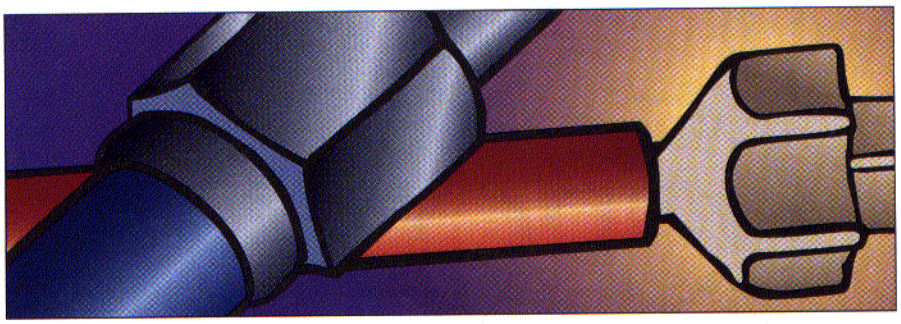
LC Troubleshooting



Priming Injections

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Why is the first injection larger or smaller than subsequent ones?

When a liquid chromatography (LC) system is in equilibrium, replicate injections of the same sample should produce identical results. Most LC methods follow this principle. However, in many methods the first injection does not produce the same results as the second, although subsequent injections behave as expected. In these cases, the first injection somehow conditions the system so that it will behave consistently. LC column preconditioning, often called priming, is the topic of this installment of "LC Troubleshooting."

The use of a priming injection is common practice for many chromatographers because the first injection often differs from subsequent injections. Analysts may observe differences in retention time, peak area, peak shape, and even resolution. Although the magnitude of the changes often is small, the fact that changes can occur with repetitive injections from the same sample vial can be disconcerting. Concern can escalate in a regulated environment, such as when system-suitability checks are used to ensure a quality control assay is working properly. In this setting, generally it is unacceptable to reject the results from the first injection simply because they differ from the other injections. Even if a priming injection is specified in the method, analysts should have a scientific basis for using this technique.

FIRST PEAK IS SMALLER

The most common observation about priming injections is that the first injection produces a smaller peak than the second (and sometimes even the third) injection. Subsequent injections, however, produce results that fall within the expected variation of the method. This phenomenon is understandable when you consider the nature of the column surface. With a reversed-phase column, the bonded phase covers approximately half of the surface; the balance of the surface comprises unbonded silanol (Si–OH) groups. Although it is convenient to think of reversed-phase retention occurring strictly as an interaction between sample molecules and the bonded phase, many molecules, especially organic bases, interact quite strongly with the exposed silica surface. This dual retention mechanism leads to peak tailing and other unwanted chromatographic behaviors.

One common practice to reduce peak tailing is to add triethylamine to the mobile phase at a 25 mM level. The strong interaction between the triethylamine and the silanol groups reduces or blocks interactions between sample molecules and the silanol groups. The reversed-phase retention mechanism predominates, and peak tailing is reduced. Additives such as triethylamine work more effectively if they are added continuously by mixing them into the mobile phase. However, some benefit is observed if triethylamine is injected with the sample instead of adding it to the mobile phase. If analysts use a longer-chain silanol suppressor, such as nonylamine, the effect builds up more slowly, but lasts longer than with the use of triethylamine. In addition to the polar interactions between the amine function and the silanol group, these larger molecules also are retained by reversed-phase interactions between the nonpolar portion of the molecule and the stationary phase.

In a manner similar to the amine additives, sample molecules can interact strongly with silanol groups on the stationary phase. Consider a hypothetical case in which the sample interaction is very slow and the column has 100 interaction sites. If you injected 400 sample molecules, 25% of these molecules might bind to the active sites (assuming a 1:1 interaction), and the resulting chromatographic peak would be 75% of the expected response. During the next run, however, the stationary phase would not be able to bind an additional 100 molecules, so a peak equal to 100% of the expected response would result. The same phenomenon occurs with real samples, although the magnitude of the effect is sample specific.

Proteins often require priming injections for consistent results. One common situation arises when an older column is replaced with a newer one. The initial injections on the new column yield lower-than-expected peak areas. Before panicking, a user observes that each subsequent injection produces a larger peak area. Finally a plateau is reached, whereupon the areas remain constant. We assume the new column now is conditioned and will behave like the column being replaced. At this point, we conclude that the active sites now are saturated with protein. The priming requirement for proteins may be fulfilled by a nonsample molecule, just as triethylamine suppressed silanol interactions for other molecules. For example, a large injection of a readily available protein such as bovine serum albumin may suffice to mask unwanted interactions with other proteins.

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The priming phenomenon is not limited to reversed-phase columns. Analysts often observe the same phenomenon with size-exclusion columns used for protein separations. Two factors are responsible for this observation. First, most analytical size-exclusion columns are larger than other analytical columns; for example, 250 mm × 9.6 mm instead of 150 × 4.6 mm. Larger size generally translates to a higher total stationary-phase surface area. This increase potentially exposes the solutes to more adsorptive sites during transit through the column and, thus, a more

noticeable loss of sample for the initial injections. A second reason is related to the difference between size exclusion and reversed-phase packings. To ensure separation by size, the solute should have minimal secondary interactions with the stationary phase. For aqueous mobile phases, one design approach is to bond a polyhydroyxlated stationary phase to silica particles to minimize direct contact between sample molecules and the silica surface. However, chromatographic observations indicate that significant solute–silica interactions persist even in the presence of a polar stationary phase.

The instructions accompanying most size exclusion columns show that column manufacturers are aware of this phenomenon. Several suggest making repetitive injections of a concentrated protein solution such as a 10-mg/mL solution of bovine serum albumin. In addition to the saturation of active sites, this technique allows users to demonstrate the consistency of the peak area and retention with an inexpensive probe before injecting a possibly valuable protein. The ability of a nonspecific priming solute to block specific solute–stationary phase interactions is open to conjecture. However, many analysts make it a routine procedure. It also is unclear whether this procedure should be employed routinely only with new columns or whether it would apply to any column, even used ones, that have been flushed and left unused for a period of time. Reader feedback on this practice is encouraged via the "Chromatography Forum" web site (enter through http://www.lcgcmag.com).

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FIRST PEAK IS LARGER

Although the smaller first injection can be rationalized with a column conditioning argument, a larger first injection isn't quite so obvious. A reader recently contacted us with the following description.

Q: I have a problem related to a gel permeation chromatography assay that my laboratory is using to monitor for aggregate formation in a protein product. Historically, we have reported only the relative percent monomer, so absolute areas have not been important. We know that for the first injection of the test sample in our assay, the resulting peak-area count may be the same as, higher than, or lower than that for a subsequent injection. Because we report only the percent monomer, we haven't been too concerned unless the variation was so great that it affected the end result. For a couple different products we use a priming injection, and then all subsequent injections are very consistent in retention time, peak area, and peak shape.

Now we are validating the method and have discovered that the variation in peak area has an adverse effect on linearity. If we don't perform a priming injection of sample, the initial injection always yields areas that are too large. This observation puzzles me. I understand the principle of a low area count on the first injection due to protein sticking to the column, but why would the initial injection produce the highest area? For one product, the initial injection can be 30% higher than the next. For the protein we are validating now, the first injection is 10% high. In both cases, the following three injections have a relative standard deviation of less than 1% in area. Can you explain what is happening here?

A: A higher-than-expected first injection is difficult to explain in terms of sample sticking to active sites. We can only speculate that the

protein is adhering to sites in the system, and under constant conditions (temperature, pressure, mobile phase, and so forth), a consistent amount of protein is bound within the system, hence constant areas are obtained after the first injection. Somehow disrupting the system, perhaps through shutting off the system and restarting, changes the amount of protein bound. A subsequent injection might displace this loosened protein, thus yielding a larger peak. To test this concept, we suggest a couple simple experiments. First, determine if a change in the system, such as shutting off the pumps for a period of time, always results in the larger first-injection peak. Does the concentration of protein affect the first injection? Injecting different sample mass and/or volume may provide some hints. Finally, what happens when a brand new column is used?

The reader tried some of these suggestions and responded as follows.

Q: We prepared a new column as usual by making four injections of ovalbumin, our system-suitability sample. The first actual assay on this column was a linearity study, so after equilibration, two zero-volume injections established the baseline appearance, then two injections of ovalbumin, and finally three injections of a 50% preparation of our protein sample. Total protein area counts were 1,730,038; 1,560,512; and 1,542,800; which yield a relative standard deviation of 6.4% — much too high for our requirements. Subsequent triplicate injections in the linearity assay had relative standard deviations of less than 1%.

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The same experiments produced comparable results for a used column (4.1% relative standard deviation for the first three sample injections). This observation of high area count for the initial sample injection is consistent over many columns and two LC systems with two models of detectors.

We also tried diluting the sample, but diluting the sample by half made no difference. Also we checked to see if the monomer or aggregate area was affected more, but the relative change in area was the same for both sample components. We did not test the impact of shutting off the system. Do these tests help formulate a better explanation of the phenomena we observe?

A: We didn't realize that the column was being subjected to ovalbumin as the system-suitability check. The data provided suggest that the ovalbumin primes the column, blocking active sites as expected. However, the injection of sample protein appears to displace ovalbumin, so a peak representing some combination of sample and ovalbumin is observed on the first injection. Explanation of the response observed when ovalbumin is displaced is not simple because certainly ovalbumin is adsorbed throughout the column and is displaced over time as the sample band passes through the column. Whatever mechanism is active, the practical result is that the first sample provides larger-than-expected peak areas. After the first sample has passed through the system, a new equilibrium is established and subsequent injections produce constant areas.

With this obvious explanation of the problem source, it should be easy to justify a priming injection of protein before running samples. The results from this injection should be ignored, just as in the more common case when a smaller-than-expected peak area is observed on the first injection.

CONCLUSIONS

We have seen that in some cases unwanted column interactions can be removed in a general way by using a generic suppressor such as triethylamine. In such cases, pretreatment or continuous treatment of the column removes the unwanted behavior for sample components that have a different chemical structure. In the case of the reader's protein samples, however, the general deactivation by ovalbumin was not completely effective. Deactivation of the column in this case required treatment by the sample itself, not a surrogate. Thus, we see that priming injection requirements may be generic or quite specific in nature. We would be interested in learning other readers' experiences with priming injections. Send your comments to the "Chromatography Forum" web site at http://www.lcgcmag.com.

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