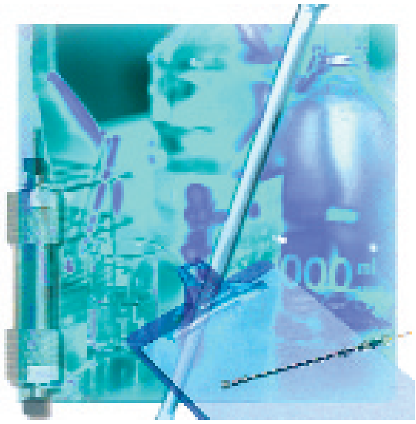

Autosampler Carryover

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Where did that peak come from? No sample was injected!

The topic of this month's "LC Troubleshooting" column is the presence of sample peaks in chromatograms when only blanks are injected. This phenomenon is commonly called carryover, in contrast with late-eluted peaks, which were the topic of last month's instalment (1). Carryover can be an important problem when analytical chemists use liquid chromatography (LC) methods to analyse a wide range of sample concentrations. Carryover peaks usually originate in the autosampler. Not all carryover problems can be solved easily, but most problems can be eliminated by performing one or more of the approaches discussed in this column.

The Problem

Carryover can manifest itself in several ways. When a standard curve is run, carryover can show up as mild or severe deviations from the expected curve. The data of Tables 1 and 2 illustrate this situation. In both instances, a hypothetical series of standards ranging from 0 to 1000 ng/mL was injected with 5% carryover from the previous injection. Table 1 shows the results if the standards are run sequentially from high to low concentrations. For all injections except the first, the error is approximately 18%. The calculated *y* intercept is roughly 9 ng/mL, and the 1000 ng/mL standard appears to be below the curve. The regression coefficient (r^2) is 0.9975 for this set of data. Table 2 shows the same series of standards injected in reverse order, from low to high. From these data the error is less than 2%, and the curve parameters are near ideal; r^2 is 1.0, and the *y* intercept is less than 0.1 ng/mL. The only difference between the two data sets is the injection order.

The carryover problem might go unnoticed in either of these first two instances because the error is constant and the curve appears to be nearly linear. When the injection sequence is rearranged so low samples follow high samples, however, the true potential of carryover problems can be recognized. The data of Table 3 show this situation. Errors range from less than 1% to more than 150% for this particular sequence of injections. This range of error is unacceptable by any standard of method performance. It is easy to imagine the effect on the accuracy of analysis if a low-level sample were injected following a high-level standard.

A Reservoir of Sample

Carryover exists because sample is being injected unintentionally; that is, if a 0 ng/mL sample (a blank) is injected, a peak still appears at the retention time of the compound of interest. For the sequences of Tables 1 and 2, the peak might be small enough that it would be unnoticed, but the sequence of Table 3 would produce a peak equivalent to 50 ng/mL for the blank injection.

The most likely source of the unintentional peak is the autosampler. A tiny reservoir of sample can remain in the autosampler after an injection and get injected with the next sample. The simplest situation is the presence of a poorly flushed portion of the autosampler plumbing. For example, a fitting that is assembled so the tube end fails to contact the bottom of the fitting port will create a small gap that may amount to 1 µL or more in volume. A 1 mm gap with 1/16 in. o.d. tubing creates a volume of approximately 3 µL. If this gap is not cleanly swept by mobile phase, some sample may remain in the gap after injection, diffuse out, contaminate the next sample and create a carryover peak.

Many workers use polyetheretherketone (PEEK) fittings and tubing because of the convenience of finger-tightened fittings. These fittings are suitable for pressures as high as 3000–4000 psi (200–275 bar), but they may slip when these pressures are exceeded. A fitting may pop apart and leak, but it can also slip slightly, especially if the

Table 1: *Injection of a hypothetical standard curve in a high-to-low sequence.*

Concentration	Response	Error (%)
1000	1000	0
300	350	17
100	117.5	18
30	35.9	20
10	11.8	18
3	3.6	20
1	1.2	18
0	0.06	—

Table 2: *Same standards as in Table 1 but injected in a low-to-high sequence.*

Concentration	Response	Error (%)
0	0	—
1	1	0
3	3.1	1.7
10	10.2	1.5
30	30.5	1.7
100	101.5	1.5
300	305.1	1.7
1000	1015.3	1.5

high pressure is momentary, such as during the rotation of an injector valve. In other instances, a poorly assembled stainless steel fitting can have the same problem of excess volume within the fitting.

When you observe carryover, first confirm that it really exists by repeating a blank injection. A second blank should drop by the same percentage as the difference between the initial injection and the first blank. For example, if the 1000 ng/mL standard were injected and followed by a blank, as in Table 3, a peak equivalent to 50 ng/mL should be observed. A second blank injection should produce a peak of the same 50:1000 ratio (50 ng/mL × (50 ÷ 1000) = 2.5 ng/mL).

Once carryover is confirmed, I recommend carefully retightening all PEEK fittings in the autosampler. For each fitting, simply loosen the nut, push the tubing fully into the fitting, and retighten the nut. If you are fortunate, this retightening will correct the problem. If the problem reoccurs, you may want to consider replacing PEEK connectors with stainless steel ones, which will withstand much higher pressures before they slip.

Cleanliness Is Key

Another possible reservoir for sample carryover is the needle-washing apparatus in the autosampler, if the autosampler is equipped with one. The design of these washing devices varies by manufacturer. Figure 1 illustrates one common design. In this situation, a small reservoir is filled with a wash solvent, and the excess flows out the waste line. The injector needle is inserted through a septum. The septum physically wipes the needle, and the outside of the needle is washed by the fluid in the reservoir. Some or all of the fluid in the reservoir is replaced during each washing cycle by wash solvent that is injected from the needle into the reservoir. Because the volume of these devices is small (e.g., 100 µL), the fluid is changed with each flushing and the needle can be cleaned effectively.

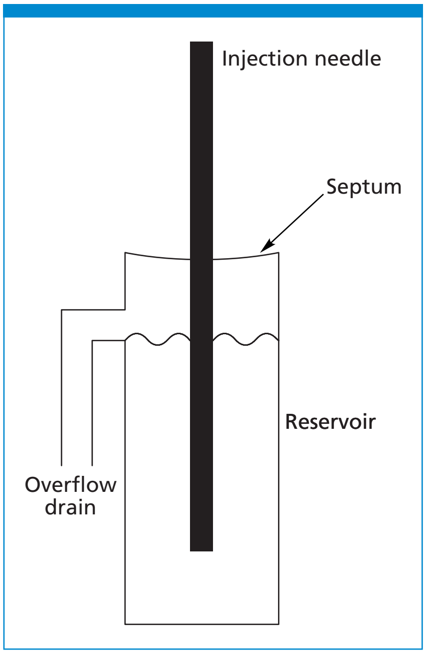

Figure 1: *Schematic of an autosampler needle-washing apparatus.*

Several possible problems can occur with the washing device. If the waste line becomes blocked or restricted, the displacement of contaminated solvent by fresh solvent can be prevented, and the washing device becomes a contamination device. Be sure that the waste line is kept open and free of deposits or kinks that could reduce its effectiveness. Similarly, if the septum becomes contaminated, it can transfer contamination to the injector needle and thus introduce it into the column with a subsequent injection.

Another common source of poor needle washing is a mismatch between the wash solvent and the sample diluent. The wash solvent should be capable of quickly and thoroughly dissolving the sample. Generally, analysts should follow the like dissolves like rule. Thus, many workers favour a mixture of organic solvent and water similar to that of the mobile phase. For example, a 50:50 acetonitrile–water mobile phase could also be used for the wash solvent. I like to avoid using buffers in the wash reservoir because buffers can precipitate and leave residues in the autosampler. If pH control is necessary, adding a volatile acid (e.g., acetic or formic acid) or base (such as ammonium hydroxide) is usually a better choice than adding a traditional buffer such as phosphate because these substances evaporate rather than crystallize when their carrier solvents are removed. Because the wash solvent is not injected, it does not have to be compatible with the mobile phase in its chromatographic characteristics, just its miscibility. A stronger solvent will flush any residual sample more readily from the injector needle than a solvent weaker than the mobile phase. For this reason, many users pick 100% strong solvent such as acetonitrile or methanol as their wash solvent of choice. Propanol or isopropanol are other options. The important thing is to select a wash solvent that will quickly dissolve unwanted sample and flush it to waste before the next injection cycle.

"Once carryover is confirmed, I recommend retightening all PEEK fittings in the autosampler."

Table 3: *Same standards as Table 1 but with a randomized injection sequence.*

Concentration	Response	Error (%)
1	1	0
1000	1000.1	0
0	50	—
3	5.5	83.3
100	100.3	0.3
30	35.0	16.7
300	301.8	0.6
10	25.1	150.9

Some autosamplers allow users to programme additional wash cycles between injections. Although most workers find a single cycle effective for needle washing, adding an extra flush or two may be the way to eliminate carryover.

Finally, be sure to check other sources of plumbing problems in the autosampler. For example, the waste line from the injection loop is designed to drain freely. If the waste line has a restriction from old sample deposits or a kink, the waste may not drain freely. Also the contents of the waste line can drain back into the loop if the waste line is not positioned properly.

Sticky, Sticky, Sticky

Some sample compounds tend to stick to the surfaces inside the autosampler. After all, the tubing and injector parts can act as chromatographic surfaces in a manner similar to the column packing. Some compounds will adsorb to stainless steel, others may prefer PEEK, and still others don't seem to be a problem. Sometimes experimenting with the tubing that connects the autosampler and the column, the internal autosampler plumbing, or the sample loop material can help you find a combination of tubing that is less prone to carryover than the standard configuration. Sample injector valves contain polymeric seals. Some compounds will adsorb on these seals and contaminate subsequent samples, which show up as carryover peaks. If you suspect the seals are the source of a problem, check with the valve manufacturer to see whether alternative seal materials are available.

Although changing the tubing or seals may be necessary, a small adjustment in the composition of the mobile phase often may be sufficient to prevent the sample from sticking to the tubing. Proteins, peptides and other large biomolecules can stick to those surfaces. The addition of a chaotropic reagent such as guanidine or urea to the sample injection solvent may counteract those interactions. Adding a small amount of organic solvent to 100% aqueous injection solvents may also reduce the interaction of hydrophobic sample components with autosampler parts.

If All Else Fails

If you've tried everything you can think of and still have carryover from one sample to the next, a couple of other options are available. None of these solutions are ideal, and depending on the precision and accuracy requirements, they may or may not be appropriate. For example, a bioanalytical method used to support drug discovery work may require accuracy of only ±15%. In these instances, 1–2% carryover may be insignificant. On the other hand, a drug potency assay that requires less than 2% error will be unusable with 2% carryover.

Because carryover is a dilution process, injecting a blank between each sample will effectively eliminate carryover from one sample to the next in most instances. For example, after an injection of standard or sample, the first blank contains 5% of the previous peak, but the second injection contains only 0.25% (5% × 5% = 0.25%). Usually the carryover in the second injection is less than the level of concern but not always. For example, the 3 ng/mL sample in Table 3 illustrates that the peak appears to be 80% larger than expected.

If you know the expected order of sample concentrations, such as with a series of samples in a pharmacokinetic experiment, you may be able to arrange the injection order so carryover is insignificant. By starting with low-level samples and working to higher levels, carryover will generally be of less significance, as was the situation for the samples in Table 2.

Another alternative is to run all the samples and then rerun those samples that give low-concentration results after the injection of high-level samples.

Conclusion

Sample carryover exists when a peak for a sample appears in a blank injection. The most common source of carryover is sample residue left in the autosampler. By adjusting tubing and fittings, selecting the best wash solvent, and perhaps adding extra wash cycles, most carryover problems can be eliminated. If all your efforts at eliminating carryover are insufficient, you may need to alter the injection sequence or perform multiple injections of each sample to obtain acceptable results.

Reference

(1) J.W. Dolan, *LC•GC Europe*, **14**(2), 76–81 (2001).

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