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FIVE KEYS TO SUCCESSFUL LC METHODS

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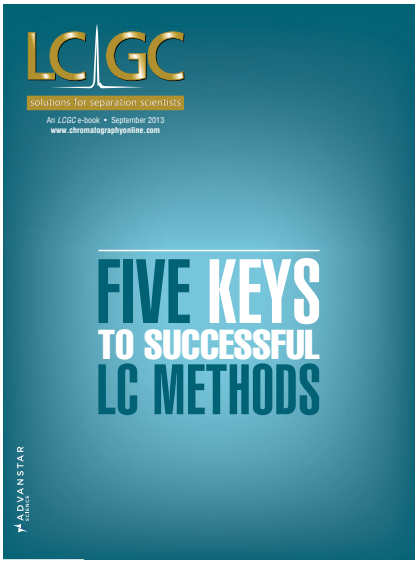

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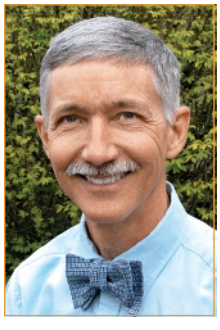
Five Keys to Successful LC Methods

I've been writing "LC Troubleshooting" now for over 30 years, which has resulted in more than 300 installments of liquid chromatography (LC) troubleshooting advice. It is interesting to look back over the topics covered and to consider what has and has not changed. When I started writing the column in 1983, instrumentation and columns had come a long way from the time I made my first injection in 1972, but compared to today's products, there was much more to do to keep your LC system running smoothly. However, even though today's instrumentation is quite reliable and columns have better performance, problems will never disappear.

Over my career of laboratory management and interaction with LC users in person, via e-mail, and in my training classes, certain practices and procedures have become central to reliable LC operation. I don't think any LC system will ever be free of failure, but there are ways to minimize the number of problems and the damage they cause. This compilation of five articles gleaned from past "LC Troubleshooting" columns encapsulates some of these procedures. They cover efficient ways to isolate problems so they can be corrected. Pressure, retention, and peak shape problems top the list, so a separate discussion of each is included. The "Best Practices" article presents a "baker's dozen" (13) of timeless techniques to help keep problems to a minimum. For those of you who want a shorter list, the "Preventive Maintenance" article reduces this to three practices, one of which is automatic on most LC systems today.

I hope you find some valuable tips in these timeless discusions that will be of practical use in your laboratory.



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Pressure Problems

What to do when the system pressure is not what it is supposed to be (and how to know what it should be)

A previous article on troubleshooting basics (1) was an overview of classifying a troubleshooting problem, and considered some rules of thumb that may be useful to isolate the source of various problems. In this article, we'll be more specific, with a look at pressure. Often the first sign that something isn't right with your liquid chromatograph is an abnormal pressure reading — the pressure is too high, too low, cycling, or erratic. In this article, we'll look at ways to estimate what normal system pressure should be, as well as some likely causes of various pressure abnormalities.

What Is Normal?

Before we can determine if there is a pressure problem, we need to know what the normal system pressure is for a given configuration of hardware, column, and mobile phase. Pressure is a result of the resistance to flow of the mobile phase through the system, and the column is the major cause of resistance. Thus, the length, diameter, and particle size of the column are important. The mobile-phase viscosity and flow rate are the other main factors. For conventional liquid chromatography (LC) systems (<6000 psi; <400 bar), the hardware (pump, autosampler, tubing, and detector) contributes little to the pressure and usually can be ignored. With ultrahigh-pressure LC (UHPLC, >6000 psi), however, narrow-bore tubing and in-line frits can result in 1000 psi or more of pressure in addition to the column, so the hardware cannot be ignored.

The easiest way to identify a pressure problem is to compare the current pressure to the normal value. I like to use two kinds of normal reference values. The first is a method-independent pressure measurement, which I'll call the "system reference pressure." To check this, install a new column that is typical of what you normally use, such as a 150 mm × 4.6 mm, 5-µm particle size (d_p) C18 column, and an easy-to-replicate mobile phase, such as 50:50 (v/v) methanol–water. Set the flow rate and column temperature at, for example, 2 mL/min and 30 °C, respectively, and allow the system to equilibrate. Record the pressure under these conditions and you can use it in the future as a reference point. To be thorough, I would also progressively disconnect the fittings at the column outlet, column inlet, in-line filter inlet (if used), and pump outlet; record the pressure after each step. Now you should have a list of pressures at various places in the flow path under these standard conditions. These reference pressures may be handy to help track down pressure abnormalities in the future.

The second reference value, which I'll call the "method reference pressure," is obtained in a similar manner, but using the normal method settings. If a gradient method is used, record the pressures under the starting conditions. You may want to shortcut the process and just record the pressure with all components installed, the column inlet disconnected, and the in-line filter (if used) disconnected; this approach will isolate the most common sources of system blockage for future reference. Because method pressure rises normally over time as frits and filters collect debris, I like to track the pressure. A convenient way to do this is to add a "starting pressure" item to the data recorded at the beginning of each batch of samples (column serial number, sample batch number, notebook reference, and so forth). These data can be used for future reference or plotted over time as a control chart to help anticipate pressure problems before they occur.

Estimating Pressure

You may want to have an estimate of what the normal system pressure should be, just as a cross-check. The technique I like for this uses equation 2.13a from reference 2:

$$P \approx (2500\, L\, \eta\, F) / (d_p^2\, d_c^2) \qquad [1]$$

where the pressure, P (psi), is a function of the column length L (mm), diameter d_c (mm), and particle size d_p (µm), as well as the mobile phase viscosity η (cP) and flow rate F (mL/min). For pressure in bar, divide by 14.5. The mobile-phase viscosity will depend on the components in the mobile phase and the temperature. Methanol and acetonitrile are the most common organic mobile-phase components for reversed-phase LC, mixed with water or buffer. Both methanol and acetonitrile are more viscous when mixed with water, with a maximum viscosity for methanol of 50% methanol in water and for acetonitrile of 10% acetonitrile in water. I have summarized the viscosities of mixtures of methanol and acetonitrile with water at several compositions and temperatures in Table I. For a more complete listing, see Table 1.5 in reference 2.

Now we can use equation 1 to estimate the pressure for a selected method. Several examples are given in Table II. I have chosen maximum-viscosity mobile phases (50:50 methanol–water and 10:90 acetonitrile–water) and 30 °C. For example, a 150 mm × 4.6 mm, 5-µm column run at 2 mL/min with the methanol mobile phase will generate approximately 2000 psi (140 bar) under these conditions. Pressure estimates, such as these,

are just that — estimates — and in my experience they may be off by ±20% in many cases, and ±50% in some cases. This is because the resistance to flow of some columns may differ because of packing techniques, and the quoted nominal particle size may not be the true value. For example, a 0.1-µm difference in a nominally 2-µm particle will make a 10% difference in the calculated pressure.

I have included the pressure calculated for the above example and several other common column configurations in Table II; in each case the columns are selected to give approximately the same plate number, *N*, so a similar separation should be obtained in each case (assuming identical column chemistry). A few general observations are in order. Acetonitrile generates approximately 60% of the pressure of methanol, which is one reason it is favored for UHPLC mobile phases. This is highlighted by comparing the last two rows of each section of Table II: 1 mL/min with a 75 mm × 2.1 mm, 1.8-µm column generates too much pressure (18,800 psi) with methanol to operate even under UHPLC conditions, whereas the 11,800 psi with acetonitrile makes this flow rate feasible. For comparison, I have also included a shell-type particle. The particle size (2.7 µm) dictates the pressure, but the efficiency of the shell configuration makes them behave like a 2-µm particle in terms of plate number. The right-hand column of Table II lists the column dead-time, t_0, which can be used to compare run times for the various columns. For example, it may come as a surprise, but a separation on the 100 mm × 4.6 mm, 3-µm column takes approximately one-third longer than the 150 mm × 4.6 mm, 5-µm column at the same pressure. Also, a 1.8-µm UHPLC column will cut the run time by about fourfold compared with a 3- or 5-µm column on a conventional LC system when all columns are run at reasonable system pressures. And finally, the shell-type 2.7-µm particle column shortens the run time by twofold when compared with a 1.8-µm UHPLC column when both are operated at the same pressure.

Now that we have a technique to approximate the column pressure, we can see how calculated values compare with the observed values under the system reference or method reference conditions. If you are using a UHPLC system, you'll need to add to the calculated value the system pressure observed when the column is removed, which may be 500–1000 psi.

Table I: Viscosity of mixtures of methanol or acetonitrile with water. Data from Table 1.5 of reference 2.

	Viscosity, η (cP)			
Methanol (%)	**0%**	**10%**	**50%**	**100%**
30 °C	0.79	1.04	1.43	0.51
40 °C	0.64	0.82	1.12	0.42
50 °C	0.54	0.71	0.94	0.37
Acetonitrile (%)	**0%**	**10%**	**50%**	**100%**
30 °C	0.79	0.90	0.74	0.32
40 °C	0.64	0.72	0.62	0.27
50 °C	0.54	0.60	0.53	0.24

Table II: Estimated pressures for several column conditions

Pressure		L	d_c	d_p	F	N‡	t_0
psi*	bar†	(mm)	(mm)	(µm)	(mL/min)		(min)
50% Methanol							
2000	140	150	4.6	5	2	9000	0.79
2000	140	100	4.6	3	1	10,000	1.06
8300	580	75	2.1	2.7	1	11,250	0.17
18,800	1290	75	2.1	1.8	1	12,500	0.17
9400	650	75	2.1	1.8	0.5	12,500	0.33
10% Acetonitrile							
1300	90	150	4.6	5	2	9000	0.79
1200	80	100	4.6	3	1	10,000	1.06
5200	360	75	2.1	2.7	1	11,250	0.17
11,800	810	75	2.1	1.8	1	12,500	0.17
5900	410	75	2.1	1.8	0.5	12,500	0.33

* Rounded to nearest 100 psi; † Rounded to nearest 10 bar; ‡ Estimated for real samples (reduced plate height ≈ 3 d_p)

High Pressure

A gradual increase in pressure over time is a normal symptom of column aging, and excessive pressure is often the first indicator that something is wrong with the system. In some cases, the pressure increase may be large enough to trigger the upper-pressure limit, and system shutdown may occur. High pressure is a symptom that something in the flow path is partly or completely blocked. The most common location for this will be the first frit after the autosampler because it accumulates debris from the sample or other sources. This is one of the reasons I strongly recommend using an in-line frit just downstream from the autosampler. Use a 0.5-µm porosity frit when columns with particles > 2 µm are used; a 0.2-µm porosity frit is used with ≤2-µm columns. This frit has smaller porosity than the frit at the head of the guard column or column, so it will become blocked first. The frit in the in-line filter is easy and inexpensive to change, making it a quick fix for the most common

high-pressure problems and a simple way to protect the expensive column from damage.

Isolate the location of the blockage by progressively loosening fittings, as described earlier, until you find the source of the pressure increase. Remember that when the column is removed, conventional systems (<6000 psi; ≤400 bar) should have negligible pressure, but UHPLC systems may normally have measurable back pressure.

If the frit at the head of the column becomes blocked, you may be able to correct the problem by back-flushing the column; this is effective about one-third of the time. Just reverse the column direction and pump 20–30 mL of mobile phase through the column to waste (not to the detector). If the pressure drops, you can leave 5-µm columns reversed. Check with the column care and use sheet for ≤3-µm columns to see if they can be safely reversed for extended use. If backflushing does not restore the column, replace the column with a new one. It may be wise to add an in-line filter or guard column (or both) if column blockage is common.

If some other component apart from the column, guard column, or in-line filter is the source of the blockage, sequentially remove connections until you isolate the location of the blockage. If the tubing is blocked, replace it. Other parts, such as injection valves, may require disassembly and reconditioning.

Low Pressure

Low pressure usually results from air in the pump, a faulty check valve, or a leak. First, check for the obvious: make sure the flow rate is set properly and that there is sufficient mobile phase in the reservoirs. Purge the pump of any bubbles by opening the purge valve and increasing the flow-rate to flush 5–10 mL of mobile phase through the pump. If this does not correct the problem, verify that the pump is working properly. Perform a simple check of pump delivery by doing a timed collection of 10 mL of mobile phase in a volumetric flask; the flow-rate should be within ±1% of the set point. If the pump still doesn't deliver properly, check to be sure there is sufficient solvent at the inlet to the pump. Remove the supply tubing at the pump inlet (or with a low-pressure mixer, the tubing at the proportioning manifold) and measure the flow in a graduated cylinder. Siphon flow should deliver at least 10 times as much solvent to the pump inlet as you need. For example, if you normally run 1–2 mL/min, expect to see at least 20 mL/min of siphon flow to the pump. If there is insufficient solvent at the pump, check for blocked frits in the reservoir or blocked tubing. Still another possible pump problem is a leaky pump seal; replacement of pump seals every 6–12 months should prevent this from happening for most applications.

After you are happy with the pump operation, check for leaks elsewhere in the system. You may have been alerted by a leak detector. If this is the case, the leak location should be easy to identify. Otherwise, check each fitting, especially upstream from the column, where the connections are under the most pressure. Look for visible signs of leaks, such as drops of mobile phase or white buffer residues left when leaked mobile phase evaporates. Sometimes a scrap of paper can be useful to help probe for leaks; thermal printer paper works best for this, but it is hard to come by today, so copier paper can be used instead. Cut a triangle of copier paper ~1 cm across the base and ~5 cm along the sides. Touch the narrow end to any suspect fittings and it will act as a wick and soak up any small leak, which should be easily visible (thermal paper will turn black). If fittings need to be tightened, it is good practice to do this with the flow off. This is especially true with finger-tightened polyether ether ketone (PEEK) fittings, because the tubing can slip in the fitting if there is pressure in the system when the fitting is adjusted. Any fittings that still leak after being tightened a quarter turn or so past their normal setting should be replaced with new parts.

Cycling or Erratic Pressure

Pressure readings that bounce around are usually the result of a faulty check valve or air in the pump. Cycling pressure usually coincides with the piston stroke of one or more pumps. The fixes to this problem are to purge the pump, clean or replace the check valves, and replace the pump seals. Persistent problems may be associated with inadequate mobile-phase degassing.

Although most of us now use automatic in-line degassers, these can fail, too. I have had several cases reported to me in the last few months where in-line degasser failure caused system shut-down, pressure fluctuations, or erratic retention times.

If you are running gradients, don't forget that the pressure will increase during a gradient. For example, with a methanol–water gradient of 0–100% methanol, the setup for the first line of Table II would give a starting pressure of ~1100 psi, which would rise to ~2000 psi mid-gradient and would end up at ~700 psi for 100% methanol.

Summary

We have looked at several pressure-related aspects of the LC system. First, we saw the importance of having a record of normal system pressures that can be used for comparison when a change in pressure is encountered. Equation 1 can be used to estimate the pressure produced by columns of different dimensions and particles sizes. We looked at some of the causes of, and corrections for, high, low, and cycling or erratic pressures. These should help you to isolate and eliminate the most common sources of pressure problems. But as one of my colleagues used to say, "Don't forget to check stupid!" It is amazing how often problems are related to something silly that we've done, such as letting the reservoir run dry or misprogramming the controller.

References

(1) J.W. Dolan, *LCGC Europe* **24**(7), 386–389 (2011).
(2) L.R. Snyder, J.J. Kirkland, and J.W. Dolan, *Introduction to Modern Liquid Chromatography* (Wiley, Hoboken, New Jersey, 3rd ed., 2010).

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Retention Problems

What to do when retention times are too long, too short, or inconsistent

In this article, we'll concentrate on problems exhibited as abnormal retention times. As a means to organize the discussion, let's look at situations where retention times are too long, too short, or inconsistent.

What Controls Retention?

Before we look at specific problems, let's take a moment to consider the things that influence retention. We can categorize these as the mobile phase, the stationary phase (column), the hardware, the environment, and the sample. Let's simplify this discussion and assume that nothing has happened to the sample, such as degradation or other chemical changes.

The mobile phase can change because of an error in formulating it, such as a mistake in volumetric measurement or adjustment of the pH. If an error in formulating the mobile phase is suspected, it is best to make a new batch to see if it fixes the problem. Some mobile phases can change over time because of chemical degradation, selective evaporation of one component, or microbial growth in highly aqueous mobile phases. Again, reformulation is the best way to verify this problem source. Most of us use on-line mixing to prepare isocratic mobile phases. An error in instrument settings or hardware failure can be the cause of errors in on-line mixing. Check for proper degassing and pump operation, as well as the correct control-program settings. Sometimes hand-mixed mobile phases can be used as a check for on-line mixing, or alternate mixing channels can be used for both isocratic and gradient methods (for example, use the C and D solvent reservoirs instead of A and B in a four-solvent LC system).

The stationary phase in the column has a finite lifetime, generally in the 500–2000 sample range (or more), depending on the nature of the sample. Every column will die eventually, and some methods are harder on columns than others. For example, mobile phases outside the pH 2–8 region accelerate the degradation of silica-based columns. Some column types have shorter lifetimes than others. For example, cyano and amino columns are unlikely to last as long as C8 or C18 columns, which tend to be quite robust. In addition to changes in retention, column failure usually is accompanied by a rise in system pressure and an increase in peak tailing. Replacement of a suspect column with a new one is the easiest way to check for column-related problems.

System hardware problems that generate symptoms of changed retention most commonly are associated with pump malfunctions or leaks. Pump problems can be checked with a simple flow-rate measurement with a stop watch and volumetric flask. A secondary symptom of pump problems may be high, low, or fluctuating pressure. Low flow may be associated with faulty check valves, worn pump seals, air bubbles in the pump, or errors in pump settings. Cleaning, component replacement, or degassing should correct such problems. High flow rates usually are a result of improper settings.

The most common environmental cause of retention changes is a change in column temperature. This effect is common if the column oven is not used or is not working properly. Methods that operate under ambient conditions are highly susceptible to failure, especially if the laboratory temperature is not well controlled. In my travels, I have encountered laboratory temperatures ranging from 10 °C (central China in January) to 35 °C (Tel Aviv in June). If we use the rule of thumb that retention can change by 2% with each 1 °C change in temperature, you can imagine the result if the same method were run in both of those laboratories under ambient conditions! Use the column oven and make sure that it is operating properly.

Two Important Measurements

One tool that can be very useful in diagnosing the source of retention problems is the retention factor (also called the capacity factor, k'). Recall that the retention factor, k, is calculated as

$$k = (t_R - t_0)/t_0 \quad [1]$$

where t_R is the retention time and t_0 (sometimes abbreviated as t_M) is the column dead time, usually measured by the first disturbance in the baseline (the "solvent front"). Another useful calculation is the selectivity, or relative retention, α:

$$\alpha = k_2/k_1 \quad [2]$$

where k_1 and k_2 are the k values for the first and second peaks of an adjacent peak pair, respectively.

Notice that changes in flow rate, whether intentional or due to a leak, will change both t_0 and t_R proportionally, so k will remain

constant for such changes. On the other hand, chemical changes will change only t_R, so the k value is changed, too. Generally, when the k value is changed it does not change exactly the same for all peaks in the chromatogram. One way to confirm chemical changes in the system is to check the α value; if α changes, a chemical source of the problem is most likely. Because k and α are so useful in distinguishing between flow-related and chemical changes, it is a good idea to make these measurements a part of the process for troubleshooting retention-time problems.

Excessive Retention

When retention times increase and k values stay constant, a flow-rate problem is indicated. Double-check the flow-rate setting to be sure you didn't make a mistake. Leaks and pump problems are the two most common remaining causes. Check for leaks throughout the system; these may or may not be accompanied by low system pressure, depending on the magnitude of the leak. Several possible problems related to the pump could be sources of increased retention. Air bubbles in the pump will also cause pressure fluctuations; thorough degassing of the mobile phase and purging the pump should correct such problems. If problems persist, check to be sure the frit in the mobile phase reservoir is not restricting flow to the pump. Faulty check valves or pump seals also can result in low flow and long retention times. Sonication of check valves will usually restore their function. Pump seal leakage often is accompanied by liquid dripping from the drain hole just behind the inlet check valve on most pumps. Check the maintenance records — if the pump seal has been in use for a year or more, replace it.

When a change in k values (and often α) is observed, you have evidence that a change in system chemistry is responsible for an increase in retention. The easiest way to check this is to make a new batch of mobile phase. If this does not correct the problem, replace the column.

A final possible source of increased retention is a drop in the column temperature. As mentioned above, a 2% increase in retention for a 1 °C drop in temperature is common. Based on the magnitude of the retention change, you can estimate the temperature change and see if it is a reasonable cause of retention. Has the oven failed, did you forget to turn it on, or are you relying on ambient operating conditions? Any of these sources can account for increased retention.

One useful tool for diagnosing the source of retention problems is the retention factor.

Retention Is Too Small

When retention times are smaller than they normally appear, a flow-rate change is highly unlikely, unless you made an error in one of the settings. This is because decreases in flow due to leaks or other malfunctions are not uncommon, but there are no corresponding causes that result in higher-than-normal flow rates that are necessary for reduced retention.

As with retention times that are too long, do a stepwise elimination of problem sources by first making up a new batch of mobile phase. If this approach doesn't fix the problem, replace the column. Temperatures that are higher than normal will cause a drop in retention; the sources of temperature problems are the same as for excess retention.

Fluctuating Retention Times

Usually, an increase or decrease in retention will not be an abrupt change. If it is, the cause is likely related to operator intervention, such as improper formulation of a new batch of mobile phase, installing the wrong column, or changing a column-oven setting. More commonly, retention will gradually increase or decrease over tens, hundreds, or thousands of samples, or it cycles over a 24-h period. Cycling retention is commonly correlated with ambient methods and a laboratory temperature that changes throughout the day and night.

Retention drift that occurs over hundreds or perhaps thousands of injections is most likely because of normal column aging. Drift from column aging usually will be accompanied by a gradual increase in pressure and an increase in peak tailing. Often, a shift in relative retention also will be observed when α values are calculated. If the column is suspected, replace it to see if the problem is corrected.

Shorter-term retention drift may be caused by the mobile phase. Although fairly rare, it is possible to selectively evaporate a volatile component of the mobile phase, especially if helium sparging is used for degassing. Retention drift resulting from a pH shift can occur if the buffer is outside its optimal buffering region, generally ±1 pH unit from its pK_a. The use of volatile buffers, as is common with LC–mass spectrometry (MS) mobile phases, may shorten the stable lifetime of the mobile phase, so daily reformulation may be a wise practice. Make up a new batch of mobile phase if the mobile phase is suspected, and be sure to adjust the pH before any organic solvent is added.

Problem Prevention

To avoid retention problems, make sure to keep the instrument in good operating condition by servicing it regularly. A routine preventive maintenance session should be done on an annual basis at a minimum, and more often for heavily used LC systems.

Because column temperature changes can have such a profound influence on retention time, it is wise to always use a column oven. Many ovens do not control

the temperature well near room temperature; a good practice is to use a minimum operating temperature of 30–35 °C so that good temperature control is ensured. It may take 30 min or longer for the column oven to reach a stable temperature. Be sure to use the solvent preheater that is included with most column ovens. The most common preheater implementation is a piece of capillary tubing that is embedded in the aluminum block of the oven.

Columns usually will last for more than 1000 injections. When this number of samples has been analyzed, the cost-per-sample for the column is less than 5% of the overall per-sample cost of analysis. My feeling is that at this point it isn't worth my time to do anything more than flush the column with strong solvent (for example, acetonitrile or methanol); if this doesn't restore the column, replace it. Guard columns or sample preparation both will extend the column life, but they have their associated costs, which may make the economics of their use questionable. A 0.5-µm in-line filter between the autosampler and column will help keep particulate matter from blocking the column inlet frit, but it will not influence retention-related problems. Another good practice for extending column life is to use a single column for each method. When the same column is used for multiple methods, sometimes unimportant sample components from one method will interfere with another method.

Mobile phases have finite lifetimes, too. My recommendation is to replace any buffer at least once a week and organic-based mobile-phase components at least monthly. It is a good idea to replace the reservoir with a clean one whenever the mobile phase is replaced so that prior contamination doesn't get transferred to the new mobile phase.

If you pay close attention to patterns in retention changes, correlations with mobile-phase use, and column history, you can establish expected replacement cycles for each component of each method. After such patterns are defined, you can put in place preventive-maintenance and component-replacement practices that will help you avoid most retention-related problems. Armed with an understanding of which variable most strongly influences retention in your particular method, you'll be able to more quickly identify and correct problems when they occur.

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Peak Shape Problems

What you need to know to address peak tailing and peak fronting

A change in peak shape is one of the most common observations of problems with a liquid chromatography (LC) method. Because of this, most system suitability tests include a measure of peak shape, so a quantitative value of peak shape can be tracked over time. Poor peak shape can compromise the results of an analysis by degrading resolution between closely eluted peaks and reducing precision and accuracy of measuring peak area, especially for small peaks. A change in peak shape is one of the first signs that the column is failing, but there are other causes of peak tailing, as well. In this article, we look at several aspects of peak tailing.

Measuring Peak Tailing

The ideal chromatographic peak will have a Gaussian shape, but it is rare that a perfectly symmetric peak is observed in real chromatograms. Most peaks tail slightly, and as the column ages, tailing typically increases. However, there are several other potential causes of peak tailing (or fronting) as well, so it is a good idea to track the peak shape over time to anticipate when practical problems will occur. As a result, nearly all system suitability tests include a measurement of peak shape.

The two most popular methods of measuring peak shape are illustrated in Figure 1. Other methods to measure peak shape are used much less often. The pharmaceutical industry uses the tailing factor, TF, which is determined by measuring the entire peak width at 5% of the height and dividing it by twice the front half-width. Nonpharmaceutical laboratories often use the asymmetry factor, A_s, which is calculated by measuring the back half-width of the peak at 10% of the peak height and dividing it by the front half-width. You can see that if the peak is perfectly symmetric, the front and back half-widths will be the same, no matter where they are measured relative to the peak height, so for such peaks, TF $\equiv$ A_s. As tailing increases, however, the two numbers diverge, with A_s growing faster than TF, but for peaks with a value less than 2 there is not a very noticeable difference. There is no inherent value in using one technique versus the other for measuring peak shape; rather, it is important to choose one technique and use it to look for changes in peak shape over time.

Most LC peaks tail or front a bit, so column manufacturers typically set their column-release specifications at $0.9 < TF < 1.2$ as normal performance. As can be seen in Figure 2, when tailing increases, several practical problems can arise. The peaks are harder to integrate because the transition from the baseline to the peak or peak to baseline is much more gradual, and on noisy or sloping baselines the peak limits are difficult to determine. Generally, the peak area stays constant, so increased peak tailing translates into shorter peaks, and peak height is the limiting factor in determining detection limits, so method limits can suffer with tailing peaks. Tailing peaks also take a larger time window to be eluted, so to achieve baseline resolution between peaks, the run time must be longer. And tailing peaks are aesthetically less pleasing. You can see that all these factors favor symmetric peaks. From a practical standpoint, peak tailing is difficult to eliminate, however, for many applications peaks with TF $\leq$ 1.5 are acceptable. When TF $\geq$ 2, usually corrective action should be taken to identify and eliminate the source of tailing.

When peak tailing occurs, it usually shows up for one or just a few peaks in the chromatogram, but sometimes all the peaks in the run tail. The appearance of peak fronting is much less common. Most often, these three behaviors are caused by three different sources. We will look at each of the three problems next.

Tailing of One or a Few Peaks

Usually, one or a few peaks in the chromatogram tail and the cause is most often chemical in nature. The problem may appear during method development, in which case you probably do not know if the peak shape ever was good. Or more often with an existing method, a peak that had acceptable shape in the past begins to tail, and tailing increases over time. If the onset of the tailing was sudden, as when a new batch of samples was run, look for some other change that coincided with the observation, such as preparation of a new batch of mobile phase or replacement of the column or guard column.

Mobile-phase changes are the easiest to check. For example, the pH of the mobile phase can have a strong influence on the peak shape, so if an error was made in pH adjustment, this could be the problem source. Mobile-phase problems will also usually cause changes in retention time. If such correlated changes are observed, carefully prepare another batch of mobile phase (or

Figure 1: Measurement of tailing factor and asymmetry factor.

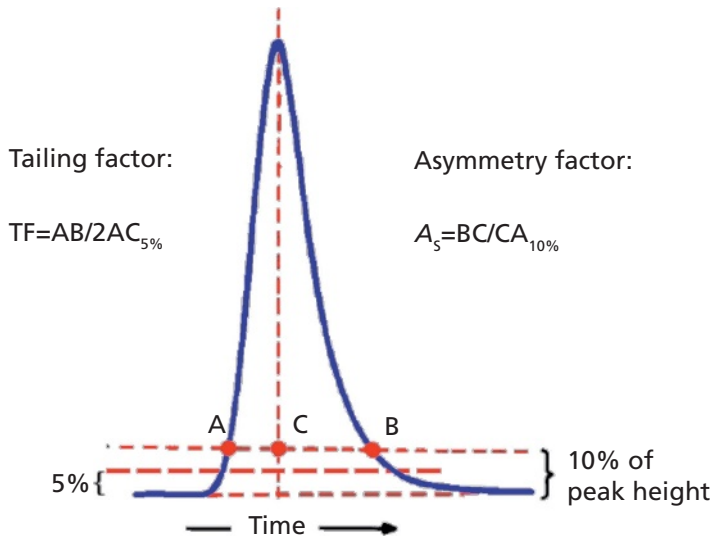
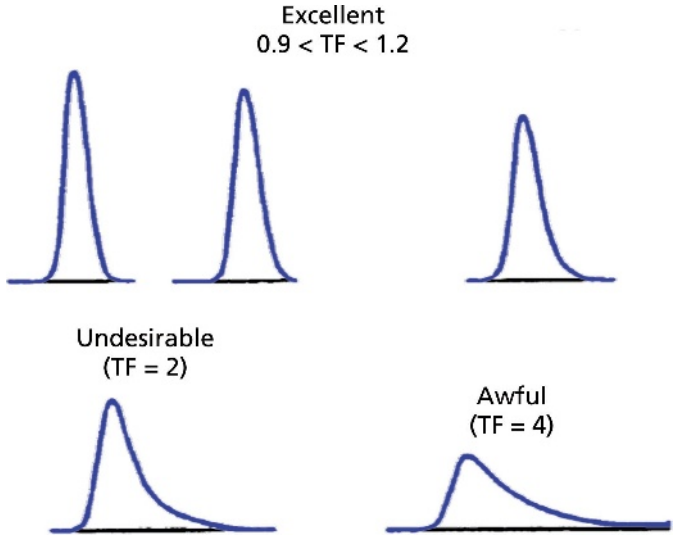


Figure 2: Examples of tailing peaks.



repeat some other change that was made) and see if that corrects the problem. Sometimes a method will be developed that is not very robust. In such cases, even small changes in temperature or other variables can cause a change in the retention or peak shape. Another mobile-phase problem that occurs occasionally is insufficient buffer concentration. Although reversed-phase separations are not strongly affected by buffer concentration, hydrophilic interaction chromatography (HILIC) and ion-exchange LC are much more sensitive to buffer effects. A buffer concentration of 5–10 mM usually is adequate to buffer the mobile phase, column, and injection solvent in reversed-phase separations. If buffer concentration problems are suspected, double the concentration and see if this fixes the peak shape.

After the mobile phase is eliminated as the problem source, look to the column. If a guard column is in use, remove it and make an injection. If the peak is OK after that is done, the guard column has failed. If the problem persists without a guard column, substituting a new column for the old one is the simplest way to check for other column problems. Column problems are more likely after ~500 or more samples have been run, but in some cases column problems can occur much earlier. Dirty samples and mobile phases outside the 2 < pH < 8 range are two common sources of rapid column deterioration. If replacing the column corrects the problem, consider improving the sample cleanup, changing the mobile-phase pH, or using a guard column to help delay the problem of column deterioration.

The source of peak tailing is not a simple process. In Figures 3a–3c, you can see the influence on peak shape of increasing amounts of mefanamic acid. Under these conditions (pH 2.8), the mefanamic acid is well below its pK_a, so it is not ionized. At low loading (Figure 3a), the tailing is dramatic. Peaks with exponentially shaped tailing are likely a result of two different retention processes going on simultaneously; for example, some molecules might be interacting with column sites that equilibrate slowly (those on the tail) and some with sites that equilibrate quickly (those on the main peak). As the mass on column increases (Figures 3b and 3c), the majority of the sample molecules are retained by a single mechanism (fast equilibration), and the peak shape improves. The slow equilibration process doesn't disappear, but it is a smaller portion of the total, so tailing is reduced. On the other hand, amitriptyline, an ionized base at pH 2.8, looks better than mefanamic acid at low concentrations (compare Figures 3a and 3d). As the injected mass of amitriptyline increases (Figures 3e and 3f), two things are observed. First, the peak tail begins to change shape until it takes on a right-triangle appearance (Figure 3f). Second, the retention time gradually gets smaller. These are

Figure 3: Peak tailing for (a–c) mefanamic acid and (d–f) amitriptyline at pH 2.8. Injection mass on column: (a,d) 3 ng; (b,e) 500 ng; and (c,f) 15 µg.

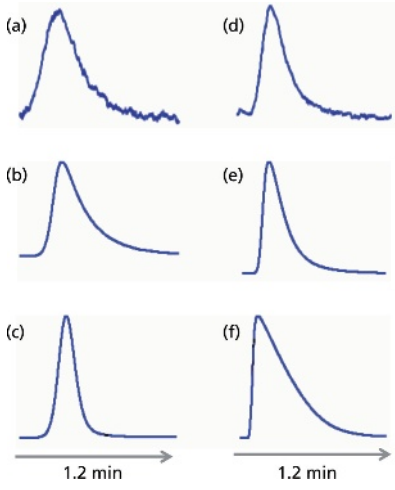


Figure 4: Peak fronting for two consecutive injections: (a) 527 and (b) 528.

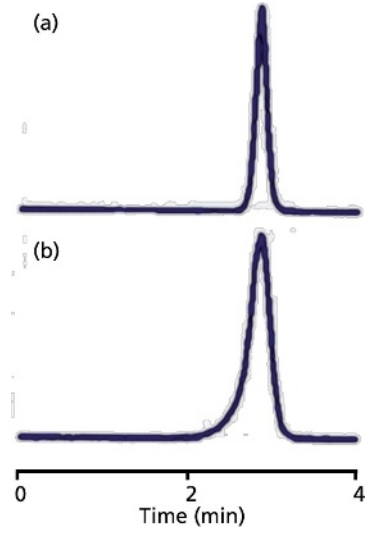
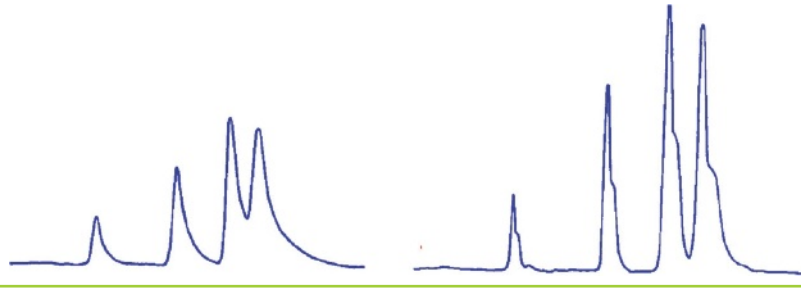


Figure 5: Peak distortion for all peaks in the chromatogram.



two classic symptoms of column overload. To verify this problem source, reduce the sample mass on column and see if retention increases and tailing improves. The cause of tailing in this case is likely because of ion exclusion. As the amount of amitriptyline adsorbed inside the pores in the column increases, the pore takes on a net positive charge (amitriptyline carries a positive charge at pH 2.8). At some point, the positive charge inside the pore is sufficient to repel another positively charged molecule, so it must travel further down the column before it finds a compatible site on the surface. The interesting thing about these two examples is that at the same pH, one analyte (mefanamic acid) gives less tailing as the sample load increases, whereas the other (amitriptyline) gets worse. It is hard to generalize about the source of peak tailing.

Peak Fronting

Peak tailing was attributed to problems related to chemical interactions on the column. One way of thinking about peak tailing is that the active sites — the places on the column where interactions between the analyte molecules and the chemical surface of the column occur — become saturated. It is possible to conceive of a similar case in which the mobile phase becomes saturated or overloaded, and in such cases, peak fronting would occur. This indeed happens, but with reasonable buffer concentrations (for example, ≥5 mM), such overload is rare in reversed-phase LC. A more common source of peak fronting is illustrated in Figure 4, where peaks from two consecutive injections are shown. The peak changes from a normal appearance in injection number 527 (Figure 4a) to badly fronting in injection 528 (Figure 4b). The most common cause of such changes is a sudden physical change in the column, usually referred to as column collapse. In the present case, the column was operated at pH 9.6 at 70 °C, but the column was designed to be used at pH ≤ 7 and ≤ 40 °C. The aggressive mobile phase conditions gradually dissolved the silica particles inside the column until they became so fragile that the minor shock of the injection valve rotating caused the internal column structure to collapse. The proper fix for such a problem would be to modify the method so that it operated with the recommended limits of the column or replace the column with a more robust one. That is, if all else fails . . . read the directions! However, for the present example, it made more sense economically to replace the column every 500 injections rather than redevelop and revalidate the method.

If All Peaks Tail

When all peaks in the chromatogram tail or are split or doubled, as in Figure 5, this is a symptom of a problem that happens at the inlet of the column before any separation takes place. The most common cause of such problems is a partially blocked inlet frit on the column. Debris from the sample, the mobile phase, or a failed pump seal or injector rotor can collect on the inlet frit. As the frit becomes partially blocked, the sample stream arriving at the column inlet is distorted, resulting in peak distortion. Because this problem happens before any sample molecules are separated, it affects all peaks in the chromatogram in the same manner. You can fix the problem about a third

of the time by reversing the column and backflushing it to waste for a few minutes. Most columns can be reversed for such flushing, but it is best to check the column care-and-use instructions to make sure it is allowed for your column. If reverse-flushing corrects the problem, you can proceed as normal. If it doesn't, replace the column. In either case, it is wise to eliminate or reduce the source of particles arriving at the top of the column. Improve the sample pretreatment by adding a centrifugation or filtration step. Replace worn pump or autosampler parts. Filter the mobile phase to remove particles. And a good safety measure is to place an in-line filter between the autosampler and the column. If you use a guard column, it acts as a filter to protect the column, but the in-line filter is less expensive than the guard column and easier to service, so I recommend using one in every system, even if you are using a guard column.

Conclusions

Changes in peak shape over time are common in the use of LC methods, but in a well-behaved method, such changes should occur gradually over hundreds or thousands of samples. Tailing of one or several peaks usually points to a problem with some chemical aspect of the system, so check the mobile phase and column for problems. When all the peaks tail or are similarly distorted, it is a sign that particulate matter is collecting at the top of the column. Better sample preparation and protection of the column will help to avoid this mode of failure. Peak fronting can result from chemical problems in the system, but is more commonly attributed to catastrophic column failure, usually resulting from using the column outside the recommended operating limits. Check the column instructions for the column limits, change to a more robust column, or expect to replace the column more often.

Although peak shape changes are a sign of problems and are difficult to avoid, if a method is developed and tested for robustness, such problems should not be a major concern for the method. A good system suitability test coupled with tracking peak shape over time should allow you to anticipate when peak shape changes will compromise the quality of the data. Take the appropriate corrective action before data are compromised, and your method should be satisfactory.

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Preventive Maintenance

How to avoid problems through a three-step preventive maintenance program

I remember reading a study on learning in the all-day short-course format. Because teaching liquid chromatography (LC) classes is a significant part of my work, my attention was captured. The writers claimed that in a 6–8 h class, only three points would be remembered. One of my LC troubleshooting classes has approximately 200 slides — what does this say about how effective a short course is at conveying critical knowledge? As the saying goes, I have tried to make lemonade out of these lemons, and use the "only three things" concept to help reinforce what I think are the key points. So, this article will use these points to form the core of a preventive maintenance program for your LC system.

Degassing

Mobile-phase degassing is the single most effective way to avoid problems with an LC system. Liquid chromatographs and air just weren't meant to be together! LC pumps are very effective at pumping liquids, but introduce an air bubble and in the best circumstances you will observe a momentary reduction in the flow rate and a drop in the system back pressure. If the bubble is large enough, the pump will not deliver any solvent, and if the pressure drops below a preset low-pressure limit, the pump will stop. Some pump designs will pass bubbles fairly well, whereas other designs will fail to operate when a bubble is present.

After a bubble passes through the pump, it usually will stay in solution due to the system pressure as it passes through the column. But on arrival at the detector, the system pressure returns to atmospheric pressure and the bubble might reappear in the detector flow cell, causing spikes in the chromatogram. This problem can be minimized by the use of a back-pressure restrictor on the detector outlet to provide sufficient pressure to keep bubbles in solution until they exit the detector. Of course, care needs to be taken not to exceed the pressure limits of the flow cell, or detector damage can occur.

Although noise spikes are the most common symptom of bubbles going through the flow cell, such as with UV detection, some detectors can be very sensitive to the presence of air. For example, dissolved oxygen has been reported to quench the fluorescence of some compounds when the fluorescence detector is used (1). In the reductive mode, the electrochemical detector is extremely sensitive to dissolved oxygen. Care must be taken to eliminate oxygen from the mobile phase and avoid oxygen-permeable tubing (such as PTFE) in the flow stream.

All of these problems related to dissolved air in the mobile phase can be avoided if proper care is taken to degas the mobile phase before it is used. For many years, the gold standard for degassing has been helium sparging. This simply involves bubbling helium through a frit placed in the mobile-phase reservoir. Helium sparging is the most effective way to remove dissolved air from the mobile phase, removing approximately 80% of the oxygen (2). With a well-distributed sparging stream, one volume of helium will remove almost all the gas that can be displaced from an equal volume of mobile phase (3). This means that 1 L of helium bubbled through 1 L of mobile phase will do the job.

Helium is the only effective way to remove sufficient oxygen from the mobile phase to avoid problems specific to dissolved oxygen, such as the fluorescence quenching or electrochemical detector problems mentioned earlier. However, if the main objective is to remove sufficient dissolved air so that bubble formation is not a problem, vacuum degassing is also effective as a degassing technique. Most of today's LC systems come with an in-line vacuum degasser either as a standard feature or an optional one. In-line degassing is simple to use, trouble-free, and effective. I give it credit for the huge reduction in bubble-related complaints that I have heard in the last few years.

Liquid chromatographs and air just weren't meant to be together!

Filter

Unless special precautions are taken, any particulate matter that enters the LC system will end up on the inlet frit of the column, eventually blocking the column, increasing the system back pressure, and distorting peaks in the chromatogram. As a consequence, any effort made to reduce the particulate load of the system will pay back in reliability. There are three major sources of particulate matter in the LC: the mobile phase, the sample, and the wear of internal components.

If the mobile phase comprises only high performance liquid chromatography (HPLC)-grade solvents, there is no need to filter the mobile phase. This is because organic solvents, such as acetonitrile or methanol, are filtered

through 0.2-µm porosity filters during the manufacturing process. Similarly, whether you buy HPLC-grade water or generate it in the laboratory with a purification system, the last step is filtration through a 0.2-µm filter. However, if there are any additives that were once solids, such as phosphate buffer, filtration of the mobile phase is a wise step to take. Although a buffer salt might be of high purity, it can contain particulate matter, such as bits of plastic generated when the lid of the bottle rubs on the edge of the bottle. In some cases, a solid additive might not dissolve completely, leaving bits of debris in the mobile phase. Any particulate matter from the mobile phase also can cause check valve leakage if it gets trapped in the check valves. Filtration of the mobile phase through a 0.5-µm porosity filter is an effective way to remove any particles from the mobile phase; 0.2-µm filters can be used, but they are not much more effective than 0.5-µm ones for this application and they filter much more slowly. Some laboratories write their mobile-phase preparation standard operating procedures (SOPs) so that mobile phases prepared only from HPLC-grade liquids do not need to be filtered, whereas all other mobile-phase compositions require filtration before use. It also is important to use a sinker frit at the inlet end of the tubing connecting the reservoir and pump. This frit typically has a porosity of >10 µm, so it is not a substitute for a mobile-phase filtration step, but it does keep dust out of the system and it holds the tubing in the bottom of the reservoir for operational reliability.

The sample is a second source of particulate matter in the LC system. Some laboratories filter all their samples through a 0.5-µm filter before loading them in the autosampler tray. This is an effective way to remove sample-related particles, but I have several concerns about this procedure that cause me to avoid sample filtration in most cases. First, it is expensive — $1 or more per filter — which can add a significant amount to sample processing costs. Also, for a validated method, you need to validate the filtration process and use filters for every sample, not just the ones you think need filtration. You never get 100% of the sample through the filter — there are always a few microliters left behind. Is there any adsorptive loss on the filter or contaminants leached from the filter? If there is loss, is it the same at all sample concentrations? If filtration is to be used, all of these issues must be addressed in the validation process, which can add work and expense to the validation procedure. I have found that an equally effective procedure for most samples is to centrifuge the sample in a benchtop centrifuge for a few minutes to settle out any particles, then transfer the supernatant to the autosampler tray.

The final major source of particulate matter in the LC system is wear of pump seals and injection valve rotors. Pump seals generally will last six months to a year in a normal laboratory. I recommend replacing these on a semiannual or annual basis as part of a preventive maintenance session. The cost is low compared with the expense of a column blocked by pump seal particles. Some pumps have frits or screens in the flow path to trap wear debris from the pump seals before it works its way further downstream. Consult your pump operation manual to find the recommended cleaning or replacement intervals for such filters. Autosampler rotor seals also wear over time, but in my experience, it takes several years of intense use before the rotor seals wear out. If your system has a function that counts injection valve cycles, you might be able to set an alarm to notify you when a specific number of valve cycles have occurred. I have heard quotes that the injector should last for 20,000 cycles, but this is only 10,000 injections — not much of a lifetime for a laboratory involved in regular sample analysis. I think they last much longer — several years in my experience. Of course, pump seal and rotor seal wear will increase in the presence of more abrasive mobile phases. Thus, if you routinely run ion-exchange gradients, such as 0–1 M sodium chloride, I would expect the seals to wear more quickly than if you use reversed-phase conditions with 10 mM phosphate buffer.

No matter what source of particulate matter I am trying to eliminate, I always use an in-line 0.5-µm porosity filter between the autosampler and the column on every system, even if a guard column is in use. This in-line filter will become blocked instead of the 2-µm filter at the head of the column, and it can be replaced in a few minutes with an inexpensive replacement frit. Just check the system pressure at the beginning of each batch of samples. When the pressure rises to a trigger point, such as a 25% or 500-psi increase, just replace the frit and you should be back in service in a few minutes.

Flush

My third key practice for reliable LC system operation is to keep it clean. If you follow the flow path through the system, you will notice several areas that can benefit from regular flushing. First, the mobile-phase reservoirs should be washed or replaced with each new batch of mobile phase. A dirty reservoir can contaminate an otherwise pure mobile phase. I don't like to use buffers longer than about a week and organic solvents for more than a month. Rather than refilling a reservoir, get in the habit of filling the reservoir, using the solvent, then replacing the reservoir when a new batch of solvent is made rather than topping it off.

Next in line is the pump. I don't like to shut off a pump for more than a few minutes if it contains a nonvolatile buffer, such as phosphate. When mobile phase evaporates, such as on the back side of the piston seal, nonvolatile materials will leave a solid deposit.

This is one of the major causes of pump seal wear. If buffered mobile phases are left in the system for extended periods, especially if acetonitrile is used, they can form precipitates, which can cause seal wear and check-valve leakage. So flush the pump with nonbuffered mobile phase before shutting it off for any extended period.

The autosampler should be cleaned on a regular basis, too. I've never seen an autosampler that was not subject to leaks and drips. These can leave deposits of buffer or sample, contaminating the system. The wash solvent in the autosampler should be treated in the same manner as the mobile phase in terms of expiration dates and regular washing or replacement of the wash reservoir.

Contaminants build up on the column over time, often being eluted as additional background noise in future chromatograms. This problem can be minimized by flushing the column with the strong solvent of the mobile phase (for example, methanol or acetonitrile) at the end of each batch of samples or whenever the column is removed from the system.

In my experience, it is easier to damage the detector than to improve it with routine cleaning efforts. For this reason, I rely on the column and system flushing procedures to remove contaminants from the detector flow cell. Only if there is some compelling reason do I take specific action to clean the flow cell for detectors that operate in the liquid state, such as UV or fluorescence detectors. Evaporative detectors, such as evaporative light scattering detectors or mass spectrometers are a different story. These detectors eventually build up a film of nonvolatile contaminants and require cleaning on a regular basis.

Summary

So there it is — degas, filter, and flush. Now you've received all the knowledge that you would have acquired in a one-day short course on preventive maintenance. Of course, it isn't quite that simple, but these three practices will go a long way toward more reliable LC system operation. Good luck!

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Best Practices

Here are 13 tips to ensure reliable and reproducible methods.

There are few things more frustrating in the laboratory than having a liquid chromatography (LC) method fail after working reliably for many hundreds or thousands of injections. For some workers, gradient elution LC methods are much more prone to problems than isocratic ones. There are some tricks of the trade that experienced chromatographers practice to help ensure reliable method operation for routine gradient assays. The LC system must perform in a reliable and reproducible manner, but it does not happen by magic. Some simple practices will help to make problems with gradient methods rare rather than frequent. This article presents a baker's dozen (13) of these tips. I hope that you find them useful in lowering your frustration quotient.

Use High-Quality Reagents

Reversed-phase gradient runs tend to concentrate nonpolar materials at the head of the column and release them as the gradient progresses. Under starting gradient conditions, the mobile phase is weak (very polar, with high water content). This means that nonpolar materials will be strongly retained. Nearly any organic impurity in the mobile phase will be held at the top of the column under these conditions. During equilibration between runs, perhaps 10 or more column volumes of weak mobile phase pass through the column. While the column is equilibrating, most of the nonpolar materials in the mobile phase will concentrate at the head of the column, only to be eluted when the gradient is run. These show up as ghost peaks in the chromatogram and can make quantification difficult and confuse interpretation of the chromatogram. For this reason, it is essential to use high performance LC (HPLC)-grade reagents for gradient work. Lower quality reagents might be suitable for isocratic applications, but with gradients, even the most minor impurities can cause problems. Discard aqueous reagents and buffers frequently (for example, weekly) to avoid contamination by microbial growth. Water impurities can be problematic, so buy HPLC-grade water or use a water purification system designed for production of HPLC-grade water.

Keep Your System Clean

Just as reagent quality is important for a minimum of interfering peaks, a clean instrument also will help avoid unwanted peaks. Thoroughly flush the system with strong solvent at the end of the day or before shutting off the system. Because switching to pure organic solvent, especially acetonitrile, can cause buffers or salts to precipitate, I recommend switching to nonbuffered mobile phase first. Just replace the buffer bottle with water and run the gradient again. Run the gradient to 100% strong solvent (usually methanol or acetonitrile) and hold it at 100% for 10–15 min to thoroughly flush the column and equipment. Do not shut off a system that contains buffers or salts — either flush the system with nonbuffered mobile phase or reduce the flow rate to 0.1 mL/min. This will help avoid formation of buffer salt crystals and microbial growth in the equipment. Check valves will work more reliably and pump seals and columns will last longer with routine flushing. If you are using high-salt mobile phases (for example, >50 mM), it is a good idea to use the seal-wash feature of your system to flush water behind the pump seals to remove any buffers or salts that leak past the seal. Clean up spills, leaks, and other potential sources of contamination. Be sure to wash or replace solvent reservoirs on a regular basis (for example, weekly).

Clean Up Your Sample

When asked how to make columns last forever, my usual answer is to never make an injection. That makes it hard to get our work done, so the next best thing is to inject clean samples. As a rule, the cleaner the samples are, the longer the column will last and the more reliable the method will be. There must be a balance, however, because it is possible to spend more time and money cleaning up the sample than you save in reliability and extended column life. You will have to figure out the economics of that yourself. However, for all methods, it is a good idea to avoid injecting any sample that contains particulate matter. Some workers like to filter samples with a syringe filter. I work primarily in the pharmaceutical industry and tend to question the value of filtering every sample. The syringe filters are expensive — a dollar or more each. Plus, you must validate the filtration process. Are any sample components selectively removed by the filter? Are any contaminants added to the sample by the filter? How much sample volume is lost during filtration? These and other questions make me want to avoid filtration. Instead, I like to centrifuge the samples in a benchtop centrifuge for a few minutes (for example, 10,000–15,000 rpm for 5 min). This settles out particulate matter that can clog column frits. Transfer the sample to a clean vial before injection. I am also a strong advocate of mounting a 0.5-µm in-line frit just downstream from the autosampler. This will trap any junk that would normally block the 2-µm frit at the top of the column and can be changed much more easily than the column frit.

Degas Your Mobile Phases

Although some gradient LC systems will operate without degassing the mobile phase, every system will operate more reliably with degassed solvents. Trapped air bubbles and solvent outgassing are two of the most common problems with gradients, and these can be largely avoided by

solvent degassing. Bubbles in the pump (or pumps) cause pressure and flow fluctuations, which can distort the gradient shape and affect retention times. Helium sparging has been the gold standard for degassing for many years, but many of today's LC systems come with in-line vacuum degassers that are much easier to use and provide effective gas removal. It is a good idea to purge the pump (or pumps) and solvent inlet lines daily by opening the purge valve (or valves) and operating at an elevated flow rate (for example, 5 mL/min) for a few minutes to remove any air bubbles that might have accumulated while the system was shut off.

Run a Blank Gradient

I recommend running a blank gradient as part of method setup to ensure that unexpected drift, noise, or peaks are not present. Simply set the autosampler to bypass the injection and run a normal gradient. (The systems in my laboratory can be programmed to make a zero-volume injection to accomplish this.) Look for peaks in the blank chromatogram. These come from reagent or water impurities. For methods run at maximum system sensitivity, it might not be possible to obtain completely smooth baselines, but the number of spurious peaks should be kept to a minimum and none should be eluted at the same retention time as sample components of interest. Although blank gradients might not be required on a daily basis for a routine method, the blank run is inexpensive insurance to help protect against loss of valuable sample data.

Have Dedicated Columns for Each Method

Each analytical method should have a column dedicated to that method. I strongly advise against sharing columns between methods, because unimportant peaks in one method can cause interferences in a second method. Dedicated columns last longer, so fewer columns will need to be purchased in the long run if each method has its own column. While we are talking about columns, remember that a column is a consumable item — it should not be expected to last forever. In my experience, with a well designed method, a column should last for at least 500 injections, even under severe mobile phase conditions. This amounts to <5% of the cost of analysis and usually is much less than the cost of other consumable items, such as solid-phase extraction cartridges. With adequate sample pretreatment and nonaggressive mobile phases, it is not unusual for a column to last for 2000 samples or more.

Equilibrate the Column Before Each Run

Before each run, the column should be equilibrated to the same extent as the other injections in the run sequence. A good rule of thumb is to allow 10 column volumes of the initial mobile phase to pass through the column for equilibration between runs. For the most common column, 150 mm × 4.6 mm, the column volume is approximately 1.5 mL, so 15–20 mL should be sufficient. Remember that during equilibration and column washing, volume is more important than time, so the equilibration time might be shortened by increasing the flow rate during this phase of the method. Recent information (for example, reference 1) shows that complete equilibration might or might not be necessary, as long as the same amount of equilibration is used between every run. Operating under partial equilibration conditions can save time by shortening the wait between injections.

Examine Your LC System Daily

It is a good idea to carefully examine the LC system each day before starting routine injection of samples. A good time to do this is during the initial equilibration of the column with a new batch of mobile phase. Just start at the mobile phase reservoirs and trace the solvent feed tubing through the mixer, connecting tubing, pump, and so forth on to the waste bottle after the detector. Are there any leaks? Is there white fuzz on any of the fittings? Do the reservoirs contain sufficient mobile phase? Is the autosampler wash solvent level sufficient? Does the waste solvent bottle have room for the anticipated waste stream? Is there anything else about the system that does not look, smell, or sound right? A few minutes each day spent examining the system pays great dividends when minor problems can be identified and corrected before they grow to the extent that they will compromise analytical results.

Make Priming Injections

Some methods will give better results if several "priming" injections are made before the first sample is injected. These injections of standards or mock samples can help to load slowly equilibrating active sites on the column so that more reproducible separations can be obtained. If you notice that it takes several runs before the retention time or peak area stabilizes, your method might be a candidate for priming injections. You can make a special priming solution that contains the analytes at high concentration or make several large volume injections at a lower concentration to load enough sample on the system to deactivate the column. Sometimes the system suitability injections serve as priming injections. Most methods do not require priming injections, but if your method does, this procedure reduces the time it takes to get reliable data from the system.

Ignore the First Injection

Because some methods require the priming process (see above) and the first injection can be equilibrated differently than subsequent injections, I advise setting up a routine method so that the first injection is not used for quantitative purposes. The second and subsequent runs will be more reliable than the first injection. By making it a formal practice to ignore the first injection, you will not have the hassle of having to perform outlier tests or other procedures to justify throwing out the first injection when it does not meet method criteria.

Conduct System Suitability Tests

Many methods that run under the oversight of regulatory agencies (FDA, EPA, OECD, and others) will require a system suitability test before sample analysis. System suitability serves as a mini-validation to show that the equipment and analytical method are operating in a fashion that will produce reliable results. Requirements for system suitability

tests vary, so the regulatory guidelines should be consulted to help select appropriate tests. Many workers use retention time, retention and area reproducibility, peak response, peak width, peak tailing, resolution, and column back pressure either alone or in combination as part of the system suitability test. The system suitability sample might be a diluted pure standard, a mock sample in extracted matrix, or some other sample selected to demonstrate system performance. Whether or not a system suitability test is required, I strongly suggest running such tests before routine analysis, even if it is just an injection of a standard to see if the retention and peak size are as expected.

Use Standards and Calibrators

For quantitative analysis, the response of unknown samples is compared with the response for standards of known concentration. The range of standard concentrations, number of replicates, and sequence of injection will depend upon the specific application. External or internal standardization might be appropriate. In any event, running at least one standard before running unknown samples will provide assurance (system suitability) that the analytical method is working properly before potentially valuable samples are injected.

Run Quality Control Samples

Analytical methods operated under regulatory oversight can require inclusion of quality control (QC) samples. QC samples are mock samples made in the sample matrix at known concentrations. Their concentration is calculated against the standard calibrators as a check to show that the method is operating in a manner that will produce reliable results. Generally, it is wise to intersperse QC samples among unknown samples to demonstrate that reliable results can be obtained throughout an analytical run. The results of QC samples can be tracked over time with a control chart. Sometimes the control chart can be helpful in anticipating failure of the system or identifying subtle problems.

Conclusions

You might realize that these tips for reliable gradient method operation already are part of your routine, either in whole or in part. Or you may have other techniques you use to ensure that the system is ready for samples. Whether required by regulations or not, you will find that your gradient methods will be much more trouble-free if you take a few minutes before injecting the first sample to make sure that the system is ready to go.

References

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