TROUBLESHOOTING

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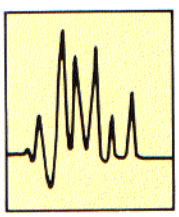

This month's Troubleshooting article considers problems associated with column equilibration, solvent changeover, and dirty sample vials. Also, methods are discussed for estimating normal column back pressure in order to determine if the column is behaving as expected.

COLUMN EQUILIBRATION

Q: I am using gradient elution with a C18 column for my analysis and find that I must "prime" the system with two blank gradient runs before running samples. Without the preliminary dry runs, the result is severe peak splitting and poor reproducibility. What causes these problems, and how can they be solved?

JWD: It appears to me that there is an equilibrium problem with the column. Aside from the fact that the column is given more time to equilibrate with the solvents, the two blank gradient runs should not have had any additional effects on the separation. You can check this by examining the chromatograms. If early peaks are not reproducible or show splitting, yet later peaks look normal, the existence of a nonequilibrium condition is probable. I expect that the problem is related to the A, or weak, solvent in the gradient. If the column has been stored in a pure organic solvent such as 100% acetonitrile and is then directly switched to 100% water as the A solvent, it may need to be flushed with 10–15 column volumes before it is fully equilibrated. Running two blank gradients merely gives the column enough time to reequilibrate with water. Try flushing the column with about 15 column volumes of the starting mobile phase instead of the blank gradients and see if it makes any difference. Another way to speed up the initial equilibration is to store the column in a mobile phase containing a little of the A solvent. For instance, instead of using 100% acetonitrile for storage, try storing the column in 95:5 acetonitrile/water. Of course, if the A solvent is a buffer or other salt, you would use water instead of buffer during storage.

Solvent demixing when ion-pair or antitailing agents are used can also contribute to the symptoms you describe. Allowing for longer equilibration times or modifying the mobile phase may be necessary to eliminate these problems.

A third cause could be late-eluting peaks. Check for late eluters by allowing enough time for all peaks to come off the column before the gradient is recycled. If these peaks are not allowed to elute during normal operation, they can build up on the column over the course of a day's run and will appear the next morning if the storage solvent is stronger than the final mobile phase in the gradient from the previous day. If this is the case, you may need to adjust the final gradient conditions. If, for example, all the desired peaks elute under a 60% strong solvent mobile phase, you may have to add an additional gradient step of 80% strong solvent to elute late peaks before the gradient is recycled.

SOLVENT CHANGEOVER

Q: Recently I needed to run a reversed-phase assay in the lab and used a chromatograph normally run by a co-worker. It never occurred to me that this chromatograph had most recently been used for a normal-phase assay. You can imagine the problems created after I'd pumped 40:60 methanol/phosphate buffer through the system and realized that there was a silica column in the system and that the pump, detector, and all the tubing were full of hexane. Several days passed before the detector quit emitting noise spikes in the chromatograms. What should I have done to correct the problem?

JWD: The problem you mention is a result of solvent immiscibility. The bulk of the old solvent is pumped out of the system with the first few system volumes of the new mobile phase. Because it is not miscible in the new solvent, however, the old solvent then tends to "stick" anywhere it can — for example, in mixers or frits, in the pores of the column packing, and in the detector cell. What ensues is the release of small droplets of old mobile phase that travel through the system, wreaking havoc with the chromatography and creating solvent "bubbles" that behave much like air bubbles in the detector cell.

The best way to deal with this problem, as you now must realize, is to avoid it in the first place. A quick visual check of the system should have raised a red flag: a silica column is not used with reversed-phase solvents. In your case, however, the silica column could have been removed before you used the chromatograph, and you'd still have had problems unless you had checked the system records or asked the previous operator for the identity of the last solvent. Another approach would be to figure out for yourself what solvent is in the pump: take a few drops of solvent from the purge valve and mix them with the new mobile phase — if the two solvents mix, there should be no problems.

If you need to change from reversed to normal phase (or vice versa), the key to a successful changeover is use of a solvent that is miscible with both reversed- and normal-phase solvents. A table of solvent miscibility can be found in reference 1. Either isopropanol or tetrahydrofuran (THF) is commonly chosen as the intermediate solvent. Simply replace the column with a piece of connecting tubing and pump ~50 mL of isopropanol (or THF) through the system, being sure to flush all the inlet lines and both positions of the injection valve. Next, rinse the isopropanol (or THF) from the system with the organic component of the desired mobile phase and then rinse with the mobile phase itself. Now install the new column, allow it to equilibrate, and start the run.

In your case — in which the damage has been done and immiscible solvents already have entered the system — isopropanol or THF can be used to remove both the old and the new solvents. First, replace the column with "jumper" tubing, flush the system with water to remove the buffer, and then flush with methanol to remove the water. Now follow the procedure described above and flush the system with isopropanol. (I would use at least 100 mL to ensure that the old solvents are removed.) I don't think there is much hope for the old silica column, but you might try the same procedure on it (be sure to bypass the detector) and see what happens.

DIRTY SAMPLE VIALS

Q: I suspect that I have a problem related to dirty sample vials. I can manually inject my samples and get good chromatograms; however, when I put the samples in vials and use the autosampler, I observe extra peaks. Is this

a common problem? Should I use a special washing procedure for sample vials?

JWD: First, be sure that dirty sample vials are actually the cause of your problem. When switching from manual to automated injections, many variables are introduced that could cause extra peaks. For example, the vial septum or even the autosampler itself could be the source of extra peaks. Also, sample carryover could be a problem if the autosampler is not rinsed properly; or late-eluting peaks could be interfering if the run time is too short.

To trace the source of the problem, first put some sample into a vial without a septum and repeat the manual injection. If extra peaks are found, confirm the source of contamination by repeating the test using a thoroughly cleaned vial or test tube. If, however, no extra peaks are observed, the vial is not the cause of the problem. Next, add a septum to the vial, then cap the vial and shake it so that the sample solution thoroughly rinses the septum. Repeat the manual injection and see if the extra peaks are present. Septa are a common source of spurious peaks. Therefore, be sure to use septa that are resistant to the solvents you are using. The best choice of a septum is one with a Teflon liner placed such that the Teflon side is toward the sample solution. In some cases, it may be necessary to use a Teflon-film septum. Although these do not seal as well as normal septa after puncturing, they do not introduce contaminants.

Once you have determined that the septa and vials are clean, place a vial of pure injection solvent in the autosampler and allow the autosampler to inject the solvent. This test will help you determine whether the sample solution becomes contaminated in the autosampler or whether you have a problem with carryover or late elution. If peaks are found at this stage, clean the autosampler thoroughly and replace the solvent in the wash reservoir. Be sure to wipe off the outside of the sample-pickup needle. When the autosampler has been eliminated as a source of the problem, again use it to inject a sample. If the extra peaks return, it is likely that sample carryover or late-eluting peaks are the problem. Check first for late eluters by increasing the run time to allow for the elution of any late peaks before the next sample is injected. Sample carryover can be checked by inserting a vial containing a solvent blank between each sample. If peaks appear in the blank injections, carryover is causing the problem. You will need to adjust the autosampler wash routine or place a wash vial between each sample vial to get rid of the carryover problem.

Now, back to the original question of what to do about dirty vials. I have found that new vials are quite clean when they are first received, although they sometimes need to be blown out with clean air or nitrogen to remove dust. You can wash the vials using one of the methods mentioned below, but I would check vials from another vendor first because washing the vials is time-consuming and expensive. If vial washing is required, try detergent washing (a covered basket permits use of an automatic dishwasher), a solvent rinse, or acid washing. The final rinse for any of these methods should be with HPLC-grade water or solvent. If you find that vial washing is required, it may be economical to reuse the vials; otherwise, discard used vials because cleaning costs usually are greater than the cost of new vials.

TABLE I: COLUMN BACK PRESSURE

d_p (μm)	Mobile Phase Composition	η (cP)	Pressure (psi) for L = 5 cm	15 cm	25 cm
3	60:40 ACN/H_2O	0.76	650	2000	3200
	60:40 MeOH/H_2O	1.4	1200	3600	6000
	Hexane	0.31	250	800	1300
5	60:40 ACN/H_2O	0.76	250	700	1200
	60:40 MeOH/H_2O	1.4	450	1300	2200
	Hexane	0.31	100	300	500

COLUMN BACK PRESSURE

Q: I use a variety of columns having different particle diameters, dimensions, flow rates, and mobile phases. How can I tell what normal column back pressure should be?

JWD: The easiest way to tell if column pressure is OK is to confirm the manufacturer's data for a new column under reference conditions for a given assay and then record the pressure. Make a note of the column pressure at the beginning of each day; you will then have a running record with which to trace changes in column pressure. This way you have all the information needed to determine if the pressure is normal (the original test) and/or how rapidly it is changing (daily records) without the trouble associated with performing the pressure calculations discussed below. If back pressure becomes much higher than would be expected from your records, you may have a blocked line, a blocked frit, or a poorly packed column. Don't forget that use of a precolumn or guard column also contributes to total back pressure. An in-depth discussion of column pressure problems can be found in the July issue of *LC Magazine* (2).

You can also determine column pressure from Equation 1, but for routine applications it is more trouble than it is worth.

$$p = \frac{15\phi L^2 \eta}{t_0 d_p^2} \qquad [1]$$

where

p = back pressure (psi)
ϕ = flow-resistance parameter
L = column length (cm)
η = mobile phase viscosity (cP)
t_0 = void time (sec)
d_p = particle diameter (μm)

The flow-resistance parameter must be determined empirically for the particular column brand because the porosity of commercial columns can vary by a factor of two, depending upon the packing technique used. Values of ϕ can vary from 500 to 1000 for "good" columns from different vendors. Fortunately, ϕ is fairly constant for all columns from a single vendor, so, once it is determined, ϕ should not change. The viscosity of pure solvents can be found in a reference manual (for example, reference 1). Table II.3 in reference 3 lists the viscosities of binary-solvent mixtures. An additional point of variation is in particle size (d_p); the average particle size can vary by as much as half a micron from the nominal size stated on the column label.

Table I gives you a general idea of the pressures to expect for LC columns using three different sample mobile phases. A value of ϕ = 750 was used to calculate values in Table I, so your column pressure values may vary by ±50%, depending upon the packing technique used. Equation 1 and the data in Table I are useful for showing the effects of several variables on pressure. For example, when smaller-diameter particles are used to increase column plate number (N), problems may arise because p increases inversely with d_p^2. In other words, when switching from a 5-μm to a 3-μm, 25-cm column, p will increase by a factor of ~2.8 ($5^2/3^2$). Under the conditions listed in Table I, pressure in the 3-μm, 25-cm column exceeds recommended operating pressures for most chromatographs. Such high pressures at relatively low flow rates are one reason that 3-μm particles are most frequently used in columns less than 15 cm in length.

A more detailed discussion of the factors contributing to column back pressure can be found in references 2 and 3.

REFERENCES

(1) *Burdick & Jackson Solvent Guide* (American Burdick & Jackson Laboratories, Muskegon, Michigan, 1982).
(2) S.R. Abbott, *LC, Liq. Chromatogr. HPLC Mag.* **3**, 568–574 (1985).
(3) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd Ed. (Wiley-Interscience, New York, 1979).

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