LC TROUBLESHOOTING

Problems with Small-Volume Columns

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Small liquid chromatographic columns have become workhorses for routine analysis in many labs, but for the uninitiated, small columns can mean big problems. Short, small-particle or microbore columns can be rendered useless by conditions that don't affect traditional 25-cm columns.

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More than ever before, workers are using small-volume (≤5 cm × ≤4.6 mm) columns packed with 3-μm particles. These columns offer fast separation (often <1 min), small peak volumes, and good sensitivity for very small sample sizes. But they also have potential problems, primarily injection problems and extracolumn band broadening. This month's "LC Troubleshooting" addresses solutions to some of the problems associated with small-volume columns.

Injection Tips for Small Columns

Responding to previous "LC Troubleshooting" coverage of injection techniques and problems (1,2), a reader (3) submitted some useful tips for those who use small-volume LC columns. The reader addressed problems he encountered when using narrow- or microbore columns, typically 3 cm × 2.1 mm columns operated at 50 μL/min; these techniques should also apply to the popular "3 × 3" columns (3–5 cm long, packed with 3-μm packing). When such small-volume columns are used, injection volume must be kept to a minimum, so techniques such as the leading-bubble technique (4) may be required in order to minimize dispersion in the injector. However, operating short columns at low flow rates can result in back pressures of 50 psi or less, insufficient to compress an injected bubble. When bubbles are injected under these conditions, they migrate through the system, passing through the detector at inopportune times. Commercial back-pressure regulators for use after the detector often are not reliable under these low pressures. In the reader's case, the requirement to collect fractions of the column effluent prohibited use of such a regulator. An alternative to back-pressure regulators that we've found useful is the use of small-i.d. PTFE tubing as a detector waste line. Select a length (for example, 1 m) of 0.010-in. i.d. tubing and connect it to the detector outlet with compression fittings. Then, with the flow off, pinch the tubing with pliers to further decrease the hole in the middle. Two or three such pinches will increase the back pressure enough to keep bubbles in solution when conventional columns are used; this should work with small columns, as well. Be sure to crimp the tubing with the flow off, because stopping the flow completely during crimping may place too much pressure on the detector cell, resulting in leaks or permanent cell damage.

To combat problems when the injection volume is critical, the reader found two methods to be helpful. First, because laminar flow problems increase with the velocity of the fluid stream, load the loop as slowly as possible. Second, you can increase the density of the injected sample by, for example, adding sucrose to the injection solvent. This creates a density boundary between the sample and the mobile phase; this boundary minimizes dispersion problems, acting much as the air bubble does in the leading-bubble technique. When the reader was assaying small volumes of proteins, 6 M guanidine hydrochloride or 8 M urea in the injection solvent was successfully used to increase sample density. These additives are compatible with reversed-phase columns, and they wash through in the void volume.

Plumbing Errors with Small Columns

When you are using small-volume columns, you must be especially careful when selecting the diameter of connecting tubing. As a general rule, you should connect small-volume columns with short lengths (as short as possible) of 0.010- or 0.007-in. i.d. tubing. In some cases, 0.005-in. i.d. tubing may be required, but this size should be used with caution because it is highly susceptible to blockage. Also, it is important to use system components that contribute a minimum of extracolumn band broadening.

Some of these problems are addressed below. In all cases a 3 × 3 column (3.3 cm × 4.6 mm packed with 3-μm C18 packing material) was used with a mobile phase of 80% methanol/water at 2 mL/min. Samples of aromatic hydrocarbons were used, as listed in the caption for Figure 1.

System incompatibility: Small-volume columns will perform best when they are mounted in liquid chromatographic (LC) systems that have minimal extracolumn dispersion characteristics. When the 3 × 3 column was tested in a well-matched LC system whose detector cell volume was ~1.5 μL, a plate number of N = 3880 was measured (for anthracene, k' = 4.4, half-height method). This is in line with our expectations for "good" 3-μm particle columns of ~ 100,000 plates/m. When this column was mounted in a system with a conventional LC detector (cell volume ~8 μL), the chromatogram of Figure 1a was obtained. The reduced column performance is obvious. First, the bands all tail, indicating extracolumn band broadening. Second, the plate number

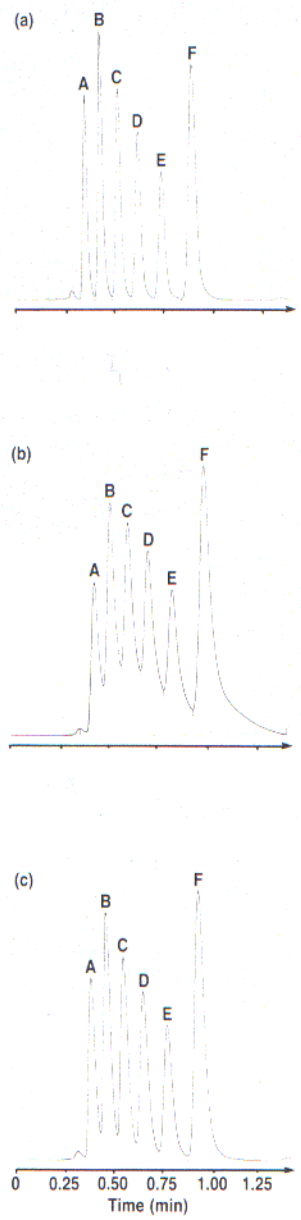

FIGURE 1: Chromatograms from a 3 × 3 column used in (a) a standard LC system with an 8-μL detector flow cell; (b) the same system as (a), but with 20 cm of 0.046-in. i.d. tubing connecting injector and column; (c) the same system as (a), but with poorly assembled fittings at inlet and outlet of system. Other conditions given in text. Peaks: A = benzene, B = toluene, C = ethylbenzene, D = isopropylbenzene, E = *t*-butylbenzene, F = anthracene.

dropped to ~2680 (see Table I). Although a 30% loss in plate number should not be ignored, the column still performs about as well as a 5-μm particle column of the same dimensions should under low-dispersion conditions. For many workers, this column, although not performing ideally, would be adequate for simple, fast separations. For example, the present six-component sample is baseline resolved in <1 min.

Connecting-tubing problems: To illustrate the magnitude of problems that can occur as a result of improper plumbing, we "accidentally" connected the 3 × 3 column to the injector with a 20-cm piece of 0.046-in. i.d. tubing that was meant to be used as a 200-μL sample loop. The change in the chromatogram (Figure 1b) is immediately obvious: the resolution has dropped and the peak tailing has increased dramatically. Less obvious in the figure is a drop in peak height by a factor of two (remember that broader bands are shorter because peak area is conserved). The plate number has dropped to half its previous value (Table I). Finally, the retention times for *all* bands in the chromatogram have increased by 0.07 min, corresponding to the delay time added by causing the sample to flow through the 200-μL connecting tube. This separation is clearly unacceptable.

This tubing substitution error is unlikely to be made because of the obvious visible difference between the 0.046-in. i.d. tubing and the ≤0.020-in. i.d. tubing that is used for most LC connections. However, "normal" errors in tubing selection can significantly degrade the separation. If a piece of 0.020-in. i.d. tubing were used instead of the one used here, the resulting chromatogram would be somewhere between those shown in Figures 1a and 1b.

Poor fitting assembly: Previous "LC Troubleshooting" columns (5,6) have covered the proper assembly of connecting fittings so that problems are minimized. To show the type of problem that can occur when small-volume columns are used with poorly assembled fittings, we intentionally misassembled the 1/16-in. fittings at the ends of our 3 × 3 column. Using finger-tightened fittings, we tightened each of the two fittings so that the tube end protruded past the end of the ferrule but did not touch the bottom of the fitting port. The resulting chromatogram can be seen in Figure 1c. Although the plate number (Table I) was not much better than that of the chromatogram of Figure 1b, the peak shape is greatly improved. There is an obvious difference in resolution between the chromatogram under standard conditions (Figure 1a) and the one with poorly assembled fittings (Figure 1c). Although the bands could be quantified by peak height, most workers would not be happy with such a separation on a routine basis.

Errors in fitting assembly are much more likely to occur than the problem of tubing selection described above, which illustrates the need to take proper precautions when using finger-tightened fittings. The following guidelines should be followed. First, assemble and tighten the fittings only when the flow is shut off. Trying to assemble the fittings with the flow on is messy, and also the mobile phase acts as a lubricant, increasing the likelihood of having the tube slip in the fitting during assembly. Second, use finger-tightened fittings only within their specified pressure rating. Although generally no physical hazard is created when a tube slips at high pressure, the void created between the tube end and the bottom of the adjoining port can cause problems similar to the one discussed above. For the most part, LC systems are operated at ≤3000 psi, so there should be no problem with these fittings if they are firmly tightened during assembly. Finally, whenever pressure within a system exceeds normal limits, it is a good idea to check all finger-tightened fittings to be sure that the tube ends are still firmly seated in the fitting port. And remember, this problem is not unique to finger-tightened fittings — similar problems can be encountered with conventional stainless steel fittings (5,6).

TABLE I: Plate Number Variation for a 3 × 3 Column

Test Conditions	*N*
Manufacturer's test (1.5-μL cell)	3880
Standard LC system (8-μL cell)	2680
Standard LC system (8-μL cell)*	1500
Standard LC system (8-μL cell)**	1740

* with large-i.d. tubing (20 cm × 0.046-in. i.d.)
** with two poorly assembled fittings

Do problems with small-volume columns mean that they're not worth using? Not at all.

Solutions

What does all this mean in practical terms? Are the problems associated with small-volume columns such that these columns aren't worth using? Not at all. In fact, we saw from the example of the 3 × 3 column that even when it was used in a less-than-ideal system, satisfactory results could be obtained. What is important is that small-volume columns are more sensitive to small changes in the operating environment. These small changes often go unnoticed when standard 15 cm or 25 cm × 4.6 mm columns are used, but they can create real problems with small-volume columns.

In general, injection problems can be avoided if the injection volume is kept below ~15% of the peak volume (find the peak vol-

ume by multiplying the baseline bandwidth in min by the flow rate in mL/min) and the sample is dissolved in mobile phase or a weaker solvent. If you suspect that injection problems are occurring, inject half as much sample and see whether the chromatogram improves (narrower peaks, better peak shape). If you see improvement, continue reducing the sample volume until you find stable conditions. If you suspect band spreading in the injector, try using the leading-bubble technique (4) or one of the injection tips discussed above.

One poorly assembled fitting can spoil an otherwise satisfactory separation when small-volume columns are used.

If you suspect tubing problems, rearrange the system to minimize tube lengths and use 0.005-in. i.d. tubing to see if the results are improved. Take extra filtering precautions to avoid blocking this small tubing.

No matter what size of tubing is used, take extra care when assembling the connecting fittings. One poorly assembled fitting can spoil an otherwise satisfactory separation when small-volume columns are used.

The use of a large-volume detector cell can cancel out the separation power of the column when small-volume peaks elute. Be sure that the detector properly matches the column. In the case of the 3 × 3 column used here, about 30% of the column efficiency was lost by using a mismatched detector. Fortunately, for the present sample, the separation was still adequate.

Microbore and short, small-particle columns may seem to have more than their fair share of problems, but they are hard to beat when you're analyzing small samples or require short (<1 min) separations. The few simple precautions discussed here will overcome the most common problems.

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