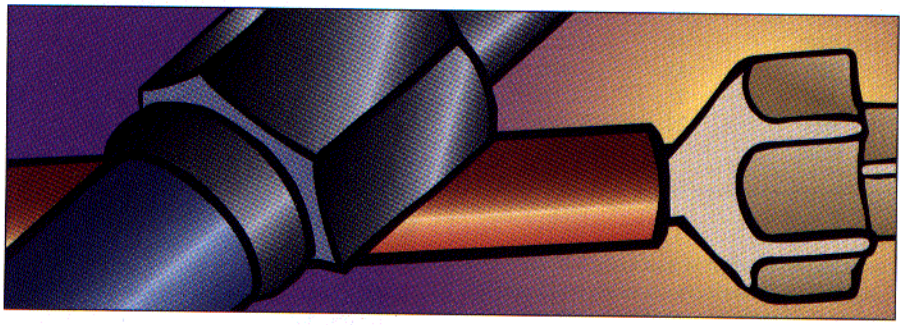
LC Troubleshooting



Sample Overload — Friend or Foe?

Nan S. Wilson, John R. Kern, and John W. Dolan

Sample overload can be frustrating, but it also can be used to your advantage.

Liquid chromatography (LC) columns have a finite capacity for sample compounds. When the injected sample mass is greater than the amount the column can handle, sample overload occurs. Generally, chromatographers like to work under conditions that avoid sample overload, so that sample behavior is predictable. Under overload conditions, retention times and peak shapes vary, and changes in peak area and peak height do not necessarily correspond. This month we'll examine the problem of sample overload and describe two case studies.

SYSTEM SUITABILITY

A method validation protocol called for the injection of 20 µL of several system-suitability standards followed by a variety of samples that were selected to demonstrate method performance. The method used a 150 mm × 4.6 mm C18 column operated at 30 °C and a flow rate of 1.5 mL/min. A gradient was performed using 0.1% trifluoroacetic acid and acetonitrile as the mobile-phase components. Development experiments showed the method produced precise and accurate results with satisfactory robustness.

The system-suitability parameters called for resolution (R_s) of 2.0, U.S. Pharmacopeia tailing factor (T_f) of 1.7 or less, a maximum peak width, and a relative standard deviation of 2% or less for five replicate runs. We are interested in two samples for the present discussion. The system-suitability standard contained the parent drug at 100% of the nominal concentration (20 µg/mL) and an impurity standard at nearly 10% of the parent level. The second sample contained several process-related compounds, including the parent drug and the impurity standard used in the system-suitability test.

Initial system-suitability parameters passed without a problem, so we performed an overnight set of runs to collect the validation data. When the data were analyzed, however, we discovered that when the system-suitability requirements were applied to the process-related sample, the requirements were not met. Table I shows the important parameters.

Figures 1 and 2 show chromatograms for the two samples. In Figure 1, the chromatograms are expanded to show the minor peak at near full scale. The difference in resolution between the two samples is obvious. In well-shaped peaks, the valley between the peaks should hit the baseline at $R_s \approx 1.5$, which is consistent with Figure 1 and the tabular data.

What was the reason for the difference in resolution? The two samples should have contained approximately the same concentration of each compound, so we expected the chromatograms to be very similar. Closer examination of the chromatograms showed the problems illustrated in Figure 2, in which the two runs of Figure 1 are overlaid with the scale adjusted for similar response of the major peak. The difference in resolution is not as obvious as in Figure 1, but Figure 2 shows that the main peak in the related substances sample is distorted. The symptoms correspond to the classic case of sample overload —

TABLE I: Resolution and Tailing Factor Requirements for a Drug Compound Analysis

Sample	R_s	T_f
System suitability	2.16	1.15
Related substances	1.54	1.72

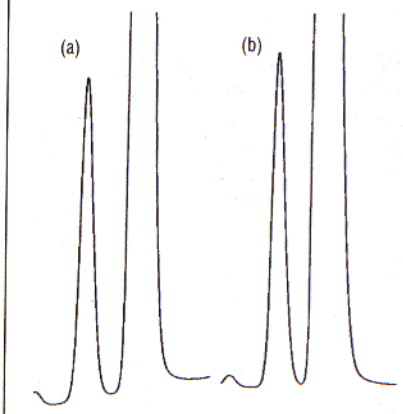


FIGURE 1: Conventional reversed-phase separations performed under (a) nonoverload and (b) sample overload conditions.

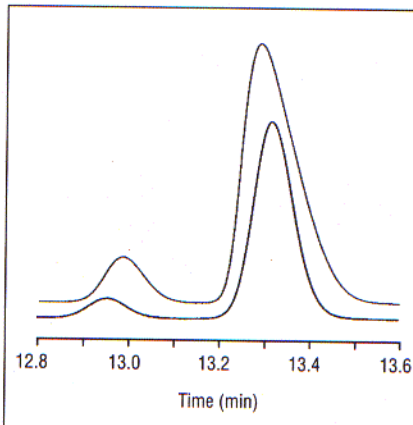


FIGURE 2: Overlaid chromatograms showing the overloaded (upper) and nonoverloaded (lower) runs from Figure 1.

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TABLE II: Retention Times, Tailing Factor, and Resolution of a Series of Drug Compound Samples

Concentration (μg/mL)	Retention Time (min)	T_f	R_s
150	14.09	1.76	1.55
100	14.08	1.53	1.76
50	14.16	1.29	2.04
25	14.15	1.07	2.20

shorter retention times and peaks that approach a right triangle in shape.

WHAT IS OVERLOAD?

It is useful at this point to examine what happens at a molecular level on the column. A physical model will help explain the process. Consider the column as a series of 100-mL beakers lined up in a row. A normal sample — for example, 50 mL in volume — is introduced onto the column by pouring it into the first beaker. Next the first beaker is poured into the second, the second into the third, and so on until the sample reaches the end of the column. At the end of the column, the sample still is in a narrow band — most of it is contained in the last beaker if we were careful with our transfers. Now take the case of a 500-mL sample. When the sample is poured on the column, the first beaker fills before the sample container is empty, so part of it is poured in the second beaker. It takes the first five beakers to contain the entire sample. To move the sample down the column, the first beaker is picked up, but beakers 2–5 have no room for the sample, so the sixth beaker is used next. In a similar manner, each sample portion leap-frogs down the column with five containers for each transfer. The net result is that the sample band is broader and the center of mass travels more quickly through the column than with the small sample.

This illustration fits the chromatographic process fairly well. The column has a finite number of sites at which a sample molecule can interact. These locations are called *active sites.* When an active site is interacting with a sample molecule, it can't interact with another (assuming a 1:1 correspondence), so the free sample molecule continues down the column until it finds a free active site. The process is very much like the beaker–column example above. Sample molecules travel more quickly down the column under overload conditions, and the bands are broader.

WHAT WENT WRONG?

The retention and peak shape of the large band in the top trace of Figure 2 show classic overload symptoms. Our examination of the sample preparation recipe revealed the source of the error. During a dilution step, a 10-mL volumetric flask was specified instead of a 50-mL flask, so the sample concentration was fivefold too high in the related-substances sample. To verify that this factor was the problem, we prepared a series of samples at different concentrations. Table II and Figure 3 show the results.

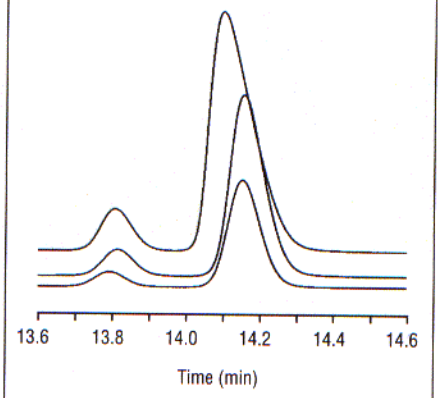

FIGURE 3: Chromatograms showing retention-time changes resulting from increasing on-column sample mass. From bottom to top, the chromatograms correspond to sample masses equal to 125% (20 μL of a 25 μg/mL solution), 250% (20 μL of a 50 μg/mL solution), and 750% (20 μL of a 150 μg/mL solution) of the nominal concentration.

The tabular data clearly show the reduction in retention time, increase in tailing factor, and reduction in resolution with increased sample loading. In this example, the 100% level for the drug was 20 μg/mL. From this limited data set, it appears that the method would pass the system-suitability requirement at 250% (50 μg/mL) of the nominal drug concentration. The chromatograms of Figure 3 confirm the tabular data, with a definite shift in retention for the largest peak (150 μg/mL).

The fix in this case was simple — correct the dilution error in the protocol and repeat the validation.

ANOTHER METHOD

The second case of overload involved a chiral method developed on a protein column. In this case, the limit of quantification needed to be at 0.1% of the nominal sample concentration with a signal-to-noise ratio of 10 or higher at this level. To pass the requirements for limit of quantification, a 20-μL injection was necessary. The lower trace of Figure 4 shows a sample chromatogram at the 0.1% level. This run yielded a signal-to-noise ratio of nearly 15, so it just passes the criterion.

After the concentration requirements were set, a sample at 100% of the nominal concentration was injected, as shown in the upper trace of Figure 4 (the upper trace is attenuated 100-fold more than the lower one). With the background in overload discussed above, analysts should be able to classify this run easily. As with the earlier example, the shorter retention time, broader peak, and increased tailing are obvious.

At first, this situation looks like an insurmountable problem — a smaller sample injection to avoid overload would make the method fail at low levels. In our experience with protein columns, sample overload at the tested levels (100% is equal to 2 μg on column) is common.

Do we have to quit because the column is overloaded at the required assay concentra-

SAMPLE VS. DETECTOR OVERLOAD

This month's "LC Troubleshooting" column discusses sample overload on the column. This situation also can be called *column overload.* Sample overload occurs when the sample mass on column is sufficiently high that the active sites on part of the column become saturated with sample. The peak shape and retention changes characteristic of sample overload result. The presence of sample overload does not necessarily compromise the performance of a method. In fact, sample overload is desirable in preparative separations, because preparative throughput can be increased by operating under overload conditions.

Don't confuse sample overload with detector overload, however. Most UV detectors are linear to 1.0 absorbance units (AU). Many manufacturers specify linearity above 1 AU, but it is best to keep the signal below this level. The detector response in area units per unit mass of sample drops off when the linear range of the detector is exceeded. It is important to understand that sample overload and detector overload are completely different phenomena. For example, the tallest peak in Figure 5 is approximately 50 mAU, which is well within the linear range of the detector, but it overloads the column. Similarly, a very strongly absorbing compound can produce a peak that exceeds 1 AU absorbance without overloading the column.

The key to differentiating between sample and detector overload is to watch the peak behavior. If retention times drop and the peak height is 1 AU or less, sample overload is the problem. If retention times are constant and the peak height is roughly 1 AU, detector overload is more likely the source of the problem.

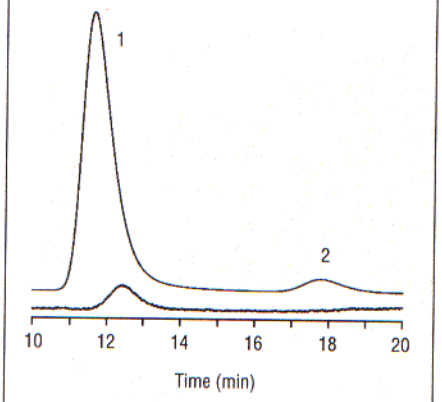

FIGURE 4: Chromatograms showing a change in retention with sample overload in a chiral separation on a protein column. The lower trace was obtained by injecting 0.1% of the nominal sample concentration used to generate the upper trace (upper trace is attenuated 100-fold). Peaks: 1 = *R* enantiomer, 2 = *S* enantiomer.

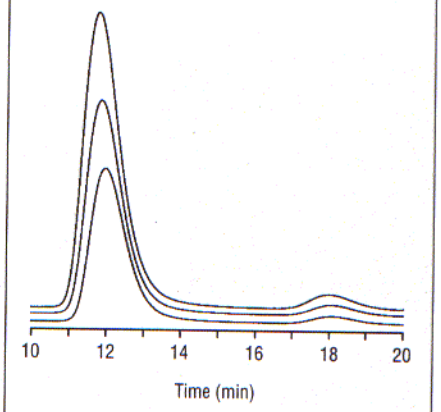

FIGURE 5: Chromatograms generated by injecting the same sample as in Figure 4 with concentrations 125% (top), 90% (middle), and 75% (bottom) of the nominal sample concentration.

tions? Not at all. The calibration curve based on area still is linear, although peak height response may not be. Figure 5 illustrates the results for standards injected at the 75%, 90%, and 125% levels. The higher concentrations produce the shorter retention times characteristic of sample overload, but the area response curve is linear.

The critical question in the present method is whether adequate resolution can be maintained under overload conditions. Fortunately, the resolution between the *R* (first) and *S* (second) enantiomers is large, so small changes in retention or peak width do not compromise the separation. It is fortunate that the overloaded peak is first in this separation, because shorter retention times have little effect on the separation. In the previous reversed-phase example, the overloaded peak was the second peak of the pair, and the resolution was barely adequate, so even small changes in retention caused by overload compromised resolution.

The column has a finite number of sites at which a sample molecule can interact — active sites. When an active site is interacting with one sample molecule, it can't interact with another.

CONCLUSIONS

Sample overload on a column never is a condition that analysts desire for an analytical separation. We described two ways to approach an overload problem. Usually, the first choice is to operate under nonoverload conditions. Analysts simply can make a series of injections at different sample concentrations to determine the sample capacity. After the sample capacity is known, it is straightforward to adjust the method so that it operates under nonoverload conditions. We also saw that overload cannot always be avoided. Because the peak area is constant for a given sample mass, regardless of the peak shape, an area-based calibration curve can yield satisfactory results even when sample overload occurs. If the method runs under these conditions, be sure to allow a sufficiently wide retention-time window so that an overloaded peak does not move out of the integration window or fail system-suitability tests.

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ERRATUM

The flow rates described in Figures 1 and 2 in the March installment of "LC Troubleshooting" (*LC•GC* **16**[3], 250 [1998]) were switched. The correct flow rate for Figure 1 is 0.5 mL/min; the correct flow rate for Figure 2 is 1.5 mL/min.