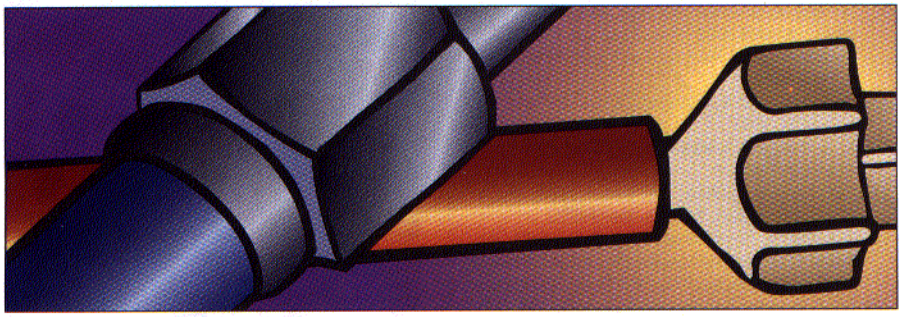
LC Troubleshooting



Reproducibility Problems

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Poor reproducibility is a sure sign of problems.

This month's "LC Troubleshooting" column responds to readers' questions about several reproducibility problems. The first question relates to problems a reader observed when analyzing system-suitability samples. Analysts use these samples to determine if liquid chromatography (LC) methods and systems are working well enough for sample analysis. Another reader suspected a problem when the peak area variation was much larger than normally observed. The final problem was identified when injections of blank sample matrix behaved differently than authentic samples. In each case, stepwise isolation of the problem should yield a satisfactory solution.

SYSTEM SUITABILITY

Q: I've experienced a puzzling problem with the performance of my system-suitability samples for one of my methods. The assay determines the stability of samples stored under various stress conditions. All standards are prepared as one solution at the beginning of the study and are frozen immediately at −78 °C. For each time point, I pull three vials of standards for system suitability and one more to use as a calibration standard. Each vial contains a standard of the analyte plus an internal standard. System-suitability testing requires triplicate injections from each of the three system-suitability standard vials. The method requires retention time reproducibility and a relative standard deviation (RSD) of the analyte–internal standard ratio (response factor) within specified limits.

I noticed an odd pattern the last two times I used the method. In each case, the response for the first two vials was the same, but the peak area for the third vial dropped significantly. Within each vial the peak areas were constant, retention times for all injections were the same, and the RSD for the response factor was satisfactory. The analysis met the system-suitability requirements, and subsequent analysis of the stressed samples yielded the expected results. The only oddity was the step change in area response for the third vial of system-suitability injections. Do you have any idea what could have happened?

A: The drop in peak areas resulted from one of two causes — either the injection volume or the sample concentration was smaller. The use of an internal standard compensates for changes in injection volume or sample loss during pretreatment. The results were satisfactory because the internal standard correctly compensated for these changes.

First, let's examine potential problems related to the standards. A few more experiments would help. For example, is the problem related to the contents of vial number 3 or its position or order of injection? If you still have the standards, it would be interesting to repeat the injection sequence for the first three vials and see if you observe the same symptoms. Then rearrange the vials (for example, number 2, number 3, and number 1) to see if the response change tracks with the vial or the injection order.

If vial number 3 contained a lower concentration of analyte and internal standard due to a dilution error, the area response would drop but the response factor would be the same. This cause seems unlikely because all the vials were prepared at the same time and stored under the same conditions. A review of the preparation procedure for these system-suitability standards might reveal a possible source of error.

If the low response tracks with the vial position rather than the vial identity, the problem might be caused by some kind of priming condition for the autosampler or column. This possibility could be checked by reinjecting samples from the first two vials after the third. If the response remains low, follow this line of logic. If the response is back to normal, reinject the third vial.

In my experience, priming the system with sample is not a step change, as you have observed, but a gradual process during several injections. For example, an analyte may have a strongly tailing peak because of interactions between column silanol groups and a basic function on the analyte. In some cases, the injection of several standards at normal or elevated concentration can provide some deactivation of the silanols and improve peak shape for subsequent injections. Similarly, if some other site in the system is adsorbing the sample, you may need to perform several injections to saturate the active sites before obtaining a normal response. In these cases of system priming, however, the response pattern is one of gradually *increasing* response, not a stepwise decrease, so I don't think this explanation will fit the present problem.

I suppose it is possible that something could be wrong with the third position on the autosampler. If this situation occurs, skip that position for future work. Also, carefully check the control program to ensure there is no instruction for a smaller injection from the third vial. Both of these problems could be checked by injecting another standard with another method. For example, by using the column test standard under standard test conditions, you could eliminate any possibility of method-specific chemical problems and focus on mechanical or electronic problems.

At this point, I'm out of ideas, and I haven't given any definitive answers. Additional ideas might emerge from a careful study of the data that looks for trends in area response, especially for the analyte and internal standard. Perhaps another reader will have some ideas to contribute.

PEAK AREA REPRODUCIBILITY

Q: My method is yielding very poor peak area reproducibility. The retention times are fine, but the peak area RSD is 5–10%, in contrast to the 1–2% RSD variation I used to see. I haven't changed anything in the system that I can correlate with this problem. The system comprises a low-pressure mixing system, an autosampler, a C8 column, and a UV detector.

The isocratic mobile phase is mixed on-line and contains pH 3 phosphate buffer and methanol.

A: Peak area reproducibility relies on consistent autosampler operation, so I suspect this factor is the source of your problem. Several parts of the autosampler could be at fault as described below. If you didn't read the recent "LC Troubleshooting" column dedicated to autosamplers (1), review it for more ideas.

First, make sure that the injected sample is consistent. You can check this factor most easily by injecting replicates of a known standard. Several problems can occur at the sample vial level.

The sample must be homogeneous. Sample matrices with high salt concentrations can cause layering in the vials, as can poorly mixed samples that have been frozen. If any chance of layering exists, make sure to mix the contents by swirling or inverting the vial.

A sample vial that is too full can produce variable results if the seal is too tight. As sample is withdrawn, a slight vacuum can form in the vial, making it hard to withdraw sample. This condition can increase sample size variability. Generally, a vial that is no more than one-half to three-quarters full will be satisfactory.

Poor sealing of the sample vial can be a problem if the sample solvent is sufficiently volatile. Evaporative loss of the sample solvent can cause the sample concentration to change from injection to injection.

A small piece of vial septum caught in the autosampler needle can act as a damper that opens and closes as the debris shifts. This situation can result in poor reproducibility or, in the extreme, complete lack of injection. If you suspect a blockage, flush the tubing and needle, clean the needle with a syringe cleaning wire, or replace it. Be sure to use a vial septum that will not core and release fragments of silicone. PTFE-faced septa seem to be less susceptible to coring than other materials.

The syringe and sampling mechanism can be susceptible to reproducibility problems if it is used improperly. For example, some autosamplers in my laboratory are designed to draw sample into a long piece of narrow-bore plastic tubing in transit from the sample vial to the injection valve. If this transfer tubing contains bubbles, the bubbles can expand and contract when they encounter resistance, which causes a variance in the amount of sample withdrawn from the vial. In my laboratory, analysts avoid this problem by degassing the autosampler wash solvent and thoroughly purging the tubing before starting a day's analyses.

Some autosamplers are designed to use different size syringes depending on the desired injection volume. If a mismatch exists between the syringe and sample size, excessive error can occur as a result of the positioning error of the syringe mechanism. Verify that the proper syringe is installed.

If the autosampler is designed to withdraw sample from the vial and transfer it to an injection valve, problems can arise at the valve. The needle must seal well at the injection port, or sample leakage and loss can occur. Try adjusting or replacing the needle seal to correct the problem. Any resistance to flow through the injection valve can create back pressure that results in leakage around the needle seal. Check each of the valve passages for resistance and take special care to ensure that the waste lines are clean. The evaporation of sample solutions and buffers can leave residues that gradually block or restrict the waste line. Misalignment of the injection valve also can create back pressure that results in needle seal leakage.

The style of injection can influence peak area reproducibility, but this situation is less of a problem with autosamplers than with manual injection. Because of fluid flow characteristics, the highest level of precision will occur when the loop is overfilled with sample by at least two to three times the loop volume. When the injector loop is only partially filled, the maximum precision will occur when the sample volume is less than one-half the loop volume (2). For this reason, you should double check to ensure that the proper loop is installed in the autosampler and that the injection volume is consistent with the required degree of precision.

Leaks also can affect peak area precision, but a leak would cause similar problems with retention time, so this case is unlikely to be the source of your error.

So you can see many possible sources for injection precision problems. I would start by carefully reviewing all the system settings to be sure you made no mistakes. Then go through the possibilities listed above to see if any of them might be an obvious source of the problem. When it comes to changing something on the autosampler to attempt a problem fix, remember to change just one thing at a time so that you can identify the true problem source. This procedure will help you understand how to avoid having the same problem in the future.

HIGH PRESSURE

Q: I observe very high pressure, sometimes enough to shut off the pump, when I inject a matrix blank for one of my LC methods. The blockage occurs in the autosampler, because if I remove the connecting fitting at the injector outlet, the pressure is high, but disconnecting the supply tubing from the pump to the injector provides normal pressure. Usually the pressure drops back to normal after a few minutes. The samples, which receive a more extensive workup, never produce this problem. The mobile phase is acetonitrile and pH 7 buffer. The sample is a biomolecule in a high-salt matrix. During sample preparation, the sample is desalted. Could the salt in the matrix blank be causing the problem?

A: It is quite possible that you are observing pressure increases that are caused by precipitation of salts in the system. It would be helpful to know the salt concentrations and the exact mobile-phase composition. Acetonitrile is notorious for its poor solubility characteristics when mixed with salts and buffers. For example, analysts in my laboratory find it difficult to operate gradient systems with acetonitrile concentrations higher than approximately 80% when the aqueous phase contains greater than 25 mM phosphate. Sometimes combinations of buffers and acetonitrile can be mixed in bulk solution with no problems, but on-line mixing produces solubility problems because the precipitation that occurs at the mixing interface causes tubing or frit blockage before it can be resolubilized effectively by mixing.

To solve your problem, I would either dilute the sample matrix before injection or run it through the same cleanup steps the samples receive. If the same mass of sample matrix must be injected, explore the combination of dilution and larger injection volume. For example, dilute the matrix fourfold and inject four times as much matrix.

Another option may be to change to methanol as the mobile-phase organic solvent. Salts and buffers have much better solubility in methanol than acetonitrile. Of course, methanol may not provide the chromatographic selectivity needed for your method, so this solution may not be viable.

Analysts should avoid conditions that facilitate the precipitation of sample or buffer in the LC system. At best, a pressure rise or a blockage, which can be flushed from the system, will occur. However, if the precipitation occurs within the column pores, you probably should replace the column because it is unlikely that this precipitate can be redissolved.

CONCLUSION

When all variables are held constant, LC methods should yield identical results for replicate injections of the same compound. When injection-to-injection variation in peak area, retention time, or some other measured parameter exceeds the normal system variation, it is a sure sign that one or more variables are being controlled insufficiently. Systematic, step-by-step isolation of the problem should lead to logical corrective measures.

REFERENCES

(1) J.W. Dolan, *LC•GC* **15**(6), 516–521 (1997).
(2) "Technical Note 3," Rheodyne (Cotati, California, 1983).

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