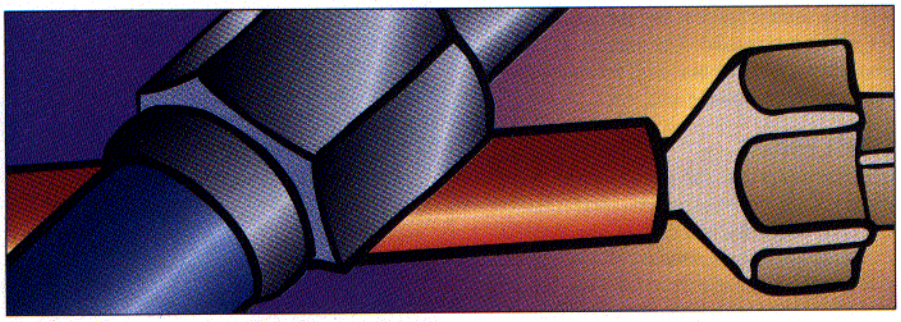
LC Troubleshooting



Autosampler Precision

John W. Dolan

Several variables can affect autosampler injection precision and may prevent the instrument from passing performance tests.

The precision of a liquid chromatography (LC) assay can depend upon the injection precision of the autosampler. I recently received an e-mail message from a reader who was having trouble getting her autosampler to pass an operational qualification–performance qualification test. The specifications for the test were that the relative standard deviation (RSD) of the peak area must be less than or equal to 0.5% for 10 consecutive injections for filled-loop (500 µL) and partially filled–loop (50 µL) injections. The filled-loop test passed without a problem, but the partial-loop injections failed. By performing several sets of runs, the autosampler could pass the test with the following typical results:

- first set of 10 injections, 2.1% RSD;
- second set of 10 injections, 1.1% RSD;
- third set of 10 injections, 0.6% RSD; and
- fourth set of 10 injections, 0.2% RSD.

However, this procedure of testing into compliance is frowned upon by regulatory agencies and certainly is not good science. The questions are what is the source of the problem and how do you correct it? Let me state before I start that the cause of the precision problem is still under investigation, but the problem allows me to make a general examination of the variables that affect autosampler precision.

FILLED VERSUS PARTIAL LOOP

One well-known phenomenon (1,2) that was covered in a previous "LC Troubleshooting" column (3) is that injection of sample from a partially filled loop generally is less precise than for filled-loop injection. This increased error arises from the occurrence of laminar flow within the loop, which can cause imprecise loop filling, particularly when the sample volume is between 50% and 300% of the loop volume. This problem is less common with autosamplers than with manual injection because the high degree of sample delivery precision in autosamplers tends to make any errors constant. With the present example, laminar flow is unlikely to be the problem source because repeated tests show improved precision, whereas precision problems due to this mechanism are not expected to improve in a systematic manner. Furthermore, the partial fill in the present case is only 10% of the loop volume, well below the 50% volume danger zone.

AIR LEAKS

Many autosamplers use a sampling mechanism in which a motorized syringe is used to withdraw sample from the sample vial through a sample needle and sometimes into a holding tube. Then the autosampler moves the needle to an injection port and dispenses the sample into the injection loop. When autosamplers of this design are used, several tubing connections have a potential for leaks. Because the leakage often occurs when the sample is withdrawn from the sample vial, leaks can allow unwanted air to enter the system instead of the sample.

The reader indicated that two syringe designs were available for her autosampler. One design has a metal end glued or fused onto the glass syringe body, and the other design has a metal end threaded onto the glass with a polymeric seal to prevent leakage. In her experience, the fused or glued design was less susceptible to leakage. However, in the present case, she observed no leakage.

A potential leak can occur anywhere a tubing connection is made in the sample dispensing hydraulics. For example, if the PTFE tip on the syringe plunger becomes worn, air can leak around it. Any slip-fit connection is especially prone to leakage, so it is a good idea to check these for air leaks as well.

Another insidious source of air in the lines is air that diffuses through the walls of PTFE tubing. Most workers have observed this phenomenon in the tubing connecting the mobile-phase reservoir to the pump. If the lines are purged carefully and the mobile phase is degassed thoroughly, one would expect the lines to remain bubble free. After several hours of operation, however, analysts often can observe a bubble at the highest point in the line. This bubble usually results from diffusion of atmospheric gases through the thin-walled PTFE tubing. The same process can occur if a PTFE tube is used in the sampling hydraulics. Any extra air in the tubing can act as a shock absorber during sampling and may result in imprecision of sample withdrawal from the sample vial.

For this reason, the standard operating procedure for autosampler start-up in my laboratory requires a complete purge of the sampling tubing before operation. Workers in my laboratory have observed occasional bubbles that originate in the autosampler wash solvent. The same standard operating procedure requires that the wash solvent is degassed before use each day. These two procedures, degassing and flushing, made significant improvements in the precision of the autosamplers. Of course, diffusion through the tubing walls is not a problem if the autosampler uses stainless steel or PEEK tubing instead of PTFE in the sampling portion of the unit.

SYRINGE SIZE

Some autosamplers have the option of changing the sampling syringe depending on the desired sample size. For example, the autosamplers in my laboratory can be used with either 500- or 2500-µL syringes. Normally I use a 500-µL syringe because typical injection volumes are less than or equal to 500 µL. If sample volumes greater than 500 µL are desired, I change to a 2500-µL syringe. Both syringes have the same stroke length (approximately 60 mm), so for the same injection volume, the plunger of one syringe would be withdrawn five times as far as the other. The mechanism that powers the syringe is

TABLE I: Positioning Error of Autosampler Syringes

		500-µL Syringe Volume	2500-µL Syringe Volume
Position (mm)	% Error	Injection Volume (µL)	Injection Volume (µL)
60	0.002	500.0	2500.0
6	0.017	50.0	250.0
1	0.100	8.3	41.7
0.2	0.500	1.7	8.3

identical, so any positioning errors are the same for both syringes. Table I illustrates the impact of the positioning error based on an assumed position error of ±0.001 mm.

Because the positioning error is constant, the volume error contribution of positioning errors will increase with smaller injection volumes. As expected, the volume percent error for a 50-µL injection with the 500-µL syringe is 10 times that for a 500-µL injection with the same syringe. The volume errors are fivefold larger for the 2500-µL syringe for the same relative fill volume. This increased error is of particular concern when small injections are made with the larger syringe. For example, the 8.3-µL injection with the 500-µL syringe generates approximately 0.1% error, whereas the same volume with the 2500-µL syringe generates approximately 0.5% error. When the error contribution from positioning approaches the desired precision of the test, it may be impossible to reach the operational qualification–performance qualification specifications. For example, the manufacturer of my laboratory's autosamplers specifies a precision of RSD less than or equal to 0.5% for a 10-µL injection using the 500-µL syringe. Typically, I observe 0.2–0.7% RSD under these conditions. It is obvious that if the 2500-µL syringe was used accidentally for the test, positioning inaccuracy alone would contribute approximately 0.5% to the error. The importance of using a syringe size that is appropriate for the sample is obvious if you want maximum autosampler performance.

BLOCKED WASTE LINE

Depending on the design of the autosampler, precision problems can occur in the waste line. The autosamplers my laboratory uses channel the purge solvent through the waste line, so the waste line receives a regular flush with wash solvent. Some other autosampler brands channel the sample loop overfill stream through a waste line that doesn't receive additional flushing. I have seen these lines become blocked with sample salts or other residue when the sample solvent evaporates. The residue restricts or blocks the waste line and can prevent proper filling of the loop. One symptom of this problem is leakage at the sample fill port during loop filling; poor injection reproducibility, even in the filled-loop mode, can result. For this reason, flushing the waste line regularly is wise if flushing is not a normal part of autosampler operation.

BLOCKED SAMPLE NEEDLE

If a piece of the septum from the vial cap becomes lodged in the sample needle, reproducibility can suffer. Depending on the size and position of the debris and the autosampler mechanism, the problem can range from irreproducible injections to no sample being injected. Sometimes the blockage can be cleared through flushing or judicious use of a syringe cleaning wire, whereas other blockages may require replacement of the needle or associated tubing. Cored septa can be caused by a rough or sharp needle tip or by use of an incompatible septum. My laboratory uses PTFE-faced silicone septa for the best results.

SAMPLE PROBLEMS

The characteristics of the sample can affect autosampler precision. Sample viscosity is a potentially important characteristic. A closely related problem can result if the sample solvent is too volatile. Most autosamplers have a setting that allows users to select a syringe fill speed. If the autosampler pulls the syringe needle back too quickly, a viscous sample may not be pulled completely into the needle. If the sample solvent is too volatile, the solvent can boil under the partial vacuum formed when the syringe attempts to withdraw sample from the vial. This process, commonly called *cavitation,* results in bubble formation in the sample and, thus, poor autosampler reproducibility. If the sample is viscous, the solution is to reduce the syringe speed so that it fills more slowly. Syringe speed reduction may or may not help with volatile solvents. Additional sample dilution with a solvent of low volatility, such as water, may help.

A rare, but perplexing problem that may be attributed to poor autosampler performance is a sample concentration gradient in the autosampler vial. This condition can occur if the sample is frozen in the vial, then placed in the autosampler tray to thaw. During the freezing process, especially with buffered solutions, the water can freeze first at the top of the vial, leaving a higher concentration of salt at the bottom of the vial, which freezes at a lower temperature. These salt gradients can cause sample concentration gradients within the vial, as well. If the vial thaws without mixing, as it would if it thawed in place on the autosampler tray, the concentration gradient could persist in solution. Now if sample from the same vial was injected several times, each injection would contain sample at a different concentration. For example, if the concentration gradient was such that the high concentration was at the bottom of the vial and the sample needle was inserted to the bottom of the vial, the first injection would have a higher concentration than the second, and so forth. Analysts can eliminate sample gradients by ensuring that the vial contents are mixed after thawing.

TABLE II: Data Error Results for Autosampler Precision Injection Problems

Problem Source	Random	Pattern
Air diffusion	✓	✓
Air leaks	✓	
Blocked sample needle	✓	
Blocked waste line	✓	
Partially filled loop	✓	
Sample gradient		✓
Sample viscosity	✓	
Syringe fill rate	✓	
Syringe size	✓	
Vial filling		✓
Volatile sample solvent	✓	

A more common sampling problem can occur if the vial is too full. If the vial is filled to the top and a new septum is used to seal the vial, sufficient vacuum can be formed when the sample is withdrawn to cause cavitation in the needle or poor precision. This problem is worse when large sample volumes are withdrawn (for example, more than 250 µL). Generally analysts don't encounter this problem if the vial is no more than three quarters of the way full — sufficient air will be present to act as a shock absorber so that vacuum formation is minimized. Some autosampler manufacturers anticipated this problem and use a second needle or some other mechanism to vent the vial when sample is removed.

SUMMARY

I've considered a variety of potential problems with autosamplers. As I try to determine the source of the reader's problem described at the beginning of this column, I need to see if a pattern exists that can help to determine the cause of failure. The reader indicated that repetitive tries at checking the system precision resulted in better results each time until the test passed. This pattern of improving results over time may be useful in matching the expected behavior of the system for each potential failure mode. Table II illustrates one possible division of the problems.

Let's briefly consider each problem source to see how it applies to the present problem. When the autosampler is used in the partially

filled–loop mode, errors are minimized because of the high degree of precision of the syringe drive mechanism. The errors tend to be random. Air leaks at fittings or the syringe should be fairly consistent, so errors will be random. Air diffusion through the tubing should be consistent with time, but this process occurs whether or not the autosampler is in use. The sampling and rinsing cycles of the autosampler tend to gradually purge bubbles from the system, so as bubbles are purged, results should improve. If this situation is the case, a pattern of improved performance should be observed, otherwise the results may be random. Errors due to installation of the wrong syringe should be constant, so the observed errors will be random and depend on mechanical precision of the drive motor. Blocked lines and sample needles can change from sample to sample and often cause very large and random changes in sample volume. Problems related to sample viscosity, syringe fill rate, or a volatile sample solvent will cause similar results with random changes in the sample volume delivered to the loop. Multiple injections from a sample vial with a sample concentration gradient will yield regularly increasing or decreasing sample areas, but the precision of multiple injections from the same vial is expected to be poor. If the vial is too full and improperly vented, the precision of sample delivery is expected to improve as the sample level in the vial drops.

For the present problem, I think the problem sources that best fit the symptoms are poorly purged sampling hydraulics or an overfilled sample vial. The balance of the sources generate random errors or don't fit the symptoms. As I mentioned at the beginning of this column, the definitive problem source has not been identified yet, and, as always, I welcome suggestions from other readers that might identify other potential problem sources.

IF ALL ELSE FAILS . . .

The reader was concerned about showing that her autosampler met the necessary precision requirements of her operational qualification–performance qualification tests, so she needs to identify and correct the problem source. A practical way to minimize autosampler errors is to use an internal standard with the sample. Quantification with an internal standard relies on the ratio of the peak area (or peak height) between the internal standard and the sample peak. Because the internal standard is added to the sample before placing the sample in the sample vial, autosampler imprecision should not affect quantification. For example, if the nominal injection volume was 10 µL and the autosampler injected four replicates from the same sample vial of 8, 10, 7, and 12 µL, the precision of injection would be terrible (approximately 25% RSD). Because the ratio of the internal standard to the sample peak is independent of the injected volume, the precision of this ratio should be quite good even though the injected volumes vary widely.

ACKNOWLEDGMENT

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