T R O U B L E S H O O T I N G

Mobile Phase Preparation

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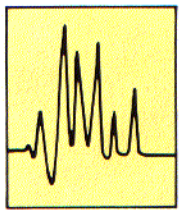

Problems that result from improper mobile phase preparation may appear in several areas of the liquid chromatographic system. Frit blockage from improper filtering or from microbial growth in buffers can occur, but deterioration in chromatographic performance is more frequently observed. Retention-time reproducibility may be poor, resolution may suffer, and baseline instability problems may arise from improper attention to mobile phase preparation. Because a standard nomenclature for mobile phases and their components is not used in practice, it is necessary to define a few terms as they will be used in the context of this column. The term *mobile phase* will be used to refer to the chemical mixture used to elute sample components from the column; *eluent* is synonymous with mobile phase. *Solvent* refers to the major pure components of the mobile phase, such as methanol, hexane, or acetonitrile. Finally, *additives* are used to change the properties of the mobile phases for the purposes of enhancing solubility, peak shape, or selectivity; examples of additives are buffers, ion-pairing agents, and amines.

SOURCES OF SOLVENTS

HPLC-grade solvents are readily available from a wide variety of sources. Some vendors specialize in selling LC solvents, whereas others sell solvents as one type of product in a line of chemicals or LC supplies. HPLC-grade solvents are purified for low spectral absorbance and low water content and are filtered through a submicron filter prior to bottling. They are stored and shipped in glass containers to minimize contamination by metals. Most vendors provide the specifications for a particular manufacturing lot either as UV-cutoff data or as spectra. Some provide chromatograms of high-sensitivity, blank-gradient assays to demonstrate low background noise and minimal drift during a gradient run. At least one vendor publishes a booklet containing the chromatographic characteristics of its solvents and gives selected references for further reading (1). The quality of all HPLC-grade solvents is generally high, so you should choose a vendor based on convenience, price, and personal preference. For very demanding analyses, such as gradient runs at low wavelengths and high sensitivity, it may be worthwhile to compare several brands of a particular solvent and select the one that produces the lowest background for your application. In any case, for consistent results it is best to buy from one vendor if possible. To help trace solvent-related problems, record the lot number of each bottle of solvent in your logbook as you use it.

For users on limited budgets or for applications in which detection sensitivity is not critical, satisfactory results often can be obtained from solvents a grade or two below the HPLC-grade solvent of a given vendor. These solvents are generally more satisfactory for isocratic than for gradient analyses because gradient conditions tend to concentrate and release impurities, which can complicate the information content of a chromatogram. To prevent contamination by particulate impurities, always filter non-HPLC-grade solvents prior to use.

Water is the most frequently used solvent in liquid chromatography, but it is also the most common source of solvent-related problems. Even though HPLC-grade water is available from those vendors who supply other LC solvents, the user is often tempted to turn on the faucet and use water from this inexpensive source. The use of poor quality water can result in a buildup of contaminants on the column that are eventually released when the breakthrough volume is reached or when mobile phase conditions are changed. It can take days to isolate and eliminate a water-related problem in an LC system. For large volumes, the most economical source of water is lab-generated, HPLC-grade water obtained from a commercially available water purification system. These water purifiers, such as the Organicpure (Barnstead Co., Boston, Massachusetts) or Milli-Q (Millipore Corp., Bedford, Massachusetts) systems, treat distilled or deionized water to remove potential LC contaminants. A particulate filter is generally followed by one or two ion-exchange cartridges and an activated-carbon cartridge. Some systems incorporate UV irradiation prior to final filtering through a 0.45- or 0.2-μm filter. The quality of the water obtained from these systems is high, and if a significant quantity of water is used, the system will quickly pay for itself. You can often clean up lesser-grade water by pumping it through a used C18 column, but caution should be taken to ensure that strongly retained material is removed from the column first, or contamination can result. In an emergency, distilled water sold in grocery stores for steam irons is quite satisfactory for most LC applications.

Three organic solvents are generally sufficient for most reversed-phase applications. Water is used as a modifier to control solvent strength. Methanol is the most frequently used organic solvent in reversed-phase applications. It provides satisfactory separations for a wide variety of solutes when used in combination with water or buffers, it has a fairly low toxicity, and it is the least expensive of the common solvents. Acetonitrile and tetrahydrofuran (THF) are the other most common reversed-phase solvents. THF may be purchased with or without stabilizers, which inhibit peroxide formation. Generally, unstabilized THF is used because the stabilizers usually increase the UV background of the THF. Unstabilized THF has a finite shelf life and opened bottles should be discarded at regular intervals to prevent accumulation of explosive peroxides. Propanol is often chosen for separations of large biomolecules because of its superior solvency for this class of compounds.

Methylene chloride, methyl *t*-butyl ether, and acetonitrile are generally chosen as the primary solvents for normal-phase applications. Hexane or FC-113 is often used to control solvent strength. Use of the Snyder solvent-selectivity triangle is recommended to the reader to aid in the choice of the proper solvent for a particular chromatographic application (2).

MOBILE PHASE ADDITIVES

Buffers are the most frequently used additives in reversed-phase systems. They act to control the ionic strength and pH of the system to enhance the chromatographic properties of the samples of interest. The stability of silica-based LC columns requires a mobile phase of pH of less than 7.5. The popular buffers are phosphate, acetate, formate, and trifluoroacetate, used either as the sodium or potassium salts or in the acid form. Phosphate, the most popular buffer, is quite stable at room temperature and has the right properties to enhance the chromatography of many solutes. Acetate buffers are used less often, although they are frequently used for PTH-amino acid analyses. Because acetate provides a good carbon source for microbial growth, special care must be taken to prevent contamination of acetate buffers. Boiling a stock solution of acetate buffer and keeping the stock refrigerated will minimize microbial growth. The stock should be diluted daily with sterilized water, and the working solution should be discarded at the end of each day. Formate and trifluoroacetate buffers are selected for use when the separated analytes are to be recovered after the chromatographic analysis because these buffers are sufficiently volatile to evaporate with the mobile phase. Caution needs to be taken during degassing, however, because it is possible to selectively volatilize the buffer components under vacuum or heating and thus to inadvertently change the mobile phase composition.

To achieve consistent results, use high-quality reagents and HPLC-grade water to prepare the buffer. It is usually most convenient to make the buffer 10–100 times more concentrated than the final level. This concentrated stock solution can be stored in a refrigerator to prolong its useful lifetime. Be sure to filter all buffers prior to use to remove both biological and physical contaminants. And finally, always measure the pH of the buffer before you add it to the organic components, because the pH of an organic solution is not very meaningful.

Growth inhibitors, such as sodium azide at the 0.004% level, can be added to buffers to minimize microbial contamination (6). If the buffer is mixed with an organic solvent to form the mobile phase, the solvent is often effective as a growth inhibitor. Adding 5% THF to a buffer, for example, greatly reduces microbial growth.

Ion-pairing agents are added to reversed-phase mobile phases to change the mode of chromatography. Ion-pairing reagents such as the alkyl sulfonates are available in high-purity form, both from LC supply vendors and general chemical supply houses. If you perform low-wavelength assays, it is worthwhile to check the purity of the ion-pairing reagent by UV spectroscopy, and then recrystallize the reagent if necessary. Knox has shown that the equilibration time of an ion-pairing agent with the stationary phase is dependent upon the chain length of the ion-pairing agent (5). For example, to obtain equilibrium conditions using a C18 column with a 0.1 m*M* ion-pair solution, lauryl sulfate requires 1500 ml of mobile phase, but octyl sulfate requires only 100 ml for equilibration. Lauryl sulfate cannot be completely rinsed from the column; however, only about 100 ml of rinse solution is required to wash off all of the octyl sulfate. Knox has also shown that ion-pairing retention is a function of the concentration of the ion-pairing agent, not of the chain length. These findings suggest that short-chain ion-pairing agents be used for the greatest operational convenience.

Amine modifiers are commonly added to reversed-phase mobile phases to reduce peak tailing. Amines appear to associate with the free silanol (Si-OH) groups at the silica surface to minimize their interaction with basic solutes. Two popular amine modifiers are triethylamine and nonylamine. Triethylamine equilibrates quickly and is also quickly rinsed off the column when a mobile phase change is made. Many chromatographers routinely add triethylamine at the level of about 5 m*M* to reduce peak tailing. Nonylamine, however, has the same problems associated with the longer chain ion-pairing agents and may take an hour or more to equilibrate, both when initially loading the column and when rinsing it out to change mobile phases. Proponents of nonylamine use claim it provides a more stable modified stationary phase than triethylamine. In either case, amine modifiers are very useful for minimizing peak tailing in reversed-phase systems.

TABLE I: EFFECT OF MOBILE PHASE PREPARATION TECHNIQUE ON TOLUENE RETENTION (3)

Volume Fraction Methanol	Retention Time (min)	Capacity Factor (k')
0.70	3.40	1.34
0.60	5.40	2.72
0.50	9.08	5.16
(0.62)*	4.82	2.32
(0.58)**	5.87	3.05

* 40 ml of water brought to 100 ml with methanol, calculated from k'
** 60 ml of methanol brought to 100 ml with water, calculated from k'

PREPARING MOBILE PHASES

Modern liquid chromatographs that provide low- or high-pressure mixing of mobile phases greatly simplify mobile phase preparation, yet for isocratic and dedicated analyses it is often cost-effective to manually premix mobile phases. In many chromatographic assays, some manual mixing is required despite the sophistication of the instruments because the mobile phase components are often mixtures of buffers or combinations of organic solvents. For applications involving refractive index detection, manual mixing is often required to produce an acceptably smooth baseline for routine operation. Differences in mobile phase preparation techniques account for some of the difficulty in repeating an assay developed in another laboratory. Measurement of the mobile phase components is straightforward: use volumetric measures for liquids and gravimetric measures for solids. Take care to make precise measurements because a 1% error in mobile phase composition can result in about a 5% change in retention (3). Add volume to volume instead of topping off the volume of one solvent with the second. Errors can arise from the expansion or contraction of the mixture relative to the component volumes. For example, a difference of 20% in retention time was seen for toluene when comparing two mobile phases of nominally the same composition (3). In Table I, it can be seen that there is a significant difference in composition between a 60:40 methanol/water mobile phase prepared by adding sufficient water to 60 ml of methanol to produce a 100-ml solution and one that is prepared by adding methanol to the water. The proper procedure — adding 60 ml of methanol to 40 ml of water — resulted in a mobile phase of intermediate composition, and volume reduction upon mixing resulted in a total volume of less than 100 ml. After the mobile phase components are combined, they should be mixed for several minutes using a magnetic stirrer or a similar method. Extended mixing should be undertaken for solutions containing THF, which mixes much less readily than most other solvents.

After the mobile phase is thoroughly mixed, it should be filtered through a 0.5-μm filter. Filters and filtration apparatus are available from a variety of vendors. Be sure to select a filter substrate that is compatible with the solvents of your mobile phase. Filtration removes dust and other physical

contaminants that are picked up in the measurement and mixing process. If only pure HPLC-grade solvents have been mixed to form the mobile phase, the filtration step can be deleted provided that the glassware is very clean.

The final step in mobile phase preparation is degassing. Depending upon the particular LC system you use, the degassing step may not be necessary, but routine degassing will result in more trouble-free operation. If filtration was performed under vacuum, the degassing that took place during filtration may be sufficient for your chromatograph. The most popular degassing methods are helium sparging, boiling, applying a vacuum, and sonication. Because there is considerable debate over which method is best, each will be briefly discussed here.

Helium sparging involves bubbling a stream of high-purity helium through the mobile phase, either continuously or in batches. The troublesome gases in solution — nitrogen and oxygen — are drawn out of solution as they seek equilibrium with the gaseous helium. Because helium has a very low solubility in most mobile phases, the solution is left in a relatively gas-free state. Capping the bottle with aluminum foil or maintaining a reduced level of sparging will usually keep the solvent in a degassed state during the chromatographic analysis. One potential problem to consider with helium degassing is the possibility of selective volatilization of mobile phase components; therefore, proper ventilation should be used because potentially toxic mobile phase components are contained in the helium waste stream. Helium is, however, relatively expensive, which may affect your choice of degassing method.

Boiling will effectively remove gas from solution and has the added advantage of sterilization if microbial growth is a concern. Boiling can, however, significantly change the composition of mobile phases with volatile components.

The application of a vacuum to the mobile phase container will effectively degas the mobile phase for most applications. Be aware of the danger of implosion of the mobile phase reservoir, selective volatilization of mobile phase components, and danger of explosion unless a spark-proof vacuum pump is used.

Degassing mobile phases by setting the container in a sonic cleaner or by inserting an ultrasonic probe into the reservoir is simple and fast, but this procedure does not remove as much gas as do other methods.

As was discussed in previous columns, degassing is often necessary for trouble-free operation of liquid chromatographs. Check valves, pumping chambers, and detectors are the parts that are most susceptible to problems created by too much dissolved gas in the mobile phase. Several instrument manufacturers have simplified degassing by including one or a combination of the techniques discussed above either as standard features or as options for their liquid chromatographs.

SOLVENT-SAVING TECHNIQUES

The cost of solvents for LC mobile phases may be a very small portion of the overall costs of analyses in a lab that does routine assays with labor-intensive sample preparation. On the other hand, the cost may be a large portion of the assay costs in a lab that does methods development or in one which labor is not a major component of the assay. Much has been written recently about the advantages of using microbore liquid chromatography to reduce solvent consumption. Some alternative ways to reduce solvent usage will now be discussed.

Recycling the mobile phase was a common practice in the early days of HPLC when detectors were not as sensitive to changes in mobile phase background absorbance as they are today. With the advent of improved detectors, recycling has become less common. Today, however, we are seeing more and more labs return to mobile phase recycling as a way to dramatically lower solvent costs. Recycling mobile phase is most useful for routine assays with moderate sample concentrations, such as in quality-assurance laboratories, and, of course, is limited to isocratic conditions. The system is set up as a closed loop with a 1–5 gal. reservoir of mobile phase. The waste line from the detector is placed in the mobile phase reservoir and, for best results, the mobile phase is stirred constantly to provide maximum homogeneity. The reservoir is kept capped to prevent dust from entering and to minimize evaporation; using a filter frit on the inlet line to the pump is a good precaution. In this system, the sample components are so dilute in the bulk mobile phase that it may take weeks before the background changes enough to adversely affect the chromatogram.

Short columns can often be used to reduce solvent consumption. Columns 3–5 cm long packed with 3- or 5-μm particles will provide enough resolution for many assays and have the added advantage of reducing analysis time. A shorter assay uses less mobile phase, resulting in a reagent cost savings. Some workers estimate that an 85% solvent savings may be realized by switching from a standard 25-cm column to a 3-cm column (4). Short columns generally do not require the specialized equipment required for microbore chromatography. Microbore columns do indeed yield significant reductions in solvent usage, but in contrast to the short columns, generally do not result in time savings. They also may require specialized pumping, injection, detection, and recording systems to provide efficient operation. Microbore chromatography may be the best choice for solvent savings in cases in which sample amounts are limited, hyphenated techniques such as LC/MS are used, or if exotic solvents are used.

The final technique to reduce solvent consumption is to reduce waste. LC systems that allow mixing of solvents on-line to form the mobile phase can result in significant solvent savings over systems in which the solvents are mixed manually before use. The main reason for this is that only mobile phase that will be used is mixed. In methods development labs where the mobile phase is premixed, it is likely that as much mobile phase is thrown away because of aging, contamination, improper labeling, and other reasons as is pumped through the chromatograph.

SUMMARY

Preparation of mobile phases for liquid chromatography should be a straightforward procedure. Start with the best reagents available, measure them carefully, use clean glassware, and take measures to prevent contamination. After thorough mixing, degas the mobile phase prior to use. If possible, use one of the methods mentioned to reduce the overall solvent consumption because it often costs more to have the waste mobile phase disposed of than it does to buy the original components. Finally, record your work in your logbook so that when problems arise you can trace the mobile phase components to the proper sources to help unravel the problem.

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Readers are invited to contribute their troubleshooting tips to this column or to submit topics or questions for discussion in future articles. Write to: The Editor, *LC Magazine*, P.O. Box 50, Springfield, OR 97477.

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