

Liquid Chromatography Problem Solving and Troubleshooting

Question

Although I am relatively proficient at adjusting eluent conditions to optimize my HPLC separations, I often have difficulty in getting started in terms of selecting an initial mobile phase. Could you provide a few tips to help me with this problem?

Answer

The first and most obvious strategy is to survey the literature to see if your analyte or mixture of analytes is similar in structure to other compounds that have been reported previously. Often, if there are not large structural differences that change the analyte's polarity too much, then experimental conditions that have been used successfully to separate similar compounds are a good starting point. However, once you have tried this and are unsuccessful, then there are two useful HPLC approaches for establishing an initial set of starting eluent conditions.

Experimentally, the quickest approach is to carry out a simple gradient-elution separation starting with a mobile phase of low eluting power and programming its strength to increase with time. A 10- to 15-min linear gradient is often a good starting point. From the gradient profile it is possible to predict starting conditions from an extrapolation of the gradient profile at the time a particular solute or group of solutes elute from the column. For reversed-phase assays the starting gradient profile is run from high aqueous conditions to high organic conditions. In order of usage and increasing elute strength, the three principle organic cosolvents are methanol, acetonitrile, and tetrahydrofuran. For normal-phase separations, the gradient begins with the nonpolar carrier solvent and ends with the nonpolar carrier solvent containing a high level of a polar modifier such as 2-propanol. In selecting the polar modifier, a useful quantitative scale (i.e., the eluotropic series) is based on the work of Snyder et al. (1,2), who examined how strongly solvent molecules adsorb to polar substrates. Although this scale was developed initially for alumina, it has been modified for silica and can be found in many chromatography books.

An initial mobile phase composition can also be established using an isocratic approach by starting with a solvent with strong eluting power that is systematically decreased in subsequent separations. When developing reversed-phase assays, the starting mobile phase should contain a high level of the organic cosolvent. It is advisable to begin with nearly 100% of the organic component for highly hydrophobic compounds, especially if more retentive surfaces such as ODS packings are being used. In the case of normal-phase separations, the scouting experiment starts with a mobile phase that contains a high level of a polar modifier such as 2-propanol (i.e., 20–30%) added to the nonpolar

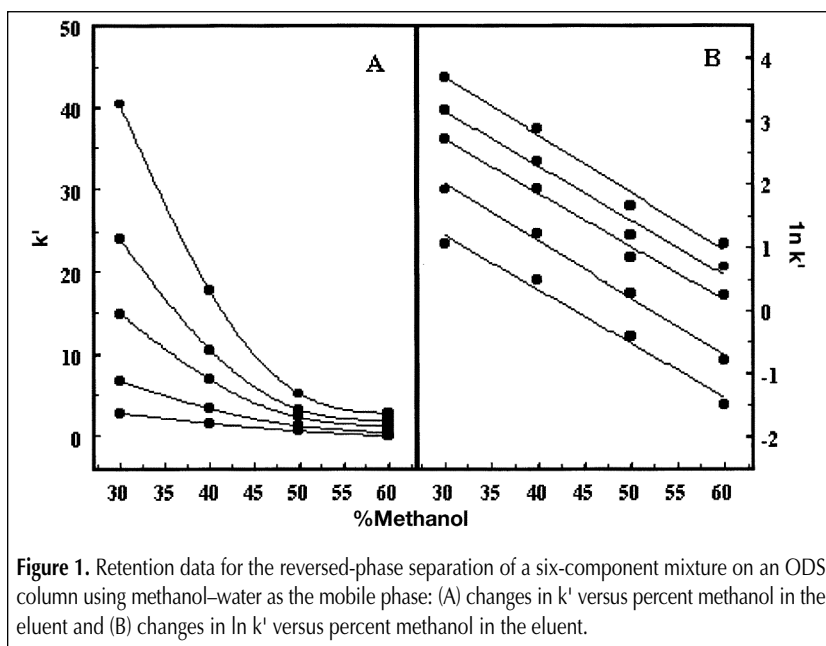


Figure 1. Retention data for the reversed-phase separation of a six-component mixture on an ODS column using methanol–water as the mobile phase: (A) changes in k' versus percent methanol in the eluent and (B) changes in $\ln k'$ versus percent methanol in the eluent.

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carrier solvent (e.g., hexane).

In carrying out both reversed- and normal-phase experiments, it is important that the strength of the initial mobile phase is high enough so that all compounds elute quickly from the column and changes in the eluent's composition are made systematically. For reversed-phase conditions, decreasing the organic component by 10% or 15% when carrying out each successive separation provides a rapid means of arriving at an appropriate range of starting conditions, and for normal-phase eluents halving or approximately halving the polar modifier's concentration provides an adequate change. Once three or four experiments have been completed, it is helpful to construct a simple plot of $\ln k'$ versus the percentage of organic component in the eluent. An extrapolation of this plot, which in many cases is linear or nearly linear, provides a convenient way of predicting the retention profile of a compound over a broader range of mobile phase compositions and thus fine-tuning the final eluent's composition. This is illustrated in Figures 1A and 1B for a mixture of six compounds separated under a typical set of reversed-phase conditions using varying compositions of methanol and water.

It is important to remember that when using either the isocratic or gradient approach to arrive at an initial set of mobile phase conditions one typically is faced with the following question: "How do I know if all of the compounds in my sample have eluted from the column?" Given a known set of analytes to be resolved, this is easily evaluated. However, when presented with a truly unknown sample or an analyte in a complex matrix, this question is more difficult to answer. Unknown/ghost peaks or slowly drifting baselines often reflect the elution of highly retained compounds. In the latter instance, it is advisable to carry out a few TLC separations as quick screening tools. This has two advantages: first, many solvent conditions can be evaluated quickly and economically including both normal- and reversed-phase modes of separation, and second and perhaps even more important, if a compound shows some level of migration (even if it is only a very small amount) under a given set of TLC development conditions, then it will be eluted from an equivalent HPLC stationary phase using a mobile phase with similar polarity. Likewise, if material is left at the point of application on the thin-layer plate, then there is a high probability that it will not elute using similar HPLC conditions.

References

1. L.R. Snyder. "Principles of Adsorption Chromatography; the Separation of Nonionic Organic Compounds". In *Chromatographic Science Series*. Marcel Dekker, New York, NY, 1968.
2. B.L. Karger, L.R. Snyder, and C. Horvath. *An Introduction to Separation Science*. John Wiley and Sons, New York, NY, 1973.