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PROBLEMS IN THE APPLICATION OF GRADIENT ELUTION TO HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The performance of a mixing chamber for low-pressure gradient mixing in high-pressure liquid chromatography is discussed. Specific problems in gradient elution with active stationary phases are demonstrated. Difficulties may arise through enrichment of the impurities in the eluent and their elution as ghost peaks during the analysis. The time necessary for the regeneration of the column depends mainly on the time required to reach equilibrium between the water adsorbed on the stationary phase and that dissolved in the eluent. A gradient elution without sample is recommended in order to identify the ghost peaks that arise from the column, the apparatus and the eluent. Between this run and the analysis, the regeneration step should be standardized.

INTRODUCTION

For the separation of complex mixtures, a systematic change in the sample distribution coefficients is required during the separation in order to achieve optimal conditions for most of the components. In liquid–solid chromatography, this can be carried out by solvent programming, which offers the greatest possibilities of the available programming techniques[1,2]. However, the technological problems to be solved with gradient elution in high-pressure liquid chromatography (HPLC) are difficult and commercially available equipment for HPLC is therefore expensive. In classical liquid–solid chromatography, many inexpensive devices have been described for the low-pressure mixing of eluents[3–6]. In the present work, the applicability of such mixing devices to HPLC has been studied, and problems with the reproducibility of analytical conditions arising from the column and/or from the eluents are discussed.

EXPERIMENTAL

Chromatographic apparatus

Home-built equipment was used. The reservoirs for the different solvents were connected to the mixing chamber by a five-way valve (Whitey SS 43 ZF 2). From the mixing chamber, a 10 cm × 1.5 mm I.D. PTFE capillary tube led to an Orlita MS 4/4 high-pressure pump. The pump and the high-pressure gauge were connected to

the injection port and the column by 0.5 mm I.D. steel capillary tubing (in the experiments in which no sample application was necessary, the injection port was by-passed). The five-way valve and the pump valves were modified so as to obtain a minimum dead volume. The dead volume of the equipment (from the five-way valve to the detector, excluding the mixing chamber and column) was 2.3 cm^3. The volume flow-rate was measured with a syphon measuring device[7].

The column was 30 cm × 4.2 mm I.D., packed either with 27-μm glass beads or silica SI-100 (Merck, Darmstadt, G.F.R.), particle diameter $\approx 10\,\mu$m.

Mixing chamber

A schematic diagram of the mixing chamber is shown in Fig. 1. By changing the diameter a and the height b, the volume of the chamber was varied between 7.7 cm^3 (a = 25 mm, b = 11 mm) and 40 cm^3 (a = 40 mm, b = 27.4 mm). No influence of the dimensions of the chamber on the reproducibility of the gradients was observed. Mixing in the chamber was achieved with a magnetic stirrer, running with the highest possible speed.

Detectors

Two different UV detectors were used simultaneously: a home-made instrument (fixed wavelength 254 nm) described recently[8], and a Zeiss PM 2 D spectrophotometer. The flow-through cells were home-made with a Z-shape, with I.D. 1 mm and depth 10 mm. Only eluents with no absorption above 230 nm were used.

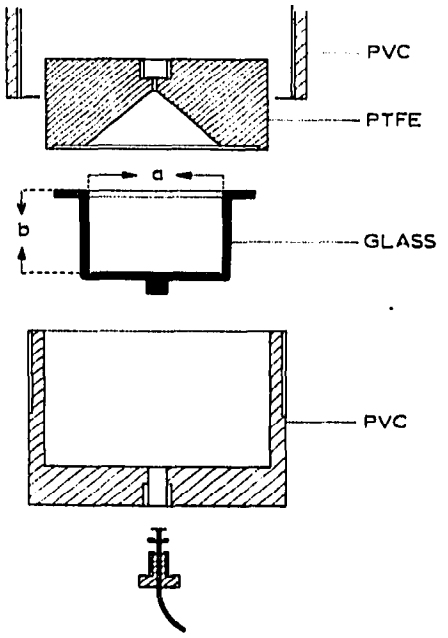

Fig. 1. Schematic diagram of the mixing chamber. Volume 7.7 cm^3: a = 25 mm, b = 11 mm. Volume 20 cm^3: a = 25 (40) mm, b = 37.8 (11.4) mm. Volume 40 cm^3: a = 40 mm, b = 27.4 mm.

Because of the construction of the cell, changes in the refractive index of the eluent could be measured with the UV detectors. By comparing the UV absorption in a spectrophotometer (Beckman Model 25), where no additive measurements of the refractive index are likely, with the results obtained with the UV detectors in HPLC, it was estimated that a change of 10^{-2} extinction units in the HPLC detector is equivalent to a change of about 10^{-2} refractive index units if eluents with no UV absorption are used. The UV detectors for HPLC function like insensitive refractometers and enable changes in the eluent composition to be monitored.

Reagents

Laboratory-grade eluents were distilled before use. Additional cleaning was achieved by filtration of the distilled eluent through highly active adsorbents in a classical chromatography column[9]. Spectroscopic-grade eluents (Uvasol, Merck) were also used.

RESULTS AND DISCUSSION

Mixing chamber

Owing to the design of the mixing chamber, it was possible to generate only convex gradients, by adding the second eluent B to the starting eluent A that fills the mixing chamber initially. The shape and the time period of the gradient depend on the volume of the mixing chamber and on the flow-rates. In order to evaluate the influence of properties of the eluent such as density and viscosity we first studied the behaviour of our mixing device by using an "ideal" system. *n*-Heptane was used as eluent A and *n*-heptane plus 100 ppm of benzene as eluent B. The form of the gradient could easily be monitored by the UV detectors. Fig. 2 shows the gradients obtained with two different mixing chambers (7.7 and 40 cm^3) at different solvent velocities (8 and 1 cm^3/min). The column was packed with glass beads. The gradient shape corresponds to a log-dilution curve (log-bottle effect). The experimental curves in Fig. 2 are identical with those calculated according to Scott[10]. The gradients are characterized by the time at the start and the time when 90% of the end value is achieved.

In order to study the influence of differences in density, the mixing behaviour of *n*-heptane (d = 0.684 g/cm^3 at 20°) with ethyl acetate (d = 0.901 g/cm^3 at 20°) or dichloromethane (d = 1.326 g/cm^3 at 20°) was studied from low to high density and *vice versa*. The viscosities of these eluents are very similar (η = 0.42–0.45 cP at 20°). Table I summarises the results. In the system *n*-heptane–ethyl acetate, no deviation from "ideal" behaviour was noticed either when moving from low to high density or when moving in the opposite direction. In the system *n*-heptane–dichloromethane, only in the largest mixing chamber (40 cm^3) is the appearance of eluent B delayed and the 90% value is reached later than in the "ideal" system at all solvent velocities. The reversed system dichloromethane–*n*-heptane, adding a low-density eluent to a high-density eluent, shows a deviation from "ideal" behaviour with all mixing chambers at all velocities. The low-density component reaches the exit at the top of the mixing chamber earlier than expected. The deviation from "ideal" behaviour increases with increasing chamber volume and with increasing flow velocity. The mixing is poor in this instance, but the gradient shape is reproducible.

The evaluation of the differences in eluent viscosity and their influence on gra-

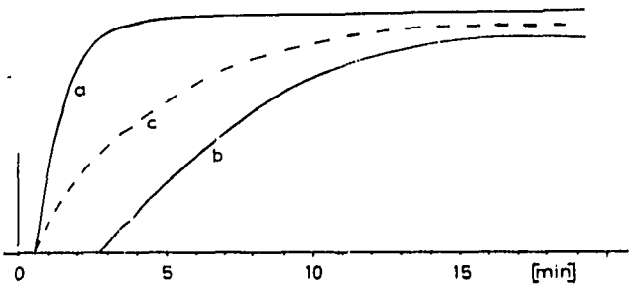

Fig. 2. Gradient form ("ideal" system: *n*-heptane–*n*-heptane + 100 ppm benzene). (a) Mixing chamber, 7.7 cm^3; flow-rate, 8 cm^3/min. (b) Mixing chamber, 7.7 cm^3; flow-rate, 1 cm^3/min. (c) Mixing chamber, 40 cm^3; flow-rate, 8 cm^3/min.

dient mixing was difficult, because in the system used the flow-rate delivered by the Orlita pump is a function of the column back-pressure, which, of course, is a function of solvent viscosity. However, no systematic deviations from "ideal" behaviour could be observed by mixing solvents with different viscosities (*n*-heptane, $\eta = 0.42$ cP; isopropanol, $\eta = 2.49$ cP at 20°).

The mixing chamber described here gives reproducible gradients of a convex shape. It is possible to use this device with all types of reciprocating pumps that deliver a continuous flow. Problems with mixing but not with reproducibility were observed only if the second eluent had a much lower density ($\Delta d > 0.3$ g/cm^3) than the first. If a large chamber volume and/or a low flow-rate is used, the linear region of the gradient is sufficient for most problems. By using a second gradient mixing vessel filled with an eluent of composition corresponding to that at the end of the linear region of the first gradient, a second linear gradient can be added.

Some problems arising from the column

When active stationary phases such as silica or alumina are used, some prob-

TABLE I

DENSITY DIFFERENCES BETWEEN ELUENTS AND DEVIATION FROM "IDEAL" BEHAVIOUR

"Ideal" system: *n*-heptane–*n*-heptane + 100 ppm benzene.

Gradient	*Volume of mixing chamber (cm^3)*			
	7.7		*40*	
	Start	*90% value*	*Start*	*90% value*
n-Heptane to ethyl acetate	No deviation	No deviation	No deviation	No deviation
n-Heptane to dichloromethane	No deviation	No deviation	Later	Later
Ethyl acetate to *n*-heptane	No deviation	No deviation	No deviation	No deviation
Dichloromethane to *n*-heptane	Earlier	Reached much earlier	Earlier	Reached much earlier

lems arise in gradient elution owing to solvent demixing, solvent impurities and column regeneration.

Solvent demixing. Solvent demixing is well known in thin-layer chromatography and also in column chromatography when eluents with large differences in polarity (or solvent strength) are used in order to generate the gradient. The more polar component will be preferentially adsorbed on the stationary phase and the breakthrough of this component is delayed against the "ideal" system. This adsorption of the polar component is undesirable as it may cause displacement of the solutes without separation. In our system with a silica column, running a gradient from *n*-heptane to isopropanol, the first breakthrough of isopropanol was delayed by about one volume of the empty column.

Solvent impurities. Solvent impurities can be collected by the stationary phase in the column. In gradient elution, these substances are eluted as sharp zones as the solvent strength of the eluent increases. The height of these peaks depends on the time the column is flushed, *i.e.*, with the weakest eluent. Surprisingly short times are sometimes required in order to obtain signals of the same magnitude as for the sample constituents. Fig. 3 shows such a "chromatogram" without any sample. After flushing a clean column with *n*-heptane (technical grade, distilled) for 15 min and running a gradient to dichloromethane, the lower curve in Fig. 3 was obtained. The column was connected directly to the pump so as to avoid difficulties arising from the septum. By cleaning the distilled *n*-heptane by filtration through active silica and alumina[9], the impurities were removed almost completely from the *n*-heptane (upper curve in Fig. 3). The last peak in both curves is due to the pressure gauge in the equipment. Traces of solvent are washed out of the dead-end gauge line. After removal of the gauge, this peak disappeared.

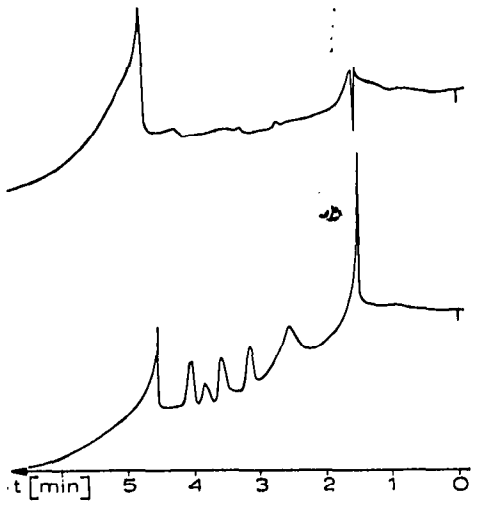

Fig. 3. Eluent impurities. Lower chromatogram: 15 min *n*-heptane (laboratory grade, distilled), then gradient to dichloromethane. Upper chromatogram: 15 min *n*-heptane (distilled, purified additionally with alumina), then gradient to dichloromethane. Column: 30 cm, 4.2 mm I.D.; silica SI-100, 10 μm (Merck). Mixing chamber, 7.7 cm^3; flow-rate, 4.6 cm^3/min; Δp, 70 atm; UV detector.

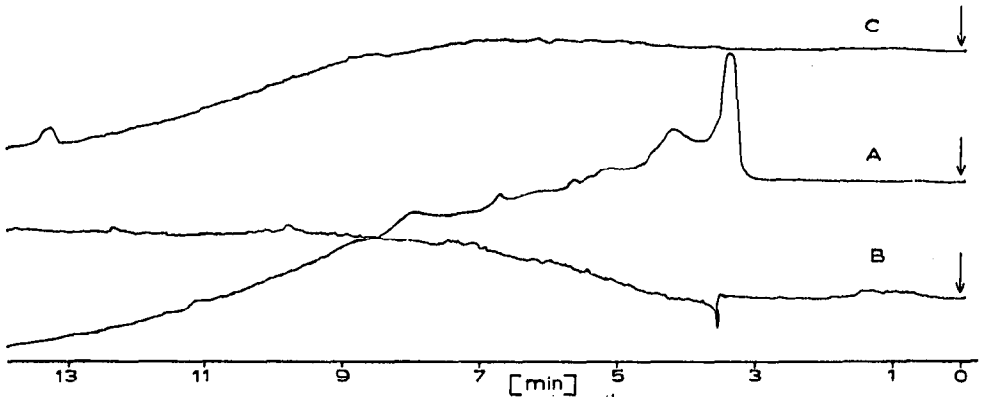

Fig. 4. Eluent impurities. A, *n*-heptane, 30 min; B, Uvasol *n*-heptane, 30 min; C, Uvasol *n*-hexane, 60 min. Gradient and other conditions as in Fig. 3.

Next, we investigated commercially available purified eluents and the results are shown in Fig. 4. When *n*-heptane (Merck, Catalogue No. 4365) was flushed for 30 min through the column, and a gradient to dichloromethane was set up, curve A in Fig. 4 was obtained. By using Uvasol *n*-heptane (Merck, Catalogue No. 4366) and a flushing period of 30 min, no significant peak appeared (curve B in Fig. 4). With Uvasol *n*-hexane (Merck, Catalogue No. 4372), the flushing period was extended to 60 min and no noticeable amounts of impurities were collected by the column (curve C in Fig. 4). Non-polar eluents of spectroscopic grade should be used in order to prevent ghost peaks from the eluent. This approach, however, is very expensive.

Some more polar eluents such as dichloromethane and chloroform were studied in a similar manner. The gradient system was either dichloromethane or chloroform and a 1:1 mixture of dichloromethane and isopropanol. With Uvasol chloroform (Merck, Catalogue No. 2447) as starting eluent (flushing time 30 min), no peak was observed during the gradient, whereas with p.a. grade (Merck, Catalogue No. 6050) and Uvasol

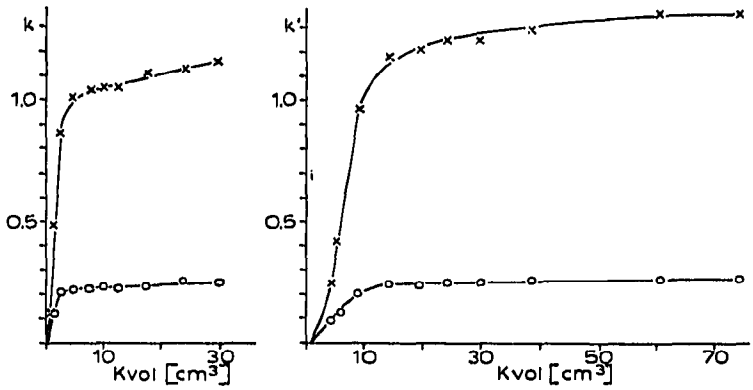

Fig. 5. Column regeneration. Direct eluent switching and "reverse" gradient, dichloromethane to *n*-heptane (water content not controlled). Samples: ○, benzene (in equilibrium $k' = 0.25$); ×, 1,2-dibenzanthracene (in equilibrium $k' = 1.35$). Column and conditions as in Fig. 3.

dichloromethane (Merck, Catalogue No. 6048) under identical conditions at least two peaks were obtained.

Column regeneration. Column regeneration, *i.e.*, returning the column to its initial state after finishing gradient elution analysis, is tedious and time consuming. In order to shorten this procedure, it was recommended always to use either "half-water-saturated" eluents[11] or a "reverse" gradient[12] instead of switching directly back to the starting eluent.

Fig. 5 shows the changes in capacity ratios (k' values) of two solutes obtained either by direct switching or by a "reverse" gradient. On switching directly from dichloromethane even after a flushing period of 30–40 empty-column volumes (volume of the empty column = 4.2 cm^3), the initial k' values of the two solutes (benzene, $k' = 0.25$; 1,2-dibenzanthracene, $k' = 1.35$) are not approached. However, on running a "reverse" gradient (from dichloromethane to *n*-heptane), the initial k' values are reached after a washing period of 30–40 empty-column volumes. In these experiments, the water content of the eluents was not controlled. The rapid increase of the curve obtained by the direct eluent switching demonstrates that in column regeneration the removal of the polar eluent is not a problem. However, organic eluents are always contaminated with water. The achievement of equilibrium between the water adsorbed on the active stationary phase and that dissolved in the eluent is more important (because it is much slower). Owing to the low solubility of water in the non-polar eluents, it takes a long time to transport water into or out of the column. When the "reverse" gradient is used, this equilibrium seems to be reached much faster.

Fig. 6 shows the regeneration procedure by a "reverse" gradient if "half-water-saturated" eluents are used. In this case, the k' values of the solutes pass through a maximum. The maximum can be explained by two opposite and superimposed mechanisms. Firstly, the polar eluent is removed from the column and the k' values increase, and secondly, because of the high water content of the eluent a heavily loaded water column[8,13] is built up and the k' values of the solutes decrease. The removal of this water from the stationary phase is demonstrated in Fig. 5. After switching from "half-water-saturated" to "dry" *n*-heptane (at the arrow in Fig. 6), the k' values of the solutes

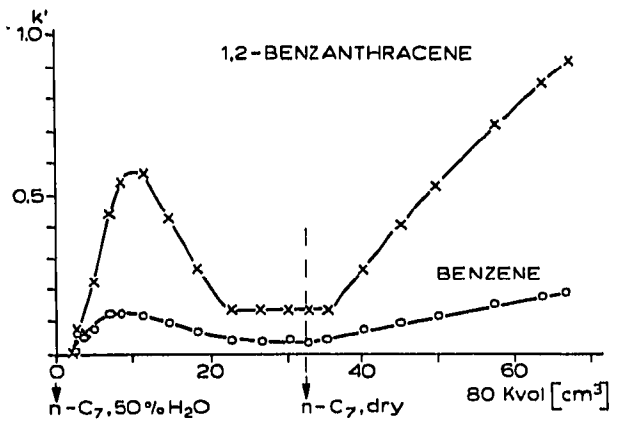

Fig. 6. Column regeneration. "Half-water-saturated" eluent. Reverse gradient to *n*-heptane, half-water-saturated. At arrow: switching to "dry" *n*-heptane. Conditions as in Fig. 3.

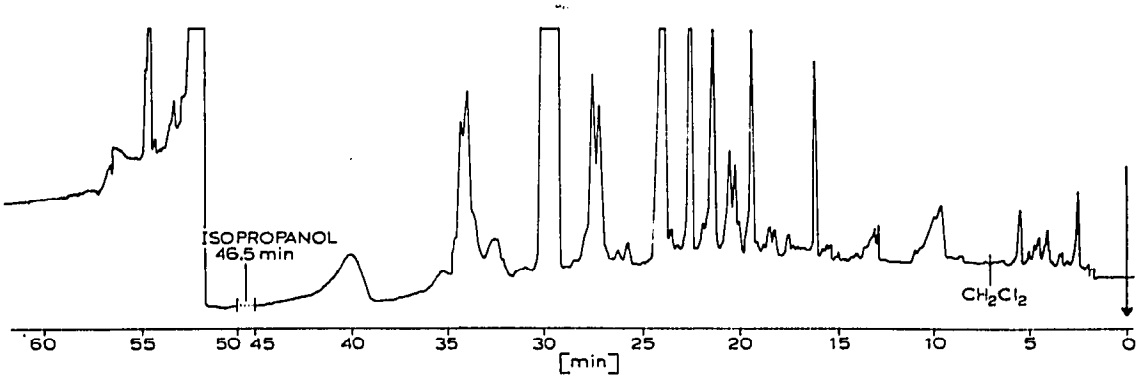

Fig. 7. Gradient elution of Bulgarian attar of roses. Gradient: *n*-heptane to dichloromethane to isopropanol. Mixing chamber: 20 cm^3. Column: 30 cm, 4.2 mm I.D., silica SI-100, 10 μm (Merck); flow-rate, 1.5 cm^3/min.

increase again (removal of the water coating) and finally reach the initial values again (*i.e.*, $k' = 0.25$ for benzene and $k' = 1.35$ for dibenzanthracene) after more than 50 empty-column volumes. The time needed in order to achieve equilibrium of the water is a function of the water content of the eluent (or the solubility of water in the eluent), of the specific surface area of the stationary phase, and, of course, of the flow-rate.

CONCLUSIONS

Gradient elution is the most versatile programming technique in HPLC. However, owing to some properties of the active stationary phase and of the eluents, the analytical results are sometimes difficult to interpret. A major problem is the enrichment of solvent impurities on the column, which are eluted during the separation and may falsify the analytical result. The purity of the eluents should, therefore, be checked before use in a gradient analysis without sample application. Another major problem is column regeneration, for which sometimes more time is necessary than for the analysis. The initial conditions should be standardized by the k' values of some solutes, or the regeneration time between two successive analyses should always be the same.

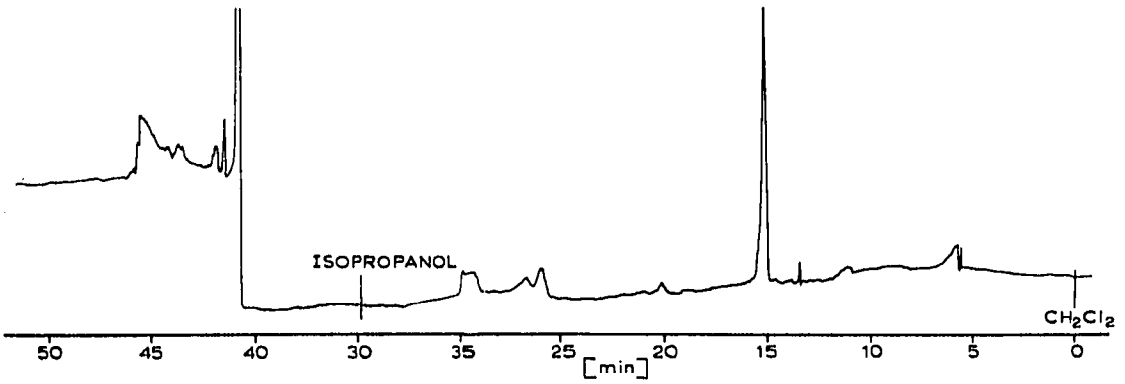

Fig. 8. Gradient elution without sample application. Conditions as in Fig. 7.

The possibilities that gradient elution offers are demonstrated in Fig. 7. With the apparatus described, and using purified eluents, Bulgarian attar of roses was separated with a gradient from *n*-heptane to dichloromethane and further to isopropanol. As a "blank", a gradient without sample application was applied under identical conditions. The results are shown in Fig. 8. Despite taking precautions, a few peaks appeared that originated from the sample injection system, which was added to the equipment before these analyses were carried out. It is therefore recommended that the eluents and the whole equipment used for gradient elution should always be "tested by a" run without sample before the analysis is performed.

As discussed above, many problems are involved in gradient elution technique. However, as long as there are no other methods available for separating samples with different retentions, one has to tolerate these problems and, if possible, to bypass them.

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