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Short communication

# Example of gradient elution in normal-phase liquid chromatography

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**Abstract**

Only a small fraction of all separations by high-performance liquid chromatography are performed at normal-phase conditions and especially on silica. Plain silica has the reputation that it is inconvenient to use due to long equilibration times. At least for solvents of low and moderate polarity this is not true, as is shown here with the reproducible separation of ten compounds with gradient elution; solvents are hexane and dichloromethane or hexane and *tert.*-butyl methyl ether. Short re-equilibration times could especially be obtained with hexane–dichloromethane gradients.

*Keywords:* Gradient elution; Silica

## 1. Introduction

Thirty years ago, in the dawn of instrumental column liquid chromatography, gradient separations on adsorbents, such as silica and alumina, were not uncommon in the literature of separation science [1–4]. Also, the later appearing books on high-performance liquid chromatography (HPLC), which became classics, present examples of normal-phase gradient separations [5–7]. Even the book of Jandera and Churácek which appeared in 1985 mentions numerous examples of such gradients as a matter of course [8].

Today most HPLC separations are performed with aqueous mobile phases, mostly on non-polar "reversed" stationary phases, but also on ion exchangers. On those systems gradients are easy to handle, rugged and reliable. Especially the typical reversed-phase methods can cope with a wide range of solute polarities. This is one of the most important features of contemporary HPLC; a look at the above-mentioned papers from the 1960s clearly shows the enormous progress in chromatographic methodology which was for the benefit of all users of HPLC.

In contrast to the ubiquitous use of reversed phases, the benefits of silica as a "normal"-phase adsorbent are often not rated at their value (with the exception of open column chromatography in organic synthesis and probably of large-scale industrial applications for preparative separations). Silica is unsurpassed in its ability to separate geometrical isomers. In the author's as well as in the experience of others [9], its performance, determined as reduced height of a theoretical plate in isocratic mode and with non-polar solutes, is higher than that of bonded phases (this is not the case with polar, especially basic solutes). Despite these beneficial properties, only a low percentage of all HPLC separations are performed on plain silica. A main reason is that its main application range is not for aqueous samples, i.e. for analyses in clinical and biological chemistry (although silica can be used with aqueous mobile


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phases, see Ref. [10] as an example). In addition, most analysts are convinced that adsorbents are not suited at all for gradient separations.

Indeed, this idea has some sound roots. If the solvents used for a gradient separation on silica (or alumina) differ strongly in their polarity, solvent demixing effects can occur which manifest themselves in the appearance of extremely narrow peaks somewhere in the chromatogram [5]. Re-equilibration times after the gradient can be long, again mainly under conditions of a wide polarity range. It can be assumed that the use of localizing solvents [11] as stronger eluent is less suitable if short equilibration times are needed.

This paper shows that reproducible gradients on silica with fast re-equilibration are possible. Its intention is not to undermine the dominant position of reversed-phase separations but to document that there exists an alternative for samples of low to medium polarity. (Another alternative, not discussed here, is the use of polar bonded phase, such as diol or nitrile, with normal-phase gradients.)

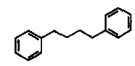
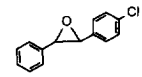
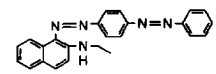
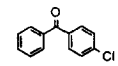
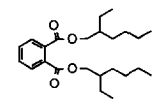
## 2. Experimental

Table 1 lists the ten compounds of a "random test mixture" which could not be separated in isocratic mode with either normal- or reversed-phase systems [12] [more solvent mixtures than the two presented there, hexane–tetrahydrofuran (99:1 v/v) and water–acetonitrile (25:75 v/v), were tried]. They are of low to medium polarity and have an aromatic ring for easy UV detection. These compounds were dissolved in hexane.

All experiments were performed on a 25 cm×3.2 mm I.D. column packed with LiChrosorb SI 60 5 μm silica (Merck, Darmstadt, Germany). This is a "type A" silica, i.e. a conventional xerogel [13,14]. Xerogels have a rather inhomogeneous surface, they are acidic and are not synthesized from chemicals of highest purity, which results in contamination with metal cations.

The solvents used for the mobile phases were Romil "Super Purity Solvent" quality (Romil, Waterbeach, Cambridge, UK); no attention was paid to their water content. The eluent flow-rate was 1 ml/min. A Jasco (Tokyo, Japan) high-pressure gradient system with UV detector set at 254 nm was used. Its dwell volume was ca. 0.6 ml. The data were evaluated with a GynkoSoft Chromatography Data System, Version 5.32 (Gynkotek, Germering, Germany). For quantitative analyses, 100 μl of sample solution were injected into a Rheodyne 7125 valve with 20-μl loop (Rheodyne, Berkeley, CA, USA).

Table 1
The test mixture

| Number | Name | Formula |
|---|---|---|
| 1 | 2-Phenylethylbromide |  |
| 2 | 1,4-Diphenylbutane |  |
| 3 | Phenetole |  |
| 4 | Nitrobenzene |  |
| 5 | trans-Chlorostilbene oxide |  |
| 6 | Sudan red 7B |  |
| 7 | 4-Chloro-benzophenone |  |
| 8 | Veratrole |  |
| 9 | Acetophenone |  |
| 10 | Phthalic acid-bis-2-ethylhexyl ester |  |

## 3. Results and discussion

Fig. 1 presents the gradient separation of the ten-component mixture with hexane and *tert.*-butyl methyl ether as the components of the eluent. The gradient profile is shown and was as follows: 0–2 min, 100% hexane; 2–15 min, 0–10% *tert.*-butyl methyl ether in the form of 0–100% B solvent (i.e. the B solvent was hexane–*tert.*-butyl methyl ether 9:1), 15–18 min, linear return to 100% hexane. The

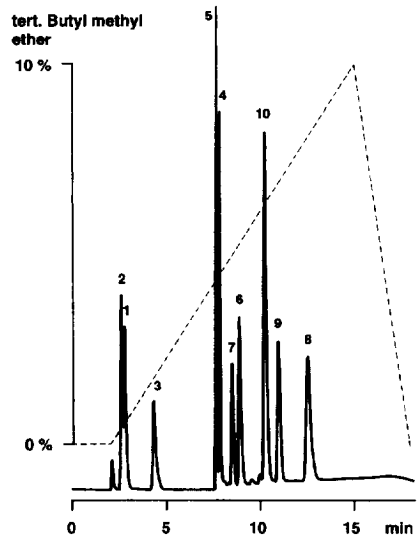



Fig. 1. Separation of the ten-compound mixture with a gradient from hexane to 10% *tert.*-butyl methyl ether in hexane. Gradient profile as shown. For the identification of the peaks see Table 1.

separation is reproducible but the peak pattern depends on the equilibration time at 100% hexane, as long as the next injection is done at less than 12 min of equilibration (or earlier than 30 min of total analysis time) which means less than ca. 8 column volumes. The chromatogram shown here was obtained after a 2-min equilibration (or 20 min after the injection of the preceding sample). The separation could be optimized by using another than a linear gradient type but this solvent system was not studied further.

Hexane–dichloromethane turned out to be a more convenient solvent system because the true equilibration time was determined as only 2 min, i.e. the chromatogram pattern shows no change if a new injection is made at 2 min (ca. 1.5 column volumes) after 100% hexane has been reached again or if it is made at a later time. Three consecutive separations with a linear gradient profile are shown in Fig. 2 (0–2 min, 100% hexane; 2–15 min, 0–100% dichloromethane; 15–18 min, 100–0% dichloromethane). The selectivity is different from the one obtained with hexane–*tert.*-butyl methyl ether. There the first half of the chromatogram had poorer resolution than the second one; now it is difficult to separate the last three peaks. Therefore, a step gradient profile was applied which resulted in the separation of Fig. 3 (0–2 min, 100% hexane; 2–8.5 min, 0–50% dichloromethane; 8.5–13.5 min, 50% dichloromethane; 13.5–20 min, 50–100% dichloromethane; 20–23 min, 100–0% dichloromethane).

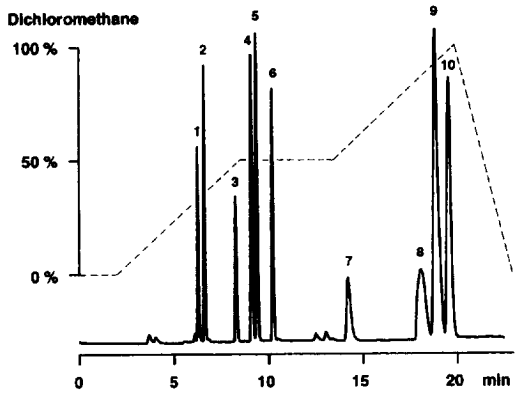



Fig. 3. Separation of the ten-compound mixture with a step gradient of hexane and dichloromethane.

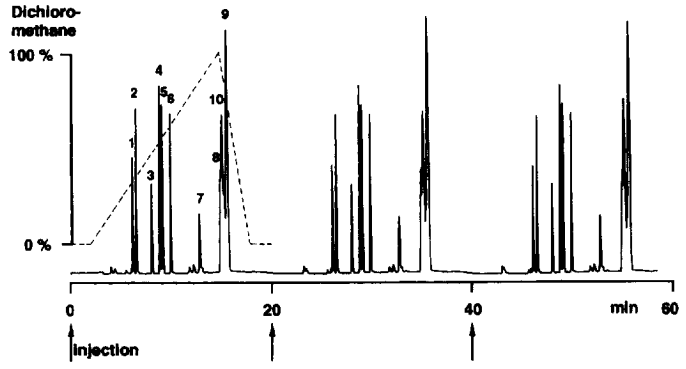



Fig. 2. Three consecutive separations with a gradient from hexane to 100% dichloromethane.

Now the peak shape of veratrole (peak 8) is unusual, which results in a high standard deviation of its height, although the precision of the area determination is excellent. The standard deviations of retention times, areas and heights of all ten peaks, separated with the hexane–dichloromethane step gradient, are listed in Table 2. Consecutive injections were made every 25 min, i.e. 2 min after 100% hexane was reached again.

A closer look at Figs. 2 and 3 reveals that future research is needed for a better understanding of normal-phase gradients. As already mentioned, peak 8 has an unsatisfactory shape and is perhaps responsible for the shoulder between peaks 10 and 9 in Fig. 2 (in the first separation on peak 10, then on peak 9). The cause could be a solvent demixing effect [5] or an unusual adsorption isotherm of veratrole (e.g., due to complexation with metal cations present in the silica). In addition, the most important question of the water content of the mobile phase needs a thorough investigation. Dry solvents have been shown to result in low column efficiency and poor peak symmetry (and, of course, in high capacity factors), especially on type A silicas [15]. Type B silicas (sol–gels) are probably advantageous for gradient separations and need to be investigated also. It can be assumed that the water content of the solvents has a strong influence on the re-equilibration time. It will also be necessary to obtain more data on the reproducibility of retention times, peak areas and peak heights with normal-phase gradients on plain silica. The data of Table 2 are poorer than obtained in many laboratories on a routine basis with gradient separations on reversed phases. It is well possible that this has to do with the fact that the water (or other polar modifier) content of the mobile phase was not controlled at all in the present study.

## 4. Conclusions

Although not a panacea, reproducible gradients with short equilibration time are possible in normal-phase chromatography on silica, at least for compounds of low to moderate polarity. The re-equilibration back to the solvent conditions from where the gradient started can be a true one, i.e. the separation is not influenced if the equilibration time is longer than stated, or it can be fictitious if one does not want to wait long enough until true equilibrium is reached. Surprisingly enough, two beneficial behaviours could be observed: First, with gradients of hexane and dichloromethane, the true equilibration time was found to be very short (2 min); and second, also fictitious re-equilibration can be used because it yields reproducible chromatograms if the time between consecutive injections is held constant. This time can be very short; the hexane–*tert.*-butyl methyl ether system was used successfully with 2 min of "equilibration" time.

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Table 2
Precision (as relative standard deviation) of five consecutive injections with the hexane–dichloromethane step gradient, as shown in Fig. 3

| Peak | Compound | Retention time | Peak area | Peak height |
|---|---|---|---|---|
| 1 | 2-Phenylethylbromide | ±1.1% | ±0.8% | ±1.7% |
| 2 | 1,4-Diphenylbutane | ±0.8% | ±1.7% | ±1.2% |
| 3 | Phenetole | ±0.2% | ±0.6% | ±0.7% |
| 4 | Nitrobenzene | ±0.1% | ±0.9% | ±1.4% |
| 5 | *trans*-Chlorostilbene oxide | ±0.2% | ±0.6% | ±1.5% |
| 6 | Sudan red 7B | ±0.4% | ±2.2% | ±1.9% |
| 7 | Chlorobenzophenone | ±1.3% | ±0.8% | ±2.5% |
| 8 | Veratrole | ±1.3% | ±0.7% | ±26% |
| 9 | Acetophenone | ±1.5% | ±0.5% | ±1.8% |
| 10 | Phthalic acid-bis-2-ethylhexyl ester | ±1.4% | ±1.5% | ±2.5% |

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