobile phase. A so-called linear optimization, *i.e.*, controlling the process throughput through a change

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in sample volume, is recommended in this instance[3]. However, if a mixture is complex and two or more components are isolated, an increase in sample mass through an increase in its volume is rarely possible. There is a possibility of increasing the sample mass without changing its volume by injection of the sample in a solvent stronger than the mobile phase, in which the samples are readily soluble. Such a procedure can result in precipitation of the sample components of poor solubility at the top of the packing. When the precipitation and progressive dissolution occur in the mobile phase, the concentrations of the sample in both phases can be constant, depending on the solubility. A section of approximately constant concentration can also appear in the outlet profile and the descent of the peak to the baseline can be delayed with respect to the analytical peak. At the same time, the front of the peak can be shifted towards the start if the concentration in the mobile phase exceeds the linear range of the isotherm. Hydrodynamic effects[4] can accompany precipitation of the sample at the top of a column.

Injection of a sample in a solvent stronger than the mobile phase need not result in precipitation of the sample, but conditions prevailing in the initial section of a column until the stronger solvent leaves this section are difficult to define. It can be anticipated that the band will be broadened owing to acceleration of migration of its frontal part caused by the stronger solvent leaving the band. The change in k' in the band will be accompanied by the change in the degree of overloading. The degree of band spreading resulting from these phenomena will depend on the volume of solvent introduced with the sample, its elution strength and the capacity factor, k', of the substance. In some papers peak distortion as result in injection of the sample in a solvent stronger than the mobile phase has been mentioned[5,6].

EXPERIMENTAL

Adsorbents

Silica gel, $d_p = 17$ μm, obtained by repeated sedimentation of H 60 gel, (Macherey, Nagel & Co., F.R.G.) was used.

Solvents

n-Hexane (analytically pure) (Reachim, U.S.S.R.), dioxane, isopropanol and methylene chloride (analytically pure) (POCH, Poland) were used as solvents.

Apparatus

The apparatus employed for preparative-scale liquid chromatography was equipped with a pump of output up to 270 ml/min and a UV-254 detector with a measuring vessel of capacity 10 mm[3]. The preparative chromatograph was laboratory constructed. Sampling of the substances was performed by means of a valve with a loop. Columns of dimensions 300 × 3.3 mm I. D. and 300 × 50 mm I.D. were used. Analytical columns were used with a KB 5113 chromatograph (Kabid, Poland).

Mixture to be separated

The main components of the post-reaction mixture were *ortho* and *para* isomers of hydroxybiphenyls.

Hydroxybiphenyls are characterized by high molar absorptivities at $\lambda = 254$

nm. An increase in the concentration of the substances in the eluent resulted in the linear dynamic range of the detector being rapidly exceeded. For this reason, the retention volume was determined for the peak front instead of at its maximum. In order to determine the concentration distribution in the band, fractions were collected and the concentration of the component was assayed chromatographically in the system silica gel/dioxane–hexane (1:9, v/v).

RESULTS AND DISCUSSION

The purpose of this work was to obtain standards of *ortho* and *para* isomers of hydroxybiphenyls from a raw mixture. A post-reaction mixture was separated on silica gel using various binary mobile phases prepared by mixing hexane and a polar solvent. It was established that the isomers were well separated using various solvent mixtures. They were also well separated from other compounds present in small amounts.

The separation of a post-reaction mixture on an analytical scale is shown on the Fig. 1. Owing to the type of detector used, three polar solvents were selected for mixing with hexane in further work: methylene chloride, dioxane and isopropanol. Values of k' and α are listed in Table I and the solubilities of the *para* isomer in the various solvents are given in Table II. It can be seen that the solubility of the *para* isomer is low, and there is therefore a problem with the preparation and injection of the sample. (The solubility of the *ortho* isomer is good; *e.g.*, in 1:99, v/v, dioxane–hexane it is 17 mg/ml). In the separation of hydroxybiphenyls, the injection of samples of large volume and low concentration would be disadvantageous in the presence of impurities with k' values slightly lower than that of the *ortho* isomer.

Fig. 2 presents the "peaks" obtained after introduction into an analytical col-

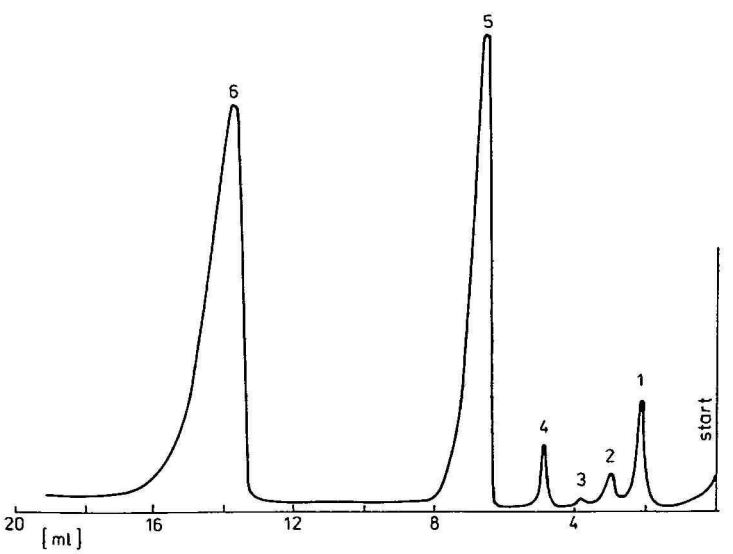

Fig. 1. Separation of hydroxybiphenyls on an analytical scale. Column, 300 × 3.3 mm I.D.; stationary phase, silica gel (d_p = 17 μm); mobile phase, dioxane–hexane (5:95, v/v). Peaks: 1–4 = unknown substances; 5 = *ortho* isomer; 6 = *para* isomer.

TABLE I

COMPARISON OF THE EFFECTS OF DIFFERENT MOBILE PHASES ON CHROMATOGRAPHIC PARAMETERS

Sample, mixture of *ortho* and *para* isomers of hydroxybiphenyls; column, 300 × 3.3 mm I.D., silica gel (d_p = 17 μm).

Composition mobile phase (v/v)	*Range of capacity factor, k'*	*Range of selectivity factor, α*
Methylene chloride–hexane (15:85–45:55)	56.6–8.7	6.9–5.6
Dioxane–hexane (1:99–7:93)	26.6–4.0	4.9–2.6
Isopropanol–hexane (0.25–99.75–1:99)	13.3–2.6	7.7–3.3

umn of 4.0 mg of the *para* isomer in 0.2 ml of the methylene chloride–dioxane–hexane (7:1:2, v/v), and in 3.0 ml of the mobile phase (*i.e.*, after diluting 0.2 ml of the sample to 3.0 ml). The elution profiles obtained with different injection volumes are very similar. The shift of the front of the peak indicates mass overloading and the lack of a distinct maximum is probably the result of poor solubility.

The concentration of a substance in the mobile phase can be calculated for a given k' value assuming that the sample at the top of the packing occupies a space corresponding to $\sqrt{N}$ plates, *i.e.*, the limiting value above which overloading begins to affect the shape of the outlet profile[7]. For example, in dioxane–hexane (1:99, v/v) mobile phase, k'_{para} = 26, with a mass of substance of 4.0 mg and $\sqrt{N}$ = 32, a concentration in the mobile phase of 2.1 mg/ml is obtained, whereas solubility is 0.89 mg/ml and the linear range of the isotherm is 0.64 mg/ml ($3.8 \cdot 10^{-3}$ *M*). These data indicate that explanation of the concentration distribution in the band described above is probably valid. A similar concentration distribution in the band was observed in hexane–isopropanol as the mobile phase.

TABLE II

SOLUBILITY OF *para* ISOMER IN DIFFERENT MOBILE PHASES

Mobile phase	*Composition (v/v)*	*Solubility (mg/ml)*	*k'*
Methylene chloride–hexane	15:85	1.22	56.6
	25:75	2.42	
	40:60	5.02	9.5
Dioxane–hexane	1:99	0.89	26.6
	2.5:97.5	2.02	
	7:93	6.15	4.0
Isopropanol–hexane	0.25:99.75	0.46	13.3
	0.5:99.5	0.91	
	1:99	1.75	2.6

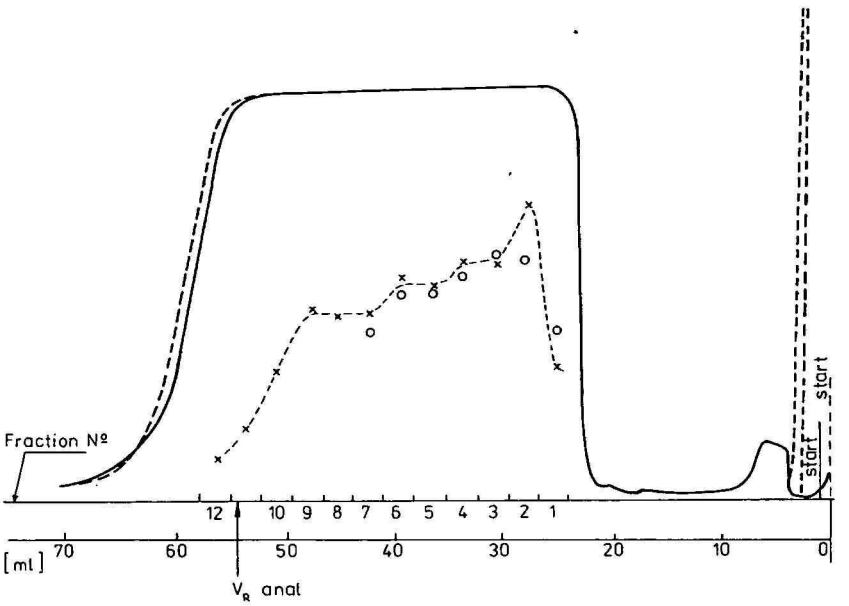

Fig. 2. Elution curves of *para* isomer. Mobile phase, dioxane–hexane (1:99, v/v). Broken line and (×), sample volume 0.2 ml; solid line and (○), sample volume 4.0 ml; sample mass, 4 mg; column as in Fig. 1.

Higher retention volumes (relative to the analytical peak) of the descending part of the peak with increasing mass of the samples, without a change in the sample volume, were observed for the system containing isopropanol. The results shown in

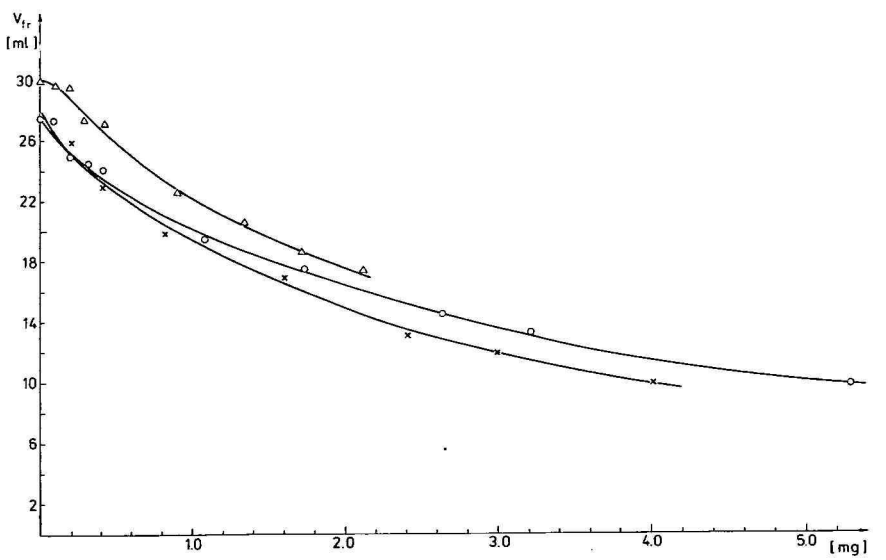

Fig. 3. Shift of the peak front *vs.* sample mass of *para* isomer. Mobile phases: △, isopropanol–hexane (0.25:99.75, v/v); ○, dioxane–hexane (2.5:97.5, v/v); ×, methylene chloride–hexane (36:64, v/v). Column as in Fig. 1.

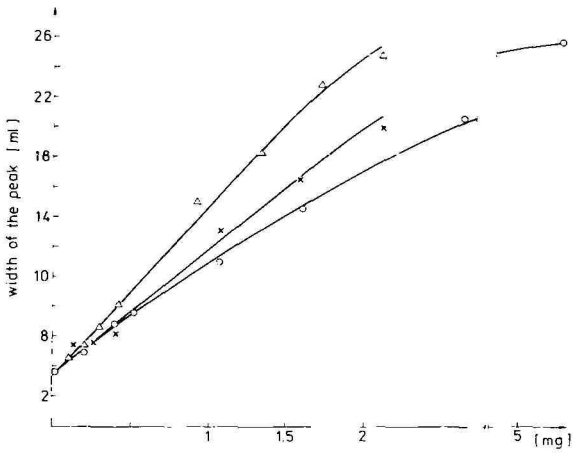

Fig. 4. Width of the peak front *vs.* sample mass of *para* isomer. Conditions and points as in Fig. 3; column as in Fig. 1.

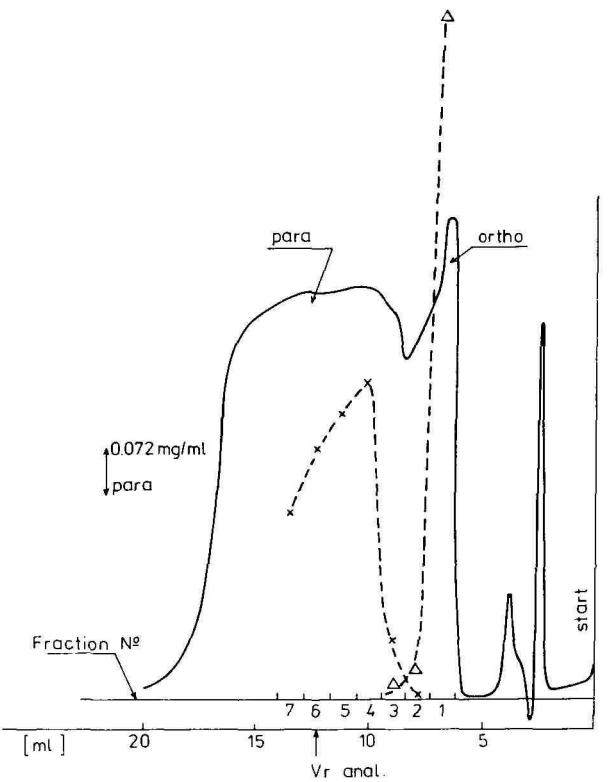

Fig. 5. Elution curves of *ortho* and *para* isomers. Mobile phase, hexane–dioxane (93:7, v/v); sample mass, 2.4 mg; sample volume, 0.2 ml; column as in Fig. 1.

Figs. 3 and 4 confirm this fact. Despite the higher rate of shift of the front of the peak in the mobile phase containing methylene chloride, its total width is still smaller than in 0.25% isopropanol, in which the peak tail was shifted by 30% with respect to the analytical peak. A shift of the back of the peak is also observed in some mobile phases containing dioxane, although to a smaller extent.

In systems containing methylene chloride or dioxane, peaks with a shape similar to a rectangular triangle were observed at higher concentrations of these solvents (Figs. 5 and 6). This can be explained by the fact that an increase in the content of the polar component in the mobile phase is accompanied by increases both in the concentration of the substance in the mobile phase (k' decreases) and in solubility. For the three polar solvents used the solubility increases most rapidly in the mobile phase containing methylene chloride. The solubility no longer influences the peak shape. When the retention of the compounds is moderate ($k'_{para} \approx 5$), for the same k' values the positions of the band fronts of both isomers are very similar. In the presence of the *para* isomer, with an increase in its amount the retention volume of the *ortho* isomer (and all other impurities except for the peak eluted with the solvent front) decreases owing to its displacement from the silica gel bed by the considerably more strongly sorbed *para* isomer. An example of the change in the retention of the *ortho* isomer is shown in Table III. The change increases with increasing retention of both isomers. In addition to the decrease in retention volume, peak narrowing approximately proportional to the decrease in retention volume is also observed.

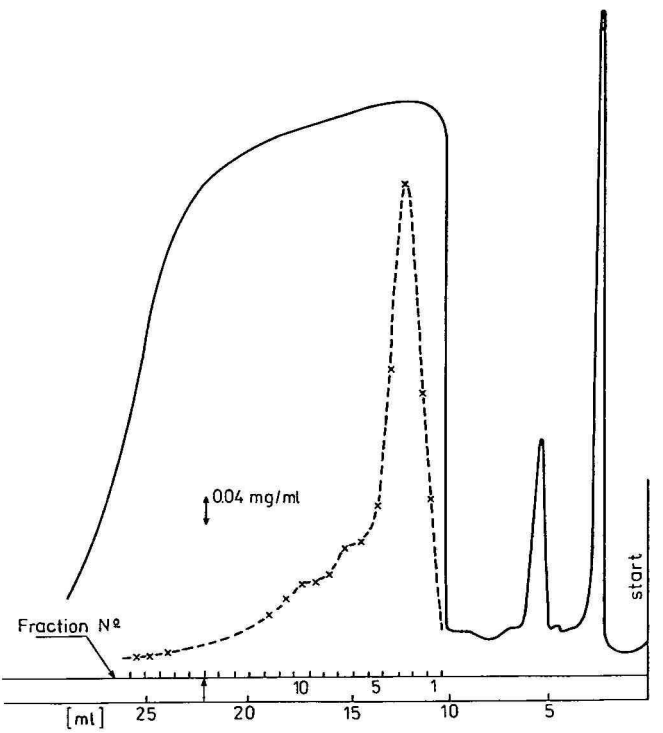

Fig. 6. Elution curve of *para* isomer. Mobile phase, methylene chloride–hexane (3:7, v/v); sample volume, 0.3 ml; sample mass, 3.0 mg; column as in Fig. 1.

TABLE III

RETENTION VOLUME OF *ortho* ISOMER ACCOMPANIED BY *para* ISOMER

Amount of para isomer in sample (mg)	*Retention volume of ortho isomer*								
	*D–H**				*C–H***		*P–H****		
	1	*2*	*3*	*4*	*1*	*2*	*1*	*2*	*3*
0.0	13.4	10.0	6.2	5.5	8.5	5.6	6.0	4.8	3.7
2.0	12.6	9.0	6.1	5.2	7.7	5.3	4.7	3.6	3.3
4.0	11.2	8.7	–	–	7.1	4.8	–	–	–
5.3	–	8.4	5.8	5.1	–	–	–	–	–

* D–H = dioxane–hexane: (1) 1:99; (2) 2.5:97.5; (3) 5:95; (4) 7:93.
** C–H = methylene chloride–hexane: (1) 30:70; (2) 45:55.
*** P–H = isopropanol–hexane: (1) 0.25:99.75; (2) 0.5:99.5; (3) 1:99.

Fig. 7 illustrates the dependence of the position of the peak front and the peak width for the *ortho* isomer on the mass of the *para* isomer in the sample in one of the mobile phases.

In order to facilitate extrapolation of the results, the dependence of the elution volume of the peak front was plotted against the logarithm of substance concentration in the sample (Fig. 8). Linear plots were obtained in the concentration range from 0.8 mg/ml ($4.7 \cdot 10^{-3}$ *M*) to 20 mg/ml (0.12 *M*) for all the investigated systems. Evidently, the slope and intercept of the straight lines no longer corresponded to eqn. 1 owing both to replacement of the retention volume of the peak maximum by the elution volume of the peak front and to the processes described earlier.

Table IV lists the throughputs of the *para* isomer determined experimentally in non-linear chromatography and those calculated for linear optimization[3]. The throughput was calculated according to $M = m_s/m_a V_t$, where m_s is the mass of the substance, m_a is the mass of the adsorbent and V_t is the total elution volume. As the

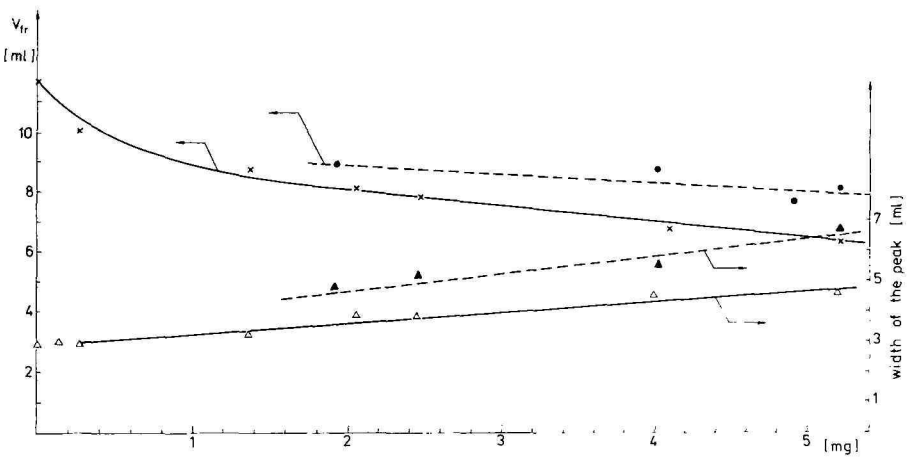

Fig. 7. Shift of the peak front and the peak width *vs.* mass of *ortho* isomer: ×, △, accompanied by *para* isomer; the masses of *ortho* and *para* isomers are equal, ●, ▲, without *para* isomer. Mobile phase, methylene chloride–hexane (25:75, v/v); column as in Fig. 1.

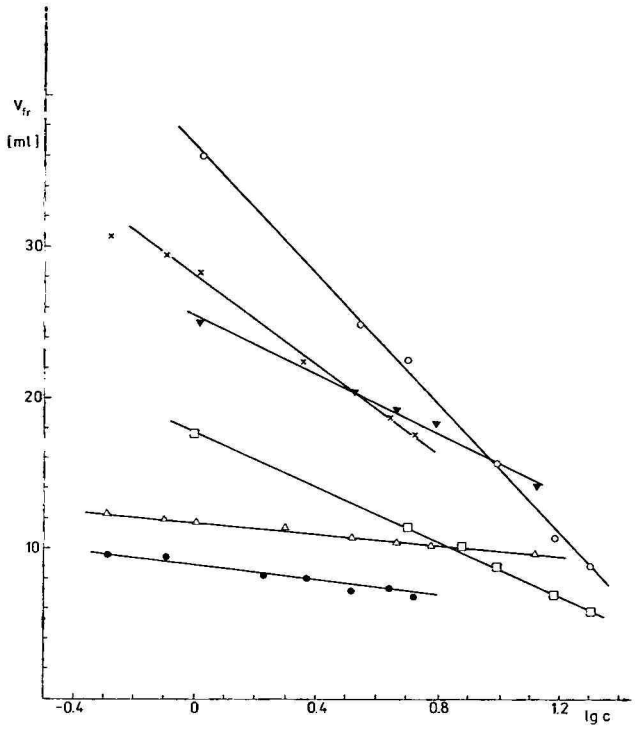

Fig. 8. Peak front elution volume *vs.* logarithm of sample concentration for different mobile phases: ●, dioxane–hexane (1:99, v/v); △, dioxane–hexane (5:99, v/v); □, methylene chloride–hexane (40:60, v/v); ▼, dioxane–hexane (2.5:97.5, v/v); ×, isopropanol–hexane (0.25:99.75, v/v); ○, methylene chloride–hexane (30:70, v/v). Column as in Fig. 1.

overload limit, a mass of the *para* isomer was selected for which the front of its peak intersected at the baseline the tangent to the descending part of the peak of the *ortho* isomer, determined for the analytial sample.

It follows from the experimental data that in chromatography of hydroxybiphenyls the throughput of the process depends to a greater extent on the value of k' than on the selectivity factor α.

In all the investigated systems a higher yield of the *para* isomer was obtained in linear chromatography. Unfortunately, in order to isolate simultaneously both isomers the sample volume, *e.g.*, in the dioxane–hexane (5:95, v/v) system, cannot exceed 1.0 ml and the output of linear chromatography decreases to 0.05 mg/ml · g. The sample can be separated in two steps, *i.e.*, isolation of the *para* isomer is followed by concentration of the sample and isolation of the *ortho* isomer. The total yield of the process is then 0.24 mg/ml · g (the time required for sample pre-concentration was not taken into account).

Tests of the described solvent compositions resulted in selection of dioxane–hexane (5:95, v/v) as the optimum mobile phase. The sample injected into an analytical column can contain at most 6.0 and 3.0 mg of *para* and *ortho* isomers, respectively, whereas the respective values for the sample injected into a preparative

TABLE IV

COMPARISON OF THROUGHPUTS OF *para* ISOMER OBTAINED WITH DIFFERENT MOBILE PHASES

M_1 is the throughput in linear chromatography, M_2 is the throughput in non-linear chromatography.

Mobile phase	*Composition (v/v)*	k'_{para}	α	M_1 *(mg/ml · g)*	M_2 *(mg/ml · g)*
Dioxane–hexane	1:99	26.6	4.9	0.13	0.05
	2.5:97.5	12.5	3.4	0.26	0.11
	5:95	5.4	2.9	0.30	0.25
	7:93	4.0	2.6	0.27	0.13
Methylene chloride–hexane	30:70	18.5	6.6	0.24	0.08
	40:60	9.5	5.5	0.32	0.12
Isopropanol–hexane	0.25:99.75	16.2	7.7	0.26	0.06
	0.5:99.5	6.0	5.0	0.35	0.14
	1:99	2.6	3.3	0.29	0.12

column are 1.3 and 0.7 g, respectively. One operation in the preparative column yielded 1.25 and 0.63 g of pure *para* and *ortho* isomers, respectively.

CONCLUSIONS

The preparation of a solution of a sample mixture in a solvent stronger than the mobile phase and injection of small sample volumes is possible and overcomes problems associated with low solubility of the sample components. If the separation of *ortho* and *para* isomers was satisfactory ($\alpha > 2$), decreasing the elution strength of the mobile phase, leading to an increase in k' of the *para* isomer to *ca.* 10, resulted in a substantial decrease of the throughput.

The best throughput was obtained in dioxane–hexane (5:95, v/v) at $k'_{para} = 5.4$ and $\alpha = 2.9$. As a displacement effect occurs, selection of the separation conditions should be carried out using a mixture and not the individual components.

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IMPROVED HYDROCARBON GROUP RESOLUTION OF OLEFINIC GASOLINES BY ADSORPTION AND CHARGE-TRANSFER HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Two rapid high-performance liquid chromatographic techniques (< 15 min) for separating light and heavy gasolines into saturated, olefinic and aromatic compounds are described. The first one involves adsorption chromatography, and the choice of stationary phase (silica gels of various specific surface areas) and mobile phase (nature, water content) is discussed in terms of chromatographic performance and the characteristic of the petroleum product (boiling point, solubility, etc.). The second method is based on charge-transfer chromatography with silver-coated silica gel or silver chemically bonded to silica gel and *n*-pentane as mobile phase. The water content is not so critical as in adsorption chromatography. Both methods offer a significant improvement in resolution, especially between saturated and olefinic compounds.

INTRODUCTION

The structural group-type analysis of petroleum products into saturated, olefinic and aromatic hydrocarbons is of great importance for the evaluation of feedstocks and refinery process control. It has been extensively studied and was briefly reviewed by Miller *et al.*[1]. The high-performance liquid chromatography (HPLC) method described by Suatoni and co-workers[2–5] is going to be adopted by the American Society for Testing and Materials and is soon to replace the fluorescent indicator adsorption method[2,7], involving displacement, gravity-flow chromatography and measurement of zone lengths stained by a fluorescent dye. Suatoni's method uses activated small particle silica gel, a perfluoroalkane as mobile phase, refractive index (RI) detection and column backflushing to elute the aromatic compounds as a single peak. Results obtained with this method have been reported by Miller *et al.*[1] and Colin and Vion[6].

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However, several difficulties remain with regard to the accuracy, especially at the often low olefin contents. These difficulties stem from the fact that the refractometric responses differ greatly within the same structural group, and also from a difficult evaluation of the baseline. This in turn may be due to backflushing, partial resolution of compounds belonging to the same group, or conversely, incomplete separation between two groups, *e.g.*, saturated compounds and olefins.

To avoid some of these drawbacks, more complicated alternative methods have recently been proposed, such as multicolumn liquid chromatography with IR detection[7] or the use of HPLC together with flame ionization detection gas chromatography[8,9]. Another trend is to make easier the location of the baseline, so that peak integration should be more accurate. In this content, we now reported on a means of improving the resolution given by adsorption HPLC between the saturated and olefinic hydrocarbon groups in gasoline samples, while keeping the peak for each group as narrow as possible. For this purpose, related parameters such as the specific surface area of the stationary phase, the mobile phase composition and water content, about which surprisingly little information is available, should be optimized. We also investigated charge-transfer HPLC using silver-modified silica, which seems to be a promising alternative.

EXPERIMENTAL

Apparatus

Experiments were performed with a liquid chromatograph assembled from a Model 302 Gilson pump (Gilson, Villiers-le-Bel, France), a 7413 Rheodyne six-port sampling valve with a 1-μl loop and two detectors: a SF 769 UV spectrophotometer (Kratos, Westwood, NJ, U.S.A.) and a R 401 Waters differential refractometer (Waters Assoc., Milford, MA, U.S.A.). Reverse flow (backflushing) was achieved with a 7010 Rheodyne valve. Ambient temperature was used throughout.

Gas chromatography was performed with a chromatograph equipped with a flame ionization detector and a Perkin-Elmer capillary column (100 m $\times$ 0.3 mm I.D.) coated with squalane. The operating conditions were: injection temperature, 250°C; detector temperature, 250°C; column temperature, linear gradient from -5 to 110°C at 1.5°C min^{-1}.

Five porous silica gels were used: LiChrosorb Si 60, 7 μm (specific surface area 550 $m^2\ g^{-1}$) from Merck (Darmstasdt, F.R.G.); Rosil, 5 μm (400 $m^2\ g^{-1}$) from Alltech, (Eke, Belgium); Nucleosil 50, 5 μm (500 $m^2\ g^{-1}$) from Macherey-Nagel (Düren, F.R.G.); a Brownlee Si 100 silica gel cartridge (300 $m^2\ g^{-1}$) from Brownlee Labs. (Santa-Clara, CA, U.S.A.) and an experimental Spherosil, 5 μm (800 $m^2\ g^{-1}$) from Rhône-Poulenc Recherches (Vitry-sur-Seine, France).

Silver(I)-modified silica gel columns were prepared by two methods, using LiChrosorb Si 60 as the base support.

In situ coating method. A 15 cm $\times$ 0.48 cm I.D. stainless-steel column was packed with LiChrosorb Si 60 by the conventional slurry technique. A 15-ml volume of a 3% (w/w) (0.138 *M*) solution of silver nitrate in acetonitrile was then percolated through the column at a flow-rate of 1 ml min^{-1}. The column was washed with 5 ml of isopropanol and finally equilibrated with 50 ml of pentane.

In situ chemical reaction. A 25 cm $\times$ 0.48 cm I.D. stainless-steel column packed

with LiChrosorb Si 60 was equilibrated with pure water. A 3% (w/w) (0.176 *M*) solution of silver nitrate in 0.5 *M* aqueous ammonia was pumped at a flow-rate of 1 ml min^{-1}. The reversal of flow was effected by rotating the backflushing valve after 18 ml of solution had percolated through the column so that a total volume of 36 ml was passed.

Then, the column was washed successively with 15 ml of water, 10 ml of isopropanol and 300 ml of chloroform. Finally, it was dried under a stream of nitrogen at 160°C for 8 h in order to remove ammonia. After this treatment, the column was equilibrated with 50 ml of pentane at a flow-rate of 1 ml min^{-1}.

Chemicals

Pentane and heptane were of Chromasol grade and purchased from SDS (Peypin, France). Fluorinert FC 72 is a perfluorinated alkane obtained from 3 M Company (St. Paul, MN, U.S.A.). 1,1,2-Trichloro-1,2,2-trifluoroethane (FC 113), spectrometric grade, was from Aldrich (Milwaukee, WI, U.S.A.). The solvents were used as received or dried over 4-Å molecular sieves. Finally their water contents were adjusted to the chosen value.

Test solutes and classical reagents were of analytical grade and purchased from well known suppliers.

For qualitative analysis, two gasoline samples from fluid catalytic cracking (FCC) were obtained from Elf (Solaize, France): the lighter one (b.p. < 220°C) contained no saturated and olefinic hydrocarbons with more than eight or nine carbon atoms; the heavier one (b.p. < 280°C) contained these hydrocarbons with up to fourteen carbon atoms. For quantitative analysis, three samples were tested: a light FCC gasoline (b.p. < 220°C), a reforming feed which does not contain light hydrocarbons and a so-called commercial gasoline containing very small quantities of olefins (b.p. ≈ 200°C).

Water content of mobile phase

The water contents of the solvents were measured coulometrically by the Karl Fischer titration method (Automat Bizot et Constant, Prolabo, Paris, France)[10]. The accuracy was about 10% for water contents of 2–10 ppm and 5% for those greater than 10 ppm.

Dead volume determination

The dead volume was measured using pentane as solute and methylene chloride as mobile phase.

RESULTS AND DISCUSSION

Liquid–solid chromatography (LSC)

Qualitative analysis

It is well known in LSC that the retention and consequently the selectivity depend, among other factors, on the specific surface area of the adsorbent, the polarity and water content of the mobile phase. Surprisingly, the specific surface area and water content were not specified in recent HPLC reports on this topic[1–7]. Like-

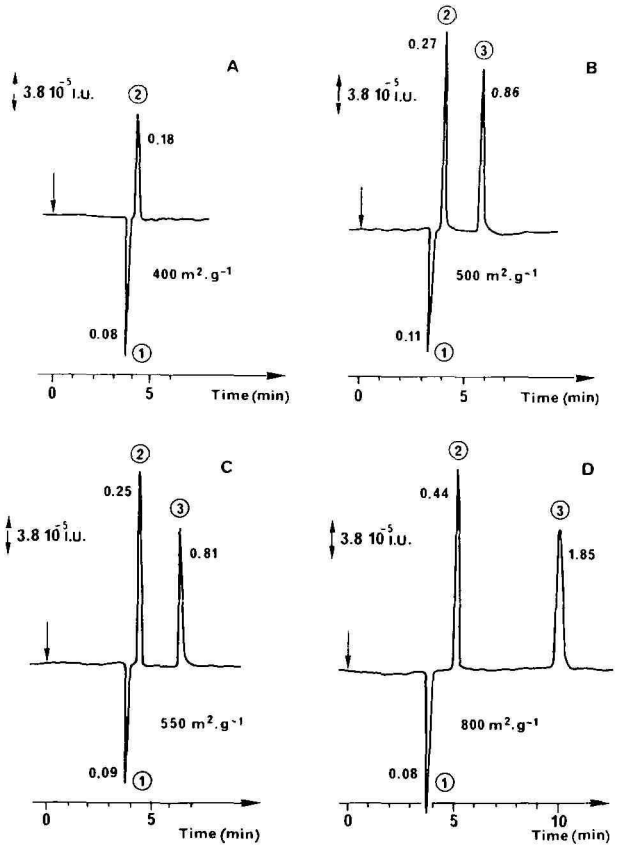

Fig. 1. Influence of the specific surface area of various silica gels on a test mixture separation. Column: 25 × 0.48 cm I.D. Stationary phases: (A) Rosil (400 $m^2\ g^{-1}$); (B) Nucleosil 50 (500 $m^2\ g^{-1}$); (C) Li-Chrosorb Si 60 (550 $m^2\ g^{-1}$); (D) experimental Spherosil (800 $m^2\ g^{-1}$). Mobile phase: heptane containing 7 ppm of water. Flow-rate: 1 ml min^{-1}. Detection: differential refractometer (Waters R 401). Solutes: 1 = pentane; 2 = cyclooctene; 3 = toluene (the k' values are given near each elution peak).

wise, the effect of silica activation on the separation between saturated compounds and olefins was described without mentioning these factors[11]. Moreover, while the advantage of perfluoroalkanes over *n*-alkanes as regards the refractometric sensitivity is clearly apparent, the choice of mobile phase with regard to a mere group-type selectivity seems less obvious: some authors have used fluoroalkanes[1,2,5,6,8], others hexane[3,4,9,11].

Effect of the specific surface area. The influence of the specific surface area of silica gel on the separations of a test mixture containing an alkane (pentane), an alkene (cyclooctene) and an aromatic compound (toluene) and of light gasoline is shown in Figs. 1 and 2, respectively. As previously demonstrated[12], the retention and selectivity increase with increasing specific surface area. Fig. 1 also shows that the efficiency remains independent of the specific surface area and lies in the range 5000–8000 theoretical plates. So, it seemed interesting to work with a silica gel having a specific surface area of over 500 $m^2\ g^{-1}$. The resolution between saturated and

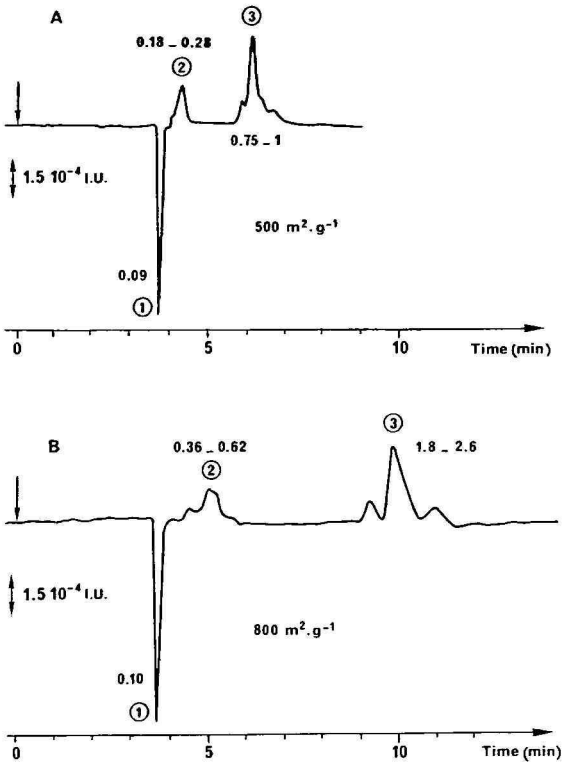

Fig. 2. Influence of the specific surface area of silica gels on a light gasoline separation. Stationary phases: (A) LiChrosorb Si 60 (550 $m^2\ g^{-1}$); (B) experimental Spherosil (800 $m^2\ g^{-1}$). Sample: light FCC gasoline. Peaks: 1 = saturated; 2 = olefinic; 3 = aromatic hydrocarbons. Other operating conditions as in Fig. 1.

olefinic compounds can be considered satisfactory on LiChrosorb Si 60 and is even better on Spherosil. However, owing to the increase in selectivity with increasing specific surface area, a partial resolution within the olefinic and aromatic compounds is observed in Fig. 2B, while the peak due to the saturated compounds became larger. This too high selectivity can constitute a major drawback for the quantitative analysis of the olefinic group because it is much easier to integrate a well defined peak than several small ones.

As regards the aromatic compounds, a backflushing technique is used in the final analytical procedure after the elution of the olefin peak and it is very easy to choose the backflushing time because a large resolution is obtained between the olefin and aromatic groups.

Thus, for the subsequent studies we selected LiChrosorb Si 60 and experimental Spherosil in order to optimize their chromatographic properties in terms of the nature of the mobile phase nature and its water content.

Effect of the nature of the mobile phase. A good separation between the saturated and olefinic groups requires a mobile phase with a very low elution strength: only alkanes and some of their fluorinated derivatives are suitable. We studied four solvents whose properties are given in Table I.

Heptane is often used as a mobile phase in LSC for heavy petroleum products

TABLE I

PROPERTIES OF SOME MOBILE PHASES FOR SEPARATION BETWEEN OLEFINIC AND SATURATED COMPOUNDS

	Pentane	*Heptane*	*FC 113*	*FC 72*
Boiling point (°C)	36.2	98	47.6	56
Viscosity at 20°C (cP)	0.23	0.41	0.71	0.39
Density at 25°C (g ml^{-1})	0.629	0.684	1.565	1.68
Refractive index at 20°C	1.3580	1.3875	1.3578	1.251
UV cut-off (nm)	195	195	231	210
Solubility of water in the solvent (ppm)	100	100	90	–
Solvent strength value on silica, ε°	0.00	0.01	0.02	≈ -0.2

because asphaltenes are generally precipitated by it. As shown in Fig. 2, its low elution strength gives an acceptable resolution of saturated compounds and olefins. However, its use is not advisable with RI detection and gasoline samples for two reasons. First, the peak due to the saturated compounds would be negative, which renders its integration more difficult; secondly, the detection sensitivity would be low since the RI of heptane is too similar to that of the major constituents of the saturated group. Naturally heptane itself is not detected at all, which alters the quantitative result. These drawbacks are partially avoided with pentane, for its RI is the lowest of the alkanes liquid at ambient temperature, and the pentane content in gasolines is low, variable, and thus of little significance. The resolving power of pentane is even a little better than that of heptane, due to its slightly lower elution strength. Fig. 3A

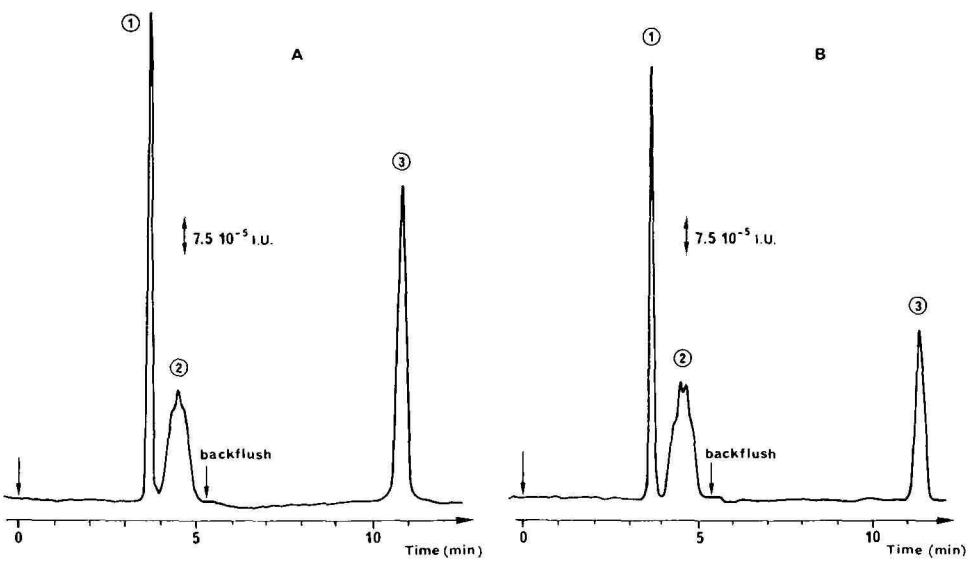

Fig. 3. Separation of light (A) and heavy (B) FCC gasoline samples by LSC with pentane (8 ppm water) as mobile phase. Stationary phase: LiChrosorb Si 60. Other operating conditions as in Fig. 1. Peak identification as in Fig. 2.

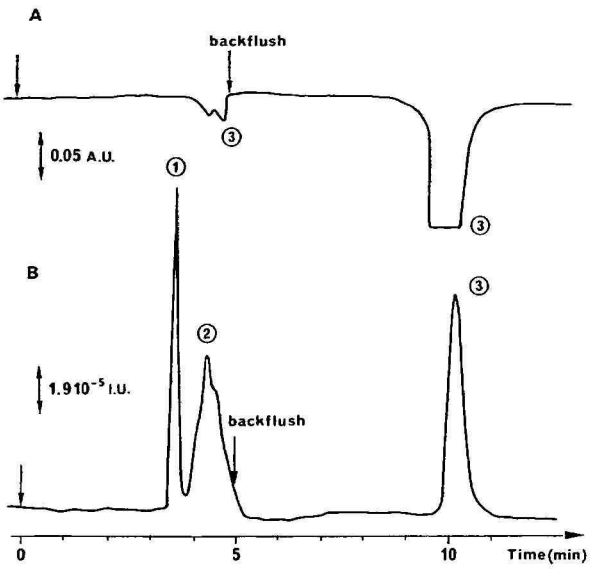

Fig. 4. Separation of a light FCC gasoline sample by LSC with FC 113 (6 ppm water) as mobile phase. Stationary phase: LiChrosorb Si 60. Other operating conditions as in Fig. 1, except detection: (A) UV at 254 nm; (B) differential refractometer. Peak identification as in Fig. 2.

and B show the comparable separations obtained for light and heavy FCC gasoline samples. The high volatility of pentane precludes working at >25°C but this does not constitute too serious a disadvantage if use is made of a tightly stated mobile phase circuit. Moreover, it has been reported[11] that the resolution between saturated and olefinic compounds increases with decreasing temperature.

1,1,2-Trichloro-1,2,2-trifluoroethane (FC 113) has recently been recommended by Glajch *et al.*[13] in LSC for its low elution strength, comparable to that of heptane or hexane, and its better solvent and miscibility properties. It has a refractive index similar to that of pentane but is not as volatile as pentane (Table I). However, as illustrated by Fig. 4, this solvent leads to slightly poorer resolution of the saturated and olefinic compounds in light gasolines, even on a well activated silica. As indicated by UV detection at 254 nm, the aromatic group also overlaps that of the olefins, which is not the case with pentane and renders the backflush time very critical. This lack of structural group resolution makes FC 113 less favourable as a mobile phase than pentane.

The last solvent investigated was FC 72 fluorinert, a mixture principally containing perfluorohexane as used in Suatoni's method[1,5,6]. Its two main advantages compared to pentane are a still lower elution strength, allowing greater resolution of petroleum compounds, and a still lower refractive index, leading to an increased sensitivity. The resolution between saturated and olefinic compounds is indeed very good (Fig. 5A), olefins are well retained, *e.g.*, $k' = 3$ for cyclooctene, and aromatics cannot be eluted without backflushing. A partial resolution also appears within the olefin group and even with the saturated group. This resolution is enhanced when Spherosil (800 $m^2\ g^{-1}$) is used instead of LiChrosorb Si 60 (550 $m^2\ g^{-1}$). An almost complete separation of the light *n*-alkanes (Fig. 5B) is observed. Such a resolution

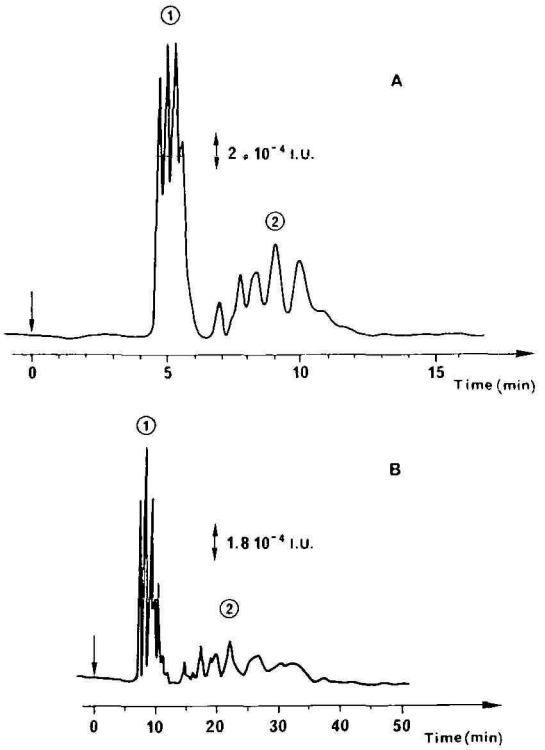

Fig. 5. Separation of a light FCC gasoline sample by LSC with FC 72 (2 ppm water) as mobile phase. Stationary phases: (A) LiChrosorb Si 60 (550 $m^2\ g^{-1}$); (B) Spherosil (800 $m^2\ g^{-1}$). Other operating conditions as in Fig. 1 and peak identification as in Fig. 2.

of compounds belonging to the same structural group does not facilitate integration of peak areas. Other difficulties may arise because of the low solubility of petroleum samples in it.

The retention of several model compounds was studied further in order to elucidate the chromatographic mechanism. Fig. 6 shows a semilogarithmic plot of the capacity factors, k', of homologous *n*-alkanes and *n*-1-alkenes, with FC 72 as mobile phase. It is seen that log k' increases linearly with carbon number at least up to the *n*-C_{16} compounds. The slopes of the straight lines are about the same for the saturated and olefinic compounds on a given stationary phase, but increased with specific surface area, as expected. A study of the retention of other model compounds under the same conditions allows one to conclude that cyclic and branched hydrocarbons are more strongly and less strongly retained, respectively, than a linear compound of equal carbon number. All these phenomena are consistent with an adsorption retention mechanism[14]. From a practical point of view, it should be noted (Fig. 6) that 1-hexene (which is probably one of the less strongly retained olefins) has the same retention as *n*-dodecane on LiChrosorb Si 60, and as *n*-undecane on Spherosil. This selectivity confirms the very good separation between saturated and olefinic compounds in the light FCC gasoline and indicates a slight overlapping of these two groups in the heavier sample.

Fig. 7 gives plots of the same kind as those in Fig. 6, but for the systems Spherosil–pentane and Spherosil–heptane. With heptane as mobile phase, the retention of *n*-olefins decreases with increasing carbon number, and the retention of alkanes with more than seven carbon atoms becomes lower than that of an unretained solute. With pentane as mobile phase, the same phenomena were observed, except that the retention of *n*-alkanes up to C_{10} increases with increasing carbon number, and decreases thereafter. These results show that for the system Spherosil–*n*-alkane the retention mechanism entails both adsorption and size exclusion, the contribution of the latter increasing with the length of the alkyl chain of the compound used as mobile phase. Exclusion in LSC was previously reported by Jinno *et al.*[11] for C_{18}–C_{20} compounds with hexane as mobile phase. Since the mean pore diameter of Spherosil is of the order of 40–50 Å, an exclusion of small saturated and unsaturated hydrocarbon molecules cannot be explained without considering the strong solvation effect of the alkane mobile phase.

From Fig. 7, a good separation between saturated and olefinic hydrocarbons in light and heavy FCC gasoline samples which do not contain compounds with more than fourteen carbon atoms (see Experimental) can be expected. Finally, a pentane mobile phase, owing to the mixed retention mechanism, seems to afford the best structural group-type selectivity.

Effect of silica gel activity. It is well known in LSC that large variations in capacity factors, selectivity and sometimes efficiency are observed with the water

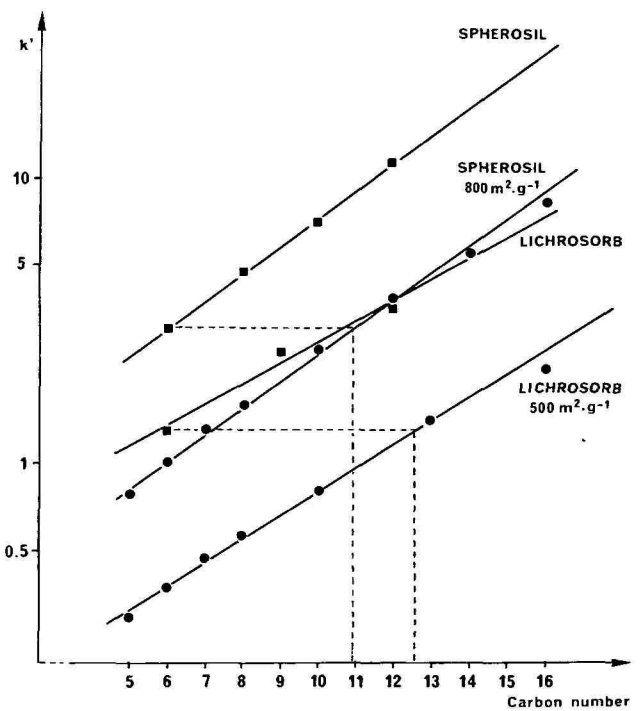

Fig. 6. Variation of the capacity factors of *n*-alkanes (●) and *n*-1-alkenes (■) with their carbon number. Stationary phases: LiChrosorb Si 60 and Spherosil. Mobile phase: FC 72.

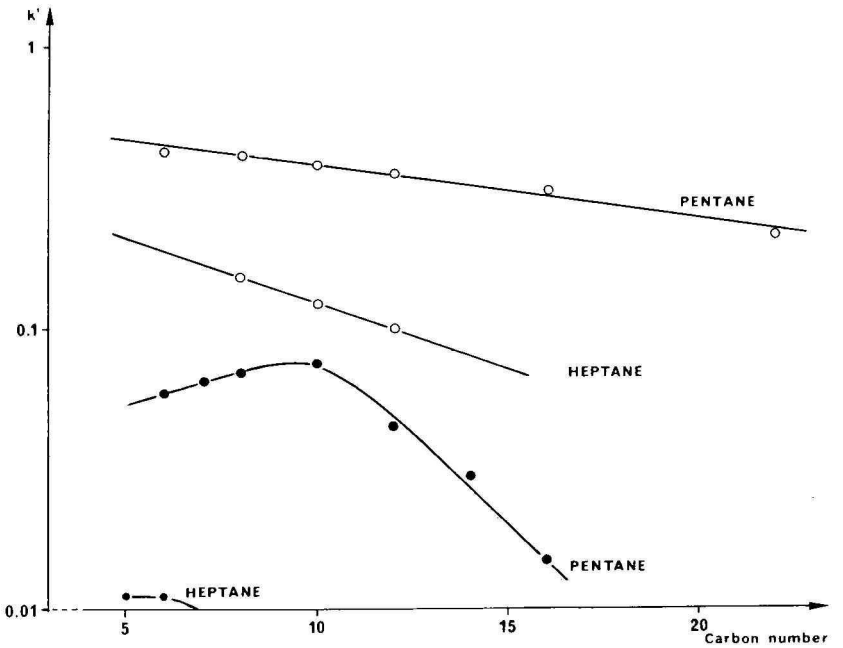

Fig. 7. Variation of the capacity factors of *n*-alkanes (●) and *n*-1-alkenes (○) with their carbon number. Stationary phase: Spherosil. Mobile phases: *n*-pentane and *n*-heptane.

content of the mobile phase[14–18]. They are particularly pronounced if class N solvents[17], *i.e.*, solvents having elution strengths in the range 0.00–0.40, are used as eluents, and the greater the activity of the adsorbent (lower water content) the higher is the resolution[19]. So isoactivating solvents (solvents having the same reduced water content for class N solvents) must be used in order to keep constant the activity of the adsorbent and to obtain reproducible results[20].

Consequently we have studied the influence of the water content in the case of *n*-pentane which is a class N solvent. Fig. 8A and B show the separation of a heavy gasoline sample on LiChrosorb Si 60 with *n*-pentane as mobile phase containing 12 and 5 ppm of water respectively. A satisfactory resolution between the saturated and olefinic hydrocarbons is observed only if *n*-pentane is sufficiently dried over a 4-Å molecular sieve and its water content lowered to 5 ppm before percolating through the chromatographic column. When equilibrium is attained (retention times of test solute are rigidly constant), pentane is kept in a closed circuit. In this way such a high resolution can be maintained for a long period of time (several weeks).

Fig. 9A and B show the same separation with the experimental Spherosil as stationary phase. A good resolution between saturated and olefinic compounds is observed even when the adsorbent is not drastically activated. Besides, a water content lower than 10 ppm is not recommended because a beginning of resolution within the olefinic group is observed. The aromatic compounds can easily be collected into a single peak by backflushing the chromatographic column.

In view of the above results, the main advantage of FC 72 Fluorinert seems to be its particularly low refractive index. As regards a purely structural group-type

selectivity, *n*-pentane (with about 5–8 ppm water) is a better mobile phase for FCC gasoline samples, if used with a silica gel having a specific surface area higher than 500 $m^2\ g^{-1}$. With still greater specific surface area, *e.g.*, 800 $m^2\ g^{-1}$, the pentane water content can be raised to about 10–15 ppm, which is more convenient in practice.

Quantitative analysis

From previous results, the phase system should be chosen according to the nature and content of the compounds included in each group.

For light gasoline in which compounds similar to pentane are present, only FC 72 can be used as the mobile phase. In that case, a silica gel with moderate specific surface area (300–500 $m^2\ g^{-1}$) must be used in order not to separate compounds belonging to the same family. For heavier samples, *n*-pentane is advocated as mobile phase because of a better sample solubility and poorer separation between the components of a particular group. In order to compensate for the higher elution strength of pentane, a silica gel having a great specific surface area (800 $m^2\ g^{-1}$) must be chosen.

Three petroleum products have been studied in order to illustrate the difficulties of quantitative analysis and to choose the most convenient mobile phase: FCC light gasoline with a substantial content of light hydrocarbons (1.9%); a reforming feed which does not contain any light and olefinic hydrocarbons; and a commercial

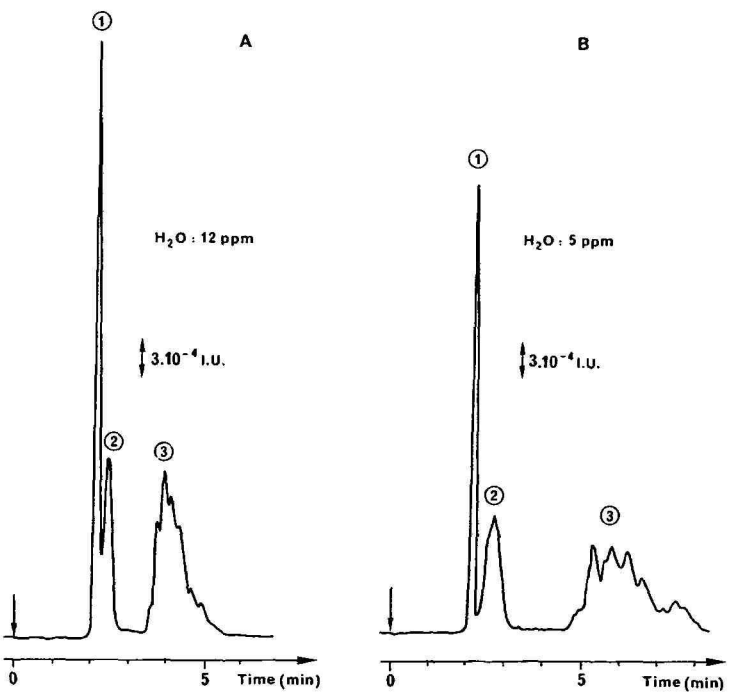

Fig. 8. Influence of mobile phase water content in LSC on the separation of a heavy FCC gasoline sample with LiChrosorb Si 60 as stationary phase. Mobile phases: *n*-pentane containing 12 ppm (A) or 5 ppm of water (B). Other operating conditions as in Fig. 1 and peak identification as in Fig. 2.

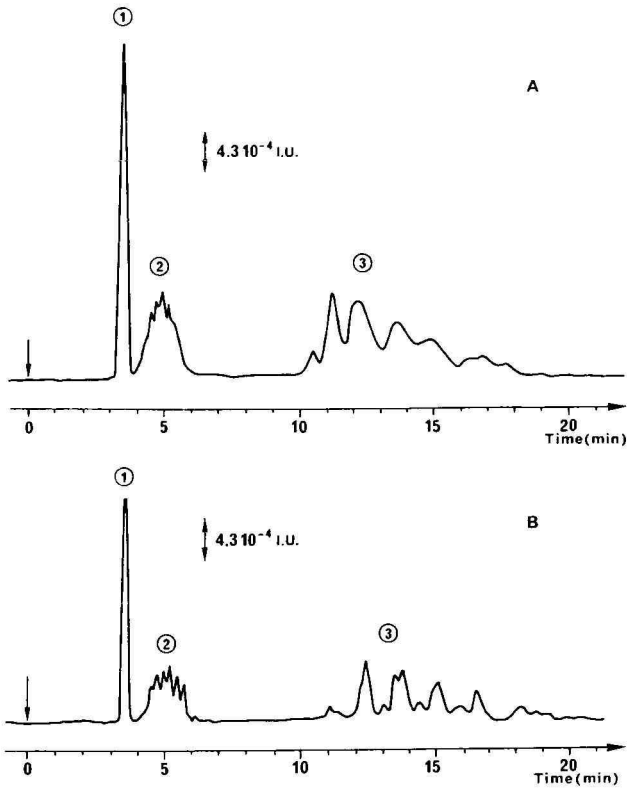

Fig. 9. Influence of mobile phase water content in LSC on the separation of a heavy FCC gasoline sample with Spherosil as stationary phase. Mobile phases: *n*-pentane containing 10 ppm (A) or 5 ppm of water (B). Other operating conditions as in Fig. 1 and peak identification as in Fig. 2.

gasoline having a low olefin content. The corresponding chromatograms are shown in Fig. 10.

The results obtained are collected in Table II. They were calculated by the following methods. In all cases, aromatic compounds have been quantified with an external standard[2]. With FC 72 as mobile phase, the contents of saturated and olefinic compounds were calculated by internal normalization using response factors[6] of 1.3 for the saturated, and 0.95 for the olefinic compounds. With *n*-pentane as mobile phase, the response factors of the two groups cannot be calculated (except in the case where light compounds are not present). So it is possible only to calculate the volume content of saturated compounds for the reforming feed sample which does not contain olefins.

From Table II, the following conclusions can be drawn:

With FC 72 as mobile phase, the results obtained for the light gasoline and reforming feed samples are in good agreement with those measured by gas chromatography (GC).

For commercial gasoline the olefinic content seems to be too high. An explanation is the low signal-to-noise ratio, resulting in a difficult integration.

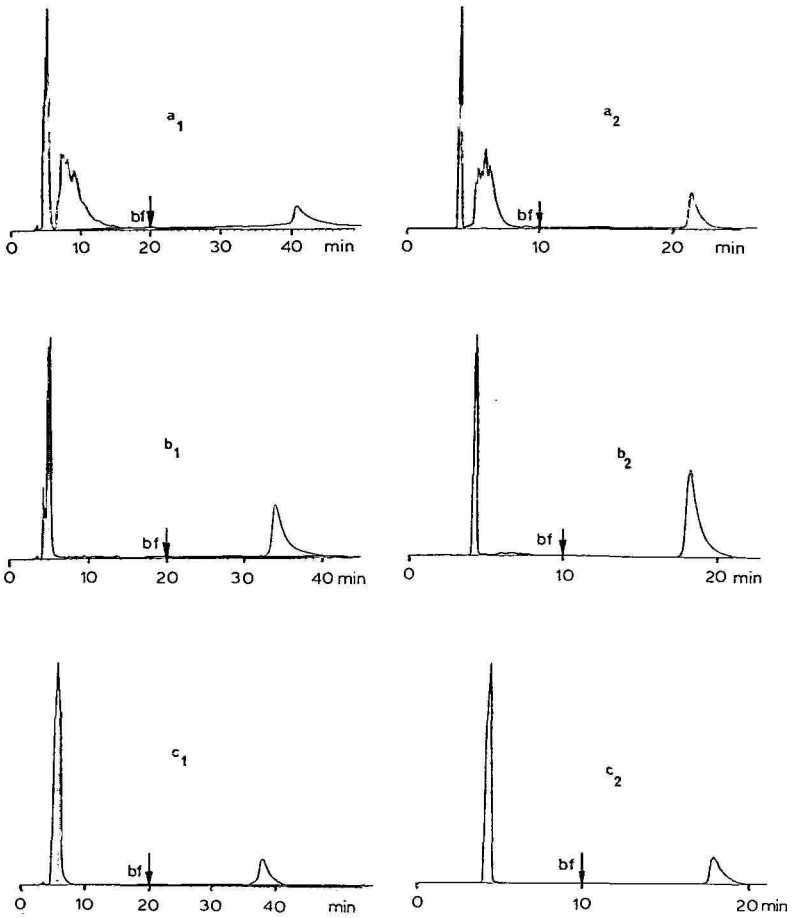

Fig. 10. Separation of three petroleum products studied for quantitative analysis. Samples: (a) FCC light gasoline; (b) commercial gasoline; (c) reforming feed. *Operating conditions: (1) Brownlee Si 100 silica gel* cartridge with FC 72; (2) experimental Spherosil with pentane as mobile phase. Flow-rate: 1 ml min^{-1}. Detection: RI. bf = Backflush.

With pentane as mobile phase the results are satisfactory for the reforming feed which does not contain light compounds.

Charge-transfer chromatography

The well known technique using silver(I) charge-transfer complexes to separate geometrical and positional isomers of olefinic compounds[21,22] has also been tested with petroleum compounds[7,23]. This technique appears to have potential for improving the resolution between saturated and olefinic groups, which remains fairly difficult in LSC despite all optimization attempts. However, the procedures described were rather intricate since they involved a separation of aromatic hydrocarbons from saturated compounds and olefins on a silica gel column (LSC), followed by a separation of saturated from olefinic hydrocarbons on a Ag^+-modified silica column. The

TABLE II

COMPARISON OF QUANTITATIVE ANALYSIS RESULTS OBTAINED BY CAPILLARY GC AND LSC

Operating conditions: GC, see Experimental; LC, Brownlee Si 100 silica gel cartridge (300 $m^2 g^{-1}$) with FC 72 or experimental Spherosil (800 $m^2 g^{-1}$) with pentane as mobile phase.

	Area (%)		*Volume (%)*		*Volume (%) GC*
	Mobile phase: FC 72	*Mobile phase: pentane*	*Mobile phase: FC 72*	*Mobile phase: pentane*	
FCC light gasoline					
Saturated compds.	35.7	27.9	44.9	★	45.8
Olefins	54.8	55.5	50.8	★	50.1
Aromatics	9.5	16.6	4.3	4.7	4.1
Commercial gasoline					
Saturated compds.	52.6	39.2	71.7	★	73.5
Olefins	5.5	2.6	5.5	★	3.8
Aromatics	41.9	58.2	22.8	24.5	22.7
Reforming feed					
Saturated compds.	83.8	77.8	90.8	89.5	90.1
Olefins	0	0	0	0	0
Aromatics	16.2	22.2	9.2	10.5	9.9

★ Response factors cannot be calculated.

procedures differ in the mobile phases to be used: cyclohexane[23] or carbon tetrachloride[7].

In this work, we investigated the silver complex method in order to separate the three groups, saturated, olefinic and aromatic compounds, on the same column using backflushing. Two problems must be solved. The first one arises from the fact that silver(I)–aromatic hydrocarbon complexes are less stable than those with monoolefinic compounds, and therefore the separations between olefinic and aromatic hydrocarbons may become difficult. The second problem is to perfect a rapid and simple procedure to prepare silver(I)-modified silica gels of known and stable characteristics. In previous studies, silver nitrate was dissolved in acetonitrile[7,22], methanol[21] or water[23] at various concentrations. The silver coating was made merely by mixing the silver solution with silica gel (batch procedure)[7,23], or by percolating the silver solution through a packed silica gel column (*in situ* procedure)[21,22]. We decided to investigate the *in situ* procedure with two different silver nitrate solutions leading either to the classical silver coating or to chemically bonded silver.

Silver-coated silica gel. By passing an acetonitrile solution of Ag^+ through a silica gel column, a mere coating is achieved:

$$\equiv Si\text{–}\underline{\bar{O}}\text{–}H + Ag(CH_3CN)_x^+, NO_3^- \rightleftharpoons \equiv Si\text{–}\bar{O}\diagup^{H}_{\cdots Ag(CH_3CN)_y^+, NO_3^-} + (x - y)CH_3CN$$

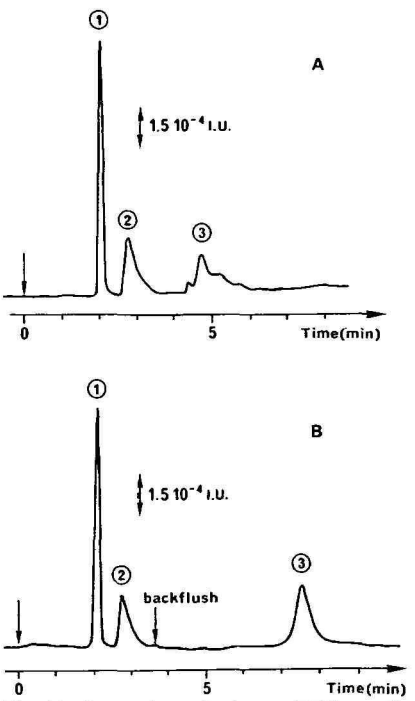

Fig. 11. Separation of a heavy FCC gasoline sample on a silver(I)-coated silica gel. Column: 15 cm × 0.48 cm I.D. Stationary phase: LiChrosorb Si 60 modified by percolating a 3% (w/w) solution of silver nitrate in acetonitrile. Mobile phase: pentane (10 ppm water). (A) Without backflush; (B) with backflush. Other operating conditions as in Fig. 1 and peak identification as in Fig. 2.

Silver ions are strongly complexed in acetonitrile and the value $x = 4$ was reported[24]. It is likely that one or several acetonitrile molecules are removed during coating so that y might be lower than x. The saturation front was monitored by RI detection and UV absorption of the nitrate ions at 313 nm. It appeared at a percolated volume of 7 ml with a 3% (w/w) silver solution and a 15 cm × 0.48 cm I.D. LiChrosorb column (dead volume, 2.0 ml; 1 g of adsorbent). So, the amount of Ag^+ coated onto the silica gel was about 0.7 mmol g^{-1}. If it is assumed that the number of silanol groups per nm^2 is near to five[25], one can estimate that about 15% of the silanol sites are covered with silver ions in this case.

After coating it seems that the major part of the remaining acetonitrile molecules should be eliminated by an appropriate column conditioning so that olefinic compounds can be retained. Fig. 11 shows a chromatogram of the heavy gasoline sample and the excellent resolution of the three structural groups. The water content used in this experiment, 10 ppm in pentane, should be noted. We ascertained that with a modified silica the influence of mobile phase water content was not as critical as in LSC, and a higher value can be tolerated.

Silver chemically bonded to silica. The operating procedure described in the Experimental is similar to that used by Foucault *et al.*[26] to prepare copper chemically

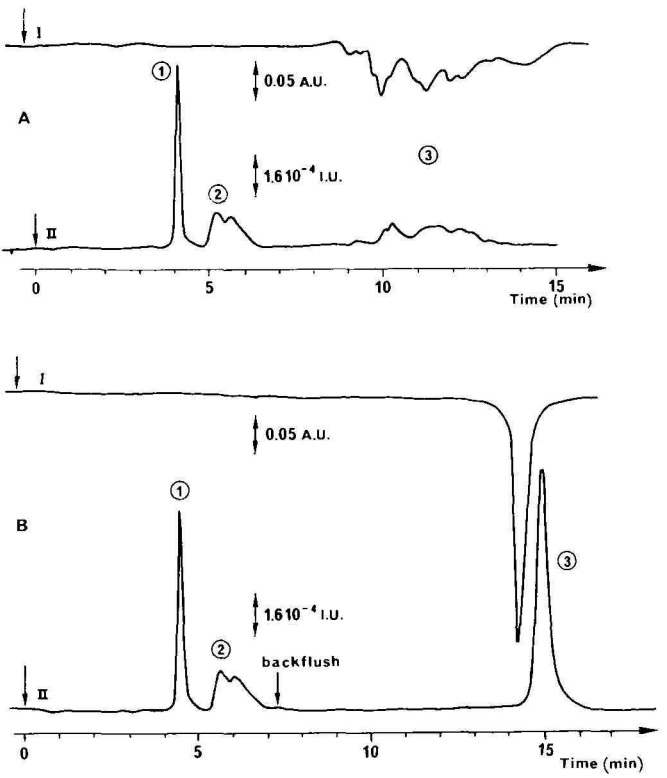

Fig. 12. Separation of a heavy FCC gasoline sample on silver chemically bonded to silica gel. Column: 25 cm × 0.48 cm I.D. Stationary phase: LiChrosorb Si 60 chemically modified by percolating a 3% (w/w) solution of silver nitrate in 0.5 *M* aqueous ammonia. Detection: (I) UV at 254 nm; (II) differential refractometer. Other operating conditions as in Fig. 11.

bonded to silica. The rôle of the aqueous ammonia solution is to make easier the exchange of an hydrogen ion by a silver ion on the silica gel surface. The most probable reaction is:

$$\equiv SiOH + Ag(NH_3)_2^+ \rightleftharpoons \equiv SiOAg(NH_3) + NH_4^+$$

Since ammonia is in excess we also have

$$\equiv SiOH + NH_3 \rightleftharpoons SiONH_4$$

and the surface of the silica is comprised of a mixture of silver and ammonium silicates. Contrary to the coating procedure, nitrate ions should not be retained with this procedure. However, with a 3% (w/w) aqueous silver solution and a 25 cm × 0.48 cm I.D. LiChrosorb column (dead volume, 3.2 ml; adsorbent mass, 1.7 g), the nitrate ion front appeared at a percolated volume of 4.5 ml. The breakthrough of Ag^+ (detected by precipitation of silver chloride) was simultaneous with that of ammonia (increase in pH of effluent) at a volume of 18 ml. A calculation similar to that given for the coated adsorbent leads here to a silver concentration of about 1.5 mmol g^{-1}, which corresponds to 30% of the silanol groups.

In order to retain unsaturated compounds, it is necessary to remove the major part of the ammonia bonded to silver atoms, which was done by washing with chloroform and drying. Fig. 12 shows a chromatogram obtained for the heavy FCC gasoline sample. As with the coated adsorbent, there is no interference between the three structural groups, and the pentane water content can be raised to about 10–15 ppm without impairing the separation.

CONCLUSIONS

In adsorption chromatography, two systems can be selected. First a silica gel having a surface area between 300 and 500 $m^2 g^{-1}$ with a perfluoroalkane as mobile phase; secondly a silica gel having a higher surface area (800 $m^2 g^{-1}$) with *n*-pentane as mobile phase. The final choice between these two systems is governed by the nature of the petroleum samples to be separated. For light samples, perfluoroalkane must be chosen; quantitative analysis is more accurate. For heavier samples, pentane must be used as mobile phase for solubility reasons.

In charge-transfer chromatography, an excellent resolution is obtained either with classically silver-coated silica gel or with silver chemically bonded to silica gel. In both cases, pentane is used as mobile phase, but its water content is not as critical as in LSC and a higher value can be tolerated. Based on these preliminary results, work is in progress in our laboratory, to optimize the selectivity of each pair of hydrocarbon groups contained in more complex mixtures such as vacuum distillates and residuals.

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IMPROVEMENTS IN METHODS FOR DETERMINATION OF ABSCISIC ACID AND INDOLE-3-ACETIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Improvements and simplifications in the early steps of the procedure for analysis of indole-3-acetic acid and abscisic acid by high-performance liquid chromatography (HPLC) are described. These include sample preparation, extraction and purification of the hormones. The method was applied to the leaves of *Fagus silvatica* and pines of *Picea abies*. With acetonitrile as extraction solvent, the hormones were recovered with high efficiency and the amounts of other substances were reduced. Partitioning of the extract between chloroform and water at different pH values obviates the need for laborious methods for prepurification of the material, simplifies the procedure and permits the use of an analytical column for further purification of the hormones by HPLC. Some terms used for characterization of the analysis are discussed.

INTRODUCTION

The growth and development of plants depends upon the balance between stimulatory and inhibitory hormones. This balance can change drastically under stress conditions, *e.g.*, water deficit, low temperature, air and soil pollution. The changes in the hormone level can also signify a disturbance in normal physiological events, and give information about these processes in the plant.

The content of hormones in plants is very low (in the range of nanograms to micrograms) and the amount of these substances is dependent not only upon environmental conditions, plant species and tissue type, but also upon the individual features of the material. Moreover, substances such as organic acids, phenolic and polyphenolic compounds present in the plant can interfere with the analysis of hormones; not only the hormone level, but also the amount of contaminants vary greatly from one species of plant or tissue type to another.

The large, and still increasing, interest in phytohormones has resulted in the elaboration of many methods for their determination. In the last 10 years, modern techniques using high-performance liquid chromatography (HPLC) have been increasingly applied to the purification and analyses of hormones in various types of

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plant material. Both abscisic acid (ABA) and indole-3-acetic acid (IAA) can be detected by UV absorption[1-5] or by electrochemical means[6]. For IAA, a more selective and sensitive means of detection is by fluorescence measurement[6-10]. Separation methods based on different adsorbents, *e.g.*, ion-exchange resin[6,9], reversed phase[10,11], ion pair–reversed phase[5,12] and silica, have also been used. Many methods elaborated by various authors are precise and selective, and have resulted in great progress in the analyses of the hormones. However, these procedures are mostly very material-specific, and not applicable to all plant materials. As mentioned earlier, the specificity of plant materials requires analyses of a very great number of samples in a short time. Most of the methods include very laborious sample preparation, *e.g.*, filtration, purification on polyvinylpyrrolidone (PVP), evaporation of large volumes of solvents, etc., making them very difficult for automatization and routine use[4,7,9,13].

This paper is concerned with some improvements in the HPLC method for analysis of the main representatives of the two various groups of hormones, ABA and IAA. Special attention was paid to the preparation of samples, extraction and purification of hormones which effectively determine the simplicity of the given method. Applications of the procedure to analyses of a large number of samples in a short time are presented. Some terms used for characterization of the analysis are discussed.

EXPERIMENTAL

The HPLC system used was a Waters 820 including two Model 510 pumps, an automatic sample injector WISP 710B, an UV absorbance detector (254 nm) Model 420 and a controlling–data processing computer Professional 350 (Digital). The UV detector was coupled in series with a fluorescence detector Model FS 970 (Kratos), excitation at 254 nm and emission at >370 nm (KV 370 filter).

Analytical grade hexane, chloroform, acetonitrile and methanol were obtained from Merck (Darmstadt, F.R.G.). Radioactive standards of *cis-trans*-abscisic acid and indole-3-acetic acid from Amersham (U.K.) were used. Water was double distilled from glass apparatus. Buffer solutions of pH = 7.0 were prepared from 5.7 g/l KH_2PO_4 and 0.2 *M* sodium hydroxide. Dissolved air was removed from already mixed solvents by direct sonification (1–2 min) with the Ultrasonifier B-12 (Branson).

Extraction of hormones from plant material

Leaves of *Fagus silvatica* L. and pines of *Picea abies* Karst. were used to test the method for hormone analyses. The plant material was harvested from trees about 20 years old, then immersed in liquid nitrogen, transported from the forest on solid carbon dioxide and stored in the laboratory at −20°C until analysis. Portions (up to 15 g) of the frozen material were placed into 250-ml centrifuge-tubes with 200 ml of liquid nitrogen and ground with Ultraturax for 3 min (20 000 rpm) with aggregate diameter 10 mm, followed by 10 min (27 000 rpm) with aggregate diameter 8 mm. After evaporation of nitrogen and thawing of the powdered plant material, samples of about 1 g were weighed in 10-ml centrifuge-tubes and lyophilized. To each dry sample, 5 ml of acetonitrile containing 200 ppm of 2,6-di-*tert.*-butyl-*p*-cresol (BHT) were added as an antioxidant. The tubes were tightly closed and shaken at 4°C to

extract the hormones. After 4 h, the samples were centrifuged at 2200 *g*, 3 ml of supernatant were pipetted from each sample into a 10-ml centrifuge-tube and evaporated to dryness under vacuum.

Purification of extracts by partitioning

To each tube containing the dry extract, 2 ml of phosphate buffer pH = 7.0 and 2 ml of chloroform were added, tightly sealed and placed horizontally on a shaker. Very gentle shaking for 1 h was required to prevent emulsion formation and to allow sufficient extraction of impurities into the chloroform. The samples were centrifuged for 10 min at 2200 *g* for better phase separation, 1.80 ml of buffer phase were transferred to a new tube and the extraction was repeated after addition of 2.00 ml of fresh chloroform. In this and the next steps of purification, the extraction process could be shortened to 5 min by vigorous shaking of the samples. A 1.60-ml volume of the buffer phase was transferred into new tubes and acidified to pH = 2.1 with 100 μl of formic acid. To each sample, 2.00 ml of chloroform were added and after shaking and separation of the phases, 1.80 ml of the organic phase containing the hormones was taken up. The procedure was twice repeated with portions of fresh chloroform.

Purification of hormone extracts by HPLC

After evaporation of chloroform, the samples were redissolved in 20.0% methanol, and 150 μl of the solution were injected onto a reversed-phase column containing LiChrospher 100 CH-18 Super (Merck, 4 μm, cartridge 125 × 4 mm) which had been equilibrated in the starting mobile phase: methanol–water–acetic acid (20:79.4:0.6). Analysis for 5 min in the starting solvent was followed by a linear gradient over 30 min to the final solvent ratio: methanol–water–acetic acid (70:29.4:0.6). A further period of 5 min in the end solvent removed the remaining impurities, and a reverse gradient over 5 min followed by 15 min of equilibration in the starting solvent prepared the column for the next injection. The flow-rate was 1 ml/min, and the pressure remained below 2000 p.s.i. Fractions corresponding to *trans-trans*-ABA, *cis-trans*-ABA and IAA were collected and evaporated to dryness under vacuum.

Analysis of hormones by HPLC

Samples were dissolved in 200 μl of 7.5% 2-propanol in hexane, and 150 μl of the solution were injected onto a silica column containing LiChrospher Si 60 Super (Merck, 4 μm, cartridge 125 × 4 mm) eluted isocratically. The mobile phase for analysis of *trans-trans*-ABA, *cis-trans*-ABA and indole-3-acetic acid was 2-propanol–hexane–formic acid (7.5:91.5:1)[7]. The flow-rate was 2 ml/min for ABA and 1 ml/min for IAA analysis. *trans-trans*-ABA and *cis-trans*-ABA were detected by their absorption at 254 nm; for detection of IAA a fluorescence detector was used (excitation at 220 nm, emission at >370 nm).

RESULTS AND DISCUSSION

The main steps of extraction and purification of hormones from plant material are presented in Fig. 1. Homogenization in methanol of materials such as pines was

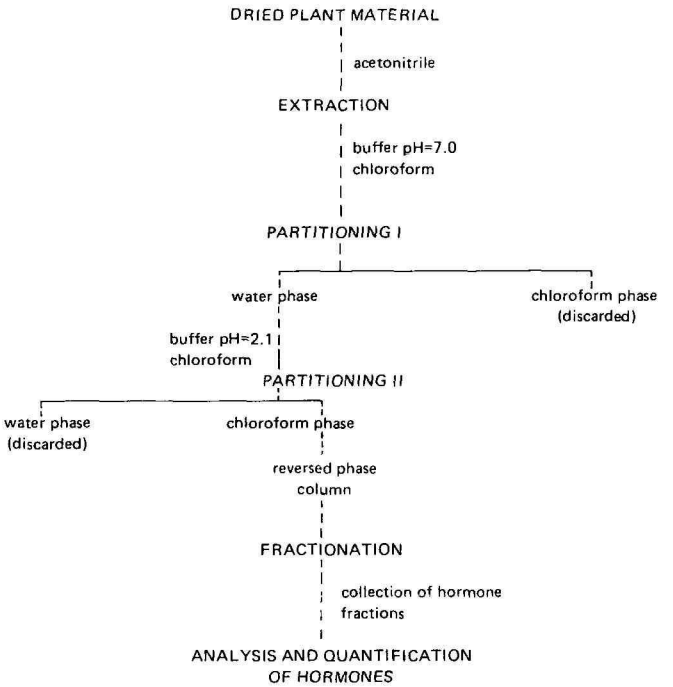

Fig. 1. Steps of the extraction and the purification of hormones.

not effective. Grinding the material in liquid nitrogen gave a fine homogenate powder, which allowed easy "penetration" of the solvent. A condensation of moisture on the frozen plant material, and loss of weight in the short time after thawing was not observed. The ground material was especially convenient for freeze drying, and for pines this was possible only after grinding. This method of grinding plant material has been used successfully for over a year on both the micro- and macroscale and the samples obtained have been divided and used for various other analyses.

In previously reported extraction procedures, methanol or methanol–water mixtures were used. The use of such polar solvents leads to extraction of many other compounds which interfere with hormone estimation, *e.g.*, phenolic acids are easily extracted with water[2]. In previous studies these polar compounds caused serious problems, and removing them required the use of laborious and often insufficient methods of purification, causing additional losses of hormones.

We found that the extraction efficiency, calculated as the recovery of radioactive standards, was increased by using less polar solvents and dry plant material (Table I). The amount of impurites extracted was proportional to the solvent polarity and amount of water. However, the use of solvents not miscible with water decreased the efficiency and reproducibility of extraction. This could be explained by less solvent "penetration" into the material and partition effects caused by traces of water. The use of acetonitrile and freeze-dried plant material gave the optimum extraction. In this step the recovery of added radioactive hormones was 97.0 ± 2% and 94.6 ± 2% for *cis-trans*-abscisic acid and indole-3-acetic acid, respectively. In comparison

TABLE I

RECOVERY OF RADIOACTIVE HORMONE STANDARDS FROM DRIED SAMPLES OF *PICEA ABIES* WITH VARIOUS EXTRACTION SOLVENTS

Solvent	*Recovery of ABA (%)*	*Recovery of IAA (%)*
Methanol	82.4	92.2
Acetonitrile	97.0	94.6
Ethyl acetate	85.5	76.1
Hexane–2-propanol (90:10)	72.6	58.1

with extraction using methanol or methanol–water, only traces of phenolic compounds were found in the extract. The time required for extraction was short, but to exclude any possible oxidation of indoles we used BHT (200 ppm) as an antioxidant. The high extraction efficiency permitted the use of only a part of the extract (3 ml from 5 ml) and eliminated the filtration step, making this procedure very simple.

The small amounts of phenols in the extracts do not require adsorption on PVP, and hormones can be purified from phenols and other impurities by a simple partition step using the large difference in partition coefficients for ABA and IAA when extracted from buffers into chloroform[11]. We found that the partitioning of hormone extracts was most effective when the one extraction solvent was applied, and the process was only pH dependent. Aqueous solutions at pH = 7.0 and pH = 2.1 with chloroform as the extraction solvent were optimal; chloroform was more effective than the dichloromethane used by Wurst *et al.*[14].

Eliminating the use of a separation funnel during partitioning by transferring a part of the sample using a precise, automatic pipette makes this normally inconvenient procedure very simple and routine. Most of the impurities are washed out from the buffer phase pH = 7.0 with chloroform, and after changing the pH to 2.1, ABA and IAA are easily extracted with chloroform leaving the more polar compounds in the water phase. The residue obtained after evaporation of the combined chloroform extracts is readily soluble in 30–40% methanol. Sandberg *et al.*[12] emphasized the great importance of the specificity of a method for successful hormone analysis. This was demonstrated by the fact that the best results were obtained by applying two different chromatographic systems, HPLC and gas–liquid chromatography (GLC)[4,11,15–17], and different chromatographic conditions for the sample preparation and analysis[1,3,6,7,9,10,18–22]. The combination of as varied methods of separation as possible alone increased the specificity of the separation method. Therefore, it is understandable that better results could be obtained when the chromatographic system used for the prepurification significantly differs from that applied to the analysis[23].

Taking into consideration the results reported in the literature and our experience, we chose as the optimum system purification on the reversed-phase C_{18} column and analysis on the silica column as described in Materials and Methods.

Chromatograms of samples from *Picea abies* and *Fagus silvatica* are shown in Fig. 2B and C.

In our procedure the impurities were present at much lower concentrations

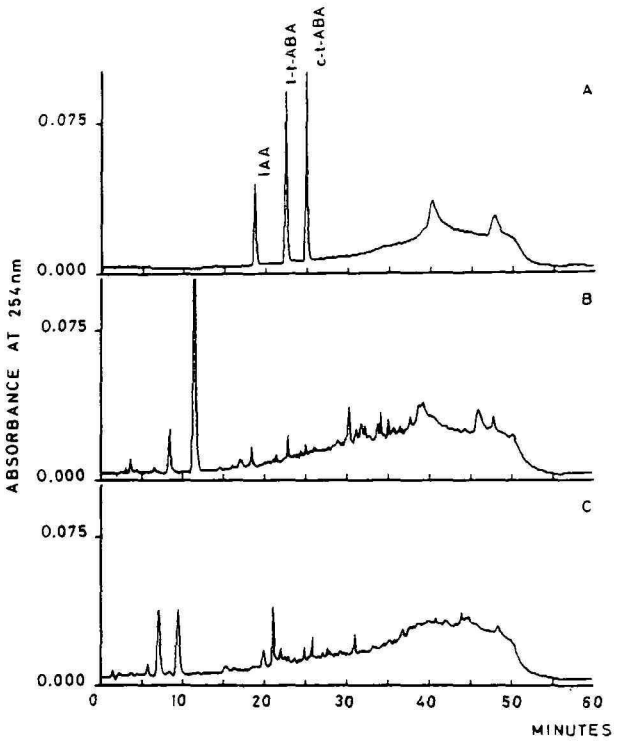

Fig. 2. Prepurification of a plant extract on a reversed-phase column. (A) Solution of standard substances; (B) sample from *Picea abies*; (C) sample from *Fagus silvatica*. Chromatographic conditions: column, LiChrospher 100 CH-18 Super (125 × 4 mm); mobile phase, gradient 20–70% methanol in 0.1 *M* acetic acid, flow-rate 1 ml/min.

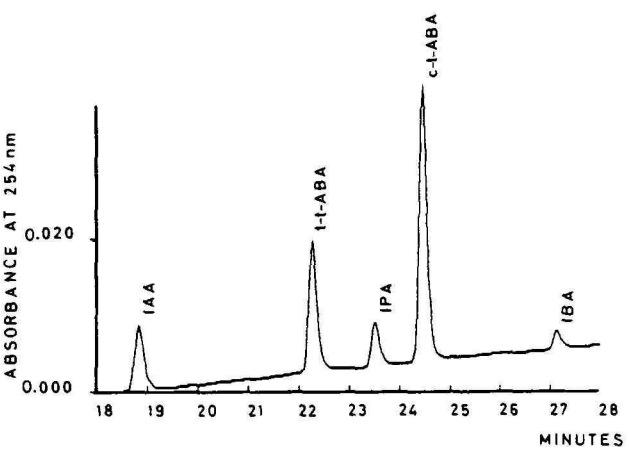

Fig. 3. Chromatogram of a standard solution of ABA, IAA, IPA and IBA. Chromatographic conditions as in Fig. 2.

than in earlier studies[13], therefore it was possible to use the analytical column instead of the preparative one for the purification step. Fractions corresponding to *trans-trans*-ABA, *cis-trans*-ABA and IAA are easy to collect (also using a programmable fraction collector), and the collection of broader fractions, if necessary, prevents losses of hormones in the event of changes in the retention time. The collection of fractions corresponding to indolepropionic acid (IPA) and indolebutyric acid (IBA) is possible as well (Fig. 3).

The same mobile phase and column for analysis of ABA and IAA allowed continuous work, without changes and reequilibration of the HPLC system (Fig. 4). Quantification by peak area or peak height measurement gave similar results.

Generally, in the analysis of compounds occurring in only very small amounts in the sample, not only the total amount of a substance, but also the correlation between this amount and the amount of impurities is important. Therefore, increasing the sample amount without increasing the efficiency of purification is not helpful. Using modern HPLC detectors it is at present possible to determine several ng of substances with the generally required accuracy.

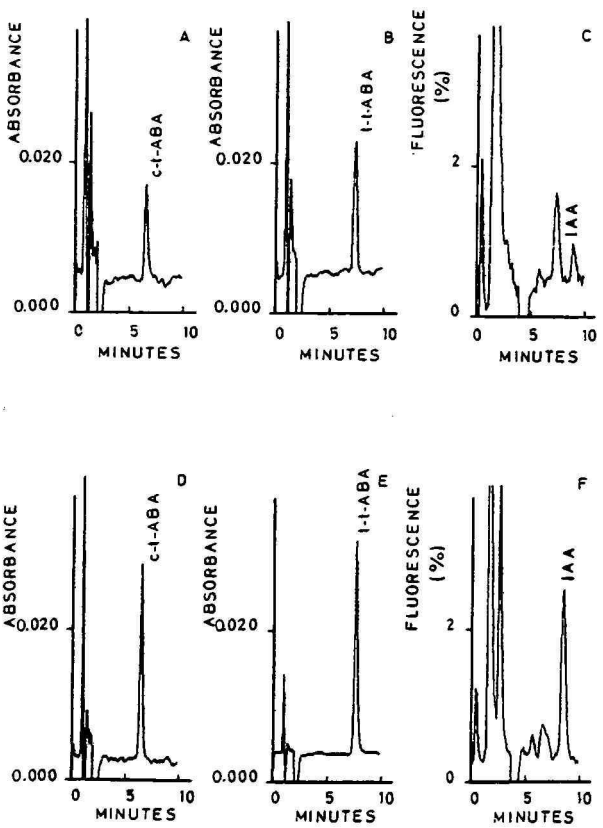

Fig. 4. Analysis of *cis-trans*-ABA, *trans-trans*-ABA and IAA by HPLC. (A) *cis-trans*-ABA from *Fagus silvatica*; (B) *trans-trans*-ABA from *Fagus silvatica*; (C) IAA from *Fagus silvatica*; (D) *cis-trans*-ABA from *Picea abies*; (E) *trans-trans*-ABA from *Picea abies*; (F) IAA from *Picea abies*. Column: 125 × 4 mm LiChrospher Si 60 Super. Mobile phase: 2-propanol–hexane–formic acid (7.5:91.5:1). Flow-rates: 2 ml/min (A, B, D, E); 1 ml/min (C, F).

The sample amount can therefore be chosen according to the concentration of substance to be analysed. It should be noted that a part of the sample is lost during processing, and the final amount of substances should be in the optimum range of determination. Loss of analysed compounds occurs in two ways: by an efficiency or yield of less than 100%, and by dilution or dividing of the sample. In the first case, the losses can be determined with radioactive or cold standards and described as 100% minus recovery (%). Some authors use this factor, without a clear explanation, to indicate the recovery of a certain step, *e.g.*, extraction, and not for the whole method. The term "recovery" in this paper means the amount of substance obtained in the last quantification step in relation to the amount of substance added to the plant material before extraction, and is expressed as per cent.

Losses by dilution or by dividing of the sample are easy to control and calculate throughout the whole method. The total dilution factor is calculated by multiplication of step dilution factors, as is normally done in the calculation of total recovery. An example of such a step dilution factor in our method is 0.6; for separating the extract from the plant material we used 3 ml from 5 ml. Losses of these types are very often characteristic of the method, and elimination of them can change a convenient routine procedure into a very laborious one (for example only part of the solution can be extracted and injected onto the column).

However, it should be noted that the recovery and the dilution factors do not describe the quality of the method, but together with the range of quantification they allow the calculation of the amount of material required for analysis, and this finally gives information about the usefulness of the method for certain problems. To determine the precision, 500 ng/g fresh weight radioactive hormone standards were added to the ground material. Analyses of six 1-g samples gave precision values of 7 and 12% for IAA and *trans-trans*-ABA respectively; the precision was equal for both plant materials. These values, sufficient for routine analyses, agreed well with previously presented data[12,14,20]. The calibration curve obtained from experiments with plant material of equal hormone concentrations to which known amounts of hormone standards were added corresponded well with the calibration curve from the pure hormone standards. The previously mentioned dependence of the systematic and random errors on the type of plant material[8] was probably the reason for the necessity of separate calibration for both plant materials. Quantification was based on peak area measurements, however, peak heights could also be used.

The limit of detection with our method was 0.1 ng, calculated for a peak signal-to-noise ratio of 3. Sandberg *et al.*[12] cautioned against the overestimation of this parameter, as it is less significant than often indicated in the literature. The definition of the detection limit which we used in accordance with other authors[10] should be understood as a characteristic parameter of the method with a strong dependence on the HPLC system used, and is useful only when calculated from the real sample. We propose to use this datum for calculation of the "determination limit", which describes the minimum amount of substance which can be quantified. The detection limit may be understood as the minimum detectable difference between two hormone concentrations and therefore corresponds to the precision of the determination. The determination limit is expressed by:

$$\text{Determination limit (Dt)} = \frac{100}{\text{precision}} \cdot \text{detection limit}$$

This means that, with 7% precision, it is possible to quantify an amount of substance 14.3 times higher than the detection limit. In this work the Dt value was 1.43 ng for IAA and 0.83 ng for ABA.

It is already possible to define the important parameter describing the method of determination, the minimum assayable amount of substance in the sample (Am):

$$\text{Am} = \frac{100}{\text{recovery (\%)} \cdot \text{dilution factor}} \cdot \text{Dt}$$

Together with the precision and accuracy as defined by Sandberg *et al.*[12], the minimum assayable amount describes the usefulness of any method for quantitation of a certain substance in a certain material. It enables the comparison of different methods and thus the possibility of finding the optimum analysis for any one problem. After taking into consideration the other important factors, *e.g.*, equipment required, time consumption or possibility of routine use, it should become easier to choose the optimum method.

The previously discussed recovery and dilution factors are presented in the Table II; the recovery achieved was better than or comparable to that in many earlier works[3,15,17,19,20]. In this study the minimum assayable amount (Am) of *cis-trans*-ABA (or *trans-trans*-ABA) in the sample was calculated as 3.63 ng; the minimum amount for analysis of IAA was 7.95 ng. This means that this amount of hormone could still be quantified with 10% precision. We used, in two examples, 1 g of material, but 200–300 mg of sample could also be used with the same precision of quantification. The Am value may also be decreased by changing the dilution factor, *i.e.*, by simply changing the volume of sample transferred during the purification procedure. We found, however, that the step dilution factors used in this work represented the optimum choice between the required Am, precision and simplicity for routine application.

The described procedure improves and simplifies the routine use of HPLC methods for plant hormone analysis. It is not specific for *Fagus silvatica* and *Picea*

TABLE II

DILUTION FACTORS CALCULATED FOR INDIVIDUAL STEPS OF THE PROCEDURE AND TOTAL RECOVERY OF THE HORMONES

Step of procedure	*Step dilution factors for ABA and IAA*	*Recovery of ABA (%)*	*Recovery of IAA (%)*
Extraction	0.60	–	–
Partitioning I (pH = 7.0)	0.80	–	–
Partitioning II (pH = 2.1)	1.00	–	–
Fractionation (HPLC)	0.75	–	–
Analysis (HPLC)	0.75	–	–
Total	0.27	84.7	66.6

abies, but could probably be applied to the determinations of ABA and IAA in any other plant material. The simplicity of operations and possibility of automatization allow the routine use of this method. With this method it is possible to extract and prepurify by partitioning at least 50 samples per day. About 20 samples can be prepurified by HPLC and analysed in 1 day. Moreover, the basic principles of the described procedure can be utilized in the determination of many other phytohormones.

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CHROM. 18 456

ENANTIOMERIC RESOLUTION OF DANSYL AMINO ACIDS BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH β-CYCLODEXTRIN INCLUSION COMPLEXES

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SUMMARY

Enantiomeric resolution of dansyl amino acids by micro high-performance liquid chromatography with β-cyclodextrin inclusion complexes was investigated. Chromatographic parameters which affect enantiomeric resolution were examined. Twelve pairs of dansyl amino acids were separated in a single chromatographic run.

INTRODUCTION

Chiral resolution has been achieved by high-performance liquid chromatography (HPLC) with either a chiral stationary phase or a mobile phase containing a chirality-recognizing reagent. Optical resolution by the latter method relies on chiral recognition by ligand exchange, ion-pair formation and inclusion complexing.

It is well known that the cyclodextrins show high stereoselectivity. They form inclusion complexes with a variety of molecules and ions. Debowski *et al.*[1,2] applied α-cyclodextrin and β-cyclodextrin as the mobile phase components for resolution of racemic mandelic acids and mandelic acid derivatives. Nobuhara *et al.*[3] resolved racemic 1-[2-(3-hydroxyphenyl)-1-phenylethyl]-4-(3-methyl-2-butenyl)piperazine with β-cyclodextrin as the mobile phase component.

Cyclodextrin-bonded phases for HPLC have recently been developed and their applications to the separation of optical, geometrical and structural isomers have been reported[4,5].

Enantiomeric resolution of dansyl amino acids by micro HPLC using β-cyclodextrin as the mobile phase component is described in this paper. The resolution of many pairs of racemic dansyl amino acids will be demonstrated after examination of the chromatographic parameters that affect the separation of enantiomers.

EXPERIMENTAL

Chromatography

Micro Feeder (Azumadenkikogyo, Tokyo, Japan) equipped with a 500-μl gas-

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tight syringe MS-GAN 050 (Terumo, Tokyo, Japan) or MPLC Micro Pump (Brownlee Labs., Santa Clara, CA, U.S.A.) was used. Most of the experiments were carried out with the former pump, although the latter was used for gradient separation. A UV spectrophotometer, UVIDEC-100II or 100V (Jasco: Japan spectroscopic, Tokyo, Japan), was equipped with a home-made flow cell (0.05–0.1 μl in volume) and used at 220 nm. An ML-422 micro valve injector (Jasco) was used to load the samples (19–20 nl). Fused-silica tubing (0.26 mm I.D.) or glass-lined stainless-steel tubing (0.3 mm I.D.) was selected as the material of the separation column. The former column was manually packed by a previously reported method[6], in which PTFE tubing was employed as the connecting tubing. The latter column could withstand high pressure and it was prepared by the high-pressure slurry-packing technique[7]; ODS-Hypersyl-3 (3 μm; Shandon, Runcorn, U.K.) was the stationary phase in this work.

Reagents

Twenty-one L-amino acids (Kit No. LAA-21), sixteen D-amino acids (Kit No. DAA-16), twenty-four D,L-amino acids (Kit No. DLAA-24) and fifteen dansyl-D,L-amino acids (Kit No. DAN-DL-15) were obtained from Sigma (St. Louis, MO, U.S.A.). β-Cyclodextrin was obtained from Tokyo Chemical Industry (Tokyo, Japan). Other reagents were from Wako (Osaka, Japan), unless otherwise noted. Dansylation of amino acids was also carried out in the laboratory at 40°C for 30 min. The pH of the mobile phase was adjusted with potassium phosphate and phosphoric acid (0.1 *M*).

RESULTS AND DISCUSSION

There are three cyclodextrins of different sizes available commercially, which differ in the number of glucose units in the ring. The cavity size of β-cyclodextrin is suitable to form inclusion complexes with compounds that have a naphthalene ring[4]. Dansyl derivatization has been widely employed for the analysis of amino acids because dansyl amino acids can be separated well by reversed-phase HPLC and detected sensitively by UV or fluorescent spectrophotometers. Enantiomers of dansyl amino acids have been successfully separated on a β-cyclodextrin-bonded phase with a mobile phase of methanol and water[4], when the L isomer of a dansyl amino acid elutes before its D isomer. This indicates that the inclusion complex with the D isomer is more stable than that with the L isomer. In the case of the separation with a mobile phase including β-cyclodextrin, the D isomer can be expected to elute before its L isomer because, as the stability of the isomer increases, the isomer is less retained on the stationary phase. In the latter case, the concentration of β-cyclodextrin in the mobile phase, the mobile phase composition and the pH of the mobile phase can affect the capacity factor and the separation factor.

Fig. 1 demonstrates the enantiomeric separation of dansyl serine with a mobile phase containing 10% (v/v) acetonitrile and different concentrations of β-cyclodextrin. As the concentration of β-cyclodextrin increases, the retention times of both isomers decrease, owing to the formation of inclusion complexes by β-cyclodextrin and the dansyl serine enantiomers. The difference in retention time between the enantiomeric isomers cannot be recognized on the chromatograms that correspond to β-cyclodextrin concentrations of less than 2.5 m*M*, while only a shoulder peak is observed for a β-cyclodextrin concentration of 5.0 m*M*.

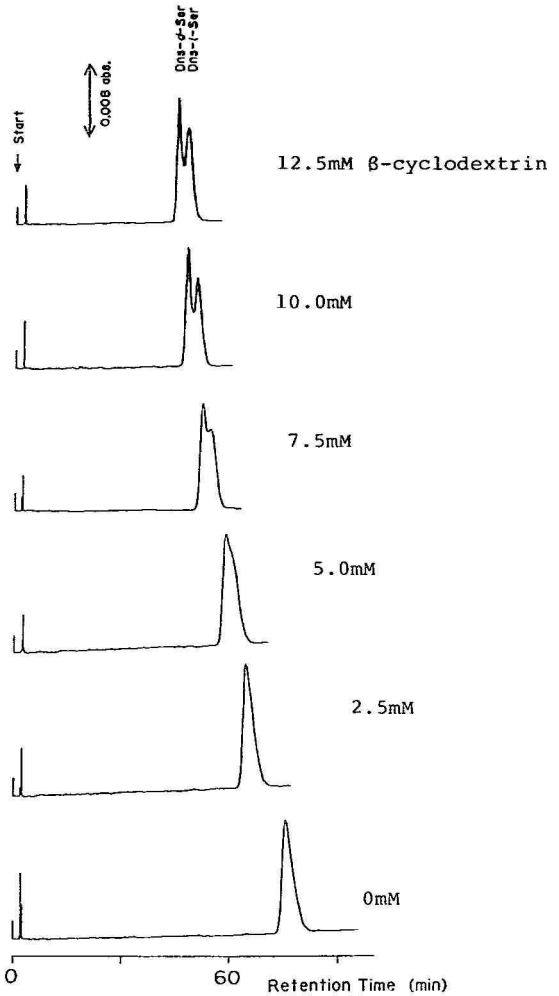
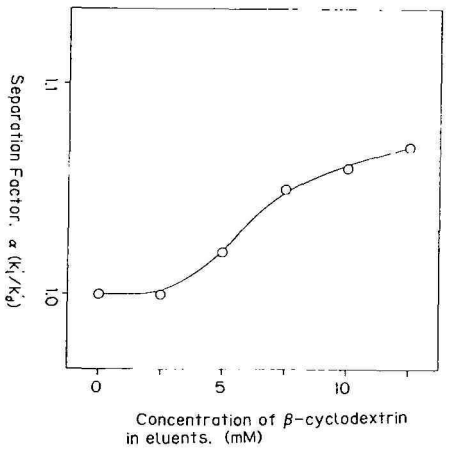


Fig. 1. Effect of the concentration of β-cyclodextrin on the enantiomeric separation of dansyl serine. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase; 10% (v/v) acetonitrile solution including different concentrations of β-cyclodextrin (pH 5.1); flow-rate, 2.1 μl/min; sample, dansyl-D,L-serine (100 pmol).

Fig. 2 shows the relationship between the separation factor and the concentration of β-cyclodextrin. The separation factor increases with increasing concentration of β-cyclodextrin up to 12.5 mM. Higher concentrations of β-cyclodextrin could not be achieved owing to solubility problems. The value of the separation factor is relatively low, but this can be compensated by the larger theoretical plate number, which can be easily attained by octadecylsilica columns. In addition, the elution order of dansyl amino acids strongly depends on their solvophobicity, and the low value of the separation factor (*ca.* 1.1) facilitates the separation of multiple pairs of enantiomers in a single chromatographic run.

Fig. 3 illustrates the effect of the concentration of acetonitrile in the mobile



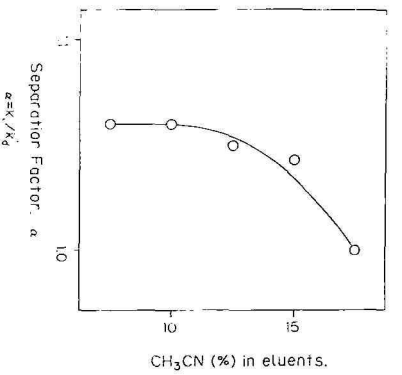


Fig. 2. Relationship between the separation factor and the concentration of β-cyclodextrin. Operating conditions as in Fig. 1.

Fig. 3. Effect of the concentration of acetonitrile on the separation factor. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer including 10 m*M* β-cyclodextrin (pH 5.0–5.1); flow-rate, 2.1 μl/min; sample, dansyl-D,L-serine (100 pmol).

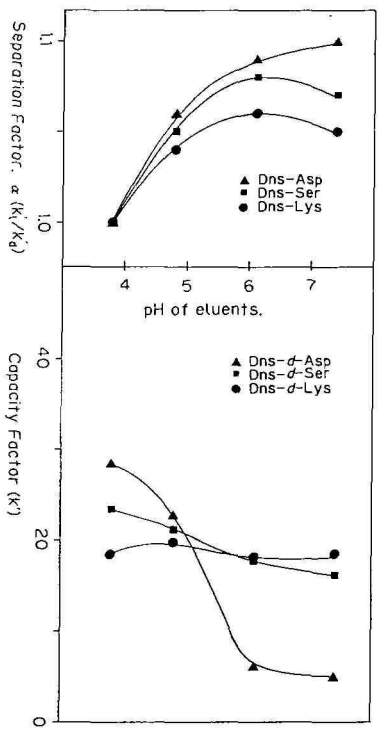


Fig. 4. Effect of the pH of the mobile phase on the separation and the capacity factor. Column, ODS-Hypersil-3 (108 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (10:90) containing 12.5 m*M* β-cyclodextrin; sample, dansyl-D,L-Ser (100 pmol), dansyl-D,L-Asp (100 pmol), and dansyl-D,L-Lys (*ca.* 50 pmol).

TABLE I

THE CAPACITY FACTOR AND THE SEPARATION FACTOR

Concentration of β-cyclodextrins, 12.5 m*M*.

Dansyl amino acid	*Mobile phase*		*Capacity factor*		*Separation factor*
	Acetonitrile (%)	*pH*	*D*	*L*	
Ala	10	6.1	28.9	30.4	1.05
Asp	10	6.1	6.4	7.1	1.10
Glu	10	6.1	7.1	7.7	1.09
Lys	10	6.1	17.6	18.7	1.06
Ser	10	6.1	18.1	19.5	1.07
Thr	10	6.1	21.7	24.3	1.12
Arg	10	6.5	21.2	22.6	1.07
Asn	10	6.5	12.5	13.2	1.06
Gln	10	6.5	17.5	18.5	1.05
α-AB	15	6.5	19.5	20.9	1.07
Met	15	6.5	83.9	87.9	1.05
Pro	15	6.5	21.0	22.1	1.05
Ile	20	6.1	18.7	20.0	1.07
Leu	20	6.1	21.7	23.2	1.07
Nle	20	6.1	30.3	31.7	1.05
Nval	20	6.1	13.5	14.1	1.05
Phe	20	6.1	30.7	32.4	1.06
Trp	20	6.1	29.6	29.6	1.00
Val	20	6.1	10.1	10.8	1.07

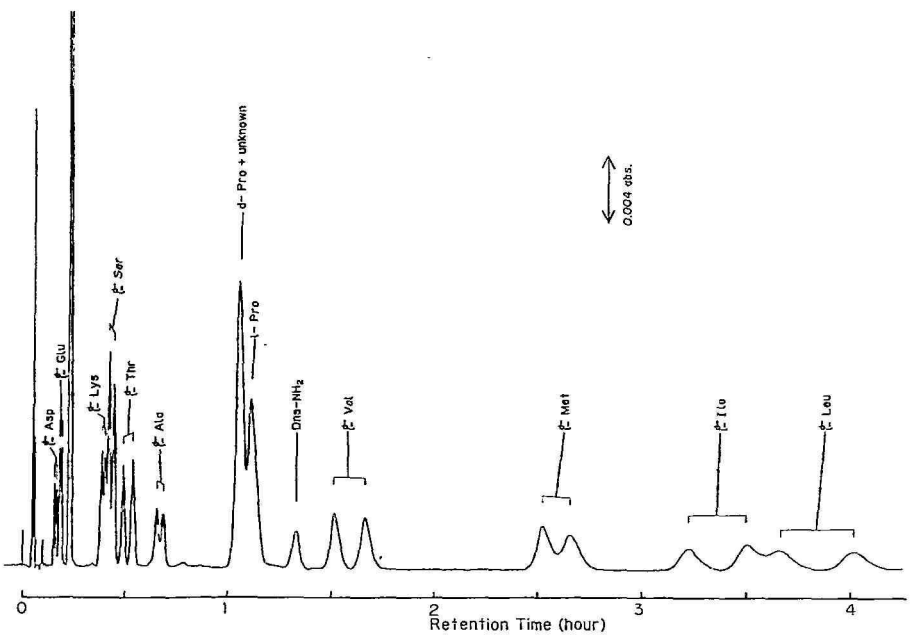


Fig. 5. Isocratic separation of racemic dansyl amino acids. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (20:80) containing 12.5 m*M* β-cyclodextrin; flow-rate, 2.1 μl/min; sample, *ca.* 20 pmol each.

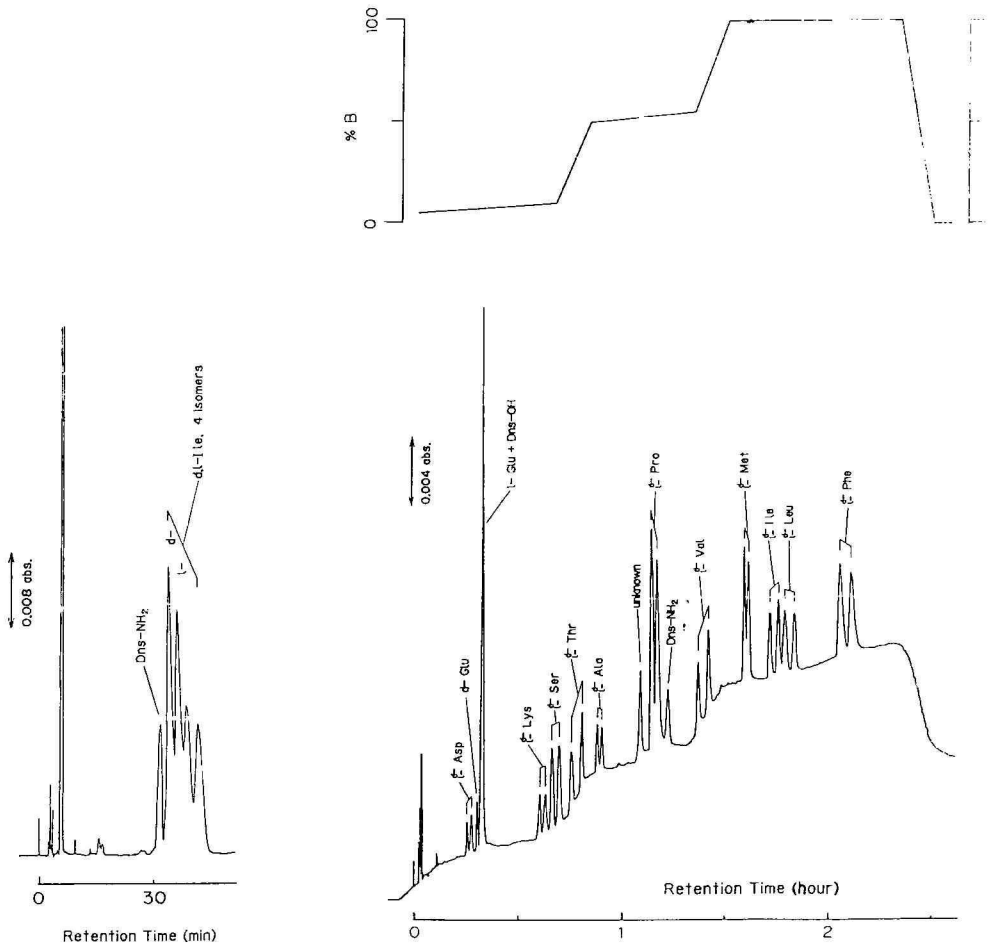


Fig. 6. Separation of dansyl isoleucine diastereomers. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (20:80) containing 12.5 m*M* β-cyclodextrin; flow-rate, 2.1 μl/min.

Fig. 7. Gradient separation of racemic dansyl amino acids. Column, ODS-Hypersil-3 (144 × 0.3 mm I.D.); mobile phase, (A) acetonitrile–phosphate buffer (10:90) containing 12.5 m*M* β-cyclodextrin (pH 6.4), (B) acetonitrile–phosphate buffer (20:80) containing 12.6 m*M* β-cyclodextrin (pH 6.4) with the gradient profile as indicated; flow-rate, 5.0 μl/min; sample, *ca.* 20 pmol each.

phase on the separation factor when using dansyl-D,L-serine as test solutes. The separation factor decreases with increasing concentrations of acetonitrile. The enantiomeric isomers could not be distinguished when the concentration of acetonitrile exceeded 17.5%, even if they were retarded on the stationary phase.

The effects of the pH of the mobile phase on the separation factor and the capacity factor are illustrated in Fig. 4. The separation factor shows a maximum around pH 6 for dansyl serine and dansyl lysine, whereas the separation factor of dansyl aspartic acid increases with increasing pH. Thus, the following experiments were carried out at pH 6–7. The variation of the capacity factor with pH depends on

the acidity of the tested solutes. Thus, the curve corresponding to dansyl aspartic acid is characteristic of acidic compounds.

Table I shows the results of enantiomeric separations of dansyl amino acids with 12.5 m*M* β-cyclodextrin. Enantiomers of all examined dansyl amino acids except tryptophan could be resolved with a separation factor of 1.05–1.12. The indole group as well as the dansyl group of tryptophan may form inclusion complexes with β-cyclodextrin. This prevents the resolution of the enantiomers. Amino acids with an aliphatic branched-chain substitution group show a larger separation factor, than those with a straight-chain group. The largest separation factor is observed for dansyl threonine.

Fig. 5 demonstrates the isocratic separation of eleven pairs of enantiomers of dansyl amino acids. The D isomer of each dansyl amino acid elutes before the L isomer, owing to the difference in the stabilities of the inclusion complexes. The elution time can be decreased by gradient separation.

Fig. 6 shows the separation of diastereomers of dansyl isoleucine. Isoleucine has two chiral centres, and the four peaks due to diastereomers are separated (Kit No. DLAA-24). Dansyl D and dansyl L isomers prepared in our laboratory gave a single peak, represented by "d" or "l".

Gradient separation of twelve pairs of dansyl amino acids is demonstrated. The separability and separation time are improved by gradient elution.

CONCLUSION

Enantiomeric resolution of dansyl amino acids by micro HPLC using β-cyclodextrin as the mobile phase additive was successfully demonstrated. Twelve pairs of enantiomers were separated in a single chromatographic run. The use of β-cyclodextrin as well as α- and γ-cyclodextrin as the mobile phase additive will extend the applicability of this technique to the chiral separation in HPLC.

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Note

Coupled high-performance liquid chromatography–capillary gas chromatography as a replacement for gas chromatography–mass spectrometry in the determination of diethylstilbestrol in bovine urine

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In many instances it is desirable to replace coupled gas chromatography–mass spectrometry (GC–MS) by cheaper methods more suitable for the routine analyses of large numbers of samples, which implies first of all automation. GC–MS is mainly used for two reasons: first, if an analysis requires highly specific detection owing to presence of interfering peaks, *i.e.*, if the sample clean-up does not isolate the compound(s) of interest sufficiently well from the remainder of the sample; second, if the available GC detection techniques, *e.g.*, flame ionization detection (FID), do not provide sufficient sensitivity. Selected ion monitoring (SIM) in MS enhances the sensitivity compared with FID by at least one order of magnitude.

Coupled high-performance liquid chromatography–gas chromatography (HPLC–GC) involving standard GC detectors is attractive for replacing GC–MS, as the highly efficient sample clean-up by HPLC often readily allows the two requirements described to be fulfilled: first, less selective detectors are suitable because of fewer interfering components and second, a larger aliquot of sample can be injected owing to the reduced intensity of sample matrix effects (interfering peaks, "dirt" effects), which enables the detection limits to be lowered.

The determination of diethylstilbestrol (DES) in bovine urine (in many countries the use of DES for accelerating growth is illegal) is a typical example of applications where in many laboratories large numbers of analyses are carried out by GC–MS, *e.g.*, according to ref. 1. Screening methods that circumvent GC–MS are available for this particular analysis, such as radioimmunoassay (RIA)[2] or pure HPLC methods (*e.g.*, ref. 3). Nevertheless, the analysis of DES was selected for experiments with HPLC–GC as it was felt that this method would be highly competitive with the existing methods. Particular emphasis was placed on a comparison of the achievable sensitivity with that obtained in our laboratory using the same sample preparation followed by GC–MS, whereby none of the methods was pushed beyond the routinely readily available to the ultimately possible sensitivity.

EXPERIMENTAL

Sample preparation

A 10-ml volume of bovine urine was hydrolysed enzymatically with

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glucuronidase–sulphatase overnight at 40°C. The hydrolysed sample was loaded on to a Sep-Pak column, rinsed with 10 ml of 40% methanol and eluted with 3 ml of peroxide-free tetrahydrofuran (THF). The THF was evaporated and the residue was dissolved in diethyl ether and dried with anhydrous sodium sulphate. The dipentafluorobenzyl ether derivative was prepared according to ref. 4 using pentafluorobenzyl bromide. The sample was evaporated to dryness and the residue dissolved in 100 μl of cyclohexane–1% THF.

HPLC separation

A 100 × 3 mm I.D. glass column was used, packed with Spherisorb S-5-W. Cyclohexane–1% THF was used as the eluent at a flow-rate of 260 μl/min. The derivatized DES was detected at 230 nm.

HPLC–GC interface

The outlet of the HPLC detector was mounted in a four-port switching valve as described in ref. 5, allowing direct introduction of the eluent into the inlet of the retention gap. The internal volume of the connection between the HPLC detector cell and the switching point was 30 μl, causing the arrival of the detected solute material at the switching point to be delayed by 6 s.

GC system

A 15 m × 0.32 mm I.D. glass capillary coated with OV-73 of 0.25 μm film thickness was fused[6] with a 50 m × 0.32 mm I.D. retention gap of glass, silylated with hexamethyldisilazane. The carrier gas (hydrogen) inlet pressure was 1.6 atm. Sample introduction occurred at 80°C, followed by solvent evaporation at 100°C (for optimization of solvent evaporation, see ref. 7) and ballistic heating to 180°C. DES eluted at 270°C after programming the oven temperature at 4°C/min.

Detector by-passing

A system resembling that proposed by Schomburg[8] was used to prevent passage of the solvent vapour through the electron-capture detector. The detector (ECD 20, Carlo Erba) was modified to allow the make-up gas (nitrogen at 40 ml/min) to enter from the top of the detector and to flush the vapours eluted from the column exit away from the detector cell into the base of the detector block and through the line usually used as the inlet for the make-up gas to the atmosphere. This was achieved by feeding the make-up gas through a four-port valve (Valco) either into the detector base as usual (for detection of solutes) or into the top of the detector (by-passing solvent).

The system avoiding passage of the solvent vapour through the detector was constructed because of concern about contaminating the detector cell. Later, dozens of injections were carried out, each time directly introducing the vapour of about 300 μl of HPLC eluent into the detector. Detector performance was not affected noticeably. The question of whether large volumes of solvent vapour cause deterioration of electron-capture detectors is still open.

Procedure

The HPLC system was optimized to elute the DES fraction with an eluent

volume of 300 μl (carried out with pure standard). Then the sample was analysed on the HPLC system under relatively high attenuation of the detector to obtain a reasonable chromatogram of the sample matrix (for a typical sample as shown in Fig. 1). This sample was spiked with a fairly large amount of DES for determining the relevant fraction in the chromatogram. The DES fraction transferred into the GC system was cut according to the HPLC trace rather than trusting in the stability of absolute retention times (a problem primarily caused by instability of the eluent flow-rate).

The GC conditions were optimized in parallel, injecting DES on-column with a syringe (as the retention gap is mounted into the on-column injector also in coupled HPLC–GC, it is sufficient to take the transfer capillary out of the injector to allow normal on-column injection with a syringe). The transfer efficiency and the overall yields were tested by comparison of the peak areas obtained by directly injecting 1 ng of DES into the GC system, injecting the same amount of DES into the HPLC system followed by transfer into the GC system and adding it to the sample before working it up. Yields were around 95 and 70% respectively.

For introduction of the DES fraction into the GC system, the valve in the transfer line was switched 6 s after the appearance of the critical point in the HPLC trace. Transfer of the fraction took 70 s.

The HPLC column was reconditioned during the GC analysis (lasting *ca.* 45 min, including cooling of the oven at the end of the analysis), using 10% THF–cyclohexane. After about 30 injections of samples the column was cleaned using THF alone.

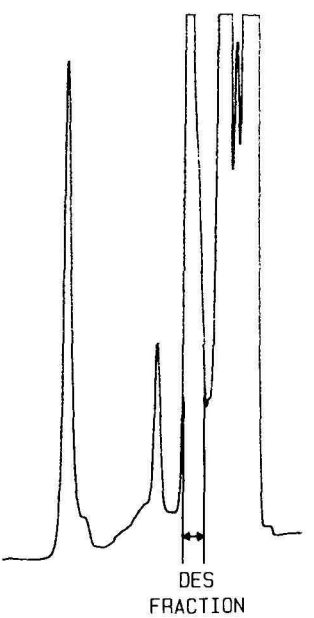

Fig. 1. HPLC trace of a typical sample after derivatization of DES to the dipentafluorobenzyl ether. UV detection at 230 nm. The transferred DES fraction almost corresponds to a peak in the HPLC trace but, of course, this peak does not represent DES.

RESULTS

Experiments involving FID

Initially, experiments were carried out using FID and preparing the heptafluorobutyro derivative of DES. These experiments provided interesting information on the sensitivity achieved with FID. However, decomposition of the derivative in the HPLC column after injection of a few samples caused severe problems, which prompted us to prepare another derivative (one of the not very common examples where a compound is perfectly stable in GC but not in HPLC!).

Fig. 2 shows the chromatogram of a sample free from DES obtained with FID. The position and height of a peak corresponding to 10 ppb* of DES, added to the same sample before the clean-up, was transferred from another chromatogram and is shown as a bar. The practical detection limit was *ca.* 3 ppb (1–4 ppb for other samples using the same criteria). This sensitivity was achieved injecting a 10-μl vol-

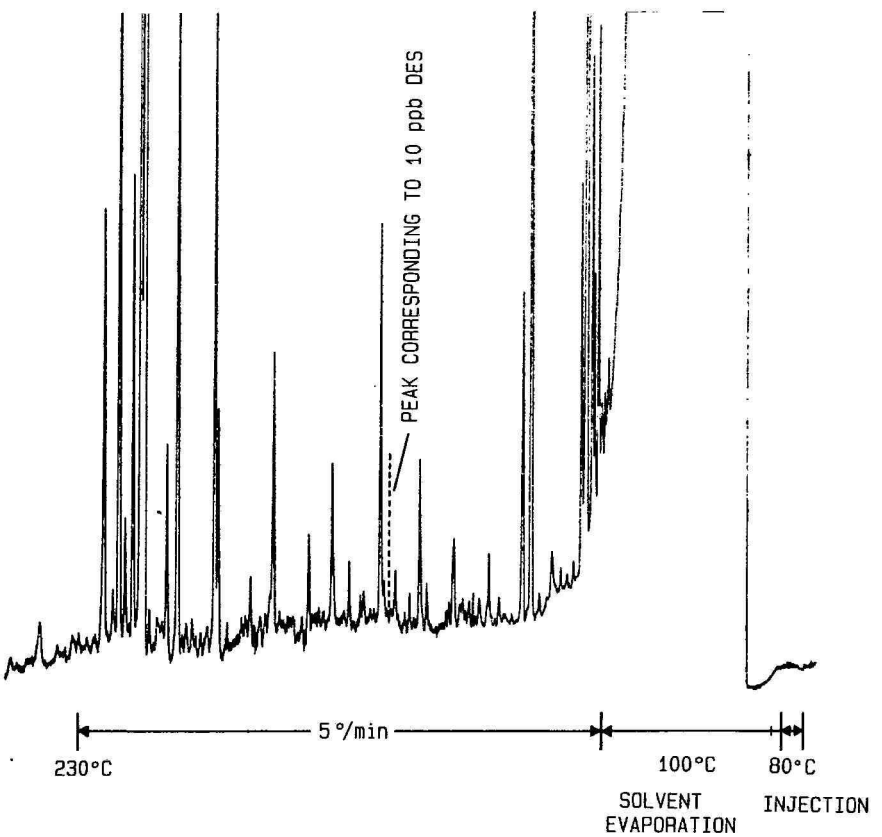

Fig. 2. FID chromatogram of the DES fraction obtained after formation of the heptafluorobutyro derivative of DES. The position and height of the (main) peak corresponding to 10 ppb of DES in urine are shown.

* Throughout this article the American billion (10^9) is meant.

ume from the 100-μl volume of prepared sample into the HPLC (a 3 ppb concentration corresponding to 3 ng of DES). This volume could easily be increased to 30–50 μl, but the reduction in the detection limit is small, and the smaller injection volume was preferred in order to reduce the load of sample by-products on the HPLC column to a minimum.

The level of sensitivity obtained with FID is similar to that routinely obtained by GC–MS using the same sample preparation except that the bis(trimethylsilyl) derivative of DES is prepared. Of course, the amounts of sample material injected were different (as the absolute sensitivity of MS is higher than that of FID). For HPLC–GC an extract corresponding to 1 ml of urine was injected (10% of the extract from a 10-ml volume of sample). For GC–MS only the extract of about 200 μl of

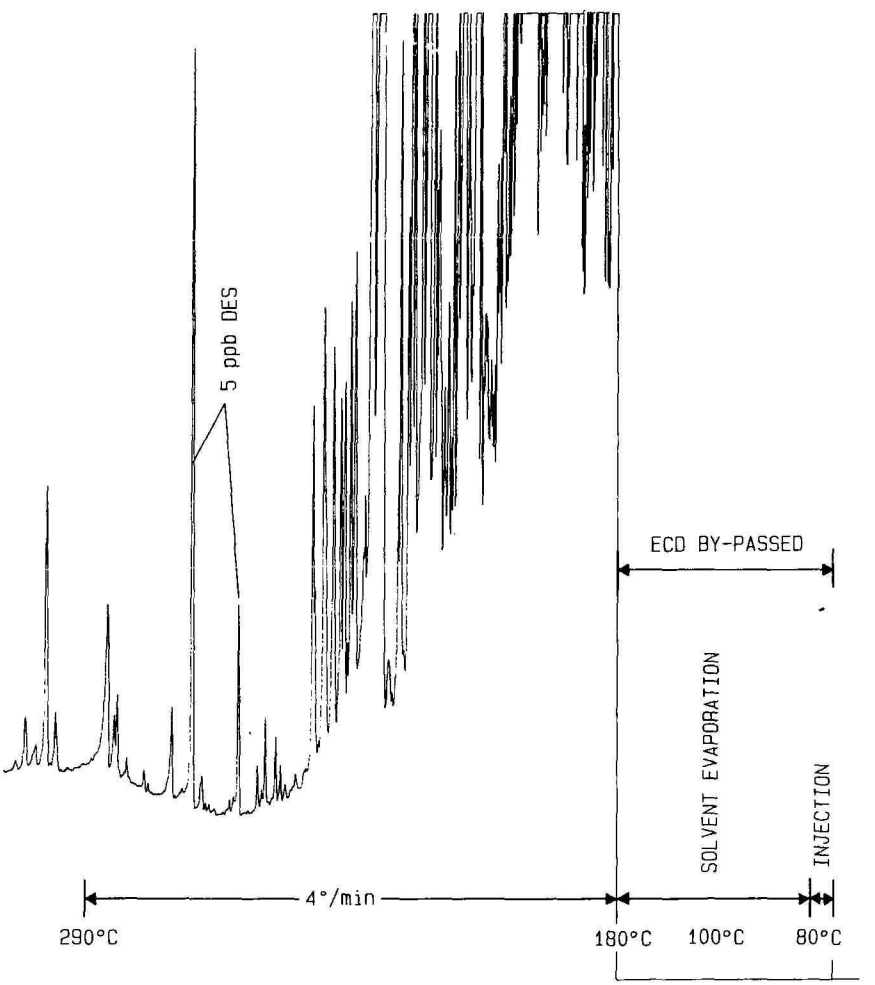

Fig. 3. ECD chromatogram of the DES fraction from HPLC, involving the pentafluorobenzyl ether derivative of the two isomers of DES. Sample spiked with 5 ppb of DES before sample preparation. During passage of the solvent vapour through the column, the column eluent was driven away from the detector cell through the line usually used for feeding the make-up gas into the detector.

urine could be injected (2 μl from 100 μl of extract). Injection of larger aliquots of sample into the GC–MS system increased the absolute peak sizes, but did not improve the detection limits owing to the simultaneously amplified sample matrix.

Experiments involving electron-capture detection (ECD)

The experiments involving ECD were carried out using the pentafluorobenzyl ether derivative of DES, as described under Experimental. The chromatogram of a typical sample spiked with 5 ppb of DES is shown in Fig. 3. The practical detection limits varied between about 0.1 and 0.3 ppb, depending on the urine analysed. There is even some room for improvement if a higher sensitivity is desirable: the injection volume (10 μl) could be increased several-fold. The detector attenuation was high, although the detector showed only about a fifth of the sensitivity of a new instrument.

DISCUSSION

HPLC–GC is an attractive method for routine analyses of trace components in complex samples as it substantially decreases the detection limits through a far more efficient isolation of the fraction of interest. It improves the sensitivity achievable primarily by permitting analyses of larger aliquots of sample (allowing the injection of larger proportions, *e.g.*, of a sample extract).

The analysis of DES by HPLC–GC–FID provided a similar sensitivity to GC–MS. This might turn out to be a generally valid rule of thumb for all kinds of samples where the sample matrix determines the practical detection limits and for which no clean-up has been carried out involving pre-separation steps of an efficiency comparable to that of HPLC.

Using ECD, the sensitivity of the HPLC–GC method exceeded that obtained by GC–MS by at least a factor of ten. At a 0.1 ppb level of DES in urine an amount of 0.1–0.2 ng of solute reached the detector, which is far from being at the limit of sensitivity of this detector.

Of course, coupled HPLC–GC–MS would be the method of choice regarding sensitivity and specificity of detection. The sensitivity obtained by HPLC–GC–MS would correspond approximately to that of HPLC–GC–ECD, but the information obtained about the solute represented by the peak at the correct retention time is more specific when detected by MS.

Another potential aspect of HPLC–GC did not materialize during our work, *viz.*, automation of the procedure. It should be easy to build a system that allows relatively dirty samples to be loaded into an HPLC autosampler and to obtain GC results automatically. Automation of the transfer step requires a power valve and a timer and has been realized by Cortes *et al.*[9].

The work described here was carried out using a long retention gap according to the concept of negligible solvent evaporation during sample introduction[10]. Simultaneously we worked on a method of introducing the HPLC eluent through a sample loop, involving concurrent solvent evaporation[11]. This method is restricted to solutes eluted at elevated column temperatures (above about 150°C), but is very convenient and allows the introduction of considerably larger eluent volumes.

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Note

High-performance size-exclusion chromatography of bovine somatotropin*

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High-performance size-exclusion chromatography (HPSEC) has been applied by numerous researchers to the separation of proteins[1–7]. Recent improvements in column packing have provided very efficient columns[8–10].

Development of a process for producing bovine somatotropin (bSt) in *E. coli* modified by recombinant DNA techniques (rbSt) prompted a need for a HPSEC method for estimation of levels of high-molecular-weight protein components in rbSt preparations. This report summarizes a survey of HPSEC conditions studied.

EXPERIMENTAL

Materials

Water was distilled and deionized. Analytical-grade reagents (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used throughout. Ultrapure guanidine hydrochloride (GnHCl) (Schwartz/Mann, Cambridge, MA, U.S.A.) and sodium dodecylsulfate (SDS) (Bio-Rad, Richmond, CA, U.S.A.) were used as denaturing agents. Molecular weight standards were obtained from Sigma (St. Louis, MO, U.S.A.), rbSt samples from Upjohn sources and pituitary bSt (pbSt) from A. F. Parlow (Harbour Medical Center, UCLA, Los Angeles, CA, U.S.A.).

Chromatography

Modular HPLC systems consisting of combinations of the following components were used (see figure captions for other details). Columns included DuPont GF-250 (DuPont, Wilmington, DE, U.S.A.), TSK 3000SW (Toyo Soda, Tokyo, Japan) and Waters I-125 (Waters Assoc., Milford, MA, U.S.A.). A Varian Vista 5500 HPLC system (Varian, Walnut Creek, CA, U.S.A.) or a Perkin-Elmer Series 10 pump (Perkin Elmer, Norwalk, CT, U.S.A.) were used. Detection was accomplished using an LDC 1203 UV monitor (Laboratory Data Control, Riviera Beach, FL, U.S.A.) with a 214-nm kit or 280-nm filter. Samples and standards were prepared in mobile phase typically at 1.0 mg/ml with subsequent 100-μl injection using a Rheodyne 7125 valve (Rheodyne, Cotate, CA, U.S.A.). Chromatographic data was collected and

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analyzed on an in-house VAX-based computer system (Digital Equipment, Merrimack, NH, U.S.A.).

RESULTS AND DISCUSSION

Two classes of high-molecular-weight protein components were of concern. First, oligomeric or aggregated forms of bSt may be present in bSt preparations due to covalent or non-covalent interactions of bSt with itself[7,11–12]. Secondly, proteins from the host bacteria used for rbSt production[13] or bovine proteins from the purification of pbSt[11] may be present as process impurities. We chose HPSEC columns designed to separate in the range of about 5000–150 000 molecular weight[14–16], to examine bSt [molecular weight = 21 816 (ref. 17)] for the presence of dimeric, trimeric and oligomeric bSt species in particular. Our studies focused mainly on the use of HPSEC systems which utilize mobile phases containing denaturing agents[3,7–9]. The column/mobile phase combinations were; GF-250/6 *M* GnHCl (Fig. 1), GF-250/0.1% SDS (Fig. 2), TSK 3000SW/6 *M* GnHCl (Fig. 3), and I-125/0.1% SDS (Fig. 4). Each of these systems separate dimer and higher-molecular-weight oligomers from bSt monomer. The GF-250 column proved to be the most efficient with about 4200 plates calculated for rbSt monomer using the band width at half-height method[18]. The TSK 3000SW and Waters I-125 column exhibited theoretical plate values for rbSt monomer of 3700 and 2900, respectively. We use the GF-250 column with either GnHCl or SDS mobile phases for routine studies of rbSt based on this superior peak shape and resolution. Coupling two GF-250 columns provided slightly better resolution of bSt oligomers from bSt monomer at the expense of doubling the separation time (*cf.* Figs. 1 and 2).

We also attempted to use the GF-250 column with a simple, non-denaturing buffer mobile phase in an attempt to study bSt in its native conformation[13]. Oligomers were also resolved on this system (Fig. 5), but significant portions of the rbSt samples did not dissolve in this buffer. Dilute ammonia was used to prepare samples

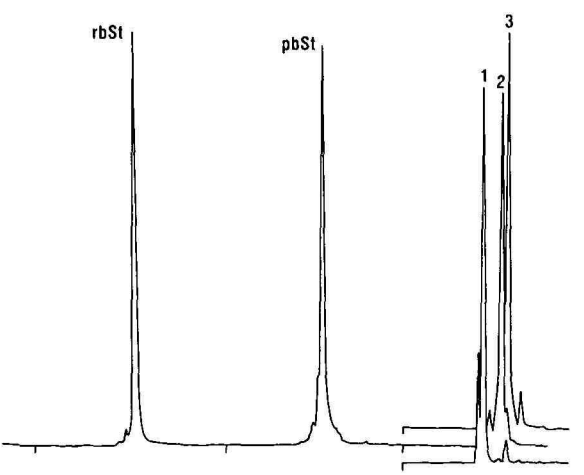

Fig. 1. Chromatograms obtained on one DuPont GF-250 column. Mobile phase, 6*M* GnHCl, 0.2 *M* sodium phosphate, pH 7.0; detection, 214 nm; flow-rate, 2.0 ml/min. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A. The retention times of peaks 1, 2, bSt and 3 were 3.40, 4.04, 4.32 and 4.40 min respectively.

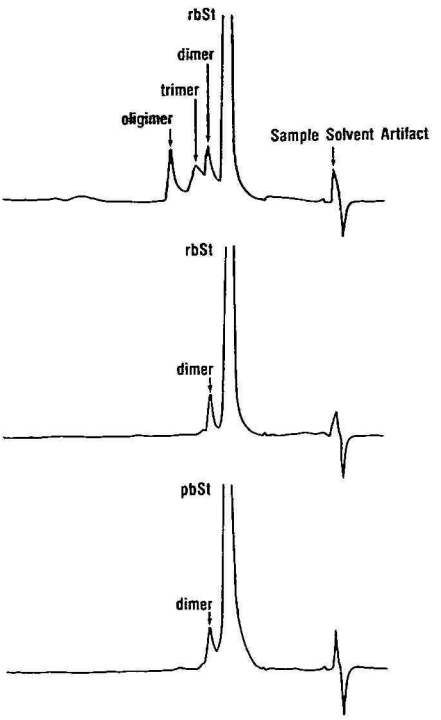

Fig. 2. Chromatograms obtained on two DuPont GF-250 columns in series. Mobile phase: 0.1% SDS, 0.1 *M* NaCl, 0.067 *M* monobasic sodium phosphate, 0.3 *M* Gly at pH 7.5; detection: 280 nm; flow-rate: 1.0 ml/min. The retention times of oligomer, trimer, olimer and bSt were 11.9, 13.9, 14.9 and 16.3 min respectively.

for injection onto the GF-250/SDS system, because the apparent dimer content doubled over 48 h when samples were prepared in ammonium bicarbonate buffers. Exposure of the GF-250 column to SDS required dedication of the column to use with this denaturing agent because SDS cannot be easily rinsed from the column[13].

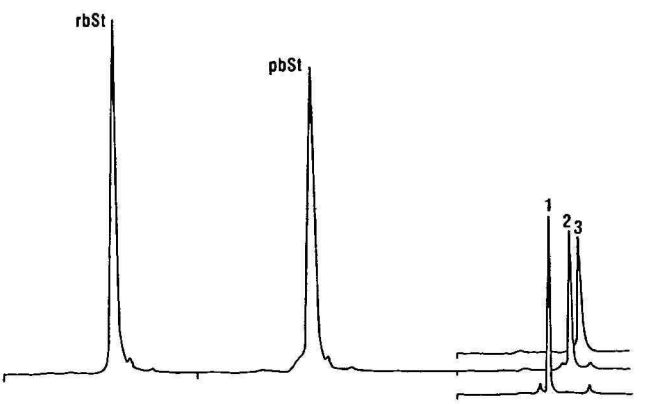

Fig. 3. Chromatograms obtained on TSK 3000SW column. Conditions as in Fig. 2. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A. The retention times of peaks 1, bSt, 2 and 3 were 7.45, 9.75, 9.98 and 10.88 min respectively.

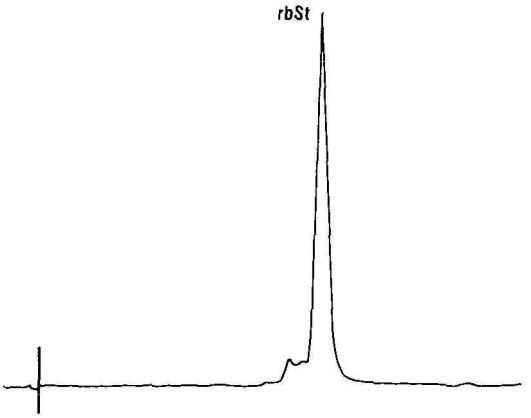

Fig. 4. Chromatograms obtained on Waters I-125 column. Conditions as in Fig. 2. The retention time of bSt was 6.5 min.

The apparent molecular weights of bSt monomer and other components were determined based on a protein standard calibration plot as shown in Fig. 6. Seven lots of bSt were examined using the GF-250/6 *M* GnHCl system and found to have apparent molecular weights ranging from 24 600 to 26 100. Precision of 0.2% relative standard deviation (R.S.D.) for molecular weight determination on five replicate injections of the same lot of material was obtained.

Recovery of bSt from the GF-250/6 *M* GnHCl system was determined to be 100% based on a comparison of 220-nm absorbance of samples before and after elution. The peak area response for bSt monomer at 214 nm for GF-250/6 *M* GnHCl and at 280 nm for GF-250/SDS was shown to be linear and to have no significant bias across the range of 0.1–1.0 mg bSt/ml in the sample preparation. Multiple in-

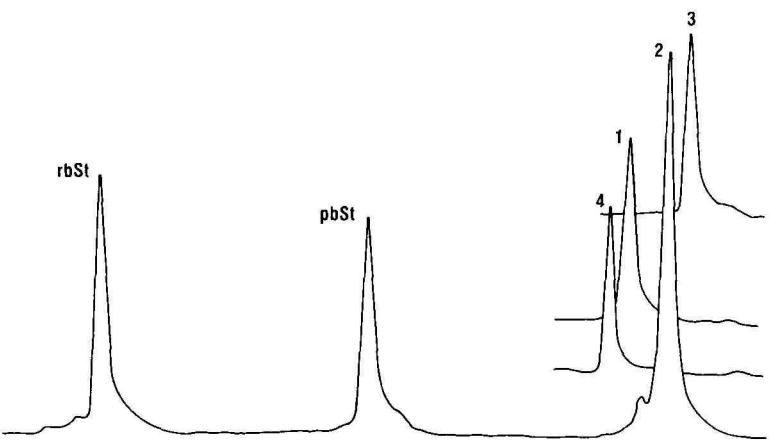

Fig. 5. Chromatograms obtained on one DuPont GF-250 column. Mobile phase: 0.2 *M* sodium phosphate, pH 6.8; detection: 214 nm; flow-rate: 1.0 ml/min. 1 = Bovine serum albumin; 2 = trypsinogen; 3 = ribonuclease A; 4 = phosphorylase A. Note: portions of bSt preparations are *not* soluble in this mobile phase. The retention times of peaks 4, 1, bSt, 2 and 3 were 6.6, 7.8, 10.1, 10.3 and 11.9 min respectively.

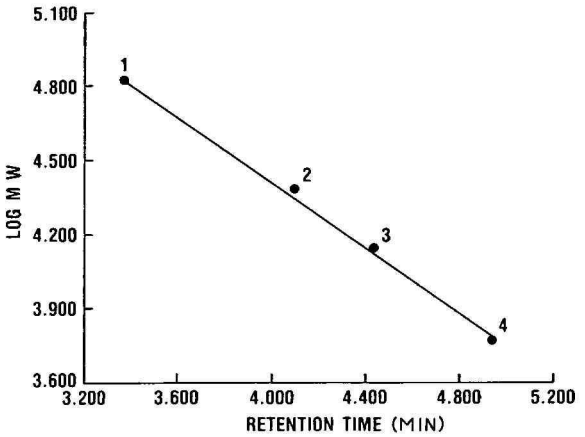

Fig. 6. Molecular weight (MW) calibration plot for DuPont GF-250 column with pH 7.0/6 *M* GnHCl mobile phase. *y*-Intercept = 7.15; slope = 0.685; correlation coefficient = 0.998. Standards: 1 = albumin (MW 66 000); 2 = trypsinogen (MW 24 000); 3 = ribonuclease A (MW 13 700); 4 = insulin (MW 5700).

jections of preparations demonstrated that the precision of quantitation of monomer was 0.5–2% R.S.D. for monomer levels of 80% (w/w) or higher and about 1.5–10% R.S.D. for dimer, trimer or oligomer levels below 20% (w/w).

CONCLUSION

HPSEC using a denaturing mobile phase and the GF-250 column provided a method for determining levels of impurities, *i.e.* non-bSt monomer, and estimating apparent molecular weights of monomer and bSt oligomers in bSt preparations.

ACKNOWLEDGEMENTS

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Note

Determination of phenoxy acid herbicides using solid-phase extraction and high-performance liquid chromatography*

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(First received December 13th, 1985; revised manuscript received February 8th, 1986)

Phenoxy acid herbicides are extensively used for weed control and ultimately find their way into lakes, streams and groundwater. Presently, the accepted procedure for determining 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2-(2,4,5-trichlorophenoxy)propionic acid (silvex) involves hydrolysis, extraction, and esterification prior to analysis by gas chromatography[1]. The main disadvantages of this method are the time-consuming sample preparation procedure and the hazards associated with handling organic solvents.

The recent introduction of solid-phase extraction materials is rapidly eliminating the need for liquid–liquid extraction in many procedures[2–5]. This paper describes a procedure for determining phenoxy acid herbicides using a Baker-10 SPE®** system for sample concentration and a high-performance liquid chromatograph for separation and quantitation.

EXPERIMENTAL

Apparatus

A Baker-10 SPE system was used to concentrate herbicide samples onto a Baker C_{18} HC (high capacity) solid-phase extraction column obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Samples were analyzed on a Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatograph equipped with two Model M6000A pumps, a Model M450 variable-wavelength detector, a WISP 710B automatic sampler, a Model 720 systems controller and a Model 730 data module. The column used was a 25 cm × 4.6 mm I.D. Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.) with a particle size of 6 μm.

Reagents

All herbicide esters were obtained from the Pesticides & Industrial Chemicals Repository [U.S. Environmental Protection Agency (USEPA), Research Triangle

* The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense (Para. 4-3, AR 360-5)

** Use of trademarked name does not imply endorsement by the US Army, but is used only to assist in identification of a specific product.

Park, NC, U.S.A.]. The herbicide-free acids were obtained from Chem Services (West Chester, PA, U.S.A.). Methanol was of UV grade obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid was of reagent grade obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Water used for preparation of solutions was first distilled and then passed through a Millipore Milli-Q (Bedford, MA, U.S.A.) water purification system.

Procedure

Stock solutions of the free herbicide acids and the esters were prepared in methanol at approximately 1000 mg/l. Serial dilutions of the herbicide acids were prepared for calibrating the HPLC system. Esters of the herbicides were prepared similarly and diluted to the range 10–250 μg/l with water. A 50-ml water sample, spiked with the appropriate ester, was hydrolyzed at pH 11 for 1 h with sodium hydroxide. The pH was then adjusted to 2.5 with concentrated hydrochloric acid, and the sample was passed through a C_8 or C_{18} SPE column at a rate of 3–5 ml/min. Two 1-ml portions of methanol were used to elute the herbicides from the column. The eluate was diluted to 5 ml with distilled/deionized water to achieve a sample concentration factor of 10. The sample's solvent strength, therefore, was weak enough to permit a large injection volume. The HPLC conditions were as follows: mobile phase, methanol–1% aqueous acetic acid (68:32); flow-rate, 1.2 ml/min; detector wavelength, 280 nm (0.005 a.u.f.s.); injection volume, 200 μl.

RESULTS AND DISCUSSION

Early experiments indicated that the methyl esters of 2,4-D, 2,4,5-T and silvex

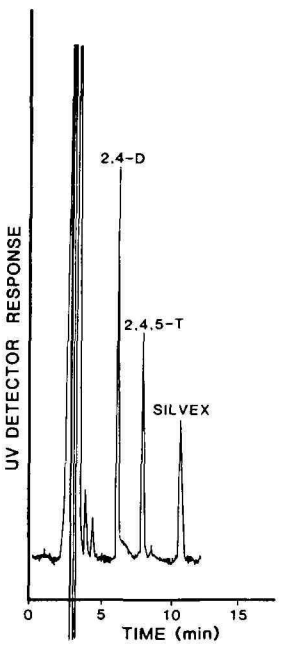

Fig. 1. High-performance liquid chromatogram of 2,4-D, 2,4,5-T and silvex standards at approximately 0.4 mg/l.

were hydrolyzed almost instantaneously at room temperature and pH 11. The 2-ethylhexyl ester of 2,4-D was also hydrolyzed in a few minutes; therefore, all samples were adjusted to pH 11, stirred and arbitrarily allowed to stand for 1 h to ensure complete hydrolysis.

Although the C_8 and C_{18} SPE columns showed comparable recoveries for the herbicides, the C_{18} HC columns were chosen because of recommendations by the supplier. Columns are normally prepared for extraction by passing 5 ml of methanol through, followed by 5 ml of water. For this study, a much cleaner baseline was obtained when the columns were first treated with 5 ml of acetone followed by the 5 ml of methanol and 5 ml of water. We have also found that columns could be re-used following this cleanup procedure with no apparent effect on their performance.

The two absorption maxima of these herbicides were both considered for UV detection. Although 235 nm gave the highest sensitivity, 280 nm was used because it produced the cleanest baseline. A typical chromatogram of the three herbicides is shown in Fig. 1.

TABLE I

RECOVERY OF PHENOXY ACID HERBICIDES FROM WATER USING 6-ml HC C_{18} SPE COLUMNS

M = Milli-Q water; R = Monocacy River water; S = Carroll Creek tributary water.

Herbicide	*Level of spike (μg/l)*	*Recovery (%)*	*R.S.D. (%) (n = 7)*	*Matrix**
2,4-D	20**	80	5.1	M
	200**	105	2.6	M
	15	29	11.4	R
	15	41	11.8	S
	50	55	1.1	R
	50	54	6.7	S
	250	63	1.9	R
	250	63	3.2	S
2,4,5-T	20**	100	6.8	M
	200**	99	3.4	M
	15	63	8.1	R
	15	53	10.6	S
	50	62	2.5	R
	50	61	5.6	S
	250	69	3.2	R
	250	68	4.6	S
Silvex	20**	100	7.9	M
	200**	95	5.0	M
	15	70	6.8	R
	15	74	10.7	S
	50	74	3.8	R
	50	74	4.4	S
	250	76	4.3	R
	250	74	6.0	S

* R and S passed through effective 1.67-μm filter prior to spiking.

** Free acids.

Sample preparation time was approximately 90 min for 15 samples in addition to 1 h for hydrolysis.

Compounds such as dicamba, chlorophenol and phenol did not interfere with the method.

Table I demonstrates the performance of the method when distilled water and local environmental water samples were spiked with each of the three herbicides. River water samples were very turbid, whereas the creek water samples were clear. The detection limit for each of the herbicides was approximately 10 μg/l which represented an observable peak of twice the level of the background noise. In tests involving distilled water, good recoveries and precision were obtained below the 100 μg/l drinking water limits set by the USEPA[6].

Evidently the herbicides were tightly bound to trace organics, when environmental water was used, as evidenced by the lower recoveries. Passing the sample through a 0.45-μm filter prior to spiking did not improve recovery. No herbicides were recovered from a second SPE column placed beneath the first. The addition of sodium chloride (300 g/l) to the sample, prior to passing it through the SPE column, did not produce an overall significant increase in recovery.

Although recoveries are somewhat low for environmental waters, this analytical method has sufficient sensitivity to be of value as a rapid scanning technique for the presence of these herbicides in drinking water. It is also less hazardous, easier to perform and less time consuming than the existing recommended gas chromatography method.

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Note

High-performance liquid chromatographic method for the simultaneous separation and determination of three additives in poly(vinyl chloride)

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(Received December 30th, 1985)

The identification and determination of low-molecular-weight components such as plasticizers and stabilizers incorporated in polymers is of great importance, particularly when the polymers are intended for medical use. *In situ* identification is often feasible if the number of additives is restricted to one or two, but the analysis becomes extremely complex as the number of additives incorporated in the polymer matrix increases. In such instances, separation by selective solvent extraction or repeated fractional precipitation, which unfortunately are tedious and lengthy, is necessary[1,2]. The use of high-performance liquid chromatographic (HPLC) methods, however, has simplified the analysis of polymer additives considerably[3] and, in recent years, chromatographic methods including size exclusion procedures have been used extensively for the analysis of common polymer additives[4–7].

This paper reports an attempt to develop a simple, rapid, HPLC method for separating and determining simultaneously three additives commonly used in poly(vinyl chloride) (PVC).

EXPERIMENTAL

The polymer additives, di-2-(ethylhexyl) phthalate (DEHP), epoxidized soyabean oil (Paraplex G62) and tris(nonylphenyl) phosphite (TNPP), were obtained from Indo-Nippon (Bombay, India) and used as received. Analytical-reagent grade carbon tetrachloride, dichloromethane and tetrahydrofuran (BDH, India) were distilled prior to use.

The chromatographic system consisted of a Waters Assoc. Model 6000A solvent delivery pump, a U6K injector, a Model 440 absorbance detector and an R-400 refractive index detector. A strip-chart recorder (Houston Instruments, U.S.A.) was used. A μPorasil column with carbon tetrachloride–dichloromethane (65:35, v/v) as the mobile phase was used for the separation. The flow-rate was 1 ml/min and the column effluents were monitored simultaneously with a UV detector (280 nm) and a refractive index detector.

Indigenously calendered PVC films containing the three additives (2% of each) were dissolved in tetrahydrofuran and the polymer was precipitated by adding meth-

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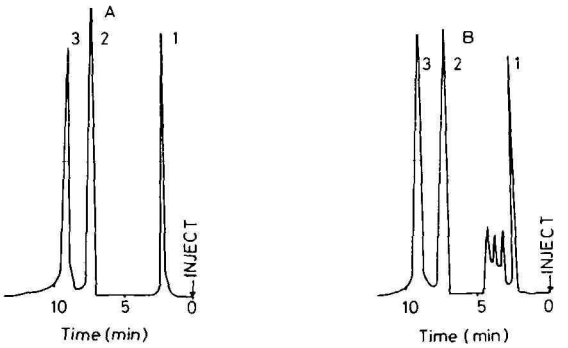

Fig. 1. (A) Chromatogram of a standard solution of three additives. Peaks: 1 = Paraplex G62; 2 = DEHP; 3 = TNPP. (B) Typical chromatogram of PVC extract. Peaks as in (A).

anol. The filtrate was evaporated to dryness in a vacuum oven. The residue was dissolved in carbon tetrachloride–dichloromethane (65:35, v/v) and used for the chromatographic analysis.

RESULTS AND DISCUSSION

Fig. 1A is a chromatogram showing the peaks of Paraplex G62, DEHP and TNPP, and a typical chromatogram of the same components extracted from the PVC sample is illustrated in Fig. 1B. The extra peaks in Fig. 1B are unidentified impurities and probably arose from the PVC resin.

Paraplex G62 (peak 1) is epoxidized soyabean oil, consisting of a mixture of components of various chain lengths. Under the chromatographic conditions used the additive is unretained and elutes together as a single peak. However, the detector response was linear with the amount of the additive in the mobile phase.

Calibration graphs of the detector response (for DEHP and TNPP 280 nm was used for Paraplex G62 a refractive index detector was used) *versus* concentration in the injected volumes of each of the samples were constructed. Quantification of the additives extracted from the PVC films was subsequently achieved from these plots. The recoveries of the additives and the corresponding retention times are shown in Table I.

Typical additive levels in PVC, excluding plasticizer, are 0.1–1% (w/w)[7]. The

TABLE I

CHROMATOGRAPHIC DATA FOR THE THREE ADDITIVES

Sample	*Retention time (min)*	*Recovery* (%)*
Paraplex G62	2.50	94.2 ± 3.1
DEHP	7.75	97.6 ± 3.8
TNPP	9.80	96.7 ± 2.9

* Average values from ten analyses with standard deviations.

involvement of many steps in the usual extraction procedures leads to further reductions in the amounts of the additives and, for reliable detection, a sensitive method is needed.

The limits of detection of the present method are 15 μg per 100 μl for DEHP, 10 μg per 100 μl for TNPP and 50 μg per 100 μl for Paraplex G62. These limits are sufficient for determination at the levels usually present.

Apart from having the required sensitivity, the present method substantially reduces the analysis time. Moreover, it offers a comparatively simple and rapid method, excluding the usual tedious extraction procedures, and could be extended to the routine analysis of commercially available polymers containing these additives.

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CHROM. 18 480

Note

Salting-out chromatography on unsubstituted Sepharose CL-6B as a convenient method for purifying proteins from dilute crude extracts

Application to horseradish peroxidase

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The purification of proteins from dilute crude extracts is often a difficult task in large-scale purification procedures. Ammonium sulphate precipitation is not convenient as it necessitates high-speed centrifugation of large volumes of sample. Ultrafiltration is relatively fast and does not concentrate salts and other low-molecular-weight substances, but expensive equipment is needed. For these reasons the absorption of the proteins on ion exchangers or on affinity materials, batchwise or in a column procedure, is the method used in many situations. However, this is not always possible because of the unavailability of specific affinity materials, their high cost and leakage of ligand and also because of the presence in the crude extract of large amounts of salt.

A technique that can be used irrespective of the protein and the composition of the extract is hydrophobic chromatography. In many instances, however, the asbsorption is irreversible or the elution requires severe conditions (high concentration of urea, chaotropic salts), which inactivate the enzymes. We realized that salting-out chromatography on unsubstituted Sepharose[1,2] can be the method of choice. As the interactions are weaker, elution is effected simply by decreasing the ionic strength. Sepharose is inexpensive and ready available.

We report here studies on the purification of horseradish peroxidase as a model enzyme. Despite a negative report[3], it was found that this enzyme bound to Sepharose CL-6B. Stepwise elution yielded the enzyme with a purity of 30–40% in a volume 100-fold smaller that that of the extract in only one step.

EXPERIMENTAL

Sepharose 6B, Sephadex G-50 Fine and Sephadex G-25 Medium were purchased from Pharmacia (Uppsala, Sweden). All chemicals were of analytical-reagent grade. Sepharose 6B was cross-linked as described in ref. 4, omitting sodium borohydride from the reaction mixture.

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The peroxidase assay

The reaction medium contained, in a final volume of 1.0 ml, 20 m*M* sodium phosphate buffer (pH 6.0), 0.4 m*M* *o*-dianisidine and 1.0 m*M* hydrogen peroxide at 37°C. The increase in absorption at 436 nm was recorded using an Eppendorf Spectralline photometer. The purity of the enzyme was also monitored after gel filtration on Sephadex G-50 Fine by calculating the ratio of the absorbance at 405 nm and 280 nm (the *RZ* value). The pure enzyme has an *RZ* value of about 3.0 (ref. 5).

Preparation of the crude extract

The horseradish was obtained from the local market. The extract (about 1 l from every kilogram of horseradish), obtained as described[5], was brought to 55% ammonium sulphate saturation. After 24 h the clear solution was carefully siphoned and filtered through filter-paper. The enzyme activity was stable for at least 1 month at room temperature, but a considerable amount of polymeric brown pigment developed, which interfered with the desalting by gel filtration and partially bound to Sepharose CL-6B.

Purification procedure using the column experiment

A 1700-ml volume of extract containing ammonium sulphate at 55% saturation was pumped at a flow-rate of 60–100 ml/h through a Sepharose CL-6B column (7.0 × 2.2 cm), equilibrated with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 60% saturation. The column was washed with the starting buffer. The enzyme was eluted with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 30% saturation, with a flow-rate of 30 ml/h.

Purification procedure using the batchwise experiment

A 1700-ml volume of extract containing ammonium sulphate at 55% saturation was efficiently stirred with 40 ml of Sepharose CL-6B. After 30 min the concentration of ammonium sulphate was gradually increased to 65% saturation during 30 min by the addition of solid salt. The enzyme activity was more than 97% absorbed. The gel was poured into a chromatographic column and washed with 0.1 *M* phosphate buffer containing ammonium sulphate at 65% saturation. The enzyme was eluted with phosphate buffer containing ammonium sulphate at 30% saturation.

Further enzyme purification

The fractions containing the enzyme, obtained by either procedure, were pooled and the peroxidase was precipitated by dialysis against a saturated solution of ammonium sulphate. After centrifugation the pellet was disolved in 0.1 *M* phosphate buffer (pH 7.0) and desalted by gel filtration. The enzyme at this stage had an *RZ* value of 1.4–1.6. The enzyme solution was brought to 50% ammonium sulphate saturation by adding solid salt. The slight precipitate was removed by centrifugation. The enzyme was absorbed on an 8.0 × 2.2 cm column of Sepharose CL-6B equilibrated with 0.1 *M* phosphate buffer (pH 7.0) containing ammonium sulphate at 60% saturation and eluted with a linear gradient generated by mixing ammonium sulphate solutions of 60% and 30% saturation in 0.1 *M* phosphate buffer (pH 7.0). The volume of the gradient was about 400 ml and the flow-rate about 30 ml/min.

Recycling of the Sepharose CL-6B

The gel (yellow to brown in colour) was suspended in distilled water and sodium hydroxide was added to a final concentration of 0.1 *M*. After 1–2 days at room temperature it was washed with a large volume of distilled water and finally with the equilibration buffer.

RESULTS AND DISCUSSION

By using either the column or the batchwise procedure, the horseradish peroxidase could be recovered in a small volume, typically 100 times smaller than the volume of the extract. In both instances the *RZ* value was 1.3–1.5, *i.e.*, the purity of the enzyme was about 30–40%. The enzyme obtained by precipitation with ammonium sulphate between 55 and 80% saturation had an *RZ* value of only 0.6–0.7. Using the column procedure the yield was lower (50–55%) because as much as 25% of the enzyme activity was not bound to the column. The batchwise procedure was noteworthy in that the yield was over 80% and the experiment could be completed in only 4 h. For further purification, the enzyme was eluted with a decreasing gradient of ammonium sulphate from a Sepharose CL-6B column (Fig. 1); 75% of the enzyme activity applied to the column was recovered in the peak fractions having an *RZ* value of 2.35–2.70. The distribution of the various isozymes and the carbohydrate content were not investigated. The contaminating proteins are relatively uniformly

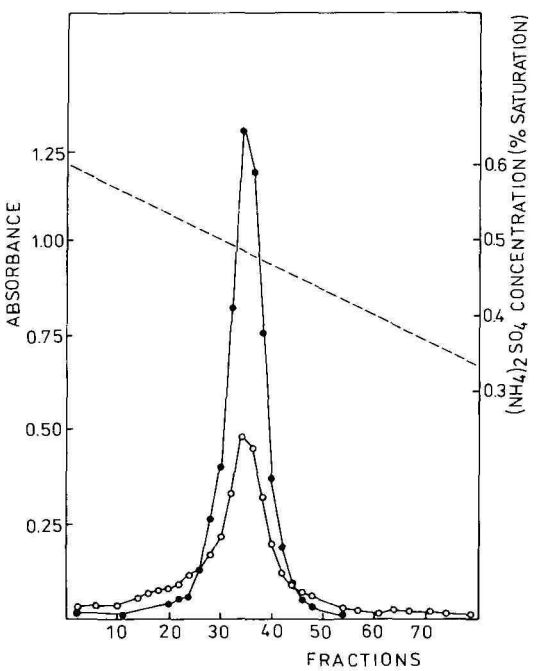

Fig. 1. Final purification of peroxidase by salting-out chromatography on Sepharose CL-6B. For details see Experimental. (○) Absorbance at 280 nm; (●) absorbance at 405 nm; (-------) slope of the ammonium sulphate gradient.

distributed so methods employing different physical properties of the enzyme are needed in order to obtain highly purified enzyme, *i.e.*, gel filtration on Sephadex G-75.

In a recent paper, Chavez and Flurkey[3] found no interaction between horseradish peroxidase and unsubstituted agarose. They used very low concentrations of enzyme and lower concentrations of ammonium sulphate that we did. Moreover, we used cross-linked Sepharose, which is probably slightly more hydrophobic than the non-cross-linked Sepharose.

The results demonstrate the usefulness of salting-out chromatography in concentrating and purifying proteins starting from crude extracts, particularly in batchwise procedures. Although the binding of proteins to unsubstituted Sepharose was noted many years ago, there have been few preparative applications[1,2]. Probably every protein binds to Sepharose in the presence of ammonium sulphate at a concentration slightly less than needed for precipitation. The proteins of rabbit muscle extract were totally absorbed on Sepharose CL-6B as the concentration of ammonium sulphate was gradually increased to 80% saturation (unpublished results). This seems to be the first report describing the use of salting-out chromatography for primary purification, *i.e.*, directly from the dilute crude extract. The advantages of this method are speed, high capacity and low cost of Sepharose, high yields and no need for costly equipment even if the purification is carried out on a fairly large scale. Potential applications of this method are the purification of plant proteins, monoclonal antibodies and other proteins from mammalian cell culture filtrates and proteins from urine.

ACKNOWLEDGEMENT

The authors express their gratitude to Eppendorf Gerätebau (Hamburg, F.R.G.) for the kind gift of the photometer used in this study.

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CHROM. 18 479

Note

Two-dimensional thin-layer chromatography for the separation and identification of nitro derivatives in explosives

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(Received January 14th, 1986)

The annual consumption of explosives in Italy (averaging not less than 50 000 tons per year) indicates their economic and commercial importance. The specific effects desired as well as police regulations regarding the handling, transport and use of explosives, necessitate a a preliminary method of characterization. Subsequent accurate detection of the components could then be undertaken by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC), coupled with mass spectrometry[1].

The present report considers the active organic components of bursting explosives. There is no lack of previous investigations into the subject. Nevertheless, those studies dealt with separation within the limits of each individual class of explosives, without trying to characterize, even to a first approximation, the various components of a mixture. Thus, descriptions have been given regarding the separation of nitroesters[2–4], nitroarenes[5–7], nitroamines[8], aromatic nitro compounds in general—mixtures of nitroarenes, nitrophenols and nitroanilines[9], mixtures of nitroesters and nitroarenes[10] and of nitroesters [nitroglycerine (NGL), ethylene glycol dinitrate], nitroamines (nitroguanidine) and nitroarenes [*p*-nitro-, 2,4-dinitro- and 2,4,6-trinitrotoluene (MNT, DNT, TNT)], but only with regard to the compounds specified in brackets[11]. More comprehensive investigations took into account various and significant components, but some did not achieve satisfactory separations[12,13], unless several solvents systems were employed[14,15].

We thought it useful to develop, for a series of components chosen according to the criterion of the highest commercial and military significance, a two-dimensional TLC separation by selecting those eluent mixtures which allowed, on the whole, the best detection. Some eluent mixtures were also found adaptable to the separation of particular components, thus being suitable for the resolution of some typical formulations.

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EXPERIMENTAL

Reagents and products

Solvents and reagents utilized were of RPE-ACS grade or the equivalent. As chromatographic standards, either analytical grade products were used where available, or commercial products recrystallized from suitable solvents[16]. The latter technique was applied for purification of DNT, TNT, HMX, RDX, PETN and TNMA (see Table I for meaning of abbreviations). NGL and NDGL were obtained as a solution of approximately known titre by Soxhlet extraction with diethyl ether from unstabilized double-base propellants.

Analytical procedure

In preliminary tests, 10 μl of a 1% solution of each product in ethanol–acetone (1:1, v/v), or in acetone (RDX, HMX), were chromatographed, under the optimum conditions described in the literature[17], on silica gel 60 plates, 20 × 20 cm (Merck, Cat. No. 5721), by utilizing either binary mixtures (light petroleum–dichloromethane, light petroleum–acetone, light petroleum–ethyl acetate, chloroform–acetone) or ternary ones (light petroleum–benzene–acetone), at various ratios. After conditioning the plates to 100°C for 10 min, detection was carried out by means of a freshly prepared solution of 1% diphenylamine in ethanol–acetone (1:1). The appearance of spots, essentially coloured and/or fluorescent to UV light, was immediate; those corresponding to HMX and RDX even became directly visible after 24 h.

For developing the two-dimensional chromatogram, a suitable mixture of all

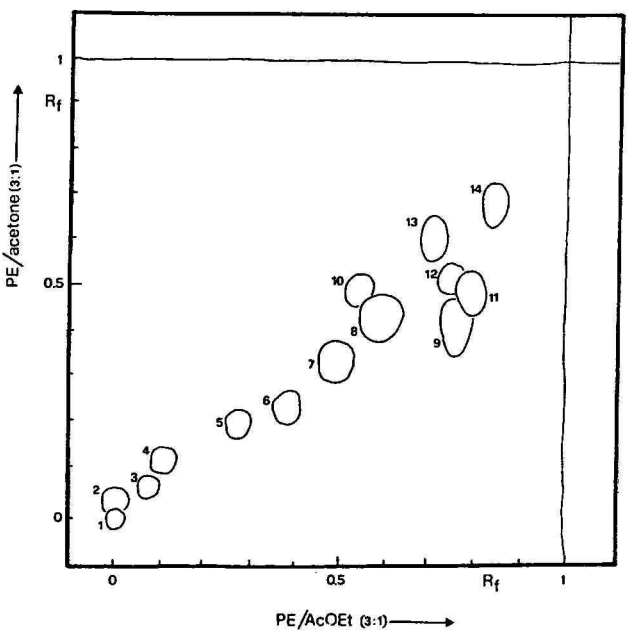

Fig. 1. Two-dimensional chromatogram on silica gel obtained for a mixture of the components listed in Table I by eluting successively with light petroleum–acetone (3:1) and light petroleum–ethyl acetate (3:1).

TABLE I

SPOT IDENTIFICATION FOR THE CHROMATOGRAM IN FIG. 1

Abbreviations for colours: y = yellow, gr = grey, b = blue, lb = light blue, br = brown, or = orange, v = violet, UV = visible in ultraviolet region.

No.	*Compound*	R_F		*Colour with diphenylamine*
		Eluent A	*Eluent B*	
1	2,4,6-Trinitrophenol (picric acid)	0	0	y (b after 24 h)
2	2,4-Dinitrophenol	0.04	0	y, lemon
3	Cyclotetramethylene-tetranitroamine (HMX)	0.07	0.07	gr-lb (after exposure to 100°C for 1/2 h), UV (v)
4	Cyclotrimethylene-trinitroamine (RDX)	0.13	0.11	gr-lb (after exposure to 100°C for 1/2 h), UV (v)
5	*p*-Nitrotoluene (*p*-MNT)	0.20	0.27	y, light
6	N-Methyl-N,2,4,6-tetranitroaniline (Tetryl or TNMA)	0.24	0.38	y-br
7	Nitrodiglycol (NDGL)	0.33	0.50	gr, light
8	Nitroglycerine (NGL)	0.43	0.60	gr (after exposure to 100°C for 1/2 h), UV (green)
9	Tetranitropentaerythritol (PETN)	0.42	0.76	gr, light (after exposure to 100°C for 1/2 h), UV (v)
10	2,4-Dinitrotoluene (DNT)	0.49	0.55	y, UV (br)
11	2,4,6-Trinitrotoluene (TNT)	0.49	0.80	or
12	*o*-Nitrotoluene (*o*-MNT)	0.51	0.76	y, light; UV (br)
13	α-Nitronaphthalene	0.60	0.72	y, straw-coloured
14	Diphenylamine	0.68	0.85	UV (v)

the products was prepared, in the same amounts as in the preliminary test; elution was carried out with light petroleum (b.p. 40–70°C)–acetone (3:1, v/v; solvent system A) in one direction and with light petroleum (b.p. 40–70°C)–ethyl acetate (3:1, v/v; solvent system B) in the other direction.

RESULTS AND DISCUSSION

Fig. 1 gives a schematic representation of the two-dimensional chromatogram obtained, under the described conditions, for the mixture of the various components. A sufficiently reliable identification of the chromatographed compounds was obtained, based not only on their respective R_F values, but also on the specific colour of the spots (Table I). The ordering of the components is basically according to their decreasing polarity, starting from picric acid which remains at the start.

Fig. 2 shows the chromatograms obtained for two typical formulations based on TNT–RDX–HMX and MNT–DNT–TNT with light petroleum–acetone (2:1) and light petroleum–ethyl acetate (8:1). By varying the eluotropic properties of the basic mixtures, optimum separations can be obtained in specific cases: thus, a slight increase in polarity of the first light petroleum mixture led to a sharper separation between RDX and HMX, whereas a decrease in the polarity of the second allowed a better resolution between *o*-MNT and TNT.

The methods described above are currently employed by us for preliminary forensic investigations, giving satisfactory results in the detection of aromatic and aliphatic nitro derivatives (nitroglycerine, nitrodiglycol, etc.; mono-, di- and trinitro-

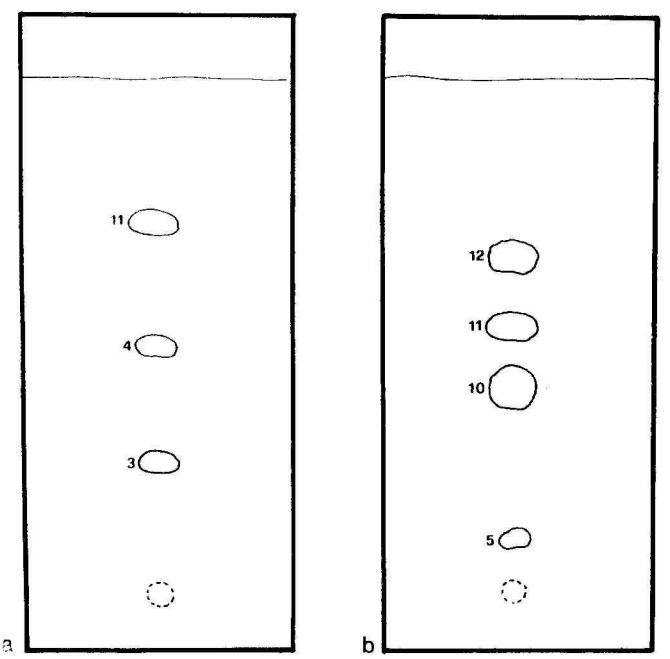

Fig. 2. Silica gel chromatograms of a (a) TNT–RDX–HMX mixture eluted with light petroleum–acetone (2:1), (b) *p*-MNT, TNT, DNT, *o*-MNT mixture eluted with light petroleum–ethyl acetate (8:1).

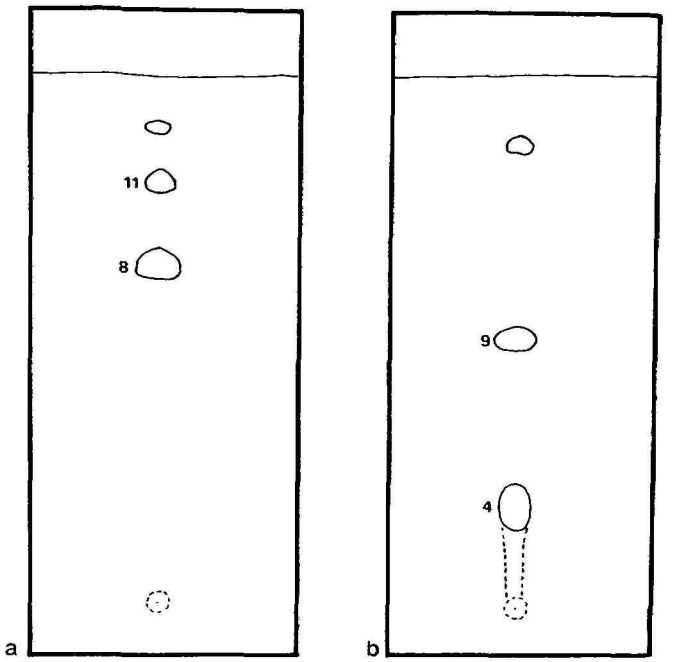

Fig. 3. Silica gel chromatogram of two commercial mixtures: (a) TNT, NGL and vaseline oil eluted with light petroleum–ethyl acetate (3:1); (b) RDX, PETN and vaseline oil eluted with light petroleum–acetone (3:1).

toluene, nitronaphthalene, etc.), as well as heterocyclic ones (cyclotrimethylene-trinitroamine and cyclotetramethylene-tetranitroamine, etc.). For example, Fig. 3 shows the chromatograms obtained from two commercial products (acetone extracts) in which also paraffin-based plasticizing agents appear (R_F = 0.88).

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Author Index

Errata

J. Chromatogr., 349 (1985) 117–130
Page 126, caption of Fig. 7, "10 μmol" should read "10 μg".
Page 127, second footnote to Table I, "(see Fig. 5)" should be deleted.

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journal of chromatography news section

NEW BOOKS

Liquid chromatography detectors; second, completely revised edition, (*Journal of Chromatography Library, Vol. 33*) by R.P.W. Scott, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1986, XVI + 272 pp., price Dfl. 175.00 (US$ 64.75), ISBN 0-444-42610-8.

Advances in capillary chromatography, edited by J. Nikelly, Hüthig, Berlin, Heidelberg, New York, 1986, 138 pp., price DM 56.00, ISBN 3-7785-1143-2.

Affinity chromatography – Practical and theoretical aspects (*Chromatographic Science Series,* Vol. 33) by P. Mohr and K. Pommerening, Marcel Dekker, New York, Basel, 1985, 320 pp., price US$ 69.25 (U.S.A. and Canada), US$ 83.50 (rest of world), ISBN 0-8247-7468-X.

Chromatographic methods, by A. Braithwaite and F.J. Smith, Chapman and Hall, London, New York, 4th ed., 1985, X + 414 pp., price £ 29.00 (hardback), £ 12.95 (paperback), ISBN 0-412-26770-5 (hardback), 0-412-25890-0 (paperback).

Gel permeation and ion-exchange chromatography of proteins and peptides (*Progress in HPLC,* Vol. 1), edited by H. Parvez, Y. Kato and S. Parvez, VNU Science Press, Utrecht, 1985, X + 223 pp., price DM 139.00, ISBN 90-6764-048-4.

Mass spectral and GC data of drugs, poisons and their metabolites. Part I: Introduction, tables, GC data. Part II: Mass spectra and indexes, by K. Pfleger, H. Maurer and A. Weber, VCH Verlagsgesellschaft, Weinheim, 1985, 208 pp. (Part I), 744 pp. (Part II), price DM 480.00, US$ 212.50, ISBN 3-527-26303-9.

Chromatography/foams/copolymers (*Advances in Polymer Science, Vol. 73/74*), by Y. Doi, G.S. Greschner, T. Keii, O. Nuyken, A. Sen, F.A. Shutov and R. Weidner, Springer, Berlin, Heidelberg, New York, 1986, IX + 263 pp., price DM 138.00, ISBN 3-540-15786-7.

Advances in steroid analysis '84 (*Analytical Chemistry Symposia Series,* Vol. 23), edited by S. Görög, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1985, XII + 604 pp., price US$ 139.00 (U.S.A. and Canada), Dfl. 375.00 (rest of world), ISBN 0-444-99533-1.

Amino acids – Neuromethods, Vol. 3, edited by A.A. Boulton, G.B. Baker and J.D. Wood, Humana Press, Clifton, NJ, Wiley, Chichester, New York, 1986, *ca.* 304 pp., price *ca.* US$ 65.40, ISBN 0-89603-077-6.

Analytical and chromatographic techniques in radiopharmaceutical chemistry, edited by D.W. Wieland, M.C. Tobes and T.J. Mangner, Springer, New York, Berlin, Heidelberg, 1986, XVIII + 300 pp., price DM 148.00, ISBN 3-540-96185-2 (Berlin, Heidelberg), 0-387-96185-2 (New York).

Cosmetic analysis: selective methods and techniques, edited by P. Boré, Marcel Dekker, New York, Basel, 1985, XIV + 552 pp., price US$ 85.00 (U.S.A. and Canada), US$ 102.00 (rest of world), ISBN 0-8247-7113-3.

Use of statistics to develop and evaluate analytical methods, by G.T. Wernimont, edited by W. Spendley, Association of Official Analytical Chemists, Arlington, VA, 1985, XVI + 183 pp., price for member US$ 47.55 (U.S.A.), US$ 50.55 (rest of world), for non-members US$ 52.50 (U.S.A.), US$ 55.50 (rest of world), ISBN 0-935594-31-5.

ANNOUNCEMENTS OF MEETINGS

PHYTOCHEMICAL SOCIETY OF EUROPE SYMPOSIUM, "BIOLOGICALLY ACTIVE NATURAL PRODUCTS", LAUSANNE, SWITZERLAND, SEPTEMBER 3–5, 1986

The scientific programme of the above-mentioned symposium will consist of twenty-three plenary lectures and poster sessions covering the following topics: isolation and structure determination of natural products, anticancer agents, immunomodulatory substances, fungicidal and other antimicrobial activities, insecticides, molluscicides, metabolites of microorganisms, marine natural products.

For further information please contact: Prof. K. Hostettmann, Institut de Pharmacognosie et Phytochimie, Ecole de Pharmacie de l'Université de Lausanne, Rue Vuillermet 2, CH-1005 Lausanne, Switzerland.

7th INTERNATIONAL SYMPOSIUM ON ANALYTICAL AND APPLIED PYROLYSIS, READING, U.K., SEPTEMBER 15–19, 1986

Pyrolysis 86, the 7th International Symposium on Analytical and Applied Pyrolysis, will be held at the University of Reading, Reading, U.K. from September 15 to 19, 1986. Pyrolysis 86 is an international conference devoted to discussion of the fundamental investigation of pyrolysis processes by chemical and physical techniques, the structural analysis and fingerprinting of synthetic and natural polymers or high-molecular-weight products, the interfacing and use of chromatographic and spectrometric methods with pyrolysis, and the computer processing of pyrolysis data.

Sessions are being planned on the following topics: natural polymers; synthetic polymers; fossil fuels; fire, propagation and retardation; instrumentation; pyrolysis mechanisms; food and tobacco; data handling; biomedicine. The lecture programme will be based on invited speakers. Contributed papers will be in the form of posters. All conference participants are encouraged to submit a poster. The results presented must be unpublished at the time of the conference. To encourage the submission of high quality posters the organising committee is offering cash prizes totalling £500 to the authors of the three posters judged, by the conference attendees, to be the "best" based on scientific content and presentation.

All contributions to the conference will be published in a special volume of the *Journal of Analytical and Applied Pyrolysis,* subject to normal refereeing procedures. Manuscripts will be expected by the conference to allow rapid refereeing and publication.

Correspondence should be addressed to the Chairman: Dr. S.C. Gutteridge, Cadburry Schweppes Plc, The Lord Zuckerman Research Centre, The University, Whiteknights, P.O. Box 234, Reading RJ6 2LA, U.K.

PROGRESS IN CAPILLARY CHROMATOGRAPHY, STOCKHOLM, SWEDEN, OCTOBER 16–17, 1986

An international symposium on capillary chromatography will be arranged by the Analytical Section of the Swedish Chemical Society, in Stockholm, October 16 and 17, 1986.

The programme will cover the following topics. *Liquid chromatography*: basic aspects, packed or open tubular columns, immobilized phases; techniques and applications; multi-dimensional approaches. *Supercritical fluid chromatography*: basic aspects, types of columns and stationary phases; techniques and applications. *Gas chromatography*: column preparation techniques and stationary phases; film thickness and diameter in capillary columns (wide bore *vs.* narrow bore); injection techniques; multi-dimensional techniques; applications.

Those interested in contributing to the symposium with a paper or a 20-min lecture should send, not later than 15 May, 1986, a preliminary abstract (approx. 500 words) to the Analytical Section of the Swedish Chemical Society. Submitted papers will be published, after reviewing, in the *Journal of Chromatography*.

For further information contact: The Analytical Section, The Swedish Chemical Society, Upplandsgatan 6A, S-111 23 Stockholm, Sweden.

EUROANALYSIS VI, EUROPEAN CONFERENCE ON ANALYTICAL CHEMISTRY, PARIS, FRANCE, SEPTEMBER 7–11, 1987

On behalf of the Working Party on Analytical Chemistry (WPAC) of the Federation of European Chemical Societies (FECS) the Presidium of EUROANALYSIS VI and the French organizing societies invite you to attend, to make oral or poster contributions to the conference, to display instruments, products, or books, on the exhibition stands at EUROANALYSIS VI. The French organizing societies include: Groupement pour l'Avancement des Méthodes Spectroscopiques et Physico-chimiques d'Analyse (G.A.M.S.), Société Française de Chimie (S.F.C.), Groupe d'ingénierie analytique de la Société de Chimie Industrielle (S.C.I.), Société Française de Métallurgie (S.F.M.), with cooperation from l'Association Technique de la Sidérurgie (A.T.S.), and the sponsorship of Comité Français de Chimie.

EUROANALYSIS conferences cover all aspects of analytical sciences. In Paris emphasis will be on the most modern techniques and advances, with the aim of demonstrating their possibilities to the widest audience. Lectures will be given on the use of lasers in analytical techniques, electrochemical detectors, ion equilibria for ion chromatography, atomic spectroscopy, trace analysis of organic mixtures, chemometrics, ultraclean laboratories, etc. Special sessions are planned on: the use and construction of analytical probes; applications of analytical methods for solving environmental problems; analysis of solid state samples; new methods of teaching analytical subjects.

The conference will consist of invited plenary and keynote lectures and contributed papers presented orally or as posters. Detailed instructions will be sent to participants wishing to contribute with the second circular (fall 1986), which will also provide details of Social Events, Visits and Excursions.

For further information, contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France.

FLOW ANALYSIS IV, AN INTERNATIONAL CONFERENCE ON FLOW ANALYSIS, LAS VEGAS, NV, U.S.A., APRIL 18–21, 1988

The 4th International Conference on Flow Analysis will be held in Las Vegas, NV, U.S.A., April 18–21, 1988. It will be organized by Dr. Gilbert Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A.

The scope of the conference will be similar to that of the Flow Analysis conferences held in Amsterdam, 1979, Lund, 1982, and Birmingham, 1985, and will cover current research on all aspects on continuous flow analysis. The topics will include: instrumentation for flow injection analysis and for continuous segmented and unsegmented flow analysis, including approaches to total automation; new detector systems and hybrid systems; theory of flow analysis; applications in the separation techniques of flow analysis; use of flow analysis in process control; use of flow analysis in electrochemical detection.

The scientific programme will consist of plenary and invited lectures, submitted research papers and posters, and working demonstrations. Authors who wish to present papers should submit abstracts before November 30, 1987. The conference language will be English. There will be an exhibition of commercial instrumentation for flow analysis.

As with the earlier conferences, the proceedings will be published in a special issue of *Analytica Chimica Acta*. Information for authors will be provided in later announcements.

The conference will be held in the beautiful Tropicana Resort Hotel in Las Vegas, NV, U.S.A. The conference registration fee will include meals, social events, and a copy of the Proceedings. For further information contact Dr. Gilbert E. Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A.

CALENDAR OF FORTHCOMING MEETINGS

May 18–23, 1986 San Francisco, CA, U.S.A.	**HPLC '86. New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A. (Further details published in Vol. 331, No. 2.)

May 26–29, 1986 Lerici, Italy	**III CAC – Meeting of the Chemometrics Society** Contact: Prof. M. Forina, Istituto di Analisi e Tecnologie Farmaceutiche ed Alimentari, Via Brigata Salerno (ponte), I-16147 Genova, Italy. Tel.: (010) 3993656. (Further details published in Vol. 330, No. 2.)
June 3–6, 1986 Munich, F.R.G.	**Analytica 86, 10th International Trade Exhibition and 10th International Conference 'Biochemical Analytics'** Contact: Dr. Rosemarie Vogel, Nymphenburgerstrasse 70, D-8000 München 2, F.R.G. (Further details published in Vol. 330, No. 2.)
June 23–27, 1986 Harrogate, U.K.	**1986 Hewlett-Packard Analytical Symposium** Contact: Tina Mears, Hewlett-Packard Ltd., Analytical Instrumentation Group, Miller House, The Ring, Berkshire, RG12 1XN, U.K. Tel.: Bracknell (0344) 424898, telex 848733. (Further details published in Vol. 347, No. 3.)
June 25–26, 1986 Bratislava, Czechoslovakia	**Interekotechnika '86** Contact: INCHEBA FTC, Drienova 24, 826 17 Bratislava, Czechoslovakia.
June 25–27, 1986 Verona, Italy	**International Conference on Developments in Analytical Methods in Pharmaceutical, Biomedical and Forensic Sciences** Contact: Dr. Alberto Frigerio, Italian Group for Mass Spectrometry in Biochemistry and Medicine, via Eustachi 36, 20129 Milan, Italy. (Further details published in Vol. 354.)
July 20–26, 1986 Bristol, U.K.	**SAC 86 – International Conference and Exhibition on Analytical Chemistry** Contact: Miss P.E. Hutchinson, Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, U.K. Tel.: (01) 734-9971.
Aug. 10–17, 1986 Ottawa, Canada	**6th International Congress of Pesticide Chemistry** Contact: T.H.G. Michael, Chemical Institute of Canada, 151 Slater Street, Suite 906, Ottawa, Ontario, Canada K1P 5H3. Tel.: (613) 233-5623. Telex: 053-4306 AIC.
Aug. 17–21, 1986 Seattle, WA, U.S.A.	**6th International Conference on Methods in Protein Sequence Analysis** Contact: MPSA, Conference Management, GH-22 University of Washington, Seattle, WA, U.S.A. Tel.: (206) 543-2300, telex: (910) 474 0096 UW UI.
Aug. 24–26, 1986 Ghent, Belgium	**2nd World Congress on Compounds in Biological and Chemical Warfare: Toxicological Evaluation – Industrial Chemical Disasters** Contact: Professor A. Heyndrickx, Head of the Department of Toxicology, State University of Ghent, Hospitaalstraat 13, B-9000 Ghent, Belgium. Tel.: (32) 91 25 10 21, telex: 11.558 A.Z. Ghent-Toxicology. (Further details published in Vol. 354.)
Aug. 25–29, 1986 Antwerp, Belgium	**10th International Symposium on Microchemical Techniques** Contact: Dr. R. Dewolfs, University of Antwerp, Department of Chemistry, Universiteitsplein 1, B-2610 Wilrijk, Belgium. Tel.: 03/828.25.28 (ext. 204), Telex: 33646.
Aug. 27–30, 1986 Brussels, Belgium	**12th International Congress of the European Association of Poison Control Centres (EAPCC)** Contact: Administrative Secretariat, Mrs. D. Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.

Aug. 27–30, 1986 Brussels, Belgium	**3rd World Congress of the World Federation of Associations of Clinical Toxicology and Poison Control** Contact: Administrative Secretariat, Mrs. D Shanni, SDR Associated, Rue Vilain XIIII, 17a, B-1050 Brussels, Belgium. Tel.: (02) 647 87 80. Telex: 61434 SDRBRU B.
Aug. 31–Sept. 3, 1986 Ghent, Belgium	**6th International Symposium on Mass Spectrometry in Life Sciences** Contact: Professor Dr. A. De Leenheer, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 218951, ext. 324. (Further details published in Vol. 350, No. 2.)
Sept. 3–5, 1986 Maastricht, The Netherlands	**5th International Symposium on Isotachophoresis, ITP '86** Contact: Secretariat ITP '86, Th. Verheggen, Laboratory for Instrumental Analysis, Department of Chemical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands. Tel.: (040) 473014, telex 51163. (Further details published in Vol. 354.)
Sept. 3–5, 1986 Lausanne, Switzerland	**Phytochemical Society of Europe Symposium "Biologically Active Natural Products"** Contact: Prof. K. Hostettmann, Institut de Pharmacognosie et Phytochimie, Ecole de Pharmacie de l'Université de Lausanne, Rue Vuillermet 2, CH-1005 Lausanne, Switzerland.
Sept. 4–8, 1986 Szeged, Hungary	**A Symposium on New Advances in Liquid Chromatography** Contact: Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary. (Further details published in Vol. 347, No. 3.)
Sept. 8–10, 1986 Freiburg, F.R.G.	**4th International Symposium on Bioluminescence and Chemiluminescence** Contact: Dr. J. Schölmerich, Medizinische Universitätsklinik, D-7800 Freiburg, F.R.G.
Sept. 9–12, 1986 London, U.K.	**5th Meeting of the International Electrophoresis Society, "Electrophoresis '86"** Contact: Dr. M.J. Dunn, Muscle Research Unit, Royal Postgraduate Medical School, DuCane Road, London W12 0HS, U.K. Tel.: 01-743-2030 ext. 338.
Sept. 15–18, 1986 Houston, TX, U.S.A.	**22nd International Symposium on Advances in Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, 77004 TX, U.S.A. Tel.: (713) 749 2623. (Further details published in Vol. 354.)
Sept. 15–19, 1986 Reading, U.K.	**7th International Symposium on Analytical and Applied Pyrolysis** Contact: Dr. C.S. Gutteridge, Cadbury Scheppes Plc, The Lord Zuckerman Research Centre, The University, Whiteknights, P.O. Box 234, Reading RJ6 2LA, U.K.
Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: GAMS, 88 bd Malesherbes, 75008 Paris, France. Tel.: (1) 563-9304. (Further details published in Vol. 331, No. 2).
Sept. 29–Oct. 3, 1986 St. Louis, MO, U.S.A.	**FACSS '86, Federation of Analytical Chemistry and Spectroscopy Societies 1986 Meeting** Contact: Dr. Marshall Fishman, U.S. Department of Agriculture, 600 E. Mermaid Lane, Wyndmoor, PA 19118, U.S.A. Tel.: (215) 233-6450. (Further details published in Vol. 354.)

Oct. 7–9, 1986 Tokyo, Japan	**23rd International Symposium Advances in Chromatography** Contact: Prof. N. Ikekawa, Department of Chemistry, Tokyo Institute of Technology, Ohokayama, Meguro-ku, Tokyo 152, Japan; or Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. (Further details published in Vol. 347, No. 3.)
Oct. 8–10, 1986 Palma de Mallorca, Spain	**3rd Symposium on Handling of Environmental and Biological Samples in Chromatography** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379. (Further details published in Vol. 354.)
Oct. 12–16, 1986 Washington, DC, U.S.A.	**100th Annual AOAC International Meeting** Contact: Margaret Ridgell, AOAC, 1111 North 19th Street, Suite 210, Arlington, VA 22209, U.S.A. Tel.: (703) 522-3032.
Oct. 16–17, 1986 Stockholm, Sweden	**International Symposium on Progress in Capillary Chromatography** Contact: The Analytical Section, The Swedish Chemical Society, Upplandsgaten 6A, S-111 23 Stockholm, Sweden.
Oct. 19–22, 1986 Boston, MA, U.S.A.	**4th International Symposium on Laboratory Robotics** Contact: Gerald L. Hawk, Ph.D. and Janet Strimaitis, Zymark Corporation, Zymark Center, Hopkinton, MA 01748, U.S.A. Tel.: (617) 435-9501.
Oct. 20–22, 1986 Baden-Baden, F.R.G.	**6th International Symposium on High-Performance Liquid Chromatography of Proteins, Peptides and Polynucleotides** Contact: Secretariat 6th ISPPP, P.O. Box 3980, D-6500 Mainz, F.R.G. (Further details published in Vol. 354.)
Oct. 20–24, 1986 New York, NY, U.S.A.	**The Silver Jubilee Eastern Analytical Symposium** Contact: Dr. S. David Klein, EAS Publicity, 642 Cranbury Cross Road, North Brunswick, NJ 08902, U.S.A. Tel.: (201) 846-1582. (Further details published in Vol. 347, No. 3.)
Oct. 22–24, 1986 Montreux, Switzerland	**4th Symposium on Liquid Chromatography–Mass Spectrometry and Mass Spectrometry–Mass Spectrometry** Contact: Professor Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 5485379. (Further details published in Vol. 354.)
Oct. 28–30, 1986 Tokyo, Japan	**7th Conference on Liquid Chromatography** Contact: Professor N. Okuyama, Tokyo Metropolitan University, Faculty of Science, Setagaya-ku, Tokyo 158, Japan.
April 28–May 1, 1987 Sydney, Australia	**9th Australian Symposium on Analytical Chemistry** Contact: The Secretary 9AC, Mr. John Eames, P.O. Box 137, North Ryde, N.S.W. 2133, Australia. Tel.: (02) 887-8688. (Further details published in Vol. 350, No. 2.)

May 11–14, 1987 Ghent, Belgium	**2nd International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. W. Baeyens, State University of Ghent, Laboratory of Pharmaceutical Chemistry and Drug Quality Control, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 354.)
June 21–26, 1987 Toronto, Canada	**XXV Colloquium Spectroscopium Internationale** Contact: Mr. L. Forget, Executive Secretary CSI XXV, National Research Council of Canada, Ottawa, K1A 0R6 Canada. Tel.: (613) 993-9009, telex: 053-3145. (Further details published in Vol. 330, No. 2.)
June 28–July 4, 1987 Amsterdam, The Netherlands	**HPLC '87, 11th International Symposium on Column Liquid Chromatography** Contact: Organisatie Bureau Amsterdam bv, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (31) 20-440807, telex: 13499 raico nl. (Further details published in Vol. 331, No. 2.)
August 17–21, 1987 Oberammergau, F.R.G.	**7th International Symposium on Affinity Chromatography and Interfacial Macromolecular Interactions** Contact: Prof. Dr. H.P. Jennissen, Institut für Physiologie, Physiologische Chemie und Ernährungsphysiologie, Universität München, Veterinärstr. 13, D-8000 München 22, F.R.G.
Aug. 25–30, 1987 Beijing, China	**8th International Conference on Computers in Chemical Research and Education** Contact: Cheng Qian, 345 Lingling Road, 200032 Shanghai, China. Telex: 33354 SIOC CN.
Sept. 7–11, 1987 Paris, France	**Euroanalysis VI, European Conference on all Aspects of Analytical Sciences** Contact: G.A.M.S., 88 Boulevard Malesherbes, 75008 Paris, France.
April 18–21, 1988 Las Vegas, NV, U.S.A.	**Flow Analysis IV, An International Conference on Flow Analysis** Contact: Dr. Gilbert E. Pacey, Department of Chemistry, Miami University, Oxford, OH 45056, U.S.A.
Aug. 27–31, 1990 Vienna, Austria	**7th European Conference on Analytical Chemistry, "Euroanalysis 7"** Contact: Prof. Robert Kellner, Austrian Society for Microchemistry and Analytical Chemistry, Institute for Analytical Chemistry, Technical University of Vienna, Getreidemarkt 1, A-1060 Vienna, Austria.

PUBLICATION SCHEDULE FOR 1986

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	O 1985	N 1985	D 1985	J	F	M	A	M	J	
Journal of Chromatography	346 347/1	347/2 347/3 348/1	348/2 349/1 349/2 350/1 350/2	351/1 351/2 351/3	352 353 354	355/1 355/2 356/1	356/2 356/3 357/1	357/2 357/3 358/1 358/2 359		The publication schedule for further issues will be published later
Chromatographic Reviews								373/1		
Bibliography Section					372/1		372/2		372/3	
Biomedical Applications				374/1 374/2	375/1	375/2	376 377	378/1	378/2 379	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 329, No. 3, pp. 449–452. 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 review articles, 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.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. *Full-length papers and Review articles should have a summary of 50–100 words which clearly* and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. *Please check a recent issue for the lay*out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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