Troubleshooting the mobile phase of your HPLC system

The first component to examine when considering liquid chromatography from the systems approach is the mobile phase and solvent reservoir. The development of HPLC over the last 13 years has clearly placed increased emphasis on the qualities and handling of the mobile phase. As in all forms of chromatography, users know that the quality and proper handling of the carrier (gas, mobile phase, developing solvent) are as critical as any other part of the chromatographic process. Yet it is all too common for the user to take the carrier for granted and attend to more technically significant system components, such as the solvent delivery system and the detector. It is simple to take a bottle of labeled reagent grade solvent, pour it into the solvent reservoir, and start the chromatograph; however, when a problem develops with the mobile phase, it may not be easily or immediately identified. Once it is, the entire system may already be affected.

The most frequently occurring problems can be avoided or reduced by taking care in choosing the solvent(s), preparing and changing the mobile phase, and handling the solvent reservoir.

Excerpted from the book Maintaining and Troubleshooting HPLC Systems *by Dennis J. Runser.*

CHOICE OF SOLVENTS

The choice of solvent is usually made by selecting one of the four major areas of HPLC (liquid-solid, liquid-liquid, ion exchange, or size exclusion) that is expected to provide the desired separation. The strategy for selecting the right solvent system to achieve the desired separation has been covered by other authors (1–7). The following paragraphs, however, describe a process of selecting solvents based on prevention of system problems.

System compatibility: The HPLC system itself needs to be considered when separation solvents are selected. The solvent system must be miscible with the previously used mobile phase. If it is not, an intermediate solvent needs to be considered first — one that is soluble with both the previous mobile phase and the new one. This will require flushing the system and introducing the new solvent, which may take up to 30 min. If the solvents are miscible, significantly less system rinsing time will be needed — from 5 to 15 min. Godfrey (8) has developed a method for predicting the miscibility of various organic solvent pairs, and additional tables are published elsewhere (9).

Ignoring miscibility will result in the formation of immiscible pools throughout the system, which may be very difficult to clean out and can cause detector readout variations and drifts. These pools may become entrapped in the system and pass through at an inopportune time, ruining needed data.

When changing columns, whether or not an intermediate flushing solvent is needed, simply removing the column and adding a piece of stainless steel tubing between the pump (or sample valve) and the detector will facilitate a quick system flush. With pumps that allow for quick draining from the solvent reservoir and have low dead volumes this may only take 5 min.

If the same column is to be used, it can be disconnected and the pump and sample introduction area of the HPLC system can be purged quickly. In either case the column will have to be purged with the new (or possibly intermediate) mobile phase. Usually 5 to 10 column volumes will be needed. It is important to note what mobile phase has last been used in the column. Once the entire system is running, the detector base line can be used to indicate whether or not the system has stabilized by noting the absence of drifting and short-term noise.

DENNIS J. RUNSER
Marion Laboratories

Column: Solvent compatibility with the column is important once the mode of HPLC to be used has been chosen, particularly with certain chemically bonded liquid-liquid partition columns. Most columns today use packings with surface-reacted or "permanently" bonded phases, which usually present no problems except for extremes in pH. For size-exclusion columns, the type of solvent is critical with regard to column bed swelling, which will seriously affect the interstitial volume as well as the bed volume in the column itself.

The choice of the column should be based on knowledge of column packing, type of bonding, and so on. Ignoring these factors may result in the creation of voids in the column, producing peak broadening and loss of resolution as a result of column deterioration.

Gradient elution: With gradient elution systems at least two solvents (A and B) are used and often a binary mixture, which is varied 20% to 80% of its A/B ratio. Again, miscibility and the ability to return to the desired starting condition are important. The larger the percent concentration variation, the longer will be the time needed to return to initial conditions. Ideally, reversing the gradient and slowly returning to the starting conditions is preferred for maintaining longer column life.

Solubility with the sample: If the sample is not soluble in the mobile phase or if components of the sample are insoluble in the mobile phase, several problems may arise. Precipitation may occur, clogging the syringe or sampling valve and may damage the sampling valve by acting as grit and creating crossport scratches. These particulates can also build up at the head of the column, restricting the flow of the mobile phase and increasing the back pressure on the system. This can create a problem of limited flow rate in HPLC systems, particularly those of limited pressure capability (3000 psi or less). Such a contaminant buildup can also cause spurious peaks.

Samples of limited solubility or materials dissolved in a very soluble solvent, such as MeOH, can precipitate out on the column. With subsequent injections the insoluble material can be redissolved and can be either partially carried through the column and then reprecipitated, or eventually eluted, causing random and spurious peaks to be observed on the detector. This insolubility can also cause impurity buildup in detector cells or create drifting.

Although insolubility of the sample in the mobile phase may not create a problem, the chromatographer must be aware of its potential hazard. Preparation of the sample in the mobile phase itself is ideal for preventing this problem. In binary and tertiary systems direct solubility may be difficult to obtain. If this occurs, the material can be dissolved in the more soluble solvent and diluted to the mobile phase concentration with the other solvent(s). Another approach is to prepare a more concentrated solution in the more soluble solvent. Pipette a small aliquot into a volumetric flask and bring it to volume with the mobile phase, or evaporate the concentrate to dryness and then bring it to volume with the mobile phase.

The use of a different solvent for sample preparation can add to the void volume peak, which may obscure early eluting peaks.

Solvent quality: In the early 1970s, little emphasis was placed on selecting solvents, other than discriminating between reagent grade and spectroscopic grade. The obvious technical and practical grades were seldom used. However, differences in system repeatability may occur when using solvents from different manufacturers. Overcoming this problem may require validating a specific procedure with another manufacturer's solvent(s) before changing sources. Differences in water content and organic impurities are the most common problems. A solvent simply labeled spectral or pesticide grade will not necessarily be free of contaminants for today's columns.

Knowing what stabilizers are present in the solvent can be critical; for example, chloroform usually contains up to 1.0% (v/v) methanol or ethanol, and tetrahydrofuran may contain butylated hydroxytoluene. There is, in essence, already a binary system in the "one" solvent. As a method is developed, note the grade, the concentration of the stabilizer(s), if present, and any other attributes of the solvents used for other chromatographers to attain repro-

ducibility with your conditions. The use of a solvent with a stabilizer as opposed to one without can reverse the elution pattern of two solutes. This may occur if an HPLC system with stabilizer-free chloroform is used, with a subsequent change to chloroform with a stabilizer. Ethyl acetate with water present can deactivate a liquid-solid column. This concentration of water can vary from bottle to bottle, thus changing k'. On the other hand, the lack of a stabilizer or the hygroscopic nature of a solvent can cause changes in the stock solvent, which can also change the k' of the system, as in solvents such as dioxane and chloroform.

Water: Water has become one of the most widely used solvents in HPLC as a result of the ever-increasing use of reversed-phase chromatography and, of course, ion-exchange and gel-filtration systems.

Plastic carboys, if used to hold distilled water, will foster microbiological growth if not cleaned and stored properly. The multitude of such colonies of bacteria, mold and/or fungus in reversed-phase, aqueous size-exclusion (gel-filtration), and ion-exchange systems will clog column interstitial spaces. The water standing overnight in the column may support such growth, increasing column back pressures. This can be prevented by cleaning the carboys, filtering the water through a 2-μm filter, or by adding 0.02% sodium azide or acetonitrile (present already in many reversed-phase systems) to the water.

The material of the container used to store the water also can be a problem. Plasticizers will leach into the water from plastic containers and interfere with gradient reversed-phase systems or contaminate the column. Metal ions can leach from the surface of glass containers and increase water resistivity measurements when this is used as a criterion for water purity. Glass leaching, which also occurs with glass distillation equipment, is usually not as much a problem as organic impurities.

Many chromatographers prepare or buy commercially available HPLC water to avoid this problem. Distilling the laboratory supply of water with simple commercial glass distillation equipment is suitable for the average HPLC laboratory. The water can be checked periodically by using the resistance measurement specification of 1–2 MΩ as a measure of the still quality. These laboratory stills usually produce water at a rate of 1–4 L/hr.

For gradient reversed phase there is no guarantee that volatile organics will not distill over. In this case several other cleanup procedures exist. The water can first be passed through a 2-ft C18 porous silica column overnight as a cleanup step; the organics will be retained. Store the water in glass and not in plastic. Commercial water purification systems such as Millipore's Milli-Q/Milli-RO systems are frequently used.

If impurities in the water are suspected, the following procedure can be used to check for purity(11):

- Pump 100 ml of water through a 2-mm i.d. × 61-cm C18 porous silica column.
- Run a linear gradient from 0% to 100% methanol at 1 ml/min for 10 min and hold for 15 min with a UV detector in-line.
- If the UV baseline shift at 0.08 AUFS is less than 10% and very few peaks of less than 3% to 5% full-scale deflections are observed, the water is pure enough for most applications. With bad water ≥ 10 peaks can be observed to run off scale at 0.08 AUFS.

Table 1 gives a comprehensive list of typical effects of impurities in HPLC solvents (10).

MOBILE PHASE PREPARATION

The preparation of the mobile phase is simple and relatively trouble-free. Some problems, however, can arise that may cause difficulties for the chromatographer.

Filtering and degassing of solvents: A routine practice often neglected when the chromatographer is in a hurry is filtering the solvent before adding it to the solvent reservoir or solvent delivery system. Even if the HPLC system has filters in-line, this simple preventative maintenance practice can prevent filter clogging, excessive changing of filters and associated expense, and can possibly decrease downtime.

Commercially purchased HPLC solvents seldom have particulates. However, they may contain particles even if these are not visible because of small size or the solvent's refractive index. Dirt accumulation at the head of the column can cause a variety of problems, such as:

- changes in V_R
- changes in k'
- decreases in selectivity
- artifacts (spurious peaks)
- drifting with solvent composition changes
- irreversible adsorption and shortening of the column lifetime.

Particulates can also lodge in the solvent delivery system, causing pump wear and flow rates that are not reproducible. This form of contamination can score the bodies of sample valve rotors, producing crossport leaks that will shorten valve lifetime.

This contamination is easily removed by filtering the solvent first. Although laboratory filter paper may be adequate, filter paper fibers can pass through with the solvent and later accumulate in filters and at the head of the column. Both commercially available filters — made of mixed cellulose esters with a 0.22-μm pore size for aqueous media and of polytetrafluoroethylene with a 0.2-μm pore size for organic media — and HPLC solvent filtration kits work well. A critical factor in filter selection is chemical compatibility. Filter media may contain materials such as surfactants or glycerin that can wash through with the filtrate or may be hydrophobic, thereby preventing filtration of aqueous solutions. Any filter to be used should be prerinsed and the first passes of solvent discarded in order to wash the filter clean of any potential soluble contamination. Using a different filter for each solvent is recommended.

The filtering step, if carried out with an aspirator or a vacuum pump in an evaporating flask, can perform another preparatory function: degassing. With all aqueous solvents this is important, although not always essential with straight organic solvents. The lack of degassing, particularly with aqueous systems, can cause bubbles to form upon degassing at the outlet of the system. Under pressure, the solubility of oxygen and nitrogen increases, which seldom causes problems except during elution at the detector, where the pressure dramatically drops to about 100 psi or atmospheric conditions. The dissolved gas leaves the solvent and forms a bubble, causing erroneous results and havoc with the detector. With aqueous systems, simple aspiration for 5 to 20 min, depending on your needs, can be sufficient.

Multiple phase systems: The mobile phase is usually a binary or tertiary system. If the preparation procedure of the mobile phase is intended to be repeated by others, a clear description of the process is crucial, especially if the values of α are close.

Contaminant	Possible Source	Effect	Removal
Particulate matter (dust, etc.)	During transfer, unclean vessels	May block in-line filters, lodge in pump seals, or accumulate at column head	Filter through membrane filter
Water	Glassware, solvent preparation, or manufacture	Variable column activity, k' variation, stability of silicate ester bonded phases	Dry over molecular sieve or anhydrous sodium sulfate
Alcohol	Stabilizer in $CHCl_3$, impurity in hydrocarbons	Similar to water	From hydrocarbons, pass through activated silica; from $CHCl_3$, extract with water, dry with Na_2SO_4
Hydrocarbons (in water)	Organic matter	Baseline instability during gradient elution	Pass through porous polymer column or C18 bonded phase
Peroxides (in ethers)	Degradation	Oxidation of bonded phase (e.g., $-NH_2$ to $-NO_2$), reaction with sample, column deactivation or degradation (polystyrene based)	Distill or pass through activated silica gel or alumina
HCl, HBr (halogenated solvents)	Degradation	Column degradation, especially bonded phases, UV absorbance (bromide), stainless steel attack	Pass through activated silica or $CaCO_3$ chips
BHT, hydroquinone	Antioxidants in THF	UV-absorbing	Distill
Dissolved oxygen	Solvent preparation	Degrades polystyrene-based packings, oxidizes β,β'-oxydipropionitrile, may react with sample	Degas solvent with vacuum or heat
Unknown UV-absorbing	From manufacture	Baseline instability or drift during gradient elution, high detector background	Use activated silica or alumina, or distill for organics, recrystallize or pass over ion-exchange column for inorganics
High boiling compounds	From solvent manufacture	Contaminates collected sample in preparative HPLC	Distill
Algae in water	Growth during prolonged storage	Can plug in-line filters, column entrance frits	Distill from alkaline permanganate or discard

Printed with permission from the Association of Official Analytical Chemists.

TABLE I: Effects of Typical Impurities in HPLC Solvents.

It is good practice to filter each solvent prior to mixing. When solvents are filtered as a mixture, differences in individual solvents' partial pressures can cause compositional changes; the user therefore may be unaware of the changes in the A/B concentration ratio.

The manner in which solvents are mixed can be crucial; either of the following methods may be employed:

- Using clean 1-L volumetric glass cylinders, measure the volume of solvent A and add this to the solvent reservoir. Then measure solvent B in a separate 1-L volumetric glass cylinder and add this to the solvent reservoir; for example, 400 ml of chloroform and 600 ml of *n*-hexane. The total volume should be 1000 ml. This is addition method 1, A + B.
- Measure solvent A and bring the volume to 1000 ml with solvent B. This is addition method 2, A + B (q.s.).

The difference between the methods is particularly noticeable with water-alcohol systems. The addition of 50 ml of water to 50 ml of methanol does not, of course, produce 100 ml of a 50/50 solution, but rather a solution less than 100 ml. Therefore if A + B (q.s.) is used, the solvent ratio is different than when A + B is used. A slight percent difference in the water/organic solvent ratio can be critical in HPLC systems. Either method can be used, but consistency in your laboratory, written procedures, and published works will help you in your methods development and allow others to duplicate your systems. A final point: prepare the mobile phase fresh daily. Usually 1 L will last 8 hr for most systems. Daily preparation assures that nothing in the mobile phase has changed.

SOLVENT RESERVOIR

A variety of containers — from solvent bottles to Erlenmeyer flasks to internally contained solvent delivery system reservoirs — are used as solvent reservoirs. Some important considerations in selection and use of reservoirs follow:

- Use a container with a small covered opening. This prevents or lessens evaporation, which can change the mobile phase A/B ratio (binary system), and prevents changes in k'. The reservoir opening can be covered with aluminum foil, for example. Aluminum foil is lint-free and shapes to the contour of the container. A hole is easily made through the foil for the solvent line (tube) to the solvent delivery system. If solvent bottles and teflon delivery tubing are being used, drill out a hole in the bottle cap slightly less than the size of the tubing and pressure fit the tubing through the hole. Be careful not to constrict the tubing because this can cause flow restrictions and possible pump starvation. A few caps such as this can be used many times. This procedure also reduces vapors in the room, which is critical to meeting today's safety standards.
- Be aware of the material from which the container is made. Avoid plastic containers because of the leaching problems described earlier. Glass can also be leached, for example, of metal ions. This is another reason, particularly with aqueous systems and those at pH > 8, why mobile phase daily preparation is important. For extreme pH aqueous systems a nonglass reservoir such as stainless steel is recommended.
- Maintain a nitrogen blanket over the mobile phase when using stabilizer-free solvents. It may also be needed with readily oxidizable solvents.
- Mix the mobile phase continuously to maintain homogeneity. A magnetic stir bar and stirrer are recommended. Fast mixing speeds ensure good mixing, but may generate heat with the consequences of compositional changes from evaporation resulting from the solvent's partial pressure, or increased and variable temperatures in the incoming mobile phase resulting in long-term drifting observed at the detector. Slow speeds avoid heat buildup, but may not ensure good mixing. The objective is homogeneity of the mobile phase at both 1000 ml and 100 ml. It is not always possible for the final milliliters in a solvent reservoir to be representative of the overall mobile phase because concentration gradients and evaporation can occur no matter how careful the user is. For this reason, using the last 100 ml to 500 ml is not always recommended — drifting and changes in k' may occur. Good mixing or large volumes of mobile phase, for example, 2 L a day when 1 L is needed, can ensure homogeneity. Finally, do not place the solvent reservoir directly on the magnetic stirrer. Magnetic stirrers generate heat, which can affect the mobile phase composition as mentioned previously. Placing a cork ring between the solvent reservoir and the magnetic stirrer works well and does not affect the stirring if adequate space is provided for dissipating heat from the stirrer.
- Be careful in selecting a position for the solvent reservoir in the laboratory. Do not position the reservoir or the HPLC system directly under the ventilation ducts in the ceiling or in the line of drafts from windows, doors, or hoods because of potential temperature gradients that might affect the mobile phase.
- Teflon or stainless steel are suitable materials for the tubing from the reservoir. Teflon varies in wall thickness and can allow air through its walls. This is not always critical at this point in the HPLC system, since it is under very little pressure.
- Keep the reservoir above the solvent delivery system. This assures a good siphon feed to the pump and avoids starving the pumping system.
- Always use a solvent reservoir filter. This will not impede mobile phase flow to the pump (unless clogged) and can prevent particles from entering the HPLC system. Filters can be cleaned by back flushing with suitable solvents.
- Always label the reservoir with the composition of the mobile phase, percent concentration, and date of preparation. Labeling your solvent preparation equipment is helpful if the same solvents are to be used daily and many users share the lab.

CHANGING MOBILE PHASES

Changing solvents is as simple as the preparation step; however, be aware of differences in solubility from solvent to solvent and from mobile phase to mobile phase. If dramatic changes in the mobile phase are called for, purge the system with a solvent having an intermediate polarity. Immiscible solvent pools can produce erratic short-term noise and take time to remove; moreover, these pools may affect the lifetime of the column.

REFERENCES

(1) E.L. Johnson and R. Stevenson, *Basic Liquid Chromatography* (Varian Associates, Palo Alto, California, 1978).

(2) L.R. Snyder, *Principles of Adsorption Chromatography* (Marcel Dekker, New York, 1968).

(3) *Modern Practice of Liquid Chromatography*, J.J. Kirkland, ed. (Wiley-Interscience, John Wiley & Sons, Inc., New York, 1971).

(4) D.H. Rodgers, *Developing an Analytical Method by High Efficiency Liquid Chromatography* (Perkin-Elmer Corporation, Norwalk, Connecticut, 1974).

(5) R.W. Yost and R.D. Conlon, *Chromat. Newsl.* **1**(1), 5-9 (1972).

(6) L.R. Snyder, *J. Chromatogr.* **92**, 223-230 (1974).

(7) D.L. Saunders, *Anal. Chem.* **46** (3), 470-473 (1974).

(8) N.B. Godfrey, *CHEMTECH*, 359-363 (1972).

(9) *Handbook of Chemistry and Physics*, 56th ed. (The Chemical Rubber Publishing Company, Cleveland, Ohio, 1975).

(10) R.E. Majors, *J. Assoc. Off. Anal. Chem.* **60** (1), 186-205 (1977).

(11) *Modes of Chromatography: Liquid-Liquid* (Waters Associates, Milford, Massachusetts, 1974). ■