Readers' Questions

JOHN W. DOLAN

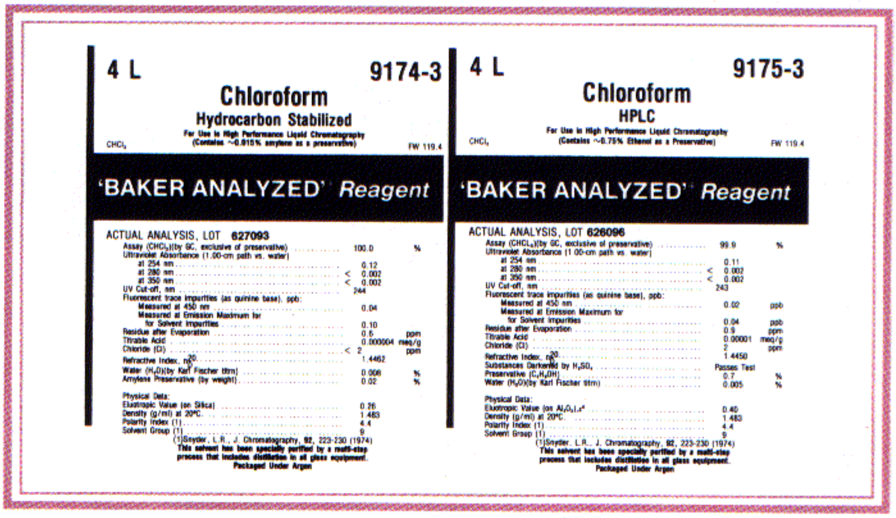
September's Troubleshooting column features input from readers about unintended mobile phase additives and presents two possible solutions to a reader's problem that was reported in the April (Volume 4, Number 4) column. A question on preservation of column performance also is discussed. Readers are invited to contribute troubleshooting tips or to submit questions for discussion in future Troubleshooting articles. Write to: The Editor, *LC•GC,* P.O. Box 50, Springfield, OR 97477.

MOBILE PHASE SELECTION FOR NORMAL-PHASE LC

If you are trying to repeat methods that have been developed in another lab, be aware of a problem reported by one reader (1). In this case, a method for separating a group of basic drugs had been developed on a silica column using 80:20 (v/v) chloroform/hexane as the mobile phase. The results were satisfactory, and assay results were within the accepted limits of reproducibility. When the "same" method was transferred to another lab, however, the chemist was unable to get the sample bands off the column. The mobile phase was modified, but still no peaks eluted, even with 100% chloroform. HPLC-grade solvents from another vendor were tried, and a satisfactory separation was obtained.

It was discovered that the difference between the two batches of solvents lay with the preservative used for fixing the chloroform. The "good" chloroform contained ethanol; the "bad" chloroform did not. Water or other polar modifiers (for example, methanol, acetonitrile, or isopropanol) often are added to liquid–solid (silica) chromatography mobile phases to control selectivity (2). Polar additives can improve column plate number (sharpen peaks), reduce retention, and improve retention-time reproducibility. In the present case, the method was developed with ethanol used, in effect, as an unintended mobile phase modifer; when dry solvents were used, the method no longer worked.

It is good to be aware that HPLC-grade chloroform (and some other solvents as well) might vary compositionally from one manufacturer to the next. Manufacturers' literature (for example, see references 3 and 4) should be consulted for detailed content assays of specific solvents. Much of the same information is included on solvent-bottle labels (Figure 1). In the case of chloroform, common preservatives are ethanol and amylene (2-methyl-2-butene) at levels of about 0.5%–1.0% and 0.01%–0.02%, respectively; 0.5% ethanol is sufficient to modify significantly the retention characteristics of a chloroform–hexane mobile phase with a silica column. Thus, we can see that "equivalent" mobile phases made up from the chloroform shown in Figures 1a and 1b are not the same — even though they contain HPLC-grade chloroform from the same vendor.



4 L 9174-3

Chloroform

Hydrocarbon Stabilized

For Use in High Performance Liquid Chromatography
(Contains ~0.015% amylene as a preservative)

$CHCl_3$ FW 119.4

'BAKER ANALYZED' Reagent

ACTUAL ANALYSIS, LOT **627093**

Assay ($CHCl_3$)(by GC, exclusive of preservative)		100.0	%
Ultraviolet Absorbance (1.00-cm path vs. water)			
at 254 nm		0.12	
at 280 nm	<	0.002	
at 350 nm	<	0.002	
UV Cut-off, nm		244	
Fluorescent trace impurities (as quinine base), ppb:			
Measured at 450 nm		0.04	
Measured at Emission Maximum for for Solvent Impurities		0.10	
Residue after Evaporation		0.6	ppm
Titrable Acid		0.000004	meq/g
Chloride (Cl)	<	2	ppm
Refractive Index, n_D^{20}		1.4462	
Water (H_2O)(by Karl Fischer titrn)		0.008	%
Amylene Preservative (by weight)		0.02	%
Physical Data:			
Eluotropic Value (on Silica)		0.26	
Density (g/ml) at 20°C.		1.483	
Polarity Index (1)		4.4	
Solvent Group (1)		9	

(1)Snyder, L.R., J. Chromatography, 92, 223-230 (1974)
This solvent has been specially purified by a multi-step process that includes distillation in all glass equipment.
Packaged Under Argon

4 L 9175-3

Chloroform

HPLC

For Use in High Performance Liquid Chromatography
(Contains ~0.75% Ethanol as a Preservative)

$CHCl_3$ FW 119.4

'BAKER ANALYZED' Reagent

ACTUAL ANALYSIS, LOT **626096**

Assay ($CHCl_3$)(by GC, exclusive of preservative)		99.9	%
Ultraviolet Absorbance (1.00-cm path vs. water)			
at 254 nm		0.11	
at 280 nm	<	0.002	
at 350 nm	<	0.002	
UV Cut-off, nm		243	
Fluorescent trace impurities (as quinine base), ppb:			
Measured at 450 nm		0.02	ppb
Measured at Emission Maximum for for Solvent Impurities		0.04	ppb
Residue after Evaporation		0.9	ppm
Titrable Acid		0.00001	meq/g
Chloride (Cl)		2	ppm
Refractive Index, n_D^{20}		1.4450	
Substances Darkened by H_2SO_4		Passes Test	
Preservative (C_2H_5OH)		0.7	%
Water (H_2O)(by Karl Fischer titrn)		0.005	%
Physical Data:			
Eluotropic Value (on Al_2O_3),$\epsilon°$		0.40	
Density (g/ml) at 20°C.		1.483	
Polarity Index (1)		4.4	
Solvent Group (1)		9	

(1)Snyder, L.R., J. Chromatography, 92, 223-230 (1974)
This solvent has been specially purified by a multi-step process that includes distillation in all glass equipment.
Packaged Under Argon

FIGURE 1: Labels for HPLC-grade chloroform containing (a) amylene and (b) ethanol as a preservative. (Courtesy of J.T. Baker Chemical Co.)

Solvent stabilizers generally are not important in affecting the selectivity of reversed-phase mobile phases. The major problem with reversed-phase solvent preservatives is their UV absorbance. For example, butylated hydroxy toluene (BHT) is added to tetrahydrofuran (THF) to prevent peroxide formation, but the aromatic nature of BHT prevents the use of stabilized THF *and* UV detection. As a result, unstabilized THF is recommended for use with UV detectors.

MAXIMIZING COLUMN LIFE

Q: What is the best way to keep C8-bonded reversed-phase columns "good"? In my liquid chromatograph, I use on-line mixing with 800 mL water plus 5 mL acetic acid as the A solvent and 100% methanol as the B solvent. The final mobile phase is 90% A and 10% B at a flow rate of 2.5 mL/min. Run time is 30 min. How often should the column be flushed, and in which direction? Should I use 100% methanol? Is volume more important than flow?

JWD: You should concentrate on two areas to get the most out of your LC column. First, try to keep it from becoming contaminated, and second, remove contaminants regularly. Let's look at each of those areas.

Prevention of column contamination starts with sample preparation, the economics of which must be balanced against the cost of replacing the column. This means that if you try to save time (money) by minimizing sample preparation, you may end up losing any savings because of increased column-replacement costs. The reverse is also true. For example, needless filtering of every sample is a waste of time and money. Disposable sample-preparation columns can be used to remove proteins, lipids, and pigments that might otherwise foul an LC column. Only samples that are opalescent or contain visible particulates should be filtered regularly.

A second major source of column contamination is the mobile phase. Be sure to use HPLC-grade solvents whenever possible and filter all salt- or buffer-containing solvents (for example, your A solvent) through a 0.45-µm filter before use. Use an inlet filter on the reservoir end of the solvent inlet lines to prevent dust from entering the system. Finally, change the pump seals regularly (every three months or so) to avoid contamination by particulates arising from seal wear.

Once you have done all you can to prevent contamination of the system, try to keep those contaminants that do get in from reaching the analytical column. The key step here is use of a guard column. Approximately 75% of the readers polled in a recent *LC Magazine* User Survey indicated that they use guard columns (5), the benefits of which are widely accepted. The guard column acts as a "superfilter" that traps not only particulate matter but also chemical contaminants *before* they reach the analytical column. The lower-priced guard column is then replaced, and the more expensive analytical column is preserved.

Now that we've discussed ways to prevent contaminants from reaching the column, let's consider how to treat the column for maximum column life. First, remove any salt- or buffer-containing mobile phases from the system at the end of each day. This prevents salt-deposit buildup, precipitation of crystals, and corrosion of LC hardware. If mobile phases that contain only HPLC-grade solvent and water are used, the mobile phase generally can be left in the system overnight. For longer storage, return the column to the mobile phase that it contained when you first acquired it (or 100% methanol). In your case, however, you should flush the acetic acid from the system; that is, replace the A solvent with HPLC-grade water, and pump about 20 column volumes (~ 50 mL) of 90% A (water)–10% B (methanol) to remove residual acid. Next, switch to 100% B for another 20 column volumes to remove any strongly retained materials; then turn the pump off. You may now leave the system until its next use. It is the *volume* of solvent that is important when flushing the column, not the flow rate, but flow should be adjusted to give a reasonable pressure (for example, <2000 psi).

Strongly retained material that is not readily removed using methanol — for example, very late eluting bands or bands showing a gradual decrease in column plate number — can be removed by a strong-solvent flush. Most modern LC columns are stable under reverse flushing (if you are in doubt, check with the manufacturer), and I recommend reverse flushing to remove contaminants more quickly if a strong solvent is used. The outlet of the column (or old inlet in the case of reverse flushing) should, of course, go directly to waste instead of to the detector. Use 20 column volumes each of the following solvents in the given order, then reverse the order until you return to your starting solvent: methanol (or acetonitrile), then 2-propanol, then methylene chloride. In your case, this would be 50 mL methanol, 50 mL 2-propanol, 50 mL methylene chloride, 50 mL 2-propanol, and, finally, 50 mL methanol. To remove most of the contaminants, 2-propanol often will be of sufficient strength. Methylene chloride should be used with care because it is not miscible with most reversed-phase solvent mixtures. (For more ways to prevent column problems, see reference 6.)

READERS' SUGGESTIONS

In the April Troubleshooting column (7), a reader reported a problem with a late-eluting peak from a protein assay on a Waters Protein Pak DEAE 5 PW column (Waters Chromatography Division, Millipore Corp., Milford, Massachusetts). The peak in question showed up when the sample was dissolved in a diluent (75 m*M* sodium chloride + 1 m*M* calcium chloride), but not when it was dissolved in water or in the starting buffer (5 m*M* Tris–acetate + 5 m*M* calcium acetate, pH 7.5). Reagents from a variety of sources were tried, with no improvement, and the problem peak also occurred without the protein sample. Two readers have submitted possible solutions to the problem.

Sodium chloride: One reader suggested that the problem peak actually is a strongly retained peak (from previous samples) that is eluted with sodium chloride (8). Late-eluting bands are hard to correlate with a specific sample, especially in gradient elution, because the widths of all the bands are similar. If the peak were retained from a previous sample, it could show up even if no protein sample were present. The reader also noticed that the late peak occurred only when sodium chloride was present. A specific interaction between sodium chloride and the late peak could result in its release from the column, which suggests that the addition of sodium chloride to the mobile phase can aid either in earlier elution of the peak or in prevention of its buildup on the column.

Sample preparation: Another reader had a similar problem that was the result of unexpected contamination during sample preparation (9). A similar "ghost" peak occurred on an irregular basis, with no apparent correlation with samples or reagents. The problem was finally traced to a contaminant (wetting agent?) leaching from the 0.2-μm membrane filters that were used during sample preparation. The filters from one manufacturer caused problems, whereas two other manufacturers' products did not, although all three were nominally equivalent. The irregular occurrence of the problem was a result of using the same filter for several replicates of the same sample; the first sample received the highest "dose" of contaminant, and later samples had little or no contaminant present. Once the replicate sample vials were mixed on the autosampler tray, the problem peak was a completely random occurrence.

REFERENCES

(1) E. Ciurczak, personal communication.

(2) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography,* 2nd Ed. (John Wiley & Sons, New York, 1979).

(3) "HPLC Solvent Reference Manual," J.T. Baker Chemical Co. (Philipsburg, New Jersey, 1985).

(4) "Solvent Guide," 2nd Ed. (Burdick & Jackson Laboratories, Muskegon, Michigan, 1984).

(5) *LC User Survey V* (Aster Publishing, Springfield, Oregon, March 1985).

(6) J.W. Dolan, *LC, Liq. Chromatogr. HPLC Mag.* **3,** 420–423 (1985).

(7) J.W. Dolan, *LC•GC* **4,** 310–312 (1986).

(8) R.P. Manley, personal communication.

(9) S. Trump, personal communication.

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