Liquid Chromatography Problem Solving and Troubleshooting

Question

Although I have been doing other types of QC work for several years including GC assays, I have not been involved in running HPLC methods until recently as a result of our only LC person leaving my company. Currently, I have been given the responsibility of an isocratic reversed-phase method that apparently has been used reliably for several years to assay the active ingredients in one of our products. Unfortunately, each time I have used the procedure, I have obtained fairly large variations in retention. Sometimes it increases and sometimes it decreases for no apparent reason from one day to the next. I really don't understand what I could be doing wrong, because I am using a simple single-solvent pump equipped with a fixed wavelength UV detector, a commercial C_{18} column, and a 55:45 mixture of methanol–water as the mobile phase, which I carefully prepare fresh each time I run the assay.

Answer

Although there can be a number of causes for variations in retention, the important parts of your question are that: (*a*) you have observed both positive and negative changes in solute retention when using the assay on different days and (*b*) you prepare your mobile phase fresh each time you carry out the assay.

A common cause of positive and negative fluctuations in a solute's retention on a day-to-day basis (i.e., from one run to the next after preparing a new mobile phase) is inconsistent preparation of the eluent. Although problems associated with eluent preparation can arise from several factors, one of these that is associated with hydro-organic eluents results from small volume changes when an organic solvent and water are mixed. These volume changes can cause either increases or decreases in retention depending on the order of addition of the cosolvents if a single volumetric vessel such as a graduated cylinder is used to prepare the eluent.

When methanol is combined with an equal volume of water the final composition is 50:50; however, the resulting volume after complete mixing is less than the sum of the individual solvent volumes by approximately 3%. As a result of this decrease in volume, composition errors can arise in solvent bending if the eluent is prepared in a single volumetric vessel by combining one solvent to a given volume of the other. For example, if 50 mL of methanol is first added to a graduated cylinder and then water is poured directly into the vessel until a final volume of 100 mL is obtained, the resulting binary mixture will not be exactly 50:50 methanol–water, but it will be preferentially rich in water. If complete mixing occurs during the addition of the second solvent, the resulting eluent will be 48.5:51.5 methanol–water. Conversely, if the solvents are added in the reverse order, the composition will be 51.5:48.5 methanol–water.

As a means of providing you, as well as other readers, with a better understanding of how changes in solvent composition influence reversed-phase retention, the elution profiles of four simple aromatic

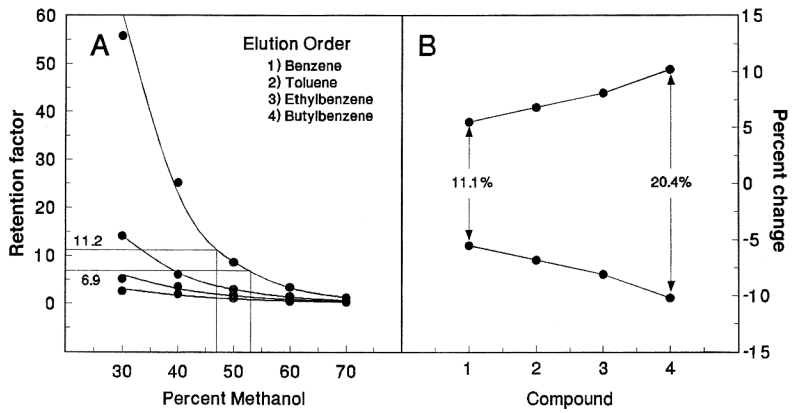

Figure 1. Influence of mixing errors on reversed-phase retention: (A) changes in the retention factor of simple aromatic solutes separated using methanol–water as the eluent and (B) relative percent change in k' expected for an angstrom 1% absolute mixing error in the eluent for these same solutes.

The purpose of *Chromatography Problem Solving and Troubleshooting* is to have selected experts answer chromatographic questions in any of the various separation fields (GC, GC–MS, HPLC, TLC, SFC, HPTLC, open column, etc.). If you have questions or problems that you would like answered, please forward these to the *Journal* editorial office with all pertinent details: instrument operating conditions, temperatures, pressures, columns, support materials, liquid phases, carrier gas, mobile phases, detectors, example chromatograms, etc. In addition, if you would like to share your expertise or experience in the form of a particular question accompanied by the answer, please forward it to: JCS Associate Editor, *Chromatography Problem Solving and Troubleshooting*, P.O. Box 48312, Niles, IL 60714. All questions/answers are reviewed to ensure completeness. The *Journal* reserves the right not to publish submitted questions/answers.

Roger K. Gilpin
Associate Editor

compounds chromatographed under relatively standard conditions are shown in Figure 1A. Also, illustrated in the same diagram by the narrow solid vertical and horizontal lines are the retention factors for one of these compounds (butylbenzene) when the separation is carried out using 47:53 methanol–water (k' = 11.2) versus 53:47 methanol–water (k' = 6.9). Changes in retention in this example are approximately twice as large as those that could result from the mixing problems discussed previously.

Illustrated in Figure 1B is the percent relative change in the retention factor (i.e., relative day-to-day fluctuations in k') for the four solutes given in Figure 1A, assuming the eluent's composition is preferentially rich in either methanol or water at approximately 1% as the result of day-to-day mixing differences. This would cause what would appear to be relatively random changes in the k' values that would vary between approximately 10% to 20%, depending on the slightly different k' values versus the percent methanol profiles shown in Figure 1A for the four example solutes. Although such eluent preparation errors would not be made by those familiar with the volume changes associated with solvent mixing, they are a relatively common mistake for those that are not accustomed to carrying out HPLC separations. Thus, the problem you are experiencing is simple to fix if you remember to carefully measure out each of the cosolvents prior to blending. Also, don't forget that the overall quality of your results will depend on the volumetric accuracy of your glassware (i.e., graduated cylinders are not considered to be highly accurate glassware).