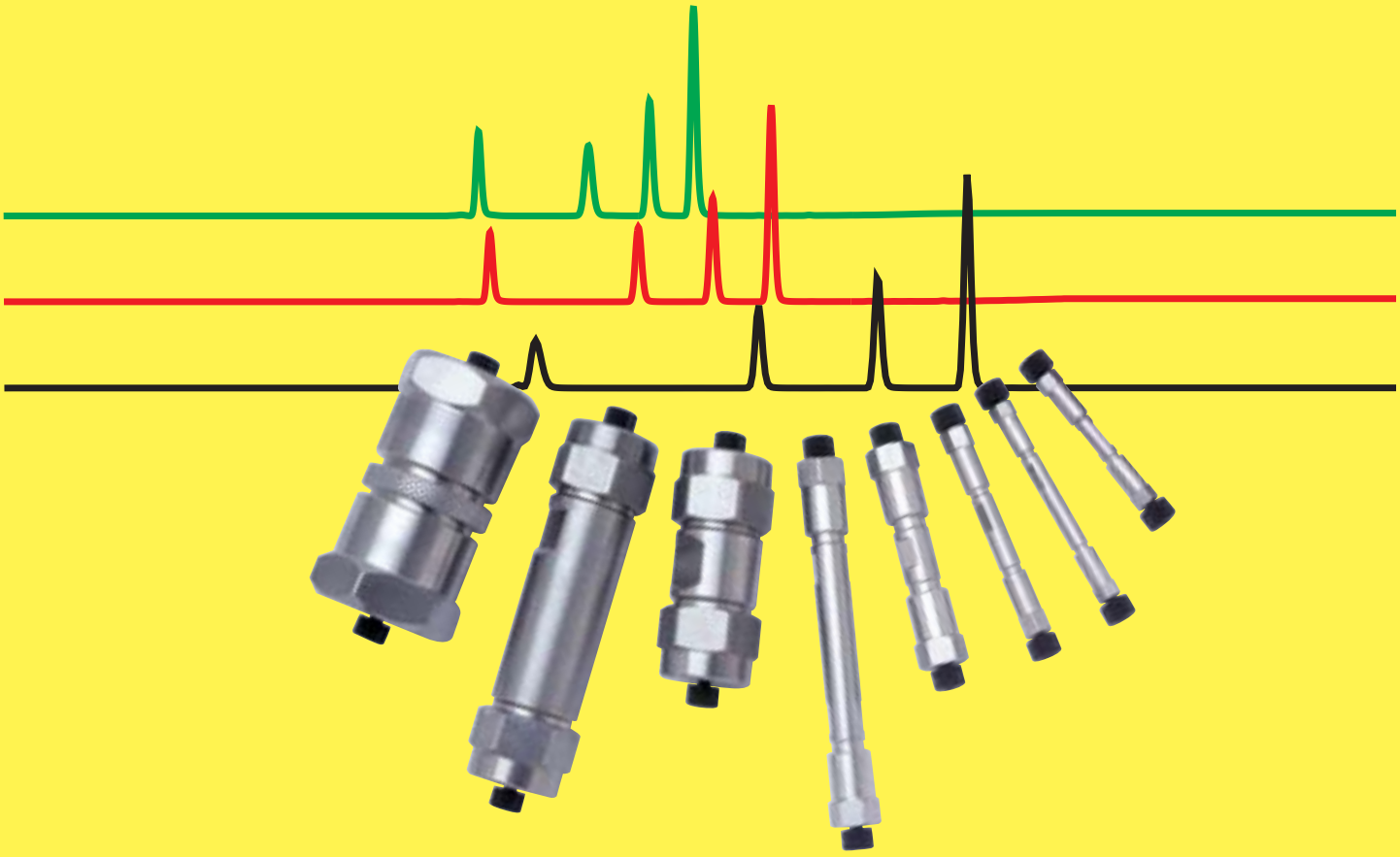
HPLC Troubleshooting Guide



Waters

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HPLC Troubleshooting

Uwe D. Neue, Waters Corporation

1. Column Life-Time

Q.: My column lasted only for about 100 injections. After that time, the peaks became distorted and the plate-counts were very low. What's wrong?

A.: 100 injections is indeed a short life-time. Under normal circumstances, one can expect a column to be in service much longer. In order to determine what is wrong, we need to establish first, if short column life is the rule for your application or not.

There are two fundamental cases:

1. previously columns used for the same assay lasted much longer.
2. all columns used for this application die after about the same amount of use.

In the first case, one would explore if the assay has remained truly constant. Has the sample composition changed? Strongly adsorbed contaminants in your sample can destroy column performance. Are the seals in the fluid path of your instrument in a good condition? Shedding seals can clog column filters and the top layers of the packing and thus effect the distribution of the sample.

If one can be reasonably assured that there are no changes in the chromatographic conditions, one can safely assume that the cause of the problem is a mechanical weakness of the packed bed. This can be induced by rough handling of the column in your lab (did you drop it?) or during shipment, or it could be a manufacturing defect. Such a defect can not be detected by standard column QC and could show up only after some use of the column. In this case, column manufacturers will replace your column free of charge.

Q.: That is nice of these manufacturers, but this is not my problem. My columns always last only a short time. Sometimes it's 100 injections, sometimes 200. I could live with 200 injections, but only 100 is not good enough. This really is getting expensive. What can I do?

A.: I agree with you 100%. What we need to do together is to find the cause of your problem and then see, what we can do about it.

The most likely cause of your problem is adsorption of sample constituents on the top of the column. They may either precipitate because of a low solubility in the mobile phase or they may be strongly adsorbed. As you inject more and more samples, these contaminants build up on the top of the column and prevent the sample to properly adsorb and distribute. This results in a distortion of the peak profile. Often this problem is accompanied by an increase in back pressure.

Q.: OK, that could be it. How do I get around this problem?

A.: There are several ways to prevent this from happening. One is to clean up the sample with a suitable sample preparation technique. Solid phase extraction using a SPE cartridge with a similar chemistry as the separation column works well for this problem.

Another and more powerful approach is to use a guard column. The precolumn serves as a sacrificial column top that is replaced when the problem occurs. For best performance, you should use a guard column that contains exactly the same packing as the analytical column and is packed with the same high performance packing technique as the analytical column. If you use precolumns made with a different brand of packing, you will not get the optimal performance both in separation capability and in protection of your analytical column. Also, do not use a larger particle size. Larger particles or badly packed precolumns can result in a deterioration of the separation due to band- broadening in the precolumn.

Q.: To use a guard column sounds ok. Do you have any other solutions?

A.: Well, not really. There are a few other possibilities, but they all have their drawbacks.

I am not an advocate of column "washing" with solvents that are supposed to dissolve the contaminants on the top of the column. In many cases, this process simply does not work. For example, if the contaminants are proteins that have precipitated on the column top, by the time you try to wash them off they have aged a lot by denaturation and maybe even cross-linking that it may be impossible to solubilize them again. Furthermore, every washing will also remove hydrolyzed bonded phase, which otherwise remains in a local equilibrium at the site where the hydrolysis occurred. Consequently, a repetitive washing can actually result in an accelerated aging of the column. Also, after this washing you have to re-equilibrate your column with the mobile phase, which in some cases like in ion-pair chromatography may be quite time consuming.

Another approach that is often tried is column backflushing. If you do it with a different solvent than your mobile phase, the same objections hold as for column washing. If you do it just with mobile phase, it will take a lot of time until the contaminants are removed or it may not work at all. Also, any backflushing weakens the column. Although today's columns are packed well enough that they can withstand backflushing, I would still recommend to not make this a standard practice.

Q.: So your best recommendation is to use a precolumn?

A.: Absolutely. They don't cost that much - depending on the brand and type between $ 10 and $ 50 - and they protect a column that usually costs about ten times as much. Furthermore, they protect your column also from other sources of contaminants that may be more difficult to trace. The source of your column problem could for example be dust in the mobile phase or debris from shedding pump seals. Or it could be the adsorption of contaminants from the mobile phase. Some of these may be very difficult to

troubleshoot, but the guard column will simply take care of them.

Q.: Are there any other causes of short column life?

A.: Yes, but they are less likely if you follow the manufacturer's recommendations.
One possibility is that the column is collapsing due to a mobile phase pH outside the recommended range. This can also be caused by a sample dissolved in a strongly acidic or alkaline solution.

Furthermore, there are a few items that are specific to certain columns.

Amino columns for example react with aldehydes and ketones. Amino columns in an unbuffered aqueous solution generate a strongly alkaline pH that leads to a partial dissolution of the silica.

Columns can also collapse when exposed to the wrong solvents. The reason for this is that columns are fundamentally very loose structures. They are partially held together by the adhesion of the particles to each other. When you put them into mobile phases that break this adhesion, there is an increased chance of a collapse of the bed. This happens occasionally to CN columns in solvents of intermediate polarity or to Phenyl columns in THF.
One more reason to stay away from column "washing".

2. Variable Retention Times

Q.: The retention times of my peaks are inconsistent. What is the problem?

A.: First, we need to find what the pattern of this variation is. This will tell us a lot about the potential source of the problem. If the retention times change randomly from one run to the next, then I would first check out the pump(s) and the solvent mixing devices. To verify that the pumps are working, measure the flow-rate with a graduated cylinder and a stopwatch. To verify that the mobile phase composition is not changing, you can add a tracer to one of the solvents and observe the baseline. If the mobile phase composition is constant, the baseline will be steady. If there is a shift in the composition, you will observe corresponding shifts in the baseline. For example, if you use a reversed phase method with UV-detection, you can add 0.1% acetone to the organic solvent and monitor the baseline at 254 nm. Alternatively, you can prepare the mobile phase manually and bypass the solvent mixing devices. If the retention time fluctuations go away, the source of your problem was the mixing device.

Q.: The retention times are pretty consistent from run to run, but they vary from day to day.

A.: In this case, the instrument itself is less likely to be the culprit. The most likely source of variation is the composition of the mobile phase. In reversed phase chromatography, there is an exponential relationship between the retention factor *k* and the volume fraction of the organic solvent in the mobile phase. As a rule of thumb, if you make an error of 1% in the amount of organic solvent, the retention time can change by between 5% and 15%, typically by about 10%. This means that you have to measure the amount of solvent very carefully. The best approach is to prepare the mobile phase gravimetrically rather than volumetrically.

Also, how you degas your mobile phase may contribute to variability. The best degassing method is the simultaneous application of vacuum and ultrasound for about one minute. This results in good degassing with a minimal amount of evaporation of the solvent. An alternative good method is the helium sparging method. After the initial equilibration of the mobile phase with helium, the flow of helium should be turned down. Otherwise the helium will carry solvent vapors with it and the solvent composition can change due to evaporation.

If your sample constituents are ionic or ionizable, then the control of the pH of the mobile phase is very important. A change of as little as 0.1 pH units can result in a retention time shift of 10%. So it is very important to measure the pH accurately and to keep the pH meter well calibrated. In reversed phase, the retention of acids decreases and the retention of bases increases with increasing pH.

By the way, my chemistry teacher always said: "A buffer is called a buffer because it's supposed to buffer the pH. If it doesn't do that, it isn't a buffer." For example a solution of ammonium acetate is just that, a solution of ammonium acetate. An acetate buffer contains the acetate ion and acetic acid. If they are present in equal amounts, the resulting pH is the pK of the buffer, 4.75 in the case of acetate. A buffer always has its highest buffering capacity at its pK.

Q.: I did not realize how closely one needs to control the mobile phase composition to get reproducible results. Are there any other variables that one must pay attention to?

A.: Another important factor is temperature. You would suspect that temperature is the cause of the fluctuations, if the retention of all peaks is moving in the same direction. The rule of thumb is that retention times change by about 1% to 2% per 1° Celsius. That isn't that much under normal circumstances, but in many places the heat or air conditioning are shut down over the weekend. And then you wonder why the analyses run automatically over the weekend show different retention times than the ones run during the week. This can obviously be avoided when a column thermostat is used.

In reversed phase chromatography using ionic or ionizable sample compounds, the retention is also influenced by the ionic strength of the buffer, but the influence is so small that it is usually negligible. Typical changes are under 1% for a 20% change in molarity of the buffer. Since the buffer constituents are always weighed, there is no way that such a large error could happen.

Q.: Are there any special cases where additional parameters need to be considered?

A.: Yes. In ion-pair chromatography, the concentration of the ion-pairing agent influences the retention of ionic sample constituents. The retention of analytes that are oppositely charged to the ion-pairing agents increases, the retention of analytes of equal charge decreases. Neutral compounds are practically unaffected. At low concentration, i.e. below 5 mM/L the retention of the sample constituents that interact with the agent changes in proportion to the concentration of the ion-pair reagent. At high concentration of the ion-pairing reagent, i.e. around 10 mM/L, the surface of the adsorbent is saturated with the reagent, and a change in concentration of the reagent does not result in an appreciable change in the retention time of the analytes. This is something to think about during methods development. If you can make the method insensitive to one of the variables, you should do so.

In normal phase chromatography the retention times are very sensitive to the concentration of polar constituents in the mobile phase. One of these constituents that is always there whether one wants it or not is water. So variable amounts of water can lead to variable retention. One trick that has been used to get around this problem is to use non-polar solvents like hexane or methylene chloride that are half saturated with water. To accomplish this you take a volume of the solvent and saturate it with water by adding some water to it and stir it for a while, then you mix this water-saturated solvent and mix it with an equal volume of "dry" solvent. This procedure results in reasonably good reproducibility of the water-content of the non-polar solvents. It also helps to prevent drifting retention times, but this is the subject of a future section.

3. Drifting Retention Times

Q.: The retention times of the peaks in my chromatogram are drifting. What is the problem?

A.: This is an interesting problem. There could be many different causes for this. Let us go through them one by one.
People most commonly assume that drifting retention times are an equilibration problem. If you are doing normal phase chromatography on unmodified silica columns this is also the most likely problem. The retention times in normal phase chromatography are very susceptible to the amount of water adsorbed on the silica surface, which in turn is a function of the water dissolved in the mobile phase. Since the solubility of water in solvents like hexane or methylene chloride is extremely low, it takes a long time for the columns to equilibrate. I have seen cases were the retention times in very dry hexane were still shifting after a week of equilibration. I therefore recommend to avoid very dry solvents. A common solution to the equilibration problem of silica with water is to use solvents that are "half-saturated" with water. They are prepared by saturating a given volume of hydrophobic solvent with water and then mixing it 1:1 with "dry" solvent. This approach speeds equilibration times up tremendously.

In reversed-phase chromatography, equilibration is usually very fast. A few (5 to 10) column volumes of mobile phase are usually sufficient for equilibration. This is however not always the case. A notable exception is the equilibration of a column with ion-pairing reagent in ion-pair chromatography. The ion-pairing reagents are typically used at a concentration of about 2 to 5 mmol/L or less. They adsorb onto the surface of the reversed-phase packing at a surface concentration of between 0.5 to 2 $\mu mol/m^2$. A 4.6mm x 250mm column contains about 3 g of packing with a surface area of 330 m^2/g. That means that there are 1000 m^2 of surface in the column. At a surface concentration of 2 $\mu mol/m^2$ 2 mmol of reagent need to be pumped into the column for complete equilibration. At a concentration of 2 mmol/L this takes a liter of mobile phase. This is clearly an extreme case, but don't be surprised if it takes a few hundred milliliters of mobile phase to equilibrate the column. It is therefore unwise to convert columns used in ion-pair chromatography to an organic solvent for overnight storage, because during this process the ion-pairing reagent is washed off and the next day you have to go through a lengthy reequilibration procedure.

Q.: Both of these phenomena have a common factor: low mobile phase concentrations of an agent that is strongly adsorbed. Is this the most common cause of drifting retention times?

A.: I would say yes. Other phenomena are not as common. But the strongly adsorbed agent could also come from the sample. In this case it would slowly build up during repeated injections and thus change the chemical properties of the column. An example of this would be the adsorption of excipients from a drug sample.

One can tell whether a contamination is coming from the mobile phase or from the sample by looking at the rate at which retention times are changing.
An experiment could be as follows:

1. Inject the sample several times, e.g., four times for a total run-time of 1 hr.
2. Pump mobile phase for the same amount of time without injecting sample.
3. Repeat the first step.
4. Plot the retention times
   a, versus time
   b, versus number of injections.

If the first plot gives you a smooth curve, then the mobile phase is the carrier of the contamination. If the second plot yields a smooth curve, the sample is the source of the contamination.

Q.: Are there other causes of shifting retention times?

A.: Yes. It is possible that the mobile phase composition is changing with time. If you are not using the on-line mixing

capabilities of today's instruments, you may be looking at a slow evaporation of a component in the mobile phase. This is especially true when you are sparging the mobile phase with helium to avoid air-bubbles in the pump. One should keep the rate of sparging to a minimum.

An often underestimated cause of shifts in retention is the hydrolysis of the bonded phase. The manufacturers usually specify a pH-range, outside which the bonded phase is "unstable". This range is typically from pH 2 to 8 or 9. However, one has to treat these limits with a lot of caution. There is not a sharp boundary, hydrolysis depends also on other factors like temperature and organic solvent, and slow hydrolysis occurs inside these limits as well.

The hydrolytic stability of a bonded phase is best at intermediate pH-values, around pH 3 to 5 and at low temperature. Isocratic chromatographic conditions are better than gradients. While hydrolysis does occur in isocratic conditions as well, the bonded phase often adsorbs to itself and is in a local equilibrium. However, when a higher concentration of organic solvent is used - as in a gradient or during column cleaning procedures - this local equilibrium is interrupted and the hydrolyzed bonded phase is washed out of the column.

Q.: I will keep this in mind. Can temperature changes cause drifts of retention times?

A.: Yes, temperature is always suspect. If you run your samples in unattended operation overnight or over the weekend, you may get drifting retention times according to the shifts in lab temperature. In many places, the ambient temperature is maintained at a different setting during the night or on the weekend to conserve energy. As a rule of thumb, the retention times shift by about 1% to 2% per 1 ºC.
Related to the last phenomenon are shifts in retention times that are caused by an increase of back-pressure in the column. Increasing back-pressure may indicate a contamination of the column, but even a clogged frit can affect retention times. The pressure needed to push the mobile phase through the frit warms up the mobile phase by friction, and this increase in temperature can affect the retention times.

There are other causes of drifting retention times, but they are exceedingly rare.

A slow equilibration phenomenon encountered in reversed-phase chromatography is the equilibration with mobile phases that contain less than a few % of organic solvent. "Fully end-capped" packings with a high coverage of C18 are not wetted well by these mobile phases. This leads to several phenomena from loss of contact of the mobile phase with the stationary phase to "hydrophobic collapse", which is the reduction of the surface area available for interaction with the sample due to self-adsorption of the bonded phase onto itself. The columns can be regenerated rapidly using a few column volumes of organic solvent, preferentially the organic modifier in your mobile phase. This phenomenon is less pronounced or even not encountered at all with bonded phases that are not end-capped.

4. Column-to-Column and Batch-to-Batch Reproducibility

Q.: I am about to start the development of a new HPLC method. After validation, the method will be transferred to the QC-lab, where it will be used for many years. I am concerned about the long-term reproducibility of the method, especially about the long-term reproducibility of the column. What can I do to assure good reproducibility?

A.: First of all, let me compliment you for thinking about this aspect of your method before you start working on it. If it can be anticipated that a new method will be used for several years, this knowledge should be part of the column selection process. You may want to assure yourself that the column will be available for the anticipated life-time of the method. This means that you want to select a manufacturer that has a large operation and is likely to stay in business for the foreseeable future. In addition, you want to select a "standard" surface chemistry - like C18 or C8 - over novel and/or less used surface chemistries. In other words, your column selection should be conservative.

The next criterion should then be the reproducibility of the column. If you or your colleagues have had good experience with the reproducibility of a particular column in the past that is a good starting point. You should also consider the information that is available from the manufacturer. Recently some column manufacturers have started to publish their specifications and the results of their batch-to-batch reproducibility studies. You can obtain this information from the manufacturers and review it critically.

Q.: What am I looking for in this information?

A.: Let me back up a little and explain the various aspects of column-to-column and batch-to-batch reproducibility. In the following, I will talk about reversed-phase packings, since they are the most commonly used.

If you are looking at different columns that are made from the same batch of packing, you may get differences in column plate-count (peak-width), back-pressure and retention times. The retention times change in proportion to the packing density and the volume of the column, the latter depending mostly on the reproducibility of the internal diameter of the column. This variability should not be detrimental to your method, since the retention times of all peaks change proportionally to each other and resolution is preserved. However, you need to be aware of this so that you specify the elution time windows for your method correctly. Variation of retention time due to this effect is typically 3% RSD, but can be as little as 1% RSD, if the column manufacturer has good control over the column hardware.

Variation in column plate-count can affect your method, especially when you are dealing with peak-pairs that are barely baseline resolved. A typical reproducibility of plate-count is +/- 10%, although standard deviations of as little as 2 to 3% have been achieved. Remember that plate-count is a squared number; resolution depends only on the square root of plate-count. Therefore a variation of 2% in plate count means that the peak-width varies by only 1%. You should check if the column manufacturer has an upper and lower limit for plate-count or only a minimum specification. Two-sided specifications are more desirable.

Column back-pressure is very reproducible for any given packing procedure. The variation within a given batch of packing should be under +/- 5%.

Q.: How about batch-to-batch reproducibility?

A.: If you are looking at the reproducibility of chromatographic parameters over different batches of packing, the same parameters as above can vary. But more importantly, the selectivity of the separation can change with the batch of packing. This means that the relative retention of peaks can vary, i.e. their relative position in the chromatogram. This can be detrimental to the specificity of your method.

This variation is the most important issue that you want to guard yourself against. Therefore what you want to obtain from the column manufacturer is information about how batch-to-batch reproducibility is ensured. This usually entails some measurements of the physical, chemical and chromatographic properties of a packing. Among the physical measurements, the most important one is probably the specific surface area of the packing, since a variation of this parameter directly translates into variation of retention times. Therefore you would like to understand what variability of the specific surface area the manufacturer finds acceptable. One may commonly find a range of +/- 10%, but +/- 5% is achievable.

As the most important chemical parameter, you would like to understand the variability of the surface coverage for the bonded phase. This is customarily expressed as $\mu mol/m^2$ of bonded phase. Many manufacturers don't give that parameter directly, but have specifications on the carbon content of the packing. What one would like to see are once again two-sided specifications and a tight range. Better than +/- 10% may be quite common, while less than +/- 4% is achievable.
Finally, you would like to understand the chromatographic reproducibility test that the manufacturer of the packing uses. Frequently, columns are accompanied with a chromatogram of a mixture of simple neutral compounds, like toluene, naphthalene and anthracene. This is not a good batch-to-batch reproducibility test. The relative retention between neutral hydrophobic compounds is extremely reproducible and very insensitive to variations in the surface coverage of the packing. Better batch-to-batch reproducibility tests incorporate strongly basic compounds in the test mix. Well-selected basic compounds in a well-designed test interact predominantly with the "residual" silanols on a packing, while neutral compounds interact predominantly with the bonded phase. Thus the relative retention between well-selected basic compounds and neutral compounds represents a stringent test of batch-to-batch reproducibility of a reversed-phase packing. If a manufacturer uses such a test for batch-to-batch reproducibility testing and has reasonably tight specifications, your comfort level should increase.

Q.: I am not sure how the manufacturer's tests relate to my compounds in my assay. What can I do to assure good batch-to-batch reproducibility for my assay?

A.: You are absolutely correct: the information given above is a starting point that allows you to select a column that has a good chance of being reproducible. But the ultimate test is a test of the reproducibility for your assay itself. Some manufacturers provide kits for this purpose that comprise columns from different batches of packing. Other manufacturers supply columns prepared from different batches on demand. Make sure you understand what you are getting. Different column lots containing the same batch of packing are not useful, you need to get columns packed with packing from different bonding reactions. There can be some confusion if the manufacturer considers columns packed on different days with the same batch of packing to comprise different lots. So it is best to ask twice, if the manufacturer is really supplying you with what you want.

You probably want to obtain three different batches of packing for testing the reproducibility of your method. If your assay proves to be unaffected by the batch-to-batch variability, you can rest reasonably assured that your method will be reproducible for years to come.

5. Sample Preparation Problems

Q.: I am encountering some problems with my sample preparation. I am using solid-phase extraction with a reversed-phase sorbent. Usually the recovery is good, around 80% or better. But sometimes it drops down to 50% or even less. What could be the problem?

A.: The problem that you have is not unusual. I have found that most of the time low recovery can be attributed to poor methods development. Although it may be adequate for the analytical purpose of your method, 80% recovery is not a good sign from the standpoint of method ruggedness. It is highly likely that there is a single reason for the incomplete recovery that sometimes results in a loss of 20% and sometimes in a loss of 50%. What we need to do together is to find out, where the missing 20% to 50% ends up.

Q.: O.K., how do we do that?

A.: There are four possible scenarios:

1. The analyte is lost before the solid-phase extraction.
2. The analyte is not adsorbed completely during the adsorption step.
3. The analyte is partially washed out in a washing step.
4. Part of the analyte remains on the solid-phase extraction cartridge after the elution step.

To find out whether the analyte breaks through the first SPE cartridge during the adsorption step, you can simply put a second SPE cartridge behind the first one and then treat it the same way as the primary cartridge. If the fraction obtained from the second cartridge contains a fair amount of analyte, then the adsorption step is incomplete. There are several possible reasons that this may occur. First, the solvent in which the sample is dissolved may be too strong a solvent. If this is the case, you may want to dilute your sample with water, or - if your analyte is ionizable - with buffer. Second, you may verify that you are activating the SPE cartridge before you load on the sample. Reversed-phase SPE cartridges need to be activated with an organic solvent like methanol or acetonitrile, followed by an equilibration with water or buffer. Only then the sample should be applied. Many people try to avoid these tedious additional steps, but they are necessary for a correct performance of the method.

Q.: I do activate the cartridge according to the manufacturer's recommendation. So I don't think that this is the problem. I do like your proposal to use a second cartridge to verify the completeness of the adsorption step. What do I do to check the other steps of the method?

A.: To check the washing steps, you should collect all the fractions and analyze them by HPLC. This may not be easy if the washing steps are removing compounds that interfere with the quantitation of the analyte. Then the quantitation of the analyte in these fractions is by definition difficult. You may try the following technique to work around this problem. Evaporate the questionable fractions to dryness, and reconstitute them in the same solvent composition as the original sample. Then take this sample and process it on the solid-phase extraction cartridge in the same way as your original sample. If you now find another fraction of your analyte in the elution step, you know that a portion of the analyte is washed off in one of the washing steps.
Another way to do this is to use standards and run them through the sample preparation process. This is less rigorous, since the presence of the matrix may affect the behavior of the analytes.

Q.: These are some good suggestions. How do I analyze for analyte that remains on the cartridge after the elution step?

A.: You need to elute the cartridge again with more eluent after your original elution step. This may elute additional analyte. Often, you need to increase the elution strength of the eluent used in the elution step. Think about how a portion of the analyte may be retained stronger. Could it be due to interaction with residual silanols? Then you may want to change the buffer pH or buffer strength in your elution step. Is it due to hydrophobic interaction? Then increase the concentration of organic solvent in the elution step or go to a stronger organic solvent (e.g. replace methanol with acetonitrile). Is it possible that the sample interacts with residual silanols via hydrogen bonding? Then the addition of methanol to acetonitrile or tetrahydrofuran might help.

Q.: O.K. If I don't find the missing fraction in all of these steps, can I exclude the solid phase extraction step as the source of the problem?

A.: Yes. Now we need to explore, if the analyte gets lost in another step in the sample preparation. Could it be that it is strongly adsorbed to a sample vessel. A strongly hydrophobic analyte may adsorb to the walls of a polyethylene vial. Strongly basic compounds may bind to the silanols on the surface of glass vials. It is also possible that the analyte could adsorb to solids in the matrix or bind to other constituents in the matrix (e.g. proteins). These problems may be more difficult to isolate.

Fundamentally, try to get the recovery of the analyte as close to 100% as possible. This is a good assurance that your method is rugged from the start. Even then, circumstances may arise that results in irreproducible recovery, but they are less likely than if you start with a method that is already compromised.

If you can achieve good recovery from the start, then neither a normal variability of the eluent composition or of the packing material itself is likely to affect your method.
The only remaining cause of variable recovery could be the quality of the packed bed of the SPE cartridge. If a void is formed in the bed, then the flow is non-uniform, and an early breakthrough of the analyte is possible. You can guard yourself against this problem by a cursory inspection of the device. A void that would affect the method is quite obvious.

6. Sources of Peak Tailing

Q.: What can I do to get rid of peak tailing?

A.: First, we will have to find out, where the peak tailing comes from. There are many sources of peak-tailing, ranging from column problems to chemistry problems to instrument problems. The most common reasons for peak tailing are extra-column band broadening, deterioration of the packed bed, and interaction of the analytes with active sites on the packing. Obviously, what needs to be done depends on the cause of tailing.

Q.: I accept that. Now, how do I determine the cause of tailing?

A.: A quick first step is a careful examination of the chromatogram. There is a lot of information in a chromatogram that can give you clues about the nature of the problem without any knowledge about the samples or the chromatographic conditions. You can then use the additional information to test the hypotheses that you have formed based on the examination of the chromatogram.

One of the first things to examine is the height of the peaks. If a UV detector has been used and the peak-heights are in the order of 1 AUFS, a good guess is that the column is overloaded and that peak-tailing is due to overload. This judgement supposes that the extinction coefficients for the compounds is in the order of 1000, which is a reasonable rule of thumb. To confirm mass overload, you would then ask the question of how much mass has been injected onto the column. For a normal, fully porous packing with a pore-size of about 100 Å, overload starts distorting peaks at a load of about 100 µg.

These are all rules of thumb, but they can be used as reasonable guidelines for sample overload. You can test for overload by injecting about 10 x less mass on the column and see, if the peak-shape improves.

Q.: Ok. But what if tailing occurs at much lower amounts injected?

A.: Once again, a peek at the peaks in the chromatogram is helpful. If there are many peaks in the chromatogram, determine whether the peak-shape remains roughly constant or if there is a consistent change of peak-shape throughout the chromatogram. If the peaks are tailing more in the early part of the chromatogram than in the later part, one would suspect that extra-column effects are responsible. The influence of extra-column effects decreases as the peaks become wider, which is why they distort early peaks more than later peaks. Two extra-column effects should be considered:

1. extra-column band broadening
2. detector time-constant.

Band spreading in connection tubing, injectors and detectors results in tailing in the early peaks in the chromatogram. You may encounter this, if you put a column with a small diameter on an instrument that was not configured for the use of small volume columns, or if you have recently re-plumbed your system. If the latter is the case, examine the type of tubing that you have used (it should be 9/1000" i.d. throughout from injector to detector). Also inspect all the connections, if they were made properly. A common cause of extra-column band spreading is the fact that different column manufacturers use different distances between the tip of the ferrule and the end of the tubing in their column end-fittings. An extra-column band spreading with a standard deviation of only 15 µL significantly influences peak shape up to an elution volume of about 3 mL on a 5 µm column.

A large detector time constant has the same influence. More tailing is observed on early peaks than on late eluting peaks. Often, the default setting of the time constant is 1 sec. If a high-performance 5 µm column is used, a distortion of the peaks can be observed up to 2 to 3 minutes into the chromatogram. Larger time constants obviously would give larger effects.

If the peaks are all tailing pretty much to the same degree for all sample compounds in the chromatogram, there are two possibilities:

1. the packed bed is damaged
2. all sample components are chemically similar, and we are dealing with chemical effects.

In the first case, a plate-count test under standard conditions, especially under conditions recommended by the manufacturer, will reveal whether the column has deteriorated or not. The column could have been damaged by adsorption of contaminants or particles. Sometimes, it is possible to remove these contaminants with an appropriate solvent (for example THF on a reversed-phase column), but if the bed itself has shifted, there is nothing that one can do to repair the column.

In the case that all peaks are chemically similar, chemical effects are potential candidates as causes of tailing. If only some peaks in the chromatogram are tailing and other peaks give a good peak shape, chemical effects are the prime candidates as the causes of peak tailing.

Q.: What are these "chemical effects"? Please explain!

A.: There could be several effects here as well, but the most common cause is the interaction of the analytes with an energetically non-uniform surface. A typical example is the tailing of strong bases on some reversed-phase packings. These kind of compounds interact strongly with residual silanol groups on the surface of the packing as well as with the bonded phase. If the silanol groups on the surface form a non-homogeneous population, tailing can result.

Q.: What can I do to eliminate tailing due to chemical influences?

A.: First, you should consider using a column that does not exhibit this phenomenon. Some of the newer reversed-phase packings have been designed to minimize the tailing of bases. Second, this tailing can be mediated by either the pH of the mobile phase or by using organic bases in the mobile phase that compete with the analytes for active sites. This is due to the fact that silanophilic interactions are often ion-exchange interactions. At acidic pH, fewer silanols are negatively charged. Therefore, less tailing is observed for positively charged analytes. As a competing base, triethylamine is often used. But among competing bases, those with a larger hydrophobicity often work better. Examples are octylamine or tetrabutylammonium salts.

In the case that the silanophilic interaction of the analyte are not ion exchange, but hydrogen bonding, you can improve peak-shape by changing the hydrogen bonding character of your solvent. For example, methanol often

results in an improved peak-shape compared to acetonitrile as organic modifier of the mobile phase.

Similar phenomena are encountered in normal phase chromatography, and similar reasoning can be applied there to suppress tailing. Significant changes in tailing can be observed depending on whether alcohols or acetonitrile are used as polar modifier of the mobile phase.

There are still some other causes of peak-tailing, but they are comparatively rare. Adsorption of sample constituents to column frits or injector parts has been observed. It is also possible that the analyte is subject to a chemical change during the chromatographic process. Examples of this can be a degradation or a slow equilibrium between different molecular forms of the analyte.

7. Normal-Phase Chromatography

Q.: I have always worked with reversed-phase chromatography and avoided normal-phase chromatography. I did this on the advice of my colleagues that normal-phase chromatography is much more difficult than reversed-phase chromatography. Is there any truth to this?

A.: Unfortunately, there is some truth to this assessment. However, equipped with the right knowledge, you can ameliorate some of the difficulties.

The most common problem with normal-phase chromatography is retention time variability. The reason for this variability is the strong dependence of the retention on the concentration of small amounts of very polar constituents in the mobile phase. This holds especially true for the water content of the mobile phase. But it is also true for the small amounts of polar modifiers like methanol or acetonitrile that are added to the mobile phase to control retention and selectivity.

Water is present in all organic solvents to some degree. Concentrations are typically in the ppm range. Table 1 gives the solubilities of water in some non-polar solvents (1,2) that are used in normal-phase chromatography. As a consequence, the water content of the mobile phase can vary widely, if one does not take special precautions to control it. It is not usually desirable to work with dry solvents, since it may take a long time (days have been reported) before a column is in equilibrium with a dry mobile phase. Mobile phases that are saturated with water are not desirable either, since under these circumstances the water accumulates in the pores of the packing and one obtains a partitioning column. One would like to use mobile phases with an intermediate, but controlled water content. A good approach to obtain a reproducible water content has been the use of mobile phases that are half-saturated with water. The preparation is relatively straightforward. One divides the mobile phase into two equal portions, then saturates one portion with water. This is accomplished by adding 1 or 2 mL of water to 500 mL of mobile phase and stirring for approximately half an hour. The excess water is removed, and the two portions are recombined. This results in a mobile phase that is "half-saturated" with water provided that the original mobile phase was reasonably dry.

Table 1

Solvent	*Solubility of Water in Solvent (Temperature)*	
	% w/w	*(°C)*
Hexane	0.0111	(20)
Heptane	0.0091	(25)
Isooctane	0.0055	(20)
Toluene	0.0334	(25)
Dichloromethane	0.1980	(25)
Chloroform	0.0720	(23)
Carbon tetrachloride	0.0100	(24)
o-Dichlorobenzene	0.3090	(25)
Ethyl acetate	2.9400	(25)
Butyl acetate	1.8600	(20)
Diethyl ether	1.4680	(25)
Methyl-t-butyl ether	1.5000	

Equilibration with a "half-saturated" mobile phase is much faster than with dry mobile phases, but it still may take hours. Therefore it is desirable to dedicate a column to a particular mobile phase. Now, equilibration may only take minutes instead of hours.

Obviously, the concentration of other polar modifiers to the mobile phase needs to be well controlled as well. Far example, if you use methanol as a modifier, often only very small amounts (<0.5%) may be needed, and a small change can result in large changes in retention. In such a case it is desirable to use a less polar modifier, such as isopropanol or another higher alcohol. Or you may want to stay away from alcohols altogether and use the less polar ethers or esters. This of course is likely to significantly influence the selectivity of the separation.

Q.: I see that you have to pay much more attention to the mobile phase composition in normal phase chromatography than in reversed-phase chromatography. How about the stationary phase? Are some stationary phases better than others?

A.: Indeed there are differences. Silica and alumina are very hygroscopic and are therefore very sensitive to the water content in the mobile phase. Polar bonded phases such as cyano-, diol- or amino-packings, are less sensitive. From the standpoint of retention time reproducibility, they are therefore the preferred packings when a new normal-phase method is to be developed. However, there are some things that one should know about the behavior of these phases.

For example, the primary amino groups on the surface of an amino-packing can easily form Schiff-bases (imines) with aldehydes and ketones under typical normal-phase chromatographic conditions. Therefore, amino-columns should not be used with samples containing aldehydes or ketones.

Cyano columns are quite stable in non-polar solvents, but often exhibit the curious phenomenon of bed collapse when exposed to solvents of intermediate polarity like neat acetonitrile, THF or methanol. This condition may be encountered during attempts to "wash" a column that has deteriorated due to build-up of sample debris. In our experience, it is best to maintain a constant high flow through the column during such washing procedures. The bed collapse is more likely to occur at low flow rates or when the column is stored in these solvents.

Amino columns may change quite drastically, if they are exposed to aqueous eluents. The high concentration of amino-groups in the pores of the packing forms a strongly basic environment, which causes hydrolysis of the bonded phase. After returning to the normal-phase mobile phase, one should not be surprised to find a significant difference in the behavior of the packing.

These polar bonded phases are quite universally useful, just as silica itself or alumina. Therefore, one can not say that one phase is "better" than others.

They will exhibit unique selectivities that are different from silica or alumina, and they are quite different from each other. Just as silica columns show strong retention for basic analytes, amino columns will retain acids more strongly. Cyano and diol columns are neutral. Diol columns are more polar and therefore generally more retentive then cyano columns.

As in the case of reversed-phase packings, there are significant differences between different brands of the same type of bonded phase. Leaving aside the differences in the base silica, there are differences in the bonding process and in the end-capping procedure. Monofunctional, difunctional or trifunctional silanes can be used. Also, if multifunctional silanes are used, significantly different coating procedures can be used that result in "monolayer" coating or "polymeric" coating. Cyano-columns may or may not be endcapped, while amino- and diol-packings are usually not end-capped. So just like with reversed-phase packings, one cannot assume that a cyano-column from brand A will perform the same separation as a cyano-column from brand B.

References:

1. Techniques of Chemistry, Vol. II, Organic Solvents, Physical Properties and Methods of Purification, Third Edition (1970), John A. Riddick and William B. Bunger, Wiley-Interscience

2. Merck Index, Eleventh Edition (1989), Merck & Co., Inc.

8. System Volume, Dead-Volume, Dwell Volume

Q.: I often encounter terms like system band spreading, system volume, dead-volume, dwell volume. Could you explain these terms?

A.: Gladly! When we talk about these parameters, we need to clearly define them. As we will see, they are quite different and affect different elements of a chromatographic separation.

Let us start with the dead-volume. It is also called the extra-column volume, which is somewhat clearer. It comprises the volume of an HPLC-system between the point of injection and the point of detection, but excluding the part of the column that contains the packing. Therefore it includes the injection volume, the volume of the injector, the volume of the connection tubing before and after the column, the volume in the endfittings of the column, including the frits, and the detector volume. Actually, to be precise, it includes half the injection volume and half of the detector volume.

We are concerned about the extra-column volume, because it causes extra-column bandspreading. Bandspreading means that the peaks become broader as they flow through the extra-column volume. This is undesirable since it may destroy some of the separation achieved in the column. We would like to keep the extra-column bandspreading as small as possible.

Q.: How can we measure the extra-column volume and the extra-column bandspreading?

A.: The total extra-column volume and extra-column bandspreading can only be measured using special equipment. This is due to the fact that it includes the column end-fittings and the frits in the column, which are not accessible by the user. So we just have to trust that the column manufacturers have done a good job and minimized that part of the extra-column volume. What we can do easily though is measure the extra-column volume associated with the HPLC system. To that purpose we simply disconnect the column and replace it with a "zero dead-volume" union. Then we inject a small volume of sample and record the detector response. An example of the response is shown in figure 1.

From this "chromatogram" we can determine the extra-column volume and the extra-column bandspreading. The extra-column volume is strictly speaking the distance from the point of injection to the center of the gravity of the peak. To determine it we need to compute the first moment of the peak. However, we do not have to be overly precise and can for simplicity use the distance between the point of injection and the peak maximum as our measure of system dead-volume.

Figure 1

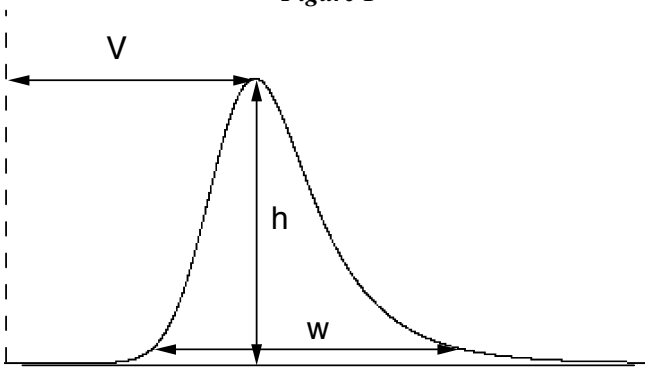

Figure 1: ***Measurement of Extra-Column Volume and Bandspreading.*** *V is the extra-column volume, w is the peak-width at 4.4% of the peak height, corresponding to 5 standard deviations.*

We can use the standard deviation of the peak as a measure of the system band-spreading. The standard deviation of the peak can be computed from the second moment of the peak. An easier way is to measure the width of the peak. Since this peak is highly asymmetric, it is best to measure the width as close to the baseline as possible to get a good measure of extra-column bandspreading. For example the width of the peak at 4.4 % of the height of the peak corresponds to a width of 5 standard deviations.

Q.: OK, but what sample and what mobile phase do I use for this measurement?

A.: You can simply use the mobile phase used in your analysis. As sample you may just utilize the standards that you use in chromatographic analysis. However, most of the time the standard is likely to be too concentrated, and you need to dilute it to make it compatible with the small extra-column bandspreading. If you do this, you get a direct idea of extra-column bandspreading under your actual chromatographic conditions. On the other hand, you may standardize the measurement in the form of a general system check that is independent of the chromatographic test that you are running. The disadvantage is that you may miss something that is specifically related to your particular analysis. I encountered a case a few years ago, where the sample was interacting strongly with a part in the injector. Although everything that we did pointed to excessive extra-column bandspreading, we could not see it in our standardized test, which used a different sample and a different mobile phase. Only when we used the actual sample under actual mobile phase conditions did we find the problem.

Q.: This covers extra-column volume and extra-column bandspreading. What about system volume and dwell volume?

A.: I do not like the term "system volume", because it is ambiguous. You may have a system dead-volume or a system dwell volume. We should obsolete the term system volume and rather use the words extra column volume and gradient dwell volume. The latter can also be called gradient delay volume. It refers to the volume of a gradient HPLC-system between the point of mixing of the gradient and the top of the column. (It exists also in isocratic chromatography, but there it is unimportant).

The gradient dwell volume comprises the volume of the gradient mixer, the connection tubing to the pump (if low-pressure mixing is used), the volume of the pump heads and check-valves, the tubing between pump and injector, the volume of the injector and the connection tubing between injector and column. As one can see, there could be a lot of volume in all these parts.

When you start a gradient, it will take some time until this volume is purged and the gradient enters the column. During this time, the peaks are subject to isocratic migration in the starting mobile phase. If there are large differences in the gradient dwell volume between different systems, this isocratic migration can be different enough to affect the chromatogram, especially the early portion of the chromatogram.

Another annoying effect is that your actual separation may be delayed significantly. If you have a system with a total delay volume of 2 mL and you run a gradient at 200 µL/min, it will take 10 minutes until the gradient reaches the top of your column. Therefore the start of your separation is delayed by 10 minutes.

Q.: How do I measure the dwell volume?

A.: Once again, you disconnect the column from your system. Then you run a step-gradient from methanol to methanol with 10 mg/L propyl paraben, using a UV detector. This will create an S-shaped detector trace. You then measure the time delay from the point at which you started the gradient to the point when half the height of the step is reached. Multiplying this time with the flow-rate gives you the gradient delay or dwell volume.

Q.: Is there a literature reference that contains these definitions?

A.: Many textbooks have sections that define the terms used in chromatography. There is however one authoritative body, the Commission on Analytical Nomenclature of the Analytical Chemistry Division of the International Union of Pure and Applied Chemistry that defines many of the terms used in HPLC and other chromatographic techniques. I believe that their latest publication containing the currently valid nomenclature has been published in Pure and Appl. Chem, Vol. 65, No. 4, pp. 819-872, 1993. Unfortunately, not all terms used are explained, for example the dwell volume is missing, and the definition of some terms still remains ambiguous.

9. Transfer of Gradient Methods

Q.: I have developed a gradient method that is very reproducible when I run it on my system, but I can't get the same results on another system. What's wrong?

A.: Occasionally, some difficulties arise when gradient methods are transferred from one HPLC system to another. Unless the systems are identical, one can usually expect some shifts in retention times. Most of the times these shifts in retention do not affect the resolution to the extent that a method becomes useless, and one can usually proceed by ignoring the differences. On the other hand, with a proper understanding of the underlying causes, one may be able to adjust the gradient to get equal performance from dissimilar HPLC systems.

In the last column we touched on one aspect of the problem: the gradient dwell volume. It is the volume between the point of mixing of the gradient and the top of the column. After you start your analysis by injecting the sample, the gradient will not reach the top of the column until the gradient dwell volume is purged. This means that your sample is subjected initially to a period of isocratic migration until the gradient catches up. Since the gradient dwell volume may be different from system to system, this isocratic migration time will be different and may result in retention time differences or even affect resolution.

Another possibility is the gradient itself. There could be compositional differences from system to system. With most HPLC systems manufactured today, this should be only of secondary concern. But generally, any gradient system delivers a composition with the highest accuracy in the mid-range of the composition, i.e. at a mixture of 50% A and 50% B. The accuracy suffers, when very disproportionate amounts of A and B are mixed, e.g. 5% A or 95% A.

Q.: What can I do to sort out, which of these possibilities causes my method transfer problem?

A.: The simplest thing to do is to compare your gradient on both systems. In order to do this, you disconnect the column and add an UV-absorber to the B-solvent of your gradient. If you are using a reversed-phase system, you can for example add 10 mg/L propyl paraben to your B-solvent. Then you run the gradient on both systems and record the baseline. You then compare the two plots to each other. You want to find the point where the gradient starts, and you also want to measure the gradient profile. If your gradient is linear, then you only need to check the slope of the gradient.

Most likely you will find that the onset of the gradient is different between the two instruments, while the profile is very similar, just off-set by some amount of time. In this case, you have a difference in the gradient dwell volume.

Q.: If this is the case, is there a simple way to compensate for the difference in dwell volume?

A.: There is a solution that works most of the time. If the gradient dwell volume is smaller on the system that you are transferring your method to, you may be able to compensate for the lack in dwell volume by programming an isocratic portion at the beginning of your gradient that compensates for the volume difference. The remainder of the gradient simply remains constant. If on the other hand the gradient dwell volume on the second system is larger than the one on the first system, the situation is more difficult. In principle, you can start the gradient, and then inject the sample after a delay time that accounts for the difference in the gradient dwell volume between both systems. But this may not be possible on an automatic system, where the injection triggers the start of the gradient. In this case, you may need to go back to ground zero and redevelop the method.

Q.: This would not be a very pleasant situation after all the time that I spent developing the method. What can I do to prevent this from happening in the future?

A.: You can avoid this situation by developing the method for the HPLC systems that will ultimately use the method. This requires some foresight and some planning, but usually is not impossible. What you need to do up front is to characterize the systems that are likely to be used for your method. There are two basic things that you need to know for each system: the gradient dwell volume and the compositional accuracy. You can get both pieces of information in a single experiment: as described above, you add an UV-absorber to your B-solvent. Then you program a multiple step gradient in increments of 5% from 0% B to 100% B. The flow-rate should be the flow-rate typically used, so most likely you will use a flow-rate of 1 mL/min. The intervals between the steps should be a few minutes, maybe 5 minutes. Now run this gradient without a column in place on the different systems and record the detector response. The time delay between the time programmed for each step and the actual occurrence of the step gives you the gradient delay time. The height of the step gives you a measure of the composition. The steps will be smeared out a little, which is a function of the mixing volume in your system.

Now that you have characterized the systems, you can design you method around the characteristics of the systems. As I mentioned before, the largest issue is usually the gradient dwell volume. If you know that the systems that you need to transfer your method to have a larger dwell volume than the system on which you are developing your method, you should automatically add an isocratic step in your methods development that will compensate for this difference. If the dwell volumes of the target systems are smaller than the one of your development system, you should be able to simply compensate for this by adding a gradient delay time to the beginning of your method when transferring the method.

If there are compositional differences in the middle of the gradient run, you could conceivably compensate for those as well by adjusting the gradient profile, but I have never encountered a situation where this was necessary.

This discussion assumes that the column is in complete equilibrium with the starting mobile phase. Occasionally I have encountered a situation, where in routine analysis the gradients follow each other so quickly that the column never returns to equilibrium in the starting mobile phase. You will see that you have this situation if your first gradient always gives results that are different from subsequent gradients. This may be advantageous for speeding up a method, but it could be a cause of difficulties when this method is transferred to another system with a different dwell volume.

10. Clogged System

Q.: My system pressure is much higher than it should be. What is the problem?

A.: First, let us check your premise. How do you know, what the pressure should be?

Q.: Previously, the same column with the same mobile phase conditions at the same temperature gave me a backpressure of about 2000 PSI. Now it has doubled. I have set the high-pressure limit of the pump at 4000 PSI, and the pump shuts off at this pressure.

A.: It is good that you have a previous reference point, we can start our troubleshooting from there. There are many different things that can lead to an increase in back-pressure. First, the viscosity of your mobile phase could not be correct. Second, a part of your system may be clogged. The latter is the more common case, but before we disassemble your system, let us check your premises.

How do you know that you are pumping the correct mobile phase? If you use automatic blending, did you put the correct solvent on the correct inlet line of the pump? For instance a mix-up of the methanol and the acetonitrile line (if your instrument is set up this way could easily give a factor of two difference in backpressure due to the viscosity difference between the mixtures of each solvent with water.

If you work at elevated temperature using a column heater, you should check that your column heater is on and that it is working. The viscosity of a solvent typically changes by 25% for 10 °C. Therefore if your solvent is supposed to be at 60 °C, but you heater does not work, you could get double the backpressure.

Q.: O.K. This is easy to check. What else can I check?

A.: I often ask the question, whether the high-pressure shut-down happened in the middle of a long series of analyses or upon start-up.

If the high pressure developed upon start-up, it is likely that some kind of an error occurred. Let us check a few things, starting with the simple things first: Is it the correct column with the correct particle size? Column backpressure changes with the square of the particle size. So if by accident you grabbed the 5 µm version of the 10 µm column that you used to run, that would explain the increased pressure.

If this is not the case, then something is clogged. First, let us disconnect the column and replace it with a union, leaving guard columns and precolumn filters in place. One possibility that we will check is an incompatibility of the mobile phase. If we would leave the analytical column in place, we could do damage to it in the subsequent operations. After disconnecting the column, we check the backpressure of the system again. If it is substantial, i.e. exceeding 1500 PSI, then one of the remaining parts is clogged and the column is most likely still O.K. We can then disconnect the remaining elements of the fluid path one by one and determine, which of the elements contributes the most to the back-pressure. Usually, it is most efficient to replace the clogged part, rather than trying to clean it. But in many cases, a replacement part is not readily available, and we may consider cleaning procedures.

It is easier to develop a cleaning procedure, if we know what we are trying to remove. In this hypothetical case, we know that the clog occurred upon start-up. We then should ask, why the clog occurred. It is possible, for example, that the mobile phase that was last used in the system was not completely flushed out and was incompatible with the new mobile phase. A buffer may have precipitated. If this is the case, you may simply wash or flush the clogged part with a solvent that will redissolve the precipitate, and you are up and running in a short time. The clog actually may even disappear, while you are still checking which part was clogged.

If it turns out that the analytical column or the guard column clogged during start-up, you may check in what solvent the column or guard column was stored. The storage solvent may be incompatible with the mobile phase and cause precipitation of a buffer. Or, the column or guard column was stored with a mobile phase that contained a salt and dried (partially) out due to loose caps. In this case, you may be able to resurrect the column or guard column by flushing it at low flow-rate with a mobile phase that will redissolve the salt.

Q.: In my case, the high-pressure shutdown happened in the middle of a series of runs. What could be the problem in this case?

A.: In this case it is most likely that a part of your system clogged due to debris that built up. The debris may come from the seals in your instrument or from the sample. It could be constituents of your sample that are insoluble in the mobile phase. It could be a component of the sample that is strongly adsorbed on the guard column, or if you do not use a guard column, on the column itself. Often, these are high-

molecular weight constituents of your sample that have not or have only partly been removed. These could be proteins in a serum sample, excipients in a pharmaceutical formulation or high-molecular weight constituents in a food sample.

If you use precolumn filters and guard columns, let us disconnect them one by one and measure the backpressure of the system without this part. This way, we should be able to quickly identify the clogged part. If it is a guard column or a precolumn filter, it is best to just replace the part. It has done the job that it was supposed to do and protected your analytical column. To attempt to resurrect that part by cleaning it is false economy: it will not work as well the next time, and there is consequently an increased likelihood that the analytical column might get damaged.

If it is the analytical column itself, it is worthwhile to do some work to attempt to clean it. However, the best remedy is prevention, and you may consider using a precolumn filter or better, a guard column in the future to protect the analytical column. If the origin of the problem is the sample itself, you may consider filtering the sample or using solid-phase extraction to remove the contaminant.

If you have a spare filter for your column, I would first attempt to replace the inlet filter of the column. If you do not have a filter, you may remove the filter and attempt to clean it in an ultrasonic bath. This is only feasible with silica-based columns. With polymeric columns, the packing is under stress and will ooze out of the column from the moment you remove the filter. If you have a spare filter handy, you can quickly replace it and close the column again. If you do not have a spare filter, you should not open a polymeric column.

If a replacement or cleaning of the inlet filter does not result in a reduction in backpressure, then most likely something is adsorbed on the surface of the packing or has precipitated in the column. In this case, the column should be flushed at slow flow-rates with something that redissolves or desorbs the suspected contaminant. Column manufacturers typically recommend a sequence of solvents of increasing solvent strength to accomplish this task. For reversed-phase columns, one might consider the following sequence: water, methanol, THF, methylene chloride, methanol and back to water. For normal-phase columns, the sequence of polarities is inverted: methylene chloride, THF, water, methanol, methylene chloride.

If this does not help, you can still attempt to back-flush the column. But at this point in time it just might be best to pick up the phone and order a new column.

11. Column Plate-Count

Q.: When I run my method and measure the plate count, I am getting about 8000 plates for my column. But the manufacturer's literature specifies 13000 plates. Can you explain this discrepancy?

A.: Columns do not have fixed plate counts. For a given column, plate counts depend on the flow rate, the viscosity of the solvent and the molecular weight of the analyte. The instrument may affect the measurement as well, but we will ignore this for the time being.

The column manufacturer has set up standardized conditions by which the quality of the column is measured. In reversed-phase chromatography, the measurement is usually done with a simple hydrophobic analyte, like toluene, naphthalene or acenaphthene. The mobile phases used with these compounds contain a high amount of organic solvent. Therefore they have a low viscosity. On the other hand, most HPLC users are dealing with more polar molecules, which require mobile phases with a higher water content. These mobile phases have a higher viscosity. Since under normal HPLC conditions and normal HPLC flow rates, the plate count decreases when the viscosity increases,the plate count is lower under practical use conditions than under column testing conditions.

Q.: If the plate counts under normal use conditions are lower than the plate counts reported by the manufacturer, aren't the manufacturers misleading the users as to the capabilities of the column?

A.: Not really. This is not the purpose of it. The column testing procedures used by manufacturers are designed to check the quality of the column, and this is best done at or close to the maximum of the plate-count capability of the column. This is the point where the column packing technique has the largest influence. Therefore, if I want to check the quality of the packing technique, this is the point at which I want to measure it.

Q.: Please explain a little bit more, why and how column plate-count varies with flow-rate, column packing etc.!

A.: Underlying all this dependence of plate count on a variety of parameters is the reduced plate height, and its dependence on the reduced velocity. I will sketch this out a little bit, but for a deeper understanding we need to look at a textbook of chromatography.

The plate count of the column is the column length L divided by the reduced plate height h and the particle size d_p:

$$N = \frac{L}{h \cdot d_p} \qquad (1)$$

The column length and the particle size are fixed (and known). The reduced plate height depends on the reduced velocity, which is defined as follows:

$$\upsilon = \frac{u \cdot d_p}{D_m} \qquad (2)$$

This equation contains the linear velocity u, the diffusion coefficient D_M of the sample in the mobile phase and once again the particle size. Most HPLC work is carried out at reduced velocities between 3 and 20.

There are several equations in the literature that describe the dependence of the reduced plate height on the reduced velocity. Within the velocity range discussed here they give identical results. Therefore, let me use the simplest one, which is based on the van-Deemter equation:

$$h = 1.5 + \frac{1}{\upsilon} + \frac{\upsilon}{6} \quad (3)$$

The coefficients are empirical and have been derived from a broad data base for reversed-phase operating conditions. The curve described by this relationship has a minimum, which is reached at a reduced velocity of around 2.5. The first coefficient is a measure of the uniformity of the packing. It therefore depends on the packing process.

The column plate count depends on the reduced velocity as follows:

$$N = \frac{L}{\left(1.5 + \frac{1}{\upsilon} + \frac{\upsilon}{6}\right) \cdot d_p} \quad (4)$$

Since the dependence of the HETP on the velocity has a minimum, the dependence of the plate count on velocity has a maximum. A little calculus shows that the maximum plate count is approximately L/2.3 dp. So we can conclude that a 15 cm column packed with 5 µm particles that has a plate count maximum of 13000 is a good column.

Bad column packing largely affects the first coefficient of the equation 3, while the other ones remain fairly much the same. The effect of this coefficient is largest, when the influence of the other ones is minimal. This is the reason why one should test columns at or close to the maximum plate count.

Let us now tackle the second part of your question, the dependence of the plate-count on flow-rate. I glossed over this a little bit by moving to the reduced velocity. First, the reduced velocity is proportional to the linear velocity, which is proportional to the flow rate. Therefore, the plate count depends on flow rate. It is low at very low flow rate and low at high flow rate. The maximum plate-count is somewhere in between. But where? As we have seen from the definition of the reduced velocity in equation 2, the reduced velocity includes the diffusion coefficient of the analyte in the mobile phase. We know from the results above, that the maximum plate-count occurs at a reduced velocity somewhere around 2 to 3. For the hydrophobic analytes typically used as column test samples with a mobile phase of 70/30 v/v acetonitrile/water, this translates to a linear velocity of 1 to 1.5 mm/sec for a 5 µm column. The flow-rate that corresponds to this linear velocity is about 0.5 to 0.7 mL/min for a column i.d. of 3.9 mm and 0.7 to 1 mL/min for a 4.6 mm column.

Since the location of the minimum of the curve depends on the diffcusion coefficient, we need to know the dependence of the diffusion coefficient as a function of the mobile phase. There are equations in the literature, which allow us to estimate the diffusion coefficient of an analyte in a particular solvent (e.g. Wilke-Chang equation). However, for the current discussion we only need to know that the diffusion coefficient is inversely proportional to the viscosity η of the mobile phase:

$$D_M \propto \frac{1}{\eta} \quad (5)$$

This means that the velocity (or flow rate) at which the maximum plate count occurs decreases with increasing viscosity. A mobile phase of 70/30 v/v acetonitrile/water has a viscosity of 0.7 cP, while the viscosity of a mobile phase of 50/50 v/v methanol/water is 1.8 cP. When we use the methanol/water moblie phase, the optimal flow rate decreases therefore to between 0.2-0.3 mL/min for the 3.9 mm column and to between 0.3 to 0.45 mL/min for the 4.6 mm column packed with 5 µm particles. This is something worth keeping in mind during methods development.

There is an interesting side aspect of the dependence of the diffusion coefficient on the solvent viscosity. One can use the following rule of thumb for estimating column performance, when the mobile phase composition is changed: the same column will give about the same plate count at the same back-pressure in different mobile phases. A useful rule to keep in mind!

12. Column Backpressure

Q.: I am using a 4.6 mm x 150 mm 5 µm C18 column. My mobile phase is 50/50 methanol/phosphate buffer pH 7. I am getting 3000 psi at 1.5 mL/min. Is this normal?

A.: It is a little high, but not outrageous. I would have expected a backpressure for the column alone of about 2400 psi, plus you have to add maybe another 200 psi for the backpressure of the connecting tubing. This would get us to 2600 psi. I think, your column may be partially clogged.

Q.: This is quite possible. I suspected this too, and this was the reason for my question. How did you arrive at the estimate of 2400 psi as the "expected" backpressure?

A.: I calculated it from the Kozeny-Carman equation. It works very well for all HPLC columns that are packed with incompressible packings like silica-based packings.
The Kozeny-Carman equation relates the pressure to the flow-rate F, viscosity η, column length L, column radius r and particle diameter d_p:

$$\Delta p = f \cdot \frac{F \cdot \eta \cdot L}{\pi \cdot r^2 \cdot d_p^2} \quad (1)$$

The pressure increases with increasing flow-rate, viscosity and column length. It decreases with the square of the column radius and the square of the particle diameter.
The proportionality factor f depends on the interstitial fraction (the "space" between particles) and therefore the packing density. Incompressible packings pack to fairly

much the same packing density, i.e. to an interstitial fraction of about 40 %. For such a column the factor *f* is about 1000.

Q.: O.K. Let me calculate the pressure for my example column. I have the flow-rate, column length and diameter, and particle size. How about the viscosity?

A.: For neat solvents, you can easily find the viscosity in handbooks. For solvent mixtures of non-associating solvents, you can estimate the viscosity by assuming a linear relationship with the volume fraction. For aqueous mixtures, this does not work. Most mixtures of organic solvents with water have a viscosity maximum. The viscosity of the most commonly used aqueous mixtures is shown in figure 1.
The viscosity of a 50/50 mixture of methanol and water is about 1.8 cP (=0.018 P) at room temperature. There is not a lot of difference between water and a dilute buffer, and I can use the viscosity of the water/methanol mixture from the chart.

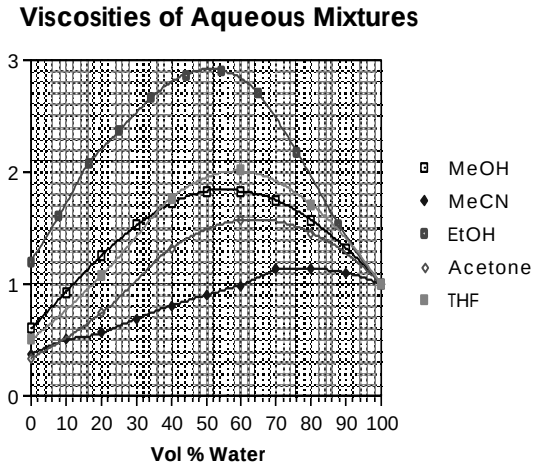

Figure 1:
Viscosity of mixtures of organic solvents with water as a function of the water content (% v/v). Appreviations: MeOH = methanol, MeCN = acetonitrile, EtOH = ethanol, THF = tetrahydrofuran

Therefore the calculation of the pressure is carried out as follows:

$$\Delta p[atm] = 1000 \cdot \frac{1.5}{60} \cdot \frac{0.018 \cdot 15}{3.1415 \cdot 0.23^2 \cdot 0.0005^2} \cdot 10^{-6}$$

The last factor (10^{-6}) is the conversion factor to atmospheres. Do not forget to divide the flow rate by 60 to obtain the flow in mL/sec. Then we have the same dimensions throughout.

To convert from atmospheres to psi, multiply by 14.7. You should get 2388 psi, i.e about 2400 psi.

Q.: This helps a lot. Now, let us go back to the column. It seems to be partially clogged. What shall I do about it?

A.: The simplest thing to do is to replace the inlet frit. Hopefully, this will reduce the pressure. You actually may also be able to clean the old frit in an ultrasonic bath. If the replacement of the frit does not result in a reduction in backpressure, the packing itself might be partially clogged. You can go through some washing cycles to remove, whatever is clogging the packing, but this is a fair amount of work. Since the pressure increase is only small, I would continue to use the column until it reaches the pressure limit of your system. I also would try to figure out, what the source of the pressure increase is and try to prevent it as much as possible. Often, the use of a guard column between injector and analytical column solves the problem or at least slows down any pressure increase.

Q.: O.K. I will try to replace the filter. In the case that his does not help and I decide to clean the column, how should I go about this?

A.: You first need to remove the buffer from the column, so you want to wash it with 5 to 10 column volumes of water. That is between 10 and 20 mL for this column. Then you switch to 100% methanol and let the column purge for a while, maybe some 15 minutes. You can do all this at high flow rate, since your column has not yet clogged completely. So I would do this at 1.5 mL/min. Let us check the pressure in methanol. Methanol has a viscosity of 0.6 cP. Therefore we would expect a pressure of about 800 psi at 1.5 mL/min in methanol. If the pressure is still high, flush the column with tetrahydrofuran (viscosity 0.5 cP) or methylene chloride (viscosity 0.4 cP) or both. The volume for each solvent should be around 20 mL. Whatever is not removed within this volume, is not likely to be easily removed in this solvent at all. After this process, you can go back to the original mobile phase. You must make sure that the mobile phases in subsequent steps are miscible. Therefore you need to go from methylene chloride to methanol to water (or 50/50 methanol/water) to 50/50 methanol/buffer. Now you need to reequilibrate the column. In your case, with this simple mobile phase, the reequilibration is fast. If you were to run a separation requiring an ion-pair reagent, you would now need to purge your column with the mobile phase for quite a while, before you are back in equilibrium.

13. Peak Area Fluctuations

Q.: What are the reasons for fluctuations in peak area, when the same sample is injected multiple times?

A.: Unfortunately, there are many different reasons, too many to count. Nearly every part of the HPLC instrument

can conceivably contribute to changes in peak area. Let us review them one by one.

The injector comes to mind first. What problems you might encounter depends to some degree on the type of injector. But in all cases, the formation of air bubbles during the measurement of the injection volume is a major problem source. If you are injecting manually, make sure that there are no air bubbles in your syringe. With fixed-loop injectors, make sure that no air is siphoned into the injector loop. In autoinjectors, air bubbles can be formed if the cap of the vial seals well around the needle, which can give rise to a vacuum when a large portion of the sample is injected. Any air bubble formation results in a random variation of the injection volume from injection to injection.

If the concentration of the analyte varies widely from sample to sample and the peak area obtained from a single sample becomes constant only after two or three injections, sample carry-over is a likely source of the problem. Clean the syringe carefully after each injection, if you are injecting manually, or check the needle wash in an autoinjector. Make sure that the solvent used for the needle wash is a good solvent for the analyte(s).

Decreases in peak area from run to run can also be caused by temperature changes, but the effect is small, under 2%. If you take a sample out of the refrigerator and put it into an autoinjector, it will slowly warm up to the injector temperature and the solvent will expand. Therefore you will inject initially a larger mass of sample than later when the sample has reached the temperature of the sample compartment.

Random variations of the flow rate can also cause fluctuations in peak area. They are typically caused by malfunctioning check valves or air or cavitation in the pump head. They are usually accompanied by fluctuations in retention times as well. You can check if this is the problem by monitoring the backpressure. What fluctuations can be tolerated depends on the width of the peak. Assume that there is a flow difference of 10% between the two heads of your pump. If your peak width is about 20 pump strokes, the fluctuation in peak area caused by the pulsation of the pump is under 1%. But it will be 10% if the peak width is about one pump stroke.

Another potential cause of flow-rate changes are leaks on the high-pressure side of the system. They should be easy to find.

Integration errors are more difficult to troubleshoot. Gross changes in the way the software integrates the peaks can be found by simple visual examination, but more subtle changes are difficult to spot. Significant integration errors often occur with tailing peaks at a signal-to-noise ratio of 100 or less. Generally, the base-line noise limits the precision of the integration, but the problem becomes worse with fronting or tailing peaks. You can try to reprocess the data with different settings of the integration parameters and see if this reduces the problem.

In complex samples which contain peaks that are only marginally resolved from the peaks of interest, the software can have difficulties determining where the baseline is. In such a case, a manual integration or an automatic integration with a forced baseline can improve the reproducibility of the integration.

The sample itself can be the source of the problem. In reversed-phase chromatography it has been observed that some proteins do not elute completely during the initial gradient. An additional quantity is eluted in subsequent blank gradients. This “ghosting” is specific to the protein and the elution protocol. If this occurs, blank gradients need to be run between analyses.

Also, proteins can be “irreversibly” adsorbed on some columns. The peak area obtained with a brandnew column may increase slowly with repetitive injections. Presumably, some protein binds to active sites on the packing. Once these sites are saturated, a reproducible peak area is obtained. It is not necessary to use the protein that you need to analyze. It has been reported that other proteins can be use as well to saturate the active sites.

The detector contributes to peak area fluctuations in an indirect way. How well the peak can be integrated depends on its signal-to-noise ratio. You can not expect a reproducibility of the peak area that is any better than the ratio of the baseline noise to the signal at the peak maximum. The mobile phase is rarely a contributor to peak area fluctuations. If there are any shifts in mobile phase composition, they will affect retention times long before they affect peak areas. Ghost peaks or negative peaks that may be generated by the mobile phase affect the integration in the same way as any other interference. They can often be avoided by dissolving the sample in the mobile phase.

Q.: What reproducibility of the peak area can I reasonably expect?

A.: Unless you are limited by the signal-to-noise ratio, the relative standard deviation of the peak area for repetitive injections should be definitely under 1%. Values of 0.2 to 0.5% are achievable. The reproducibility can be further increased by using an internal standard and calculating the ratio of the peak area of the analyte(s) to the peak area of the internal standard, but even with this method you are not likely to be able to drive the r.s.d. much under 0.1%.

14. Ghost Peaks

Q.: A peak appears when I am making a blank injection. Where does it come from?

A.: Ghost peaks can occur in several different situations. In the following, we will discuss the various causes of these ghost peaks. First, different phenomena can occur in isocratic chromatography and in gradient chromatography. Therefore we will divide the subsequent discussion into these subjects.

1. Isocratic Chromatography

Let us define what you mean by a "blank" injection. If you are injecting mobile phase, then indeed we do not expect to see a peak. But if you are injecting something else, for example the solvent in which you commonly dissolve the sample, you should not be surprised to see peaks. The injection of anything but the mobile phase disturbs the equilibrium between the mobile phase and the stationary phase, and peaks can be observed.

Recirculated Mobile Phase

If a constituent of the mobile phase has a moderate retention, it will show up as a retained peak. These peaks can be positive or negative. If you recirculate the mobile phase, and if this mobile phase has been used for awhile, you can see a negative peak profile that corresponds to the sample that you usually inject. The previously injected samples have accumulated in the recirculated mobile phase, and the stationary phase is partially coated with the analytes. When you inject a freshly prepared mobile phase or just some blank solvent, there is a concentration deficit of the sample constituents. This concentration deficit moves down the column at the same speed as the corresponding peaks, and a negative image of the normal chromatogram results. The phenomenon is called vacancy chromatography and the peaks are called vacancy peaks. This is one of the reasons why a recirculation of the mobile phase is generally not a good idea.

Carryover and Precipitation

If you inject a sample that is identical to the mobile phase and still observe a peak, we need to look at sources outside the column and the mobile phase. For example, there could be some carryover from previous injections. You should look if the needle wash in your autoinjector works well. If you use a manual injector, make sure that your syringe is properly cleaned. This includes the outside of the needle.
Is it possible that previously injected sample constituents have precipitated in the injector or are adsorbed to a part of the injector? The area of the peaks that you are observing should then decrease with every injection. Make sure that all sample constituents are soluble in the mobile phase. The best solution is to dissolve the sample in mobile phase.

Fluid Path Issues

An injection is always accompanied by a pressure pulse. If there are dead corners in the fluid path, such as a T-connection to a pressure gauge etc., the pressure pulse can cause some of the solvent in these dead corners to enter the fluid path and create a peak. Usually, these peaks are broader than peaks normally observed in the chromatogram. Inspect the fluid path and eliminate these dead corners wherever possible.

2. Gradient Chromatography

All of the previous comments applied to isocratic chromatography. If you are running gradients, then there are several other possible sources of peaks in blank runs.

Solvent Impurities

All minor constituents and impurities in the mobile phase will enrich on the column during equilibration of the column with the starting composition. As you increase the strength of the mobile phase during the gradient, these minor constituents and impurities will be desorbed from the column just like a sample. This can result in a complex chromatograms with many peaks, if your mobile phase contains many impurities. A typical source of problems is the quality of the water used in reversed-phase chromatography. Water can contain impurities from many sources. These sources include the water purification system itself, the containers in which the water is stored, and bacterial growth. The performance of a water purification system should be monitored on a regular basis using a blank reversed-phase gradient. Otherwise, the purchase of HPLC-grade water is recommended.

Even with high-quality solvents and reagents, it is possible to observe peaks due to the reagents themselves. This depends on the detector that you are using. If you are using a UV-detector, it depends on the wavelength and how sensitive the detector is to changes in the refractive index of the mobile phase. With high-quality reagents, the gradient background can appear as a hump rather than a collection of peaks.

Protein-Containing Samples

When protein-containing samples are analyzed by reversed-phase chromatography, it is also possible to observe peaks with blank gradients that stem from a previous injection of the sample. The peak area decreases rapidly with subsequent blank gradients. The phenomenon is reminiscent of sample carryover in the injector, except that no sample is injected. The injector can even be taken off-line. What is happening is that the elution of some proteins is incomplete in the first gradient. For example, under a specific set of conditions only 2/3 of the injected amount of ovalbumin is recovered in the first gradient. In every subsequent gradient approximately the same amount of the remaining ovalbumin is recovered. After a few runs the amount of ovalbumin becomes negligibly small. This phenomenon seems to be specific for proteins. To my knowledge, it has never been observed with small molecules.

Summary

Being aware of all these causes of ghost peaks will allow you to take the proper precautions to avoid these situations.

15. Dependence of Retention Times on pH

Q.: I have a separation that seems to be very sensitive to variations in pH. It is a simple reversed-phase method, and I am not using any ion-pairing reagents. I experienced a lot of changes in retention time from day to day, which I was able to trace to small variations in pH. What is the problem?

A.: From your description of the problem it appears that you are dealing with ionogenic analytes with a pKa close to the pH of the mobile phase. Therefore, you are dealing with two forms of your analytes which have significantly different retention times. This could be for example an acid in its protonated and unprotonated form. The charged form of the analyte has a much lower retention factor than the uncharged form. Both are in an equilibrium with each other that depends on the pH. Small variations in the pH of the mobile phase change the ratio of both forms of the analyte and therefore the retention time.

As a consequence of this, it is necessary to control the pH tightly. Since you have made some experiments already, you may have obtained enough information to know quantitatively the dependence of retention time on pH. If not, make a few controlled experiments to obtain this knowledge. Also, you may check the history of the method. Such information should have been generated during method robustness testing. Once you the dependence, you need to decide, what are the retention time fluctuations that you can live with. Then you calculate, what the accuracy of the pH adjustment should be. Even in difficult cases, a precision of +/- 0.02 pH units should be sufficient.

Q.: It is difficult to adjust the pH that accurately. If I add only a little bit of acid, the pH changes drastically.

A.: Unfortunately, this indicates that you are not using a buffer. The definition of a buffer is that it buffers the pH from small additions of acid or base, i.e. the pH should change very little upon the addition of acid or base. This ability to maintain the pH is called the buffer capacity. It depends on the concentration of the buffer and on the pKa of the buffering ions. Maximum buffer capacity is obtained at the pKa of the buffering ion. Table 1 gives you the pKa's of some buffers that are commonly used in HPLC. Note that they are measured in water, without the addition of an organic solvent. The pKa's will shift upon addition of an organic solvent. But generally, all pKa's of all compounds including your analyte will experience the same shift.

For the buffer concentrations commonly used in HPLC, the pH of your buffer should remain within 1.5 pH units around the pKa of the buffering ion. For very dilute buffers, under 5 mM, you may want to narrow the range even further to +/- 1 pH units around the pKa.

Unfortunately, I frequently encounter methods that completely ignore the buffer capacity. An example is a solution of potassium dihydrogen phosphate at pH 4.5. This is not a buffer, this is a salt solution. As you can see from the table, the pKa's of a phosphate are about 2 and 7. 4.5 is right in the middle, and phosphate has no buffering capacity whatsoever at this pH. To buffer the pH at 4.5, you need to use a different buffer, for example acetic acid.

Similarly, a solution of ammonium acetate at pH 7 is not a buffer, but a salt solution. The use of ammonium acetate "buffers" has become popular, since ammonium acetate is volatile and can be used with mass spectrometry detectors. But at pH 7 it is not a buffer, and you might as well leave it out of the mobile phase, unless you need a salt for a different reason.

Table: pKa Values of Commonly Used Buffer Ions (at 25° C)

Buffer	pKa
Acetate	4.75
Ammonium	9.24
Borate 1	9.24
Borate 2	12.74
Borate 3	13.80
Citrate 1	3.13
Citrate 2	4.76
Citrate 3	6.40
2-(N-morpholino)ethanesulfonic acid (MES)	6.15
Oxalate 1	1.27
Oxalate 2	4.27
Phosphate 1	2.15
Phosphate 2	7.20
Phosphate 3	12.38
Trichloroacetic acid	0.52
Triethanolamine	7.76
Triethylamine	10.72
Trifluoroacetic acid	0.50
Tris(hydroxymethyl)aminomethane (Tris)	8.08

Q.: What can I do to improve the method?

A.: If you are using the wrong buffer for the intended pH, you should simply select a more suitable one from the table. Often, the substitution does not affect the separation, otherwise you may need to make additional adjustment.

Sometimes, a substitution of the buffer is not possible due to constraints imposed by the detector. We mentioned already the need to use volatile buffers with mass spectrometers. Buffers based on carboxylic acids are less suitable for low-UV detection due to a high background absorption, which results in excessive noise. If you can not find a detection compatible buffer other than the one that you are using, you are in trouble. You can either live with the retention variability as it is, or you should redevelop the method at or near the pKa of the buffer that you are using. This will clearly change the chromatogram drastically, and you need to reoptimize the method from scratch. This may not be good news, but you will be better off in the long run.

I generally recommend for methods development to select the buffer and therefore the general pH range first, then use solvent selectivity to fine-tune the separation. This methods development strategy is fast and highly efficient. It also prevents you from ending up in the wrong place with respect to buffer pH. You might want to consider this approach in your next methods development.

16. Column Equilibration

Q.: How long do I need to equilibrate my column?

A.: Column equilibration is rather simple, once the basic principles are understood. How long you need to equilibrate depends primarily on the state of the column before equilibration, the concentration of the ingredients of the mobile phase and the retention factor of these ingredients. You have to realize that the kinetics of equilibration are rather fast, and the local equilibrium in the column is reached practically instantaneously. Therefore, equilibration is primarily limited by the speed with which mobile phase constituents are transported into the column or can be removed from the column. Let us first discuss the cases where the column needs to be equilibrated with new constituents of the mobile phase.

In equilibrium, the concentration of mobile phase constituents that are adsorbed on the surface may be between 1 and 20 µmol/m^2. A typical packing has a surface area of 300 m^2/g, and a typical HPLC column contains around 0.5 g of packing per mL of column volume. Consequently, the surface area per column volume is around 150 m^2/mL and the concentration of adsorbed ingredients is 150 to 3000 µmol/mL.

Solvents usually have a high surface concentration. Let us take methanol with a molecular weight of 32 as an example. Using a stationary phase concentration of 3 mmol/mL, we need approximately 100 mg of methanol per mL of column volume to completely saturate the surface. If we start with a surface that contains no methanol whatsoever and if the concentration of methanol in the mobile phase is 10% or higher, one mL of mobile phase is sufficient to deliver the necessary amount of methanol to the column. For complete equilibration, add a factor of 2 to 3 or so, and your column is equilibrated. This means that for mobile phase ingredients that are present at high concentration, equilibration is fast. This is one of the reasons why gradient chromatography works as well as it does. On the other hand, if the concentration of mobile phase additives is low, long equilibration times result. For example, if the column has never seen methanol and the mobile phase concentration is only 1% methanol, then we need at least 10 column volumes to deliver the correct amount of methanol to the column. In such a case, other mobile phase equilibria may actually complicate things. Assume that the column was originally equilibrated with acetonitrile, and we want to equilibrate it now with a mobile phase containing 1% methanol. Methanol needs to substitute for the acetonitrile adsorbed on the surface. This equilibration is not favorable to methanol, therefore a longer equilibration time may be anticipated. But we can speed up the process by washing the column first with a mobile phase containing a high methanol concentration and then reducing the concentration to the 1% level.

Even larger equilibration volumes may be needed, if the concentration of an ingredient of the mobile phase is still lower. Such an example are ion-pairing reagents. They are typically used at concentrations of 5 mmol/L (5 µmol/mL). Luckily, their surface concentration is also usually rather low, between 1 and 3 µmol/m^2. We can go through the same calculation as before and find that we need between 150 to 450 µmol of ion-pairing reagent per mL of column volume. At the given concentration of ion-pairing reagent in the mobile phase, we need between 30 and 150 column volumes for delivering the necessary amount of ion-pairing reagent to the column. Complete equilibration will necessitate still larger volumes of mobile phase.

Consequently, in the first case under discussion, the factor limiting the equilibration time is the concentration of the relevant ingredient in the mobile phase and its final surface concentration. If we want to speed up equilibration, it might be advantageous to increase the concentration of the critical mobile-phase constituent.

Q.: Let me summarize what you said. If we need to equilibrate the column with a new constituent of the mobile phase, we need to take into consideration how much is needed to equilibrate the column and how much is dissolved in the mobile phase. Then we can estimate, how long it takes to deliver the needed amount of this constituent to the column. But what about the opposite case, when we need to remove an ingredient from the column?

A.: Correct. In the second case, the factor determining equilibration is the retention factor of the compound that is adsorbed on the surface in the new mobile phase. The organic solvents typically used in reversed-phase chromatography have a low retention factor in all typical mobile phases. This is not the case for polar solvents in normal-phase chromatography. For example, the retention factor of methanol on silica with hexane as mobile phase is very large. To remove methanol from the surface of a normal-phase sorbent, we are better off to wash the column first with a solvent of intermediate elution strength. It may be best to choose the solvent that is used to modify the elution strength of hexane. If ethyl acetate is the polar constituent of our hexane-based mobile phase, we should first wash the column with ethyl acetate to remove the methanol. Only then should we equilibrate it with the final hexane-ethyl acetate mobile phase.

If we understand what we need to do to equilibrate the column fast, we can design efficient equilibration protocols.

Therefore it is important that we know the history of the column.

Another example of strongly adsorbed mobile-phase constituents are ion-pairing reagents. It is generally recommended to dedicate columns used with ion-pairing reagents to ion-pairing applications because these reagents are difficult to remove from the surface. These reagents are strongly retained on reversed-phase packings for two reasons: one is hydrophobic interaction, the other is polar interaction. If we had to deal with hydrophobic interaction alone, we could wash our packing with a strong organic solvent such as THF. But ion-pairing reagents also interact strongly with surface silanols. Cationic ion-pairing reagents, such as tetrabutylammonium salts, are particularly difficult to remove. Therefore, any washing procedure must take this complex interaction into account. Without special washing procedures, a complete removal of ion-pairing reagents is practically impossible.

One of the most difficult equilibration problems is the equilibration with water in normal-phase chromatography. Water is usually present only in very small amounts, and it is strongly retained on normal-phase packings, especially silica and alumina. Due to the strong retention of water, equilibration with a dry hydrocarbon mobile phase may take several days. To remove water from silica, it is preferred to use a protocol with sequentially weaker solvents. A possible sequence is methanol, ethylacetate, methylene chloride, hexane. This is a faster way to obtain a "dry" column then to attempt to wash a "wet" column with hexane.

Q.: Therefore, if the mobile phase does not contain strongly retained components at low concentrations, I should observe fast equilibration. What is the problem, if retention times nevertheless drift slowly?

A.: In such a case I would suspect that the cause of your retention time drift is not related to equilibration per se. It is possible that the cause of the retention time drift is something other than a column phenomenon. Is the mobile phase composition slowly changing due to evaporation? Is the temperature in the lab changing slowly? Maybe some component of your sample is accumulating on the column. Maybe we are simply looking at column aging, for example through hydrolysis of the stationary phase. The latter two phenomena are usually put into the category of column conditioning rather than equilibration. We will discuss column conditioning in a separate HPLC Troubleshooting column.

17. Column Conditioning

Q.: What is column conditioning?

A.: In the last troubleshooting column we talked about column equilibration. Column equilibration comprises all phenomena that are reversible, while column conditioning changes the column irreversibly. By column conditioning you are changing the product that the manufacturer has delivered to you, and the reproducibility of this step is your responsibility. I generally advice against conditioning, but there are some circumstances where column conditioning is unavoidable.

Q.: Please give some examples of column conditioning!

A.: My first example is a commonly used procedure. However, I want to point out that I do not recommend to use this procedure. If you are using a strongly acidic mobile phase, pH 2 or less, with a fully endcapped C18 column, you will fairly rapidly hydrolyze the endcapping groups. Consequently, the column will have a much larger silanol activity than what has been delivered from the manufacturer. This may influence the selectivity of a separation. It is possible that somebody has developed a method using a strongly acidic mobile phase, and by the time that methods development was complete the column had already changed. When a brand-new column is later used for the same assay, the selectivity of the separation may be different. But the separation comes back, when the column is "conditioned" for a day or two with the acidic mobile phase. As you can see, this is a permanent change of the column outside the manufacturer's specifications. I recommend against such a procedure, but it is practiced in some labs, and detailed column conditioning protocols have been set up for new columns. Instead of changing the properties of a column with such a conditioning process, I recommend to explore the possibility to redevelop the method on a non-endcapped packing. This avoids such a conditioning step in the future.

Another example of column conditioning occurs when aminopropyl bonded phases are used in aqueous solvents. The most common application is the use of this column for the separation of carbohydrates by hydrophilic interaction chromatography. Typical mobile phases are water/acetonitrile mixtures with 60 to 90% acetonitrile. When an aminopropyl column is exposed for the first time to an aqueous eluent, the high concentration of the amino groups in the pores of the packing creates a basic pH, which results in a slow hydrolysis of the silica and the bonded phase. The amount of bonded phase that is washed off decreases exponentially with time, and soon nearly stable retention times are achieved. However, the column has changed significantly from its original properties and should not be used for normal phase separations any more without specifying the exposure to the aqueous eluents as part of the column history.

If you want to use aminopropyl bonded phases for the separation of carbohydrates, there is just no way around this conditioning problem. Some manufacturers offer columns that are dedicated for this application. In this case, the manufacturer has performed the conditioning step for you. Since this is performed with a fixed protocol to fixed specifications, you are better off to purchase a

preconditioned column instead of doing the conditioning yourself.

Another unpleasant, but apparently unavoidable conditioning phenomenon happens with columns used in the separation of proteins, very specifically with diol bonded phases used for the aqueous size-exclusion chromatography of proteins. It has been observed that for some proteins the initial injections give smaller peaks than later injections. This has been attributed to non-specific binding of protein to adsorptive sites on the packing. To avoid this, it has been proposed to inject first a large amount of a protein, for example bovine serum albumin, to condition the column and saturate the active sites. In general, I do not recommend this conditioning procedure and suggest that you should observe for yourself, whether or not your sample exhibits such a phenomenon. When you observe an increase in peak height with subsequent injections, you should make sure that this is not caused by carry-over in the injector.

The last example that I want to discuss does not fit my definition of conditioning, but is considered a conditioning step by many people. The example involves dry reversed-phase columns. It is possible that a column has dried out during storage, because the fittings were not tightened well. Also, radial compression cartridges are shipped dry. In these cases, the column needs to be wetted first with an organic solvent, such as methanol or acetonitrile. This drives the air out of the pores and wets the surface. Now you can exchange the methanol or acetonitrile for your mobile phase and obtain reproducible retention times. In the case of a column that has dried out accidentally, a problem may arise if the column has been stored in a mobile phase containing buffer or salt. The precipitated buffer may give rise to high backpressure during the reconditioning step, and you may need to re-equilibrate the column at low flow rates.

A related phenomenon is "hydrophobic collapse". Very hydrophobic, well endcapped reversed-phase packings may lose retention in highly aqueous mobile phases. It has been observed that this can happen suddenly, for example when the flow through a column is stopped. In other cases a gradual decrease in retention has been observed over the course of a few days. In both cases, retention can be restored by washing the column with an organic solvent for a few column volumes and re-equilibrating it with the mobile phase.

18. Complex Sample Matrices

Q.: I am studying the metabolism of a newly developed drug. The sample medium is blood plasma. Despite the fact that I am using a sample preparation technique, I still get a significant amount of interferences eluting in the chromatogram. In addition to this problem, the drug recovery from my spiked samples varies more than I would like. What can I do?

A.: You are dealing with one of the most difficult separation problems. Blood contains an innumerable number of compounds that can interfere with the detection of your analytes. In addition, the number of interferences increases as the analyte concentration decreases. Therefore, the sample preparation technique is an essential part of the chromatographic method and needs to be optimized together with the chromatographic method itself. The detection method for the compound(s) of interest is, of course, an important part of the method. More selective detection methods such as derivatization and mass spectrometry simplify the separation problem significantly, but these techniques are not available to everybody.

Let us discuss therefore some of the options that you have in sample preparation. Plasma samples contain a significant amount of salt and proteins that can precipitate or adsorb on reversed-phase packings. The adsorbed protein can easily foul the column, resulting in changes in the separation and ultimately clogging the column. Although packings have been designed for the direct injection of plasma samples, most analyses are performed using classical reversed-phase packings. If one desires a reasonable column life, sample preparation is unavoidable.

There are several sample preparation techniques available for the pretreatment of plasma samples. The simplest one is a protein precipitation. This technique entails the addition of an organic solvent, for example acetonitrile, to the plasma sample. At least two milliliter of acetonitrile should be added for every milliliter of plasma. A significant amount of the proteins present in the serum are precipitated in the presence of the organic solvent. However, a large fraction of the proteins remains soluble and can cause interferences with the analytes. Also, all low molecular weight compounds, for example lipids, stay in the sample.

Another sample preparation option is liquid-liquid extraction. A separation using this technique requires two immiscible solvents. Polar, water miscible solvents can not be used for the extraction. Due to this fact, liquid-liquid extraction works best for more non-polar analytes; it is not suitable for very polar analytes. Since many samples also have very polar metabolites, this method has limitations.

A third option - and the one which is most frequently used - is solid phase extraction. It is a very convenient technique, and due to its similarity to chromatography, solid phase extraction is a very popular technique with chromatographers. One can use several extraction techniques to clean the sample; reversed-phase chromatography and ion-exchange are the techniques that are most commonly employed. As a result of its versatility and simplicity, solid phase extraction is the sample preparation method that has the broadest range of applicability. Therefore, it is the sample preparation method that I commonly recommend.

Q.: I am using solid phase extraction for sample preparation. The SPE cartridge is a reversed-phase cartridge. I am experiencing low, variable analyte recoveries.

A.: Let us examine the SPE procedure in more detail. The general procedure of a reversed-phase solid-phase extraction method is as follows:

Step 1: load the sample

Step 2: remove polar interferences

Step 3: elute analytes, leaving behind the more non-polar interferences

Before loading the sample onto the SPE cartridge, we must first activate the cartridge. This is typically done by initially washing the reversed-phase cartridge with an organic solvent, usually methanol. Methanol is then replaced by a polar solvent, usually water or buffer. The buffer should be at the same pH as the plasma sample. This washing procedure preconditions the cartridge to the sample. The sample is then loaded onto the preconditioned cartridge. It is important that the cartridge does not dry between the conditioning step and the sample loading step. Cartridge drying may be one of the causes of low and variable recoveries on C18-type sorbents.

After the sample is loaded onto the cartridge, removal of the more polar interferences is necessary. Table 1 gives the constituents of a serum or plasma sample. Typical polar interferences are salts, carbohydrates, and a significant amount of the proteins. Salts and most of the carbohydrates do not adhere to a reversed-phase sorbent and are removed without difficulty. The elution solvent for most of these very polar interferences is water or a buffer solution at the pH of your choice. Some proteins are also eluted using these conditions.

To remove all the interferences that are more polar than your analytes, it is best to wash the cartridge with a solvent that is more polar than your mobile phase. In general, most protein interferences are removed using a wash of around 5% organic solvent. You can also change the retention your analytes relative to the interferences by changing the pH of the wash solution. You have to examine this step carefully to make sure that you are not loosing a portion of your analytes in this step.

Elution of the analytes requires a stronger eluent than your mobile phase. Many people use a straight organic solvent, most commonly methanol, for the elution. Methanol is easy to evaporate, thereby resulting in a sample that can be easily reconstituted in mobile phase. However, an elution step employing methanol is neither specific nor selective. In your case, a potential problem can arise from strong interactions of your analytes with surface silanols, which are not broken by methanol. If this is indeed the case and the cause of your variable recovery, you need to consider a sorbent that does not contain silanols. Reversed-phase sorbents that are not based on silica are commercially available.

Alternatively, more specific extraction procedures can be designed. A manipulation of the pH in conjunction with an increase in the organic modifier content is something to consider. You should compare the recovery of your analytes at neutral pH and at acidic pH. Of course, such an approach will work only for compounds with ionizable functional groups. However, the description of your analytical problem strongly suggests that an analyte-silanol interaction is a possible cause of your recovery problems. In such a case, an alternative to silica-based sorbents might be the best solution.

19. Hydrophobic Collapse

Q.: I have two reversed-phase C18 columns of the same type from the same manufacturer that show a significant difference (40%) in retention. What is the problem?

A.: This is obviously a serious problem. The retention time reproducibility from column to column should be significantly better, in the range of +/- 5%. For higher quality packings, the retention time reproducibility is on the order of +/- 2%, but achieving this level of reproducibility requires good temperature control as well. Knowing this, the column-to-column reproducibility observed in your case is obviously unacceptable. Let us investigate, what the problem could be. First, let me ask the question if one of the columns has been used extensively. It is not uncommon to find differences in retention times between old columns and new columns. This can be especially noted, if the old column has been used close to the limits of the bonded phase pH stability. Also, it is not unusual to observe a retention time change if sample constituents have accumulated on the column. Accumulation of sample constituents can cause a drastic change in retention. In such instances, it is necessary to examine a suitable washing protocol to remove the sample constituents from the column. So, which of the two columns has been extensively used?

Q.: Neither! Both columns are new, fresh out of the box. I would have expected to achieve much better reproducibility from brand-new columns.

A.: Indeed, column-to-column reproducibility should be much better. Even batch-to-batch reproducibility of the packing should be better than what you are observing. Batch-to-batch reproducibility should be better than +/- 10%, even better than +/- 5% if you purchase the column from a reputable manufacturer. Have you investigated whether both columns were prepared from the same batch of packing?

Q.: Yes, I have. The manufacturer declared that both columns were prepared from the same batch of packing.

A.: This makes the retention time differences even less understandable. It appears that the only difference between the two columns is the packing process. Column packing processes are sufficiently reproducible such that packing densities do not vary by more than +/- 2%. Therefore, retention times should not vary by more than +/- 2%. Even if uncertainties in the mobile phase makeup were added to the

packing process uncertainty, it still would not explain the large retention time discrepancies that you are observing.

Typically, errors in the mobile phase composition could be an explanation of your observation. If the organic content of the mobile phase varies by 1%, you may observe retention time differences of 10%. On the other hand, differences in the concentration of a mobile phase buffer of up to 20% result in retention time differences of only 1% in many cases. Another possibility would be some error in the mobile phase pH. If the pKa of your analytes is close to the pH of the mobile phase buffer, a 0.1 unit change in the pH can result in retention time differences of 10%. Therefore a careful check of the mobile phase pH is necessary.

Other external influences are less pronounced. Changes in temperature can affect retention by roughly 10% per 5 °C. Therefore, changes in temperature are not likely to be the cause of your problem. If you are dealing with an ion-pair separation, it is not unusual to unknowingly use columns that have not been completely equilibrated with the mobile phase. It may often take several 100 mL of mobile phase to equilibrate the column with an ion-pairing reagent, especially if the mobile phase concentration of the ion-pairing reagent is low.

Q.: I am not using an ion-pairing reagent. Also, let me add that both columns were tested with the identical mobile phase, and the results are reproducible. This excludes the influences that you just described, doesn't it?

A.: This makes it even more difficult to explain your results. How were the columns treated before you tested them?

Q.: Both were treated in exactly the same way. I took them out of the box and I equilibrated them with mobile phase.

A.: Hmm... This is getting difficult now. To summarize, we have two new columns, prepared from the same batch of packing material, giving significantly different chromatographic results. The only rational conclusion is that there are some differences in the column equilibration causing these changes in retention time. What is the concentration of organic solvent in the mobile phase?

Q.: The mobile phase is nearly 100% aqueous. It consists of 98% buffer and 2% methanol. I removed the columns from the box and equilibrated them with the mobile phase for one hour before I started to inject my samples.

A.: Now we are getting closer. The low concentration of organic solvent in the mobile phase raises the possibility that the differences in the retention times observed by you are due to differences in the wetting of the columns. Very hydrophobic, well endcapped C18 columns can exhibit significant differences in retention depending on their prior history. If one of the two columns has inadvertently dried or partially dried during the shipment, it can exhibit significantly lower retention times than a fully wetted C18 column. This is due to the fact that water and mobile phases with a very high water content do not wet the hydrophobic C18 surface very well. When you use a mobile phase with a high water content, only part of the C18 surface is therefore available for retention. Consequently, the retention factors are significantly lower than the ones observed on a well wetted column. You can eliminate the problem by reconditioning the column with 100% methanol, then re-equilibrating it with mobile phase. This "rewetting" step ensures that the entire surface of the packing is available for interaction with the analytes. After you have done this for both columns, the retention times should be very reproducible, perhaps as close as 1%. Based on the description of the problem and the fact that we have eliminated all other possibilities, I am very certain that this is the cause of your retention time problem.

20. Baseline Noise

Q.: I set up a method a while ago, and it was working fine for a long time. Recently, the baseline noise has increased significantly, about 5-fold compared to where it was earlier. The method is a reversed-phase method, and I am using a UV detector. What could be the problem?

A.: The very first things that come to mind in your case are either a dirty cell window or a dirty mobile phase. Let us discuss the dirty mobile phase first. Although this may be more difficult to troubleshoot, it does not require any disassembly of the detector cell for cleaning the window.

If you are using a reversed-phase method, you are likely to use an aqueous mobile phase with methanol, acetonitrile or tetrahydrofuran (THF) as the organic modifier. For any one of the organic solvents you need to make sure that they are HPLC-grade. If other grades are used, they often contain small amounts of miscellaneous chemicals that can influence the UV-background of the solvent. Especially when using THF, you need to make sure that you are using an HPLC grade. Other grades of THF contain antioxidants that can be detected by UV detectors and therefore affect the UV background. Since we are talking about THF: use of old THF may also result in a high UV background due to the formation of peroxides.

The other solvents usually do not deteriorate with time, but methanol is less suitable than acetonitrile for detection at low UV wavelengths. However, this is an intrinsic property of the solvent and not something that would change with time and show up after long use. But there are other ingredients of reversed-phase mobile phases that can create background problems. In many reversed-phase methods, buffers are used to control the pH of the mobile phase. Most inorganic buffer salts like phosphate are of sufficient purity that an increase in the UV background is not very likely. But it is generally

recommended to use high-quality reagents, for example p. a.. If you are using triethylamine or related amines as a buffer ingredient, then it is possible that the UV background of your buffer varies with the purity of the amine. The quality of amines also deteriorates with age, largely due to oxidation.

Under some circumstances, water can also be the source of the problem that you have described. Usually, water is subjected to a purification scheme such as ion exchange or reverse osmosis. Problems with impurities can occur, when the cartridges in the purification system are saturated and need to be replaced. This can increase the background noise of the chromatographic method. A good quality control scheme for the water generated by the purification device should warn the user when the useful life of the purification cartridges has been reached. This is best accomplished by running a reversed-phase gradient from water to 100% organic solvent using a dedicated reversed-phase column. The UV-trace obtained from this gradient is then compared to a standard good UV-trace.

If you are using a dual-pump gradient system, you can use an old column between the pump for the more aqueous component of your mobile phase and the point where the gradient is mixed. Impurities in your more aqueous mobile phase are adsorbed on the precolumn, and you get a cleaner baseline. Of course, it is necessary to clean the precolumn on a regular basis. Unfortunately, this elegant solution to the problem of removing mobile phase impurities is only available to users of HPLC-systems with dual-pump gradients.

Generally, you will see many more solvent impurities when your detection method uses the low UV range between 200 and 215 nm than when you are using the standard wavelength of 254 nm. This can make a significant difference in the UV background.

Q.: In this assay, I am also using 254 nm as the standard wavelength. I have already considered some of the issues discussed above, but have not yet been able to solve the problem. How about instrument issues like a dirty cell window?

A.: This is exactly the point that I want to discuss next. If we cannot find a problem with the mobile phase, the cause of the increased noise is most likely related to the detector. The most common cause of increased baseline noise is dirt in the detector cell or a dirty detector window. Of course, an air bubble in the detector cell will look exactly like dirt, but it can be removed easily by briefly pressurizing the detector cell or flushing the cell at higher flow rate. Often, a short piece (4" long) of 9/1000 tubing can be used in the waste line to prevent the formation of air bubbles in the detector cell. However, if dirt has accumulated in the detector or on the cell window, this simple trick will not change the phenomenon at all.

The only way to clean the detector cell and the detector window is to disassemble the detector cell and clean the window manually. The disassembly of the detector cell will also give you access to the body of the detector cell, and any material that has accumulated in this area can be removed without difficulty. To clean the cell window, one needs to be careful to use gentle techniques. Often, the window can be treated in an ultrasonic bath with a solvent that will remove any film on its surface. You can try a soap solution or tetrahydrofuran (HPLC grade). This is a rather mild treatment and should always be tried first. If such a simple washing does not remove the film, than one can try to clean the window by rubbing it with a suitable piece of wet paper or cloth. You can get suitable tools by going to a camera store and ask for lens cleaning tissue and fluid. This should solve the problem.

Since this procedure is involved, it is better to prevent a contamination of the detector window. Unfortunately, there can be many different causes for dirty detector cell windows, and in many cases it is not at all clear, what causes the contamination of the window. But generally, one should prevent precipitation by using the same precautions as for a column.

If the detector cell is clean and the problem still persists, then the likelihood is high that the detector lamp has aged. You should consult the detector manual to determine, if you can replace the lamp by yourself or if you need technical service. In most cases, the replacement of a UV detector lamp is something that can be carried out by the HPLC operator without difficulty.

If the problem persists after cleaning of the cell and replacement of the detector lamp, the increased noise could be due to issues with other parts of the HPLC equipment such as the integrator or data system. In such a case, it is best to have the system checked by trained service personnel.

21. Narrow-bore Columns

Q.: I understand that solvent consumption is dependent on the column diameter. I would like to reduce the solvent consumption in the laboratory and therefore would like to use 2 mm columns. Unfortunately, the first 2 mm columns that I purchased are not performing as well as the standard columns. What is the problem?

A.: Unfortunately, your experience is not unique. I get similar comments from many people who are trying small volume columns for the first time. However, we should not assume that the problem is necessarily a result of poor column performance. More frequently, the lower-than-expected column performance is due to instrument issues,

more specifically the instrument bandspreading of a standard HPLC instrument.

Before I explain this in detail, I would like to point out a quick way in which you can determine if system bandspreading plays a role in your standard assays. I guarantee that you will be surprised about the magnitude of the instrument effects.

Let us assume that you are running an isocratic method, and that your assay contains several analytes that elute at different retention factors. Check the plate-count that your instrument measures for the different analytes. In many cases you will observe that the plate-count increases with increased retention. Now note that there is no rule in HPLC theory that predicts a higher plate-count at increased retention. Therefore, the observed increase in column performance for the peaks with higher retention values is most often due to a decrease of the influence of extra-column bandspreading.
Since this effect is usually present to some degree with columns of standard diameters (4.6 mm, 4.0 mm and 3.9 mm), it will play a larger role with smaller diameter columns. In the following, I will discuss this effect in more detail and give you the equations that allow you to calculate the deterioration of column performance due to instrument influence.

In isocratic chromatography, the square of the peak width observed by the detector w^2_t is the sum of the square of the peak width inside the column w^2_c and the square of the peak width of all effects outside the column w^2_o:

$$w_t^2 = w_c^2 + w_o^2 \qquad (1)$$

The peak width contribution from the column alone increases with retention. The peak width contribution outside the column remains constant. This system contribution is also called extra-column bandspreading or extra-column effect.

The measurement of the extra-column bandspreading is very straightforward: disconnect the column and inject sample directly into the detector, using the same mobile phase and flow rate as you use for the HPLC analysis. You may need to dilute the sample, if the peak height exceeds the dynamic range of the detector.

In order to go further, we need to specify the contribution from the extra-column effect. We will assume that the peak width contributions are all measured the same way, specifically by either the tangent or the 4-sigma method. For this method, the contribution of the column to the total peak width is:

$$w_c^2 = 16 \cdot \frac{V_R^2}{N_C} \qquad (2)$$

In this equation, N_C is the column plate count and V_R is the retention volume of the peak of interest. What we are interested in is the apparent plate count N_a that the combination of instrument and column delivers. This can be derived from the previous two equations:

$$\frac{N_a}{N_C} = \frac{w_R^2}{w_c^2 + w_o^2} = \frac{16 \cdot V_R^2}{16 \cdot V_R^2 + w_o^2 \cdot N_C} \qquad (3)$$

If this ratio approaches 1, then the apparent column performance is similar to the true column performance. This is realized, when the extra-column bandspreading wo becomes negligible.

Q.: Can you show me with some concrete example calculations how the extra-column bandspreading deteriorates the column performance?

A.: Yes. Let us look at figure 1. In this figure, I have plotted the relationship between the plate-count ratio (equation 3) and the retention volume. I have assumed for this calculation that the column plate-count is 10,000, and that the extra-column bandspreading (measured as the peak width by the tangent method) is 80 µL. One can see that the true plate-count of the column is never reached, even at an elution volume of 10 mL. An elution volume of 10 mL translates to roughly a k' of 4 for a 4.6 mm x 150 mm column. The graph also shows tha at an elution volume of about 2 mL, which is roughly the point of elution of an unretained peak on this column, the real plate-count is only half of the maximally achievable plate-count.

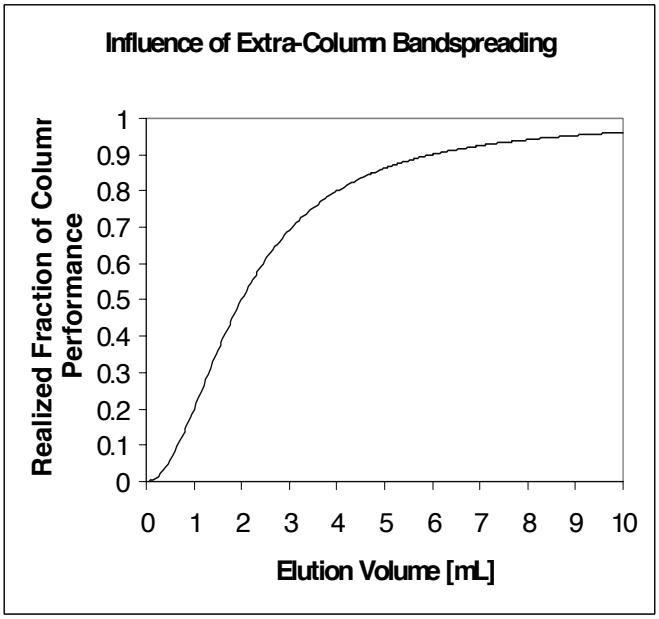

***Figure 1**: Influence of extra-column bandspreading on column performance*

Let us consider now a reduction of the column diameter to 2 mm. The column dead volume of such a column is only about 0.33 mL. This is the elution volume at which our first peak is eluting. At such a low elution volume, the extra-column effects are dominating the peak width, and less than 1/30th of the true column plate-count can be realized. The

elution volume of about 2 mL discussed above now corresponds to a k' of about 5. As we have seen above, one obtains approximately only half the plates of which the column is capable at this k'. Only at a retention factor of about 10 or higher, equivalent to elution volumes in excess of 5 mL, are we achieving good column performance again. For most chromatographic applications, a retention factor of 5 or lower is normal, and under these circumstances the performance of the column is reduced drastically by the extra-column effects. In summary, this is the fundamental reason for the low performance of 2 mm i.d. columns on normal HPLC instruments. Therefore, it is usually not the column that is the problem, but the bandspreading of the standard HPLC instrument.

Q.: This is fairly much in line with my experience. What can I do to improve the situation?

A.: We have to reduce the extra-column bandspreading to a significant level. To achieve roughly the same performance level you have experienced with the large diameter column, you need to reduce the extra-column bandspreading by the same factor as the change in column volume. In the case where you go from a 4.6 mm column to a 2 mm column, you should reduce the extra-column bandspreading effects roughly by a factor of 5. This means that if you had good performance on a standard instrument with a bandspreading of around 80 µL, you should strive for a system bandspreading of around 16 µL for use with a 2 mm column.

To do this, you usually have to reduce the length of the tubing from the injector to the column and from the column to the detector. Also, one usually needs to reduce the diameter of the connection tubing from the standard 9/1000" tubing to a 5/1000" tubing. Naturally, you should reduce the injection volume in proportion to the column volume. If your injection volume for the standard column was around 25 µL, it should be only around 5 µL for the 2 mm column. The next issue is the reduction of the bandspreading in the detector cell. You should use a detector cell that is designed for the smaller diameter column. This cell should have a significantly smaller volume than your standard detector cell. Commonly, 3 µL cells are readily available. Of course, that does not get you quite to the factor of 5 in volume reduction, but it is a start. The other thing that you have to realize is that a reduction in the detector cell volume usually increases the detector noise. Therefore if you need high sensitivity and have enough sample to inject, the step to a smaller diameter column is actually ill advised. Of course, your intentions were to reduce solvent consumption, and this goal can indeed be accomplished with smaller diameter columns.

22. Sample Solvent

Q.: Despite sample preparation, the concentration of my sample analyte is very low, and I need to achieve high sensitivity. When I inject 100 µL of the sample, I see already an unacceptable deterioration of the separation. What can I do to improve the sensitivity of the assay?

A.: There are several different techniques you can employ to increase the sensitivity of an assay. One of the first things to consider is the choice of the detector or the detection wavelength, if a UV detector is used. If several detection principles are possible, and if the different detectors are available in your laboratory, this might be a first approach. For example, fluorescence detectors are several orders of magnitude more sensitive than UV detectors, if fluorescence is an option. Also, detection in the low UV range can be significantly more sensitive than detection at higher wavelength. Of course, one also gets more signal from interferences in the low UV range, which can be counterproductive. Electrochemical detection can be very specific and highly sensitive, but electrochemical detectors are not readily available in many laboratories. Post-column and precolumn derivatization techniques can improve the detection limits of an analyte quite significantly, but the complexity of the procedures and/or the instrumentation makes this approach less desirable.

Q.: I have already explored the possibility of other detection methods. The analyte does not fluoresce, and the best UV-wavelength has already been chosen. Other detectors are not available in my lab.

A.: The next approach would be to preconcentrate the sample using either a liquid-liquid extraction or a solid-phase extraction technique. Solid-phase extraction is generally simpler and more predictable than liquid-liquid extraction, especially if you are using the same type of sorbent as you use for the HPLC assay. Therefore, if your HPLC assay uses a reversed-phase method, it is convenient to use a reversed-phase solid-phase extraction technique to enrich your analyte.

Q.: This is exactly what I am doing. The sample is concentrated on a reversed-phase sorbent. Then interferences are removed during the wash step, and finally the analyte is eluted from the sample preparation device. It is then injected into the HPLC instrument.

A.: May I ask you, which solvent you are using to elute the sample from the SPE device and what the HPLC mobile phase composition is?

Q.: I am using 1 mL of methanol to elute the analyte from the SPE device. The HPLC mobile phase is 50 : 50 acetonitrile : phosphate buffer, pH 7.2.

A.: This sheds some light on your problem. You said earlier that you experience a deterioration of the separation when you inject 100 µL of sample. Since the sample is dissolved in methanol, this is not a case of column overload, but a case of

inappropriate sample solvent. Methanol is a stronger eluent than the mobile phase. Therefore, the analyte will move more rapidly down the column as long as there is a high concentration of methanol around the analyte band. At low injection volumes, the sample gets diluted with mobile phase in the injector, the connection tubing, the column frit and the column top. The analyte then enriches rapidly on the column top and is separated from the excess methanol due to the fact that the analyte is moving down the column much more slowly than the methanol. At large injection volumes, the initial dilution of the sample with mobile phase becomes ineffective, and the sample band moves with the methanol at a higher velocity down the column. This leads to a distortion of the peak shapes, which in turn forces you to inject only 100 µL or less.

Q.: This is indeed the case. What can I do to solve this problem?

A.: There are two possibilities. The first suggestion is to evaporate the sample to dryness and then redissolve it in the mobile phase or a solvent composition whose elution strength is weaker than that of the mobile phase, for example 30% acetonitrile : 70% buffer. This is best done in the presence of an internal standard to monitor the concentration of the analyte, but you probably use an internal standard anyway for the sample preparation step. This is the classical approach used to eliminate strong solvents that interfere with the HPLC assay.

Another option is to dilute the sample with water (or buffer) and then inject more (1). For example, if the sample is diluted 1:1 or 2:1 with water, you can probably inject the entire sample (2 or 3 mL) without peak distortion. Assuming that you want to keep some of the sample for reanalysis, you should dilute the sample 2:1 with water and then inject 1.5 mL of the sample. Under these circumstances, the sample enriches on the top of the column and is then eluted as a sharp band with roughly 5-fold increased peak height compared to the current situation, without any distortion of the peaks. If your injection system lets you do this, this is clearly the more convenient approach.

Reference:

1. Uwe D. Neue and Ed Serowik, "Sample Dilution Increases Sensitivity and Resolution", Waters Column VI, 2 (1996), pp. 8-11

23. Gradient Scaling

Q.: I have developed a separation on a 150 mm x 4.6 mm column. There is one pair that is not well resolved, and I hoped to get better results from a longer 250 mm column. Unfortunately, this did not turn out to be true: the resolution of the critical peak pair is worse, and also other peaks have shifted in the chromatogram. The manufacturer says that both columns are from the same batch of packing. What is the problem?

A.: If the packing in the 15 cm column and in the 25 cm column is indeed identical, you should get the same separation but with improved resolution using the longer column. Your observation that the position of peaks is shifting indicates that the packing is not the same in both columns. Since the manufacturer insists that the packing in both columns comes from the same batch and since you have used the 15 cm column already for a while, one would suspect that the 15 cm column has aged during your method development and is not representative of the packing material anymore. This situation is not uncommon and is often the cause of significant frustration on the part of the chromatographer. For this reason I always recommend to verify and validate a new method after methods development using a new column.

Q.: I indeed have another 15 cm column of the same packing around. When I ran the same gradient on this column, I got practically identical results as on the other 15 cm column. This should prove that the packing has not aged, doesn't it?

A.: This is indeed a good possibility. But you said that you are using a gradient method. This complicates the discussion somewhat. How did you scale the gradient from the shorter column to the longer column?

Q.: I did not scale the gradient. I ran the same gradient on the short column and on the longer column. I used the same flow rate and the same gradient time.

A.: This is most likely the cause of the problem. When you use a gradient method on different columns, the gradient volume should be scaled in proportion to the column volume to give you results with identical elution patterns. Of course, this increases the analysis time for the longer column, just as in isocratic chromatography. There are a few additional complications that can occur due to the system delay volume. In the following, I will discuss the scaling of gradient methods in a little more detail.

To obtain the identical gradient profile between different columns, the gradient volume should be changed in direct proportion to the column volume. You started with a 15 cm column and wanted to obtain the same results on a 25 cm column. Both columns had the same internal diameter. Therefore, your gradient volume should be 25/15 = 5/3 larger for the longer column. If you keep the same flow rate on both columns, the gradient duration and other gradient event times should increase by 5/3.

The rule to change the gradient volume in proportion to the column volume also holds, when you change column diameter. If you want to scale a gradient from a 150 mm x 4.6 mm column to a 150 mm x 3 mm column you should reduce the gradient volume by a factor of 2.35. Generally, this will be automatic, since you are reducing the flow rate in

direct proportion to the column volume anyway. Under those circumstances, you can keep the gradient profile constant and get the same results.

All of the calculations until now assumed that the gradient delay volume of the instrument that you are using is negligible and/or has no influence on the separation. This is in general true for well retained compounds that elute late in the gradient. However, this is not necessarily the case for early eluting compounds. In the commonly used single-pump gradient systems, the gradient is generated on the low-pressure side of the pump, and on older systems there is a significant delay until the gradient reaches the top of the column. The elution pattern of early eluting compounds can be affected by this gradient delay volume. The ratio of the gradient delay volume to the column volume should be held constant, when we change column dimensions. Unfortunately, this can be a significant problem, when one wants to scale a gradient from a larger volume column to a smaller volume column. Fortunately, you want to scale the gradient from a smaller volume column to a larger volume column, which simplifies the situation.

In order to adjust the gradient delay volume, you first have to measure it for the system that you are using. This is best done by running a step gradient at 1 mL/min from methanol to methanol with a small amount of a UV absorber, for example 1 % acetone in methanol, without a column in place. One measures the time from the start of the program to the time that the step has reached half the height of the final concentration. Multiplying this time with the flow rate results in the delay volume of the instrument. Since the start of the programmed gradient at the column top will be delayed by this volume on the small column, we need to delay the gradient on the larger column by the same ratio of delay volume to column volume to obtain exactly the same gradient profile on both columns. For example, if the system delay volume was 1 mL when the 150 mm x 4.6 mm column was used, it should be 1.66 mL for the 250 mm x 4.6 mm column. Therefore we need to add an isocratic delay of 0.66 mL to the beginning of the gradient program for the 250 mm column to fully account for the differences in the gradient profile between the actual gradient obtained on the 150 mm column and the 250 mm column. After this is done, identical elution profiles are obtained on both columns.

24. Column Storage

Q.: I asked my colleagues how I should store my HPLC columns. Unfortunately, everybody gave me a different answer. What should I do?

A.: In most cases, there are many different ways in which an HPLC column can be stored that have little effect on column longevity. Therefore, it is quite possible that many of the answers that you got were correct. However, the best way of storing columns depends on the type of the stationary phase. Also, some column storage conditions are more convenient than others. Let me first discuss this in general terms, then I will discuss specific and special considerations that depend on the nature of the packing.

The most convenient way to store a column is in the mobile phase in which it is commonly used. The biggest advantage of this approach is that reequilibration of the column with the mobile phase is very fast. Therefore, one can get reproducible results within a short time after start-up. This approach is especially recommended for normal-phase chromatography, where a change to a storage solvent different from the mobile phase can result in lengthy reequilibration times. However, one needs to think this approach through very carefully. Bonded-phase columns often change slowly in the commonly used mobile phases. Therefore, the convenience of storing the column in the mobile phase needs to be balanced against the reduction in column life.

In the following, I will discuss storage conditions for various HPLC packings based on the nature of the packing. Let me first talk about silica and alumina. Both of these packings are very stable in the mobile phases in which they are commonly used. These mobile phases commonly comprise organic solvents with small amounts of polar modifiers, including water. Due to the low concentration of the polar modifiers, it often takes a considerable time to equilibrate the columns with mobile phase. Therefore it is best and most convenient to store the columns in mobile phase.

The situation is similar for polar bonded phases used in normal phase chromatography. Equilibration with mobile phase is somewhat more rapid than with silica or alumina; nevertheless, it is still fastest to store the columns in mobile phase. Some mobile phase ingredients are not suitable for certain columns. Therefore, they also should not be considered for column cleaning or column storage. An example is the incompatibility of amino columns with acetone.

Polydivinylbenzene-based packings such as SEC packings and ion-exchangers are chemically very stable, but they swell and shrink in different solvents. The manufacturers usually supply you with information about which solvents are compatible with a particular column. General statements can not be made, since the stability of the columns depends on the packing conditions. However, the best storage solvent for these columns is the solvent in which the columns are used.

For silica-based bonded-phase columns used in reversed-phase chromatography, the situation is more complicated. Water attacks the bonded phase, but the process is very slow in the commonly used pH range from pH 2 to pH 8. In addition, it depends on the nature of the bonded phase. The commonly used C18 and C8 columns are sufficiently stable to be used for several months with little change in their hydrophobicity. However, bonded phases based on shorter chains can hydrolyze measurably within a few weeks. Therefore, the best storage conditions for reversed-phase columns depend on the frequency of their use. If they are used every day, or even every few days, it is most convenient

to store them in the commonly used mobile phase. On the other hand, if they will not be used for an extended period of time, it is best to store them in a solvent that prevents hydrolysis, commonly acetonitrile or methanol.

However, some reversed-phase columns are not stable in these solvents. Some cyano columns can void when stored in organic solvents. For these columns it is best to use the mobile phase for storage, despite the danger of faster hydrolysis. Read the manufacturer's recommendations!

Amino columns are often used in acetonitrile/water mobile phases for the analysis of carbohydrates. Unfortunately, the amino group creates a basic pH in the pores of the packing, which leads to a slow loss of the functional group. Therefore, amino columns used for this application are best stored in acetonitrile instead of the mobile phase, at least for long term storage.

Additional considerations need to be made for the long-term storage of columns in highly aqueous mobile phases that allow the growth of algae or bacteria. Often, the addition of sodium azide to the storage buffer is recommended. If feasible, organic solvents are better solvents for long-term storage.

As a final remark, it should be emphasized that it is always a good idea to record the storage solvent in a permanent file. This can be done conveniently by recording the column type and the serial number. When you create such a file, it is also worthwhile to record the date and time of the last use of the column, as well as the number of analyses run since the last storage. Such a column history file gives you a permanent record that allows you to go back and check column use and column life time. An alternative and less efficient way is to mark the column with your choice of the storage solvent. Of course, this approach works best if you always use the same storage solvent for this particular column.

25. Paired-Ion Chromatography

Q.: I am using a chromatographic method on a C18 column that employs an ion-pairing reagent, octyl sulfonic acid. The mobile phase consists of 20% methanol and 80% of the aqueous buffer solution. The aqueous buffer consists of 5 mM of the ion pairing reagent and 50 mM acetate buffer; the pH of the aqueous solution is adjusted to pH 4.0 with acetic acid. What bothers me is the lengthy equilibration time necessary to get consistent retention times. What's wrong?

A.: Most likely nothing is wrong. Long equilibration times are typical when ion-pairing reagents are used. Let me discuss this in detail. Once we understand what the issues are, we also should be able to find a faster protocol.

Ion-pairing reagents are used with reversed-phase columns to add ion-exchange properties to the stationary phase. Purely hydrophobic interaction is influenced very little as the ion-pairing reagent is added to the mobile phase. Therefore, the ion-pairing reagent opens a new dimension to the separation.

The columns used in ion-pair chromatography are mostly C18 columns, just as in your case, but C8 columns often work as well. The ion-pairing reagent is adsorbed on the surface of the reversed-phase packing material. The concentration of the ion-pairing reagent on the surface of the packing depends on its concentration in the mobile phase. The retention times of compounds that interact with the ion-pairing reagent depend on its surface concentration. If the mobile phase concentration is low, the retention of analytes with a charge opposite to the charge of the pairing reagent increases in direct proportion to the concentration of the ion-pairing reagent. If the concentration is high, around 10 mmol/L, the retention of such analytes often becomes independent of the concentration of the ion-pairing reagent. The retention of non-ionic compounds is nearly unaffected by the concentration of the ion-pairing reagent. Therefore, one can use the concentration of the ion-pairing reagent to influence the retention of ionic compounds relative to non-interacting compounds. This makes it possible to affect the selectivity of a separation.

These observations are only true for columns that are fully equilibrated with the ion-pairing reagent. Since the ion-pairing reagent is adsorbed onto the surface of the packing, it may take some time until the column is equilibrated with the reagent. The surface concentration of the ion-pairing reagent depends on its mobile phase concentration as well as on the organic content in the mobile phase. But we can make an estimation on the amount of reagent that is adsorbed on the column by assuming that the surface concentration is around 1 µmol/m^2. A column may contain around 2 g of packing material, with a specific surface area of 300 m^2/g. This means that it contains around 0.6 mmol of ion-pairing reagent at full equilibration. If the mobile phase concentration of the ion-pairing reagent is 5 mmol/L, you need about 120 mL of mobile phase to send enough ion-pairing reagent to the column to achieve a surface concentration of 1 µmol/m^2. In reality, a complete equilibration may actually take somewhat longer, maybe 150 mL of mobile phase. If you equilibrate the column at 1.5 mL/min, this means that you need to wait for 100 minutes - nearly two hours - before the column is equilibrated and ready for analyses.

Q.: This appears to be the problem that I am running into. What is the solution?

A.: What you are probably doing is to convert the column to an organic solvent for storage. While this is a good approach for normal reversed-phase separations, it is a problem when you are using ion-pairing reagents. The best solution to the problem is to store the column in mobile phase, at least for overnight storage and for storage over weekends. If you do this, the column should be completely equilibrated with the mobile phase after only a short purge of 10 or less column volumes. Of course, since you are using a buffered mobile

phase, the fittings and end-caps of the column should be well tightened to prevent the column from drying out during storage.

Usually, I recommend storing a column in an organic solvent, if the column is not used for a period of time longer than a weekend. However, in the case of ion pairing reagents, I generally recommend to store the column in mobile phase due to the lengthy equilibration times. Only if you intend to not use the column for an extended period of time, maybe around a month or so, should you consider storing the column to an organic solvent.

Q.: Does this apply also to reagents like triethylamine, which are often used to suppress the tailing of basic compounds?

A.: No. These reagents are primarily constituents of mobile phase buffers. They are adsorbed on the packing, but due to the high concentration in which they are typically used, there is little concern about lengthy equilibration. Buffers are commonly used at concentrations around 50 mmol/L, and the concentration of the buffering reagents on the surface of the packing are much lower than the common concentrations of ion-pairing reagents. Therefore, the equilibration of the column with the buffering reagent is much faster than the equilibration with the ion-pairing reagents. Since the issue of long equilibration times does not exist with these simple buffering reagents, columns should be stored in an organic solvent if they will not be used for several days or longer.

26. Hydrolytic Stability of Reversed-Phase Packings

Q.: Some manufacturers claim that their silica-based reversed-phase columns can be used to pH 9 or 10, while others recommend not to use a pH above 8. I recently was forced to use a reversed-phase column at pH 9, since this was the only condition which separated all the compounds of interest without interferences. While this was outside the recommended use range of the manufacturer, column life time was quite acceptable. I now wonder how seriously one should take the manufacturers' recommendation about the pH range over which their columns should be used.

A.: This is indeed a good question. It is probably best answered by your experiments: if you are happy with the lifetime that you are able to achieve than there is nothing wrong with the use of a higher pH than the manufacturer is recommending. However, I would make sure that the same separation can be obtained on a brand-new column as on a column that has been used for a while under your conditions. If this is indeed the case, then there is little reason to expect trouble.

The pH stability of packings is a more complex issue than can be stated in a simple rule. Let me explain this in a little more detail to provide a better understanding.

At alkaline pH, hydroxyl ions (OH^-) can attack and dissolve the silica. The speed of the process depends on the concentration of the hydroxyl ions in the mobile phase, their access to the surface of the silica, and the solubility of the dissolved silica in the mobile phase. As you can see, the concentration of the hydroxyl ions, which is determined by the pH of the mobile phase, is only part of the story. In addition, the speed of all these processes is a function of the temperature. What works well at room temperature may represent an unacceptably short column life at 60 ºC.

The access of the hydroxyl ions to the silica plays a crucial role in the stability of a packing. A dense coverage of the surface of the silica with a C18 or C8 ligand improves the stability substantially. Also, the protection of the surface through a good endcapping process is important. The total hydrophobic ligand density is probably a reasonable measure of the protection of the silica surface from the attack of the hydrophilic hydroxyl ions. Consequently, one can expect that modern packings with a high surface coverage are more stable than packings with a low surface coverage. In addition, the quality of the endcapping process may play an important role.

At alkaline pH, the silica itself dissolves. Therefore, the nature of the ligand plays a secondary role only. The stability of a bonded phase based on a monofunctional silane is not dissimilar to the stability of a bonded phase based on a trifunctional silane, at equal coating level. However, the access of the hydroxyl ions to the silica surface plays a crucial role. Therefore, the stability of a monofunctional bonded phase with a bulky isopropyl side chain is inferior to a standard bonded phase, simply because the maximally achievable surface coverage is lower.

If the column is run continuously under the same mobile phase conditions and is never washed with an organic solvent, the desorption and dissolution of the bonded ligand can be very slow. Therefore, retention time changes can be very small. Nevertheless, the underlying silica is dissolving slowly. As a consequence, a sudden collapse of the column can be experienced, with little indication before this event as to the slow deterioration of the column.

Of course, the silica density is a crucial factor in such a case. Silicas with a high specific pore volume are less stable than silicas with a low specific pore volume, simply due to the fact that they have a weaker skeleton. The porosity of a silica may range from 40% to 70%, but the change in strength may easily be 10-fold. Therefore, one can expect substantial differences in the properties of a bonded phase simply based on the density of the parent silica. In addition, the specific surface area decreases as the pore size of the packing increases. Therefore, packings with a larger pore size are more stable than packings with a small pore size, all other parameters kept constant (1).

The nature of the buffer ingredients is also crucial to the stability of the packing. At equal pH, organic buffers such as a Tris, citrate or HEPES buffer are much less aggressive than the commonly used phosphate buffers (2). Also, borate and

glycine buffers have been shown to be less aggressive even at pH 10.

It should be pointed out that in the literature studies available the stability of a packing is commonly tested under isocratic operating conditions. If you are forced to switch occasionally to an organic solvent to clean the column from contaminants, you may also wash off unbonded but adsorbed ligand. Therefore, cleaning cycles may impact the stability of a column quite drastically.

All of these observations hold for the commonly used C18 and C8 ligands. More polar ligands such as those used for the preparation of CN packings exhibit significantly lower stability, even under normal operating conditions. At pH 7, the hydrolysis of a CN packing can be by a factor of 1000 faster than that of a C18 or C8 packing.

As you can see, you can get reasonable column life at higher pH than commonly recommended, if you are using the correct combination of operating conditions. The most stable columns are based on a high-density silica, have a dense coverage of a C18 or C8 bonded phase and are endcapped. The nature of the buffer ingredients can have a drastic effect on column life and should be chosen carefully. However, if your separation demands it, and if you achieve a column life that is acceptable to you, there is nothing wrong with an exploration of the frontiers of column stability.

References:

1. T. Walter, B. Alden, P. Casellini, paper presented at HPLC '97, Birmingham, UK
2. H. A. Claessens, M. A. van Straten, J. J. Kirkland, J. Chromatogr. A. 728 (1996), 259-270

27. Optimal Flow Rates

Q.: I am always using my reversed-phase column (5 µm, 4.6 mm x 150 mm) at a flow rate of 1 mL/min. A colleague told me that I can get better resolution at 0.5 mL/min. Upon reducing my flow rate according to his suggestion, I do not see much of an improvement in my separation. Additionally, the runtime has been increased 2 fold. Nevertheless, I would like to understand the concept and would appreciate, if you would discuss the influence of flow rate on resolution.

A.: Gladly! Hidden behind your question is the dependence of the height-equivalent to a theoretical plate on the linear velocity. Both are terms of chromatographic theory that most practitioners forget the moment they complete the introductory course to chromatography. What I will try to do in the following is explain the phenomena in simpler terms.
In isocratic chromatography, resolution usually decreases as the flow rate is increased and increases as the flow rate decreases (see figure 1). However, at a particular flow rate, which is analyte and mobile phase specific, the resolution reaches a maximum. If I decrease the flow rate any further, the resolution will decrease again. Obviously, this region of the curve is not a good place to be in, since increased analysis time is accompanied by decreased resolution. When you use a 5 micron 4.6 mm column at 1 mL/min with a low viscosity mobile phase, you may be fairly close to this point of decreasing resolution with increasing analysis time, but the exact location of this point depends on your analytes and the mobile phase composition. For common reversed-phase mobile phases and common reversed-phase columns, you may be far away from the point of maximal performance. Let me examine this in more detail.

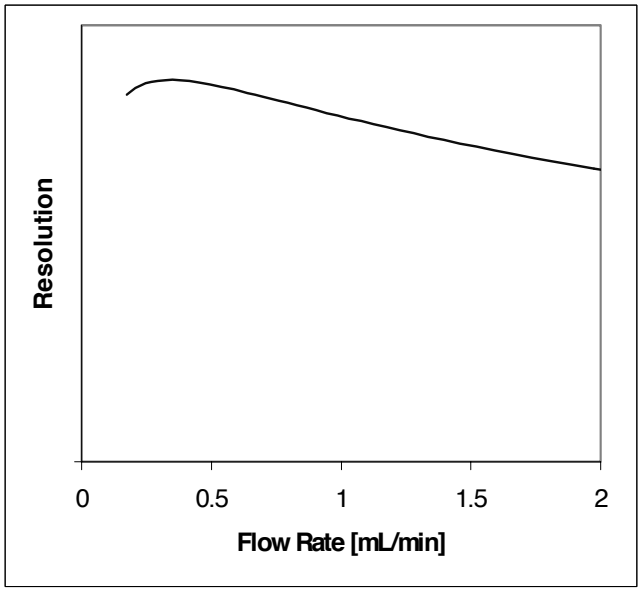

Figure 1
Resolution as a function of flow rate for a 5 µm 15 cm column for the conditions assumed here.

First, let us explore the rules that determine the position of the maximum column performance. It can be calculated by using the classical van Deemter equation, given here in its reduced form:

$$h = A + \frac{B}{\upsilon} + C \cdot \upsilon \quad (1)$$

h is the reduced plate height, υ is the reduced velocity, and A, **B**, and C are coefficients describing the packed bed quality, the diffusion of the sample in the packed bed and the mass transfer of the sample into the packing, respectively. We really do not need to spend a lot of time on this equation. What we need to do is find the minimum of this equation, which is simply derived by calculating the first derivative and equating it to 0. When we do this we obtain for the reduced velocity at the minimum:

$$\upsilon_{\min} = \sqrt{\frac{B}{C}} \quad (2)$$

The value of B is typically about 1.5, and C is typically 1 / 6, making the coefficient under the square root about 9 (1). Using this information and converting from the reduced velocity to the linear velocity, we obtain

$$u_{\min} = 3 \cdot \frac{D_m}{dp} \qquad (3)$$

u_{min} is the linear velocity, at which maximum column performance is reached. From the last equation, one can see that the linear velocity for achieving maximum performance depends on the diffusion coefficient of the analyte, D_m, and the particle size of the column, dp. The smaller the particle size, the higher is the linear velocity at which maximum column performance is reached. Also, one can see that this velocity depends on the diffusion coefficient of the sample, which in turn depends on the mobile phase composition.

Q.: Thank you for your nice theoretical exploration! So, what am I going to do with this now? I don't know anything about the diffusion coefficient of my analytes.

A.: Luckily, you don't need to know anything about it. There is a way around this issue that is very practical. Just bear with me one more moment. The theories of diffusion in liquids relate the diffusion coefficient of an analyte to the viscosity of the solvent. With a few assumptions, one can relate the diffusion coefficient in an aqueous or polar solvent at room temperature to the viscosity of the mobile phase using the following equation:

$$D_m \approx 1.7 \cdot 10^{-6} \cdot \frac{1}{\eta \cdot V^{0.6}} \qquad (4)$$

η is the viscosity of the mobile phase in Poise, and V is the molar volume of the solute, in mL. Most analytes have a molecular weight between 200 and 500 Dalton, which results in diffusion coefficients ranging from $D_m \sim 0.4*10^{-7} * 1/\eta$ to $D_m \sim 0.7*10^{-7} * 1/\eta$. Let us just assume that a typical diffusion coefficient is about $D_m \sim 0.6*10^{-7} * 1/\eta$. Therefore, the optimum linear velocity is

$$u_{\min} = 1.8 \cdot 10^{-7} \cdot \frac{1}{\eta \cdot dp} \qquad (5)$$

This means that the optimum linear velocity is inversely proportional to the viscosity of the mobile phase. The higher the viscosity, the lower the velocity! This is worth knowing, but is still not yet anything that we can deal with easily in practice. However, the column backpressure depends on the viscosity of the mobile phase, and this is something that we can read off the instrument. The relationship between the linear velocity and the backpressure can be obtained from the Kozeny-Carman equation:

$$u = \frac{\Delta p \cdot dp^2}{1000 \cdot \varepsilon_t \cdot \eta \cdot L} \qquad (6)$$

Substituting this into equation 5 and assuming that the typical total column porosity is about $\varepsilon_t = 0.7$, we obtain

$$\Delta p = 1.25 \cdot 10^{-4} \cdot \frac{L}{dp^3} \; [\text{ dyn / cm}^2] \qquad (7)$$

The column backpressure at the point of optimum column performance depends only on the column length and the particle size. This is a beautifully simple result after this tour-de-force through three independent branches of column theory. The last step is really a simple conversion to the dimensions commonly used in the US. I also rounded the numbers to get a simple formula that is easy to remember. If you use the particle size in µm, the column length in mm and you want to get the pressure at the optimal linear velocity in psi, you should use the following simple formula:

$$\Delta p[\,psi] = 200 \cdot \frac{L}{dp^3} \qquad (8)$$

For example, if I want to know the pressure at the optimum performance point for a 150 mm column packed with 5 µm particles, I get 240 psi.

Q.: This looks like a good rule of thumb. Should I use the column at this pressure?

A.: These considerations represent a simple way to get you calibrated for the point at which maximum column performance is expected for normal analytes. But you have to realize the limitations of this estimation. If your analytes are smaller than about 200 Dalton or larger than about 500 Dalton, this estimate is not going to work. For smaller analytes, the maximum plate count is reached at a higher pressure, and for larger analytes at lower pressure.

In addition, I generally would not run the column at the maximum plate-count, but at a linear velocity that is about a factor of 2 higher than this estimate. This is generally the point of the best compromise between column performance and analysis time. Therefore, I would select the flow rate that gives me a backpressure of about 500 psi. This is the best choice from the standpoint of overall column performance.

Of course, many separations do not need the best column performance. However, if you have optimized your separation around this point, you can then select other conditions that might speed up your separation. You can increase the flow rate or use a shorter column, or a combination of both.

Reference:

1. U. D. Neue, in "HPLC-Columns, Theory, Technology and Practice", Wiley-VCH, 1997

28. Carbon Load

Q.: I always thought that the carbon load of a reversed-phase packing determines the retention of a compound - at least for compounds that interact with the packing mainly by hydrophobic interaction. A colleague told me that this is an oversimplified view. I would appreciate a good explanation of the dependence of retention on carbon load.

A.: Your colleague is correct: the commonly quoted "carbon load" of a packing is not the factor that determines retention. The story is a little bit more complicated. Let us discuss the simple case of purely hydrophobic interaction first!

The question of retention can be easily analyzed, if we view reversed-phase chromatography simply as a partitioning

mechanism. In this mechanism, the retention factor k of a compound is determined by its distribution coefficient K between the stationary phase and the mobile phase and the volume of the stationary phase V_S and the mobile phase V_M:

$$k = K \cdot \frac{V_S}{V_M} \qquad (1)$$

The ratio of the volume of the stationary phase to the volume of the mobile phase is called the phase ratio. The volume of the mobile phase in the column is just the retention volume of an unretained peak. Therefore the adjusted retention volume V_R' of a peak is:

$$V_R' = V_R - V_M = V_S \cdot K \qquad (2)$$

This means that the retention volume of a compound is simply proportional to the volume of the stationary phase in the column. The volume of the stationary phase in the column is however not proportional to the commonly quoted carbon load. This is due to the fact that the specific pore volume of different silicas is different. A silica with a small specific pore volume is denser than a silica with a high specific pore volume. Therefore, more silica can be packed into a column, and more stationary phase is packed into a column as well. The amount of stationary phase in the column can be calculated as follows:

$$V_S = V_C \cdot (1 - \varepsilon_i) \cdot f \qquad (3)$$

where V_C is the column volume, and ε_I is the interstitial fraction in the column, which is constant for all practical purposes. The factor f contains all the components that vary with the type of packing:

$$f = \frac{V_{st}/g}{V_{pore}/g + V_{silica}/g} \qquad (4)$$

g is the number of grams in a column, V_{st} is the amount of volume of the stationary phase in the column, proportional to the % carbon given in the literature, V_{pore}/g is the specific pore volume, and the last factor V_{silica}/g is the inverse of the skeleton density of silica, which is 2.2 g/mL. This factor f determines the retention of the packing. All the components of this factor are commonly given in the manufacturers' literature, so it can be used without difficulty to estimate the retentivity of one packing compared to another.

Let me show this in a simple example! Let us compare three different C_{18} packings: Nova-Pak® C18, Spherisorb® ODS2 and Lichrosorb® RP18. The carbon content of the three packings is significantly different (data from Phase Separations HPLC Columns and Supplies Catalog): 7.3% for Nova-Pak® C18, 11.5% for Spherisorb® ODS2 and 16.2% for Lichrosorb® RP18. Thus if retention was determined by the carbon content of the packing, one would expect 2.5 times more retention for Lichrosorb® RP18 than for Nova-Pak® C18, with Spherisorb® ODS2 somewhere in between. However, the packing density of the different packings is significantly different, because of the differences in the specific pore volume between the three packings (table 1). Consequently, if one calculates the factor given in equation 4, one will discover that this factor is very similar for the three packings, and that therefore the hydrophobic retention for the three packings is very similar as well.

Table 1
Example calculation

	% Carbon	V_{pore}/g	Factor f
Nova-Pak C18	7.3%	0.3	0.10
Spherisorb ODS2	11.5%	0.5	0.12
Lichrosorb RP18	16.2%	1	0.11

In summary: the carbon content alone cannot be used to compare the retention of packings with significantly different porosities. However, all the information that one needs for a good estimate of the hydrophobic retention of a packing is readily available in the manufacturers' literature.

Q.: This is enlightening. It would be nice if the column manufacturers would use this factor in their literature. If would make the comparison of packings easier. You mentioned that this applies to hydrophobic retention only. I assume that the other factor to consider is the silanol activity of a packing?

A.: Yes, you are correct. The silanol activity of a packing is the second important factor determining retention on reversed-phase packings, especially for basic analytes. Of course, the influence of silanols on the retention of an analyte depends on the properties of an analyte. For neutral hydrophobic analytes, the activity of silanols on the surface of a packing is unimportant. For basic compounds, the retention factor can increase 10-fold due to the activity of silanols. For other polar compounds, more subtle differences in selectivity can be observed. Therefore, a knowledge of the silanol activity of a packing plays a role in our judgement of the usefulness of this packing for a separation. One can get a first impression of the silanol activity of a packing by checking whether a packing is endcapped or not. Non-endcapped packings have a higher silanol activity than endcapped packings.

Most of the modern reversed-phase packings are well endcapped. Thorough endcapping results in good peak shape for basic analytes without the need of mobile phase modifiers. Nevertheless, packings with a high silanol activity can be used even for basic analytes to obtain different selectivities than those available with the fully endcapped packings. Of course, modifiers such as triethylamine or octylamine often must be added to the mobile phase to improve peak shapes for basic compounds. This is a nuisance to many chromatographers, and the reason why fully endcapped packings are preferable for most applications.

Whether a packing is endcapped or not unfortunately gives only a very rough impression on the activity of the silanol groups of different packings. Therefore, the catalogues of some column suppliers contain additional, more detailed information on the activity of silanols (1, 2). This information can then be used to select packings that are significantly different from each other for your next

applications development. After all, the differences between different columns are an advantage in methods development.

References:

1. Phase Separations HPLC Columns and Supplies Catalog
2. Alltech Chromatography Catalogue 400

29. pH Control

Q.: I have recently read an article that recommends the use of mobile phases with an acidic pH for acidic compounds. It was suggested that using acidic mobile phases reduces the ionization of the acidic analytes and the silanols. This is supposed to improve the peak shape and reduce tailing. Can you explain the reason for this phenomenon?

A.: In general, tailing peaks are observed quite commonly on reversed-phase packings with basic analytes at neutral pH. This phenomenon is due to the interaction of the bases with the silanols on reversed-phase packings. Tailing of acidic analytes on the other hand is quite rare, at least under circumstances where complicating factors such as complexation or size-exclusion phenomena can be excluded. There is no reason to expect an interaction between the negatively charged acidic analytes and the negatively charged surface silanols could cause such a phenomenon. Therefore, the recommendation to exclusively use acidic mobile phases for the separation of acidic analytes seems to be quite limiting. Can you give me an example of this improvement in peak shape?

Q.: Yes. In the article that I read, the example analyte was ibuprofen, which is a simple hydrophobic analyte with an acidic functional group. A C18 column was used, and the mobile phase was a mixture of acetonitrile and buffer. The buffer was a 5 mM phosphate buffer at pH 4.4. As you can see from the chromatogram (Figure 1a), significant tailing was observed. In the article, the pH of the mobile phase was then changed from pH 4.4 to pH 3.0, using the same 5 mM phosphate buffer, and a significant improvement in the peak shape was observed. Additional improvements were shown at pH 2.5 with 0.1% trifluoroacetic acid. This demonstrates quite clearly the improvement in peak shape for ibuprofen at acidic pH, doesn't it?

A.: I strongly disagree. This set of experiments does not demonstrate at all, that you need an acidic mobile phase to suppress the tailing of acidic analytes. The only thing that this demonstrates is the fact that one should use a buffer in the mobile phase. Phosphate has two pKa values, one is around 2, the other around 7. Therefore, phosphate buffers have their optimal buffering capacity around pH 2 and around pH 7. At pH 4.4, phosphate has no buffering capacity whatsoever. Therefore, the tailing of the ibuprofen peak with the phosphate "buffer" at pH 4.4 is due to the lack of pH control under these conditions.

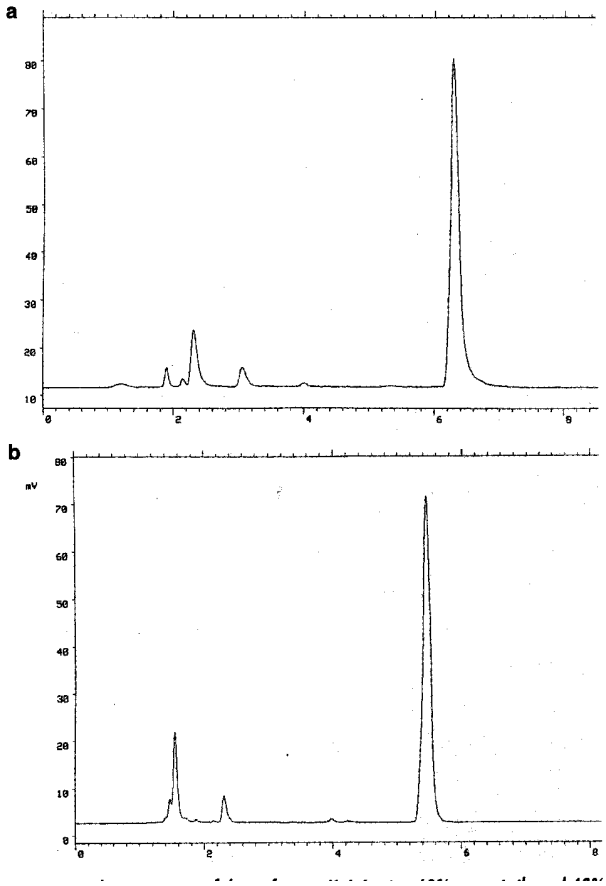

Figure 1: *Chromatogram of ibuprofen at pH 4.4 using 60% acetonitrile and 40% of*
a. a 5 mM phosphate solution and
b. a 5 mM acetate buffer.

This can easily be demonstrated in a simple experiment. In this experiment, we keep the pH at the same value as in your example, at pH 4.4, but we use a true buffer with good buffering capacity at this pH. This can readily be accomplished utilizing an acetate buffer. Figure 1b shows the resulting chromatogram of ibuprofen at pH 4.4 using a 5 mM acetate buffer. As you can see, good peak symmetry has been achieved, and no tailing is noticed. This demonstrates clearly that the original cause of the tailing is <u>not</u> due to the mobile phase pH, but rather due to the fact that the mobile phase is not buffered. Consequently, good peak shapes and good results are achievable with acidic analytes at any pH value, provided that the mobile phase is properly buffered. This gives you a wider choice of options in the optimization of a separation.

If you are dealing with ionizable compounds, the manipulation of the pH value is a very powerful tool in the development of a separation (1). Frequently, as the pH is changed, the elution order of peaks changes. The effects that

pH changes have on the selectivity of a separation are commonly much more powerful than the variation of the organic solvent. Therefore, the suggestion that you should not use the tool of pH manipulation in your methods development is counterproductive. For many compounds, but especially for acidic analytes, an interaction with surface silanols is unlikely to cause a problem. If you use one of the newer reversed-phase stationary phases based on high-purity silicas, then you often won't encounter tailing peaks even at neutral pH using basic analytes.

As we have seen in this example, the proper control of mobile phase pH is not only important for the peak shape, but it is also vital for the ruggedness of the separation. How well the pH is controlled, depends on the buffering capacity of a buffer. In turn, the buffering capacity is a function of the buffer concentration and the difference between the pH and the pKa of the buffering ions. In most HPLC separations, the concentration of a buffer is around 50 mM. At this concentration, the buffer can be used to control the mobile phase pH in a range of +/- 1.5 pH units around the pKa of the buffer, but a range of +/- 1 pH unit is preferred. If you need to work at lower buffer concentrations, it is definitely better to stay within the smaller range. This is due to the fact that the buffering capacity of a buffer is a function of both the concentration of the buffer and the difference between the pH and the pKa of the buffer. Therefore, lower buffer concentrations correspond to a narrower buffering range.

In summary: there is no reason whatsoever to limit yourself to acidic mobile phases when running acidic analytes. A good control of the mobile phase pH by using the correct buffers at the correct concentration is important for the reproducibility of your method and the peak shape of your analytes. And don't forget: A buffer is a buffer if it buffers the pH. If it doesn't, it isn't.

Reference:

1. M. Zoubair El Fallah, "HPLC Methods Development", in Uwe D. Neue, "HPLC Columns - Theory, Technology and Practice", Wiley-VCH (1997)

30. Mobile Phase Composition

Q.: We recently converted an isocratic method from an older two-pump gradient system to a single pump HPLC system. We were surprised to find significant differences in the retention times between both systems. The differences are consistent and reproducible. What is the reason?

A.: The most likely reason for your observations is the contraction (or expansion) of the solvents upon mixing. This is an old issue in the preparation of reversed-phase mobile phases. It is due to the significant volumetric contraction of the commonly used mixtures of water or buffer with methanol, acetonitrile and tetrahydrofuran. I rarely get any questions about this subject today, possibly due to the fact that the HPLC systems in use today commonly use similar principles for solvent mixing.

Q.: Our method is indeed a reversed-phase method. We use a mobile phase of 50% methanol and 50% water.

A.: Methanol-water mixtures represent the worst case of solvent contraction in HPLC. You can observe significant differences in retention, depending on how you mix the solvents. You need to understand that if you mix 500 mL of water with 500 mL of methanol or if you take 500 mL of one solvent and fill the graduate with the other solvent you do not obtain the same composition. Since you do not get the same composition, the elution strength of both mixtures is different. Since the elution strength is not the same, you observe different retention times. Examples of this effect have been published in the literature (1), so let me use the literature example to demonstrate the effect.

The separation used to demonstrate the mixing effect is a separation of explosives dissolved in acetone. It was carried out using a 4.6 mm x 250 mm C18 column using a mobile phase of "40% water and 60% methanol". The details of the method are given in the legend to the graph that show the results of the experiment.

Four different ways to prepare the mobile phase of "40% water and 60% methanol" were used. In the first case, 400 mL of water were put into a 1 L volumetric flask, and the flask was then topped off with methanol. Due to the contraction of the mixture during the combination of the tow solvents, more than 600 mL of methanol are added to the flask. Consequently, the elution strength of this mobile phase preparation is the highest, and the retention times are shorter than with the other preparation techniques.

In the second case, 400 mL of methanol and 600 mL of water were measured out separately and combined in a flask. The retention times were longer than in the previous case. As we will discuss below in more detail, this is the most preferred approach. It is also the way that mobile phases are prepared in most laboratories.

In the third case, the gradient was formed by two pumps delivering the correct quantities of either methanol or water. The mixing of the gradient was done at high pressure. In this case, the solvent composition is identical to the solvent composition of the second case. However, the contraction of the solvent mixture occurs behind the pump, resulting in an actual flow rate of less than 1 mL/min. Therefore, the slower flow rate results in longer retention times, while the solvent composition is accurate.

The fourth case is the inverse of the first case: 600 mL of methanol were added to the volumetric flask, which was then topped off with water. Due to the contraction of the mixture, more than 400 mL of water were needed to fill the flask. Consequently, this mobile phase contains the highest water concentration, and the retention times are longer than in the other cases.

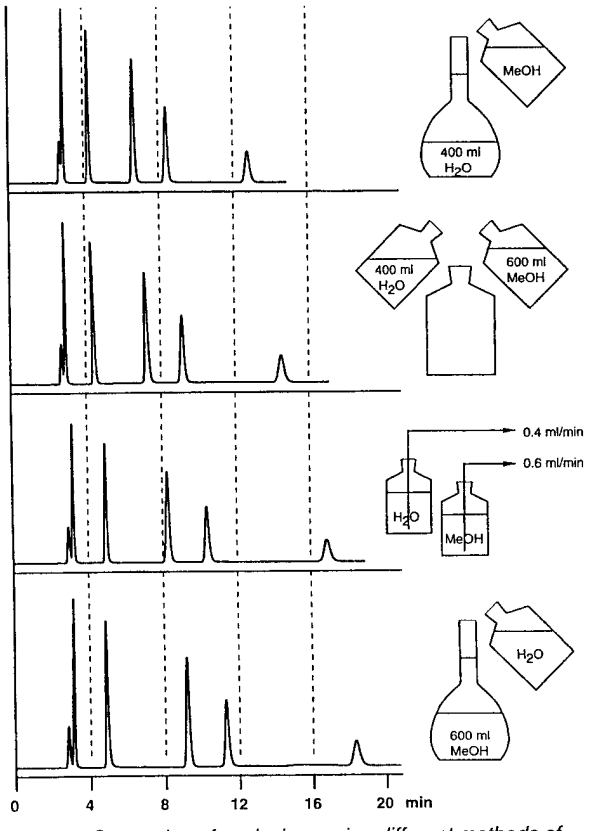

Figure 1: Separation of explosives using different methods of mobile phase preparation.
Sample: explosives dissolved in acetone (octogen, hexogen, tetryl, trinitrotoluene, nitropenta); *column:* Grom-Sil 80 ODS-7 PH, 4 µm, 4.6 mm x 250 mm; *detection*: UV at 220 nm; mobile phase: "40% water and 60% methanol"; *flow rate:* 1 mL/min; *(reprinted from reference 1 with permission of the publisher and the author)*

The second approach is the most preferred way of mobile phase preparation. On one hand, it is the most similar to the commonly used low-pressure gradient mixing systems. On the other hand, it does not require a volumetric measurement after mixing. Therefore, this approach avoids problems which arise from the heating or cooling of the solvent mixture upon mixing. It represents the practice that is commonly recommended in HPLC training courses. I would therefore believe that this is the method that you used as well.

Q.: This is indeed the case. From your description of the problem, the differences that we observe in the retention times on our different instruments are related to the different ways of preparing the mobile phases due to the fact that we are comparing a two-pump system to a single-pump system. This is identical to the second and third case in your examples. If I understand you correctly, the composition of the mobile phase is identical in case two and case three, and the difference is the flow rate delivered by the system. Is that correct?

A.: Yes, it is. The mobile phase composition is identical in these two cases. Therefore, the selectivity of the separation is not influenced at all. The only difference between the second and the third method is the actual flow rate in the HPLC system. Therefore, the retention factors and the selectivity of the separation are absolutely identical. From this standpoint, the transfer of the method from one system to the other is successful. The minor differences in the actual flow rate between both systems is nothing to worry about. You have successfully transferred the method from your two-pump system to your single pump system.

Reference*:*
1. Veronika R. Meyer, "Pitfalls and Errors of HPLC in Pictures", 1997, Hüthig, Heidelberg - Oxford, CT, page 51

31. Column Contamination

Q.: My column lifetime is not very long. After only about 500 injections, the peaks broaden and begin to tail. Previously, the column lifetime was approximately 1000 injections. What is wrong with the column?

A.: It is quite possible that absolutely nothing is wrong with the column. Before we discuss this in detail, let me ask you a question. Are you using a guard column?

Q.: No, I am not using a guard column. But I use a precolumn filter. This should protect the column, shouldn't it?

A.: Unfortunately, precolumn filters are only a partial protection for the column. They serve to remove particulates from the mobile phase stream. Particulate derive either from your sample or from the moving parts of the HPLC instrument. Precolumn filters can not protect your column from material that is smaller than the filter and may adsorb on the surface of the packing. Most of the time, but not all of the time, the origin of such material is the sample. What is your sample?

Q.: It is a plasma extract from a solid-phase extraction procedure. Admittedly, plasma components can reduce a column's lifetime fairly rapidly, but the chromatogram is fairly clean, with few background interferences. More importantly, the column lasted previously for over 1000 injections, and only now do we observe the more rapid deterioration.

A.: In many cases, the sample preparation procedure is not entirely perfect. After all, some background interferences are still present in the chromatogram. Similarly, some protein

may be left in your sample despite your best efforts. Proteins are often strongly adsorbed on the packing material and can slowly accumulate on the top of the column. In addition, the concentration of these background contaminants may vary slightly with the samples. Therefore, a decrease in the column lifetime by a factor of two does not shock me. My recommendation would be to use a guard column to protect your analytical column from this contamination.

Q.: I have tried guard columns before. The results were not good. The peaks were distorted right from the beginning. Therefore, I don't think that employing a guard column is a good solution.

A.: A lot depends on your choice of a guard column. I generally recommend to use a guard column that is packed with the identical packing material as the analytical column. If you do this, the guard column works as an extension of your analytical column and retains the contaminants that would normally adsorb to your main column. If this guard column is reasonably well packed, the deterioration of the separation performance can be minimized. In some cases, you may actually gain a little bit in overall performance. However, this is not exciting; the important part is the protection of your analytical column.

If you choose a guard column that does not contain the same packing as your analytical column, you can get significant peak distortion. In addition, you can not predict how well this guard column is protecting your analytical column. Both of these effects are due to the fact that the retention properties of different packing materials are different. Therefore, it is always better to match the packing material in the guard column to the packing material in the analytical column. This allows you both the best possible column protection and the least amount of peak distortion.

Q.: The problem with guard columns is that they are very expensive. How about column clean-up and washing procedures?

A.: I always look at clean-up procedure as a last resort, if I don't have any other options. The problem with clean-up procedures is that they work best, if you know what is contaminating the column. In most cases, we do not have a clue, and can only make some guesses as to the nature of the contamination. Additionally, in some cases it may be very difficult to remove the contaminants. Your problem is most likely an example for this case. I suspect that the contamination consists mostly of small amounts of proteins. To find a good column clean-up protocol is not easy. There are a few generalized cleaning protocols available in the manufacturers' literature, but they are quite involved and may or may not help you. In all of these protocols, you need to wash the column with a series of solvents that are meant to remove the contaminants. This may take a significant amount of time, and you may or may not be successful.

Therefore, I favor the use of guard columns. They are guaranteed to capture the contaminants that would otherwise foul up your column. They are not that expensive; the cost is only a fraction of the analytical column that you are trying to protect. It takes only a few minutes to replace a guard column, while a thorough column washing protocol including reequilibration of the column may take hours. Your savings in time and aggravation are significant. If you adhere to the principles that we discussed above, they are nearly guaranteed to work and protect you from many headaches.

32. Method Verification

Q.: A colleague recently passed a new QC method on to me. I bought a new column to verify his method. Unfortunately, the retention times obtained in my lab were different than the retention times that he had reported. He sent me his old column, and indeed I was able to obtain with his column the separation that he had reported. I then changed a few mobile phase parameters and was able to obtain the separation on my column too. I suspect that I am looking at batch-to-batch differences from the manufacturer, since my colleague's column is over one year old. How can I deal with such a situation in a QC department? I cannot write into the QC procedure that the method should be reoptimized every time we buy a new column!

A.: Indeed, you should not have to do that. There are possible solutions to the problem, but before I discuss them with you I would like to verify an important point: are the two columns that you have mentioned the only columns on which the method has been run?

Q.: Yes, this is the case. My colleague developed the method on his column, and the only other column on which the method has been tried is my new column.

A.: In such a case, the very first thing to do is to verify that you are indeed looking at batch-to-batch differences. It is not impossible that the differences that you are observing are due to a comparison between an old column and a new column, and not due to batch-to-batch differences. After all, I would suspect that your colleague needed to try a lot of different mobile phases before he established the method. During the method development process his column might have aged, maybe lost some bonded phase or strongly adsorbed a mobile phase additive that was used during the development of the method but was not needed for the final method. Since events like this can happen inadvertently, it is always a good idea to check a new method with a brand-new column from the same batch and verify that consistent results are obtained on both columns.

I suggest the first thing to do is to contact the manufacturer and ask him to pack you a new column from the old batch of packing material. Most manufacturers maintain a small

amount of material from every batch of packing for exactly this purpose. Since your colleague's column is only about a year old, you should be able to get a fresh column from the old batch of packing material. Depending on the manufacturer, it is even possible that the batch of packing has not changed over this period of time. This would be the simplest case, since this would prove immediately that the differences observed are not due to batch-to-batch differences.

Run the assay on the new column packed with the old batch, using your colleague's mobile phase. If you get the same results as your colleague obtained, then you are indeed looking at batch-to-batch differences, and you have your work cut out to create a rugged method. If you get the same or similar results that you obtained on your column, then the issue is that your colleague's column had aged during method development. I would then recommend that you design a column lifetime study with your new mobile phase conditions to establish how long the column is expected to last when treated only with the mobile phase used in this assay.

Q.: This is a reasonable suggestion, and I'll see that I can get a new column from the same batch that my colleague has used. But what do I do if the problem turns out to be due to a batch-to-batch difference?

A.: This, indeed, would be the worst case. First, I would try to make a judgement as to how long the assay will be needed. Sometimes, only a limited number of columns is required, and the project will discontinue at some point in the near future. Under these circumstances, the simplest thing to do would be to ask the manufacturer to reserve sufficient packing material from this batch for your project and ask them to prepare columns for you upon request. Sometimes, the manufacturer may ask you for an up-front payment for the amount of packing that they put aside for you. However, if you are in a situation where the new test will be used for an indefinite amount of time into the far future, you need to carefully think through what can be done to make the method rugged.

Having demonstrated to yourself that the packing material that your colleague has chosen does not give you reproducible results, it might be worthwhile to redevelop the method on a different packing. This is a significant amount of work, but it might very well be worth your while. There are significant differences in the reproducibility of different packings. You may discuss the problem with the manufacturer and see if he can demonstrate to you that another packing could give significant improvements in reproducibility. Or you may consider a column with a good reputation of reproducibility from another manufacturer. In all of these cases, the development of the method starts again at the bottom. You should inquire if the manufacturer can make several batches of the packing available to you, so you can verify the ruggedness of the method for yourself. Some manufacturers offer column sets specially prepared for this purpose. They consist of columns from different batches. Make sure that at least one of the columns in the set is from the same batch as the column that you used to establish the method. This way, you can check the column-to-column reproducibility of your method as well as the batch-to-batch reproducibility. This is something that I generally recommend to everyone developing a new method that is supposed to be reproducible over an extended time period.

In all of the discussion above I have assumed that the type of column that you are employing is a mainstream column, such as a C_{18} or C_8 column. If the original method was developed using a cyano or amino column, it is highly likely that the reproducibility problem is due to the limitations of the stability of the bonded phase. If this is the case, my recommendation would be to see if the method can be redeveloped using a C_{18} or C_8 column. Of course, method redevelopment is a significant amount of work, but it may be the best solution in this case.

33. Double Peaks in Sugar Separations

Q.: I am working with a column designed for sugar separations. It is a specially prepared amino column. The mobile phase is a mixture of acetonitrile and water. For the standard separation we use a mobile phase of 75% acetonitrile and 25% water. Recently, I am getting double peaks for all compounds. I thought the column is voiding, but a new column gives the same results. What is the problem?

A.: Unfortunately, the principle behind this separation is more complex than the principle of other separations. We need to discuss it in detail to get to the root of the problem.
There is a simple way in which you can rapidly check whether the columns have voided or if something else is going on. Use glycerol as your test compound! It will give lower retention than most of your sugars, but that is O.K. If you get the same double peak from glycerol as from the other carbohydrates in your chromatogram, then the column has voided. If you get a single, undistorted peak from glycerol, while you get double peaks from the other carbohydrates, then the column is O.K., and the problem is not with the column, but with the mobile phase.

Q.: Glycerol is one of the compounds in my sample mixture. It elutes early in the chromatogram. I do not see any double peak for glycerol. It actually gives a very nice sharp peak, sharper than the other peaks in the chromatogram.

A.: This demonstrates that the column is in good shape. Therefore, we have to look somewhere else for the explanation for the double peaks of your other analytes.
Sugars exist in two anomeric forms. In solution, both anomeric forms are in equilibrium with each other. These anomers are two distinct molecules that can be separated without difficulty by HPLC. Therefore, one should get two

peaks for every sugar, and indeed, this is what you are getting.

Q.: But under normal circumstances, I am getting only one peak for every sugar. Only recently did I get these double peaks….

A.: Yes, amino columns usually give only single peaks for every sugar. The reason for this is simply the pH in the pores of the packing. The rate of the conversion from one anomeric form into the other is a function of the pH. At alkaline pH, the conversion is very rapid, and one gets only a single, reasonably sharp peak. At acidic pH, the conversion is much slower, and one can get two distinct peaks for every carbohydrate. This is the prime reason for the use of amino columns for this separation. The amino groups create an alkaline environment in the pores, which speeds up the interconversion, and one gets a single peak for all carbohydrates. If for whatever reason the pH in the pores of the packing is neutral or acidic, you will get broad peaks or double peaks or even two very distinct and well separated peaks for every sugar.

Now that we understand that this is the most likely cause of your problem, we need to determine why there is a pH shift. Commonly, the reason for this behavior is the aging of the stationary phase; as the column is used, it looses some of the basic bonded phase and forms more acidic silanols. However, your description indicates that you observe the same behavior on a new column. This would speak for a shift in the pH of the mobile phase. Since the mobile phase contains only two components, acetonitrile and water, you could simply check the pH of the water alone and then of the water/acetonitrile mixture. Hopefully, this will pinpoint the source of the problem.

The next step is to "regenerate" the columns. Since most likely the columns have been neutralized by an acidic ingredient in the mobile phase, it should be possible to remove this ingredient by a wash of the column at an alkaline pH. The best approach is probably to wash the column with a dilute solution of an ethylenediamine oligomer, such as tetraethylene pentamine. This wash can regenerate the column, but avoiding the acidic contamination to begin with is still the best solution.

34. Method Control

Q.: I am working on a new HPLC procedure that will be used in our QC department. I have worked out the details of the procedure; I know the linearity, the detection limits, and the influence of mobile phase pH and organic concentration on retention and selectivity. I am now discussing with my colleagues, how to set the specifications for the method: what should be the precision with which the mobile phase is made up, what variations of the mobile phase pH are tolerable etc. Can you help us with some advice?

A.: It is good to know that you have done your homework. This will help significantly in setting up the necessary controls over the mobile phase composition and other parameters. The primary question that you have to answer is at what point of variation of every parameter the method will start to fail. Sometimes this requires a very tight control over the method parameters, sometimes the specification of the method can be varied widely without a negative effect on the quality of the results. Unfortunately, it is not possible to give you good general guidelines. You have to study all the relevant process parameters of your method. Once you understand the influence of the method parameters on the goals of the assay, you can set the limiting values for every parameter.

Let me give you some examples: a complex case that commonly requires fairly tight control of the method parameters is an impurity profile of a pharmaceutical compound. Frequently, many impurities are possible; often they are closely related to the parent drug and some peak pairs are difficult to separate. Under these circumstances, a tight control of the method parameters is required. An entirely different situation is given for a dissolution test or a compositional analysis. Under these circumstances, the interest is simply to quantitate the parent compound, which needs to be separated from the internal standard. Such an analysis is not very demanding, and one can often allow for significant variations in mobile phase composition or temperature without affecting the results. In such a case, the best approach is to specify method control parameters that are readily achievable by an average technician and leave it at that.

However, if your analysis is complex and requires tight controls, you should have been able to demonstrate the limits in the preliminary experiments. How much of a variation of the pH can you tolerate before the resolution between peaks becomes inadequate? How tightly do you need to control the organic content of the mobile phase? There are some rules of thumb that can tell you, how much variation in retention time one can expect if a given parameter is varied, but the best approach is the experiment. Since you have studied several of the important method parameters and know their effects, you should be able to set reasonable specifications. Only your experiments can provide the answer to the question of the tolerances of the method guidelines.

Yet it appears that you have studied only some of the relevant parameters. While some of the variables that you have not mentioned may have only a smaller effect on the method, it is nevertheless important to know their influence. The influence of temperature is often small, but it is worth knowing. Another commonly small effect is the buffer concentration. Nevertheless, both should be included in the method validation studies.

Another parameter that is often overlooked and can cause problems in the future is the column variability. I always recommend that columns are purchased from several batches of the packing material when a new method is established. Many manufacturers provide such a service. Of course, this

costs some money, since a few columns need to be purchased, but this is money well spent. An early check of the batch-to-batch variability of a packing can save you lots of headaches later in the process. If I were your QC manager, I would require these data from you before I would accept your method.

Another thing worth incorporating in a method is a troubleshooting procedure. Since you know all or most of the variables affecting the method, it is good to write them down in a way that allows a future investigator to find a problem rapidly. Such a procedure should also specify the criteria for replacing the column with a new one. This can be a specification of the selectivity of the separation, or criteria on the peak shape, or both. Changes in peak shape or tailing make a quantitation more difficult and are most often used as criteria for the end of the useful life of a column.

35. Mobile Phase pH

Q.: Recently, we have had discussions in my group about the adjustment of the pH of the mobile phase. The simplest and most reliable way to do this appears to be in the final mobile phase, after the organic solvent has been added. Others have advocated to measure the pH always in the pure aqueous component of the mobile phase. When comparing the two approaches, we found differences in the final pH as well as in the chromatography. What is the reason for this, and what is the best way of adjusting the pH?

A.: This is a very good question, because there is often confusion between different labs using different practices for mobile phase preparation. Let me first describe some of the problems that one encounters. This will then lead us to the best solution.

The adjustment of the pH is accomplished using a buffer solution. The capability of a buffer to maintain and control the pH is called the buffer capacity. The buffer capacity is a function of the difference between the pH and the pKa of the buffer and of the concentration of the buffer. For example, acetic acid has a pKa of 4.75 (in water), therefore acetate buffers have the best buffer capacity at this pH. Typical buffer concentrations used in HPLC vary from 20 to 50 mM, therefore good buffering capacities are achieved within +/- 1 pH units around the pKa of a buffer. To achieve reasonable buffering capacities, the pH of the buffer should never depart more than 1.5 pH units from the pKa of the buffer.

If an organic solvent is added to the buffer, the measured pH shifts to higher values. This is due to two separate effects. One is a true shift in the pH due to the shift of the concentration of the hydrogen ion in the presence of the organic solvent. The second effect is related to the pH measurement itself: since the pH meter has been calibrated and adjusted to give accurate results in water, it will exhibit a departure from the true value if the measurement is made in the presence of a large concentration of an organic solvent. However, both effects are irrelevant for the actual composition of the buffer and its buffering capacity: a buffer solution prepared from a given ratio of the acidic form and the basic form of the buffer components will still have the identical ratio of both in the presence of an organic solvent and therefore the identical buffer capacity as in pure water. Therefore, if we measure the buffer composition and its pH in water, we have a fixed constant reference point, and unquestionable tools to measure the pH value. For example, the acetate buffer has its maximum buffer capacity at pH 4.75 in water. An acetate buffer whose pH is adjusted to this value in water will be at its maximum buffering capacity independent of the pH value that is read by the pH meter in the presence of an organic solvent. For all commonly used buffers, I can look up the pKa values in water in a table. Then, I can make the adjustment of the buffer pH in water, using the pKa as the reference point. Then, as the last step, I add the organic solvent.

However, there are occasional unavoidable exceptions to this rule. If you use an older procedure developed using an older column, it may specify a long-chain amine as a component of the buffer. Sometimes, the solubility of the amine in neat water is limited, and the simplest way to prepare the buffer solution is by adding the organic solvent prior to the adjustment of the pH. There is nothing wrong with this procedure, as long as you know the titration curve and the buffering capacity of your buffer in the presence of the organic solvent. The best way of getting this information is to obtain a titration curve in the presence of the targeted concentration of the organic solvent and to construct the buffer capacity curve from this titration curve. A titration curve is a plot of the pH of the solution as a function of the amount of base added to the solution. The buffer capacity curve is a plot of the (negative) change in pH per amount of base added versus the pH. This curve has a maximum at the pKa of the buffer in the presence of the organic solvent. Since the buffer capacity is at its maximum at the pKa of the buffer, you can then use this information to prepare a buffer for your assay with an optimal capacity.

The preferred practice in the industry is to prepare the buffer in water and then to mix the pH adjusted buffer with the organic solvent. This gives unequivocal results and allows for a good and universal judgement on the capacity of the buffer. It should be noted that this can be indicated clearly in the statement of the mobile phase composition. To avoid confusion, the composition should be stated as the aqueous component including the buffer and its pH mixed with the organic solvent. Here is an example: "50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0 / methanol 40/60", or "40% 50 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.0 and 60% methanol". The inverse statement is somewhat ambiguous: "methanol / KH_2PO_4/K_2HPO_4 buffer, pH 7.0, 60/40" and should not be used.

Q.: Your proposal is different from what I had originally in my mind, but it makes sense.
What is a typical shift in pH with solvent composition?

A.: It is relevant enough to cause measurable retention changes if the buffer is prepared before or after the addition of the organic solvent. In the literature (1), upward shifts of the pKa of acids of about 1 unit have been reported between 100% water and 50% water/methanol. For the bases reported, the pKa shifted by approximately 0.5 units between 100% water and 50% water methanol. If the analytes are not completely ionized or non-ionized, this difference causes substantial shifts in their elution times. Even changes in the elution order are possible. Therefore, from a practical standpoint, the current practice of standardizing the way the mobile phase pH is specified is extremely important. I am happy that I managed to convince you to follow the same practice and to measure and specify the pH in the aqueous component of your mobile phase.

Reference:

1. E. Bosch, P. Bou, H. Alleman, M. Rosés, Anal. Chem. 68 (1996), 3651

36. Signal-to-Noise Improvements

Q.: I would like to improve the sensitivity of my HPLC method. Would you please give me some advice on what I can do?

A.: Gladly! There are many different components to the story (1). Let us first see what we can do to reduce the detector noise. First of all, we need to know that the detector is in good working condition. This means, for example, that the lamp is working at full capacity, and that the cell window of the detector is clean. The detector performance is best verified using a set of standard testing conditions.

Q.: I know that the detector is in good working order. We monitor the detector linearity, its noise and its response on a regular basis to verify that it works for routine analysis. My question is more directed towards what one can do with a fully functioning detector rather than towards detector troubleshooting.

A.: O.K. The first thing to do is probably to examine the detector response and the detector noise as a function of the wavelength. Then select the wavelength that results in the best signal-to-noise ratio. This varies with the detector, the mobile phase and the sample that you are trying to analyze.
In principle, the noise of a variable wavelength detector is inversely proportional to the amount of light the photodiode receives. This is the reason why the detector noise increases as the light source ages or the detector window becomes dirty. It is also the reason for increased noise, as the mobile phase becomes more adsorptive. For example, alcohols have a significant absorbance at 210 nm, while the absorbance of acetonitrile is very low. This is the reason for reduced noise and consequently higher sensitivity using acetonitrile in the low UV. Around 250 nm both acetonitrile and methanol work equally well.

If you need to use a mobile phase that has a background at the wavelengths where your sample has a good response, both noise and response must be measured as a function of the wavelength. With this data, you can then calculate which wavelength is best suited achieving the maximum signal-to-noise ratio. If you have a photodiode array detector, this exercise is very straightforward and quick. You simply obtain a spectrum at the peak maximum and compare it to the mobile phase background spectrum
Then you choose the wavelength that gives you the best signal-to-noise ratio. Now you have selected the best wavelength for a given mobile phase composition. If this still does not give you satisfactory results, you have your work cut out.

The simplest thing to do next is to examine an increase in the detector time constant, or the equivalent noise reduction mechanisms built into modern detectors. If you increase the time constant or its equivalent, the peaks will become broader, and the noise is reduced. If your chromatogram is fairly empty, this approach can improve the signal-to-noise ratio significantly. However, if there are many peaks in the chromatogram and the resolution between some of them is small, this approach has its limitations.

Another relatively simple thing to consider is the amount of sample that you inject. If you see disturbances in the separation long before you run out of sample, you should think about the solvent in which the sample is dissolved (2). Often, the sample can be dissolved in a solvent composition that has a much lower elution strength than the mobile phase (3), and you can inject very large sample volumes before you see any distortions of the peak shape. If your sample is dissolved in an organic solvent and your analysis is done by reversed-phase chromatography (i.e. the mobile phase is a mixture of water and methanol or acetonitrile), you will be able to inject only a small volume before the peaks become misshapen. On the other hand, if you make up the sample in a solvent composition that contains 20% more water than the mobile phase, you can inject a milliliter of sample without appreciable peak distortion. The sample simply enriches on the top of the column at the beginning of the chromatography.

Q.: This is a neat trick worth remembering. What other things should one consider?

A.: Another approach is a reduction in column volume. However, there are several things necessary to consider before implementing this option. First of all, you need to determine, if indeed the amount of sample available to you is limited, and that an injection of the total amount of sample on the standard column does not reveal any other limitations.
The simplest approach towards a reduction in column volume is to reduce the column diameter, keeping the column length and the particle size constant. For example, a reduction of the column diameter from 4.6 mm to 2.0 mm

increases the mass sensitivity by about a factor of 5. As a side benefit, it reduces the flow rate and thus the solvent consumption by about the same factor. As a tradeoff, the effects of instrument bandspreading become more significant. The instrument bandspreading is likely to reduce the resolution in the chromatogram, but you can address at least part of it.

There are two components to instrument bandspreading. The first one is the pre-column bandspreading, caused by the injection volume, the bandspreading in the injector and in the tubing leading to the column. You can reduce its influence by using the trick described above: dissolve the sample in a solvent with a lower elution strength than the mobile phase or dilute the sample with such a solvent and inject more. The second component is more bothersome. It is the post-column bandspreading, caused by the tubing leading from the column to the detector and by the detector cell itself. The length of tubing leading from the column to the detector should be kept to an absolute minimum. Since you can address the influence of the pre-column tubing volume by other means, it is best to keep the post column tubing to an absolute minimum. The question of the detector cell volume is more tricky. On one hand, you want to minimize the volume to reduce bandspreading. On the other hand, a reduction in the detector cell volume increases the detector noise. In the worst case, the whole exercise of reducing the column volume and the detector volume may result in only a marginal improvement in sensitivity.

Q.: I guess this needs to be thought through carefully. How else can I improve the signal-to-noise ratio of my assay?

A.: Another relatively simple thing might be to change the detector. Maybe a fluorescence detector or an electrochemical detector are a better choice than the standard UV detector. Of course, the purchase of a new detector is a significant expense.

There are a few more drastic changes to your assay that can improve the signal-to-noise ratio. Often, noise is caused by components of the mobile phase. Earlier I had mentioned the reduced absorption of acetonitrile compared to methanol in the low UV. Such a change requires the redevelopment of the separation. Another frequent cause of excessive detector noise are additives in the mobile phase, for example amines used to suppress peak tailing. You can eliminate the need for such mobile phase additives by using a better column. In a similar vein, if the low sensitivity is caused by peak tailing, a reduction of the tailing by using a better column can increase the sensitivity by a factor of about three. All of these suggestions represent a significant amount of work, and the improvements that you are getting are comparatively small. It is best to make these choices at the beginning of the development of a new assay.

References:

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37. Overload

Q.: When I increase the amount of sample that I am injecting the peaks start to tail and become broader. I am working with fairly low concentrations and, therefore, would not expect that the column is overloaded. I would appreciate if you would discuss the phenomena that could cause such an event.

A.: Mass overload of the column is only one of the phenomena that can cause a peak distortion upon increasing the injection size. It is also possible that the mobile phase is overloaded. Something like this can occur, if the sample is not dissolved in the mobile phase. However, let me discuss mass overload of the column first.

Mass overload of the column can be diagnosed very rapidly. For common analytes, mass overload starts at an injection of about 0.1 mg to 1 mg of sample per milliliter of column volume. If you are using a UV detector, you can use another rule. For most analytes possessing a strong UV chromophore, mass overload starts if the peak height reaches about 0.1 AUFS. Of course, these rules are rough rules of thumb, but if you are injecting significantly less sample, mass overload is not likely to be the problem.

Q.: Indeed, the amount of sample injected is much smaller. Therefore, I do not think that mass overload is the cause of the peak distortion. What else can be the problem?

A.: A common issue is the solvent in which the sample is dissolved. If the sample diluent is a stronger eluent than the mobile phase, peak distortion can occur. There can be several reasons for this. It may be that the sample originated in a solid phase extraction procedure that required a stronger solvent than the mobile phase for the elution of the sample. Under these circumstances, a simple dilution of the sample in the weaker eluent may avoid the problem. Alternatively, the sample can be evaporated to dryness and then reconstituted in mobile phase or a weaker eluent. A similar problem is caused by samples generated from dissolution tests. The acid in the sample may overload the buffer of your mobile phase and cause peak distortion. This can be fixed by either adjusting the pH of the sample or using a stronger buffer in the mobile phase. To prepare a stronger buffer, increase its concentration and adjust the pH to a value close to the pKa of the buffer. This requires a little bit of homework, but is relatively straightforward. In most dissolution tests, the number of analytes in a sample is very small, often just the compound of interest plus the internal standard.

If the sample pH is the cause of the peak distortion, a simple increase in the buffer concentration is often the solution, even if the chromatogram is fairly crowded. Typically, changes in retention are very small for even significant changes in buffer concentration. Of course, you should check at what concentration the buffer starts to precipitate.

Another reason for peak distortion problems can be low sample solubility in the mobile phase. The effects are similar to the case where the sample is dissolved in a different solvent. After all, the sample was dissolved in a different solvent than the mobile phase to get around the problem of low solubility. Fortunately, cases where this is the true problem are very rare. In such a case, the best approach may be to live with the problem, which means that the concentration range over which the sample concentration can be determined is rather narrow. Alternatively, the choice of a completely different chromatographic mode may offer a solution.

Q.: None of these issues seem to apply. What is left?

A.: One of the items that we have not yet discussed is a simple sample volume overload. If the sample is dissolved in mobile phase, and a large volume is injected, the peaks are becoming broader. This phenomenon has more of an effect on early eluting peaks, and becomes less of a problem for late eluting peaks. One can use this as a criterion to distinguish this problem from the other items that we have discussed. The solution is fairly simple: dissolve the sample in a weaker eluting solvent than the mobile phase. You can check very rapidly, if this is the problem. Dilute your sample 2x with the weaker eluent in your mobile phase, and then inject 2 times the sample volume. This will concentrate the sample at the column top, and the total amount of sample remains the same. If the peak distortion disappears, a simple volume overload is the problem. Simply dissolve your sample in the future in a solvent composition that has a lower elution strength, and the problem will disappear. Thinking carefully through what the impact of the sample solvent on the analytical results might be can solve many problems.

38. Column Durability

Q.: What can I do to make an HPLC column last longer?

A.: There are several options available to you to increase column lifetime. An acceptable column lifetime is approximately 1,000 injections. However, I have seen columns last for over 10,000 injections without any performance deterioration. The only requirement was a reasonable column care.

Q.: 10,000 injections? I have never achieved that many injections on a column. I find this difficult to believe.

A.: Today, columns prepared by the leading manufacturers are very rugged. In general, the column performance deterioration of is due to column contamination. If this is prevented, good column lifetimes will result. My coworkers and I have investigated column lifetime issues in detail. Let me explain some of our experiments, and then we can discuss other issues that affect column life.

We examined column lifetime by running the columns practically continuously using a single isocratic assay. The columns were Symmetry C_{18} columns from Waters Corporation. The sample was a mixture of pharmaceutical standards, and the mobile phase contained 79 % water, 20% methanol, and 1% acetic acid. We performed two sets of experiments. In one of them, the sample was injected directly onto the column. In the second set, the columns were protected by a guard column.

The experiments that were performed without use of a guard column resulted in an irreversible deterioration of column performance somewhere around 1,500 to 2,000 injections. While the retention times remained constant, the plate count dropped shortly before the end of the column life, the backpressure increased and, finally, double peaks were observed. Even with a clean sample, column life without column protection appears to be limited.

We then repeated the same experiments with a guard column in place. We used high-performance guard columns prepared from the same packing material as the analytical columns. Under these circumstances, the guard columns do not alter the separation and function as just an extension of the analytical column. Thus the analytical separation is not affected.

We found soon that the combination of the analytical column and the guard column exhibited a deterioration of the separation after somewhere around 800 injections at the earliest. When we replaced the guard column, the separation was restored to the same performance as we had observed initially. To avoid deterioration of column performance, we decided to regularly replace the guard column at a predetermined number of injections that was lower than the observed failure rate. When we did this, we did not experience any deterioration of the separation until over 10,000 injections. Then we stopped the experiment. The column was still in excellent condition. Neither an appreciable shift in retention nor a substantial change in plate count were observed. We also did not find a difference in performance, when we ran the same test using columns packed with different particle sizes: 3.5 µm packings performed equally well to 5 µm packings in these experiments.

This set of experiments clearly demonstrates that the durability of well-packed columns is quite excellent. The limitations in column lifetime that we all experience are not due to the column, but are caused by other factors. Some of these factors are extraneous materials from the sample, the injector, pump seals, etc., that normally accumulate on the top of the column and cause an irreversible deterioration of

the column performance. Many people believe that filters will prevent this mechanical deterioration of the columns. We used the standard filters in our HPLC system, and they simply did not prevent column deterioration. Only the sacrifice of the guard columns preserved the performance of the analytical column. The reason for this is simply that whatever is causing the deterioration of the analytical column will be retained on the guard column, independent of the nature of the cause.

We also performed similar experiments with spiked serum samples. In this case, we did a simple protein precipitation for sample cleanup. As in the experiment described above, a regular replacement of the guard column preserved the analytical column. In this case, the guard column needed to be replaced more frequently due to the nature of the sample matrix and the simple sample preparation procedure.

Unfortunately, it is not possible to give general advice as to when a guard column should be replaced. It largely depends on the sample and on the sample clean-up procedure that is used. For samples with a low amount of extraneous impurities, a guard column may be a reasonable substitute for a sample preparation and cleanup procedure. Conversely, preparation procedures for plasma or milk samples still leave a sufficient amount of endogenous material behind that the use of a guard column is absolutely necessary. However, you may be able to reduce the complexity of the sample preparation procedure knowing that the column is protected by a guard column. If the life of the guard column is less than 100 injections, I would consider incorporating a sample cleanup procedure or revising the sample preparation process. If the guard column protects your analytical column for 100 injections or more, the cost is under 50 cents per analysis. In the long run, it is cheaper to replace the guard column then to continue to buy new analytical columns.

All of the previous discussion assumed that the column is used for a single assay or in a single procedure. Under these circumstances, the column contamination is fairly predictable. If a lab uses many different HPLC procedures, I always recommend to dedicate a single column, including the guard column, to each procedure. This eliminates cross contamination of the column from one assay to another and makes the column life predictable and controllable. If your lab does not have any standard assays, this is a mute point. Even under these circumstances, I would keep the guard column and the analytical column together until a deterioration of the performance requires a replacement of the guard column.

Q.: This is good advice, but I am sure that the analytical column does not last forever even if it is protected by a guard column.

A.: This is indeed correct. There are other elements that limit column life, even when a guard column is used. Reversed-phase packings are very stable between pH 2 and 8 at room temperature. However, if you increase the temperature, especially when you are working close to the pH limits of the packing, column life may deteriorate rapidly even when a guard column is used. From a column stability standpoint, the best procedures use an intermediate pH. Also, the choice of the buffer ions plays a significant role in column degradation (1). The commonly used phosphate buffers are not the best choices. Significantly better results have been obtained with organic based buffers like TRIS or citrate buffers at pH 7-8. Also, the buffer concentration plays a role: a lower buffer concentration results in improved column life. This needs to be balanced against the ruggedness of the assay, which is improved at higher buffer concentrations. Therefore, even under more stressful conditions, good column lifetimes can be achieved.

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39. Fast Analysis and Column Backpressure

Q.: I am currently trying out a new column. It is a new 3 µm packing. It is supposed to give higher resolution and therefore faster analysis than 5 µm packings. However, I can't see how this can be faster, since the backpressure is higher on the 3 µm packing, and I actually can't run it at the same high flow rates as I used to run my 5 µm column. Isn't it wrong to think of smaller particle size columns to be faster?

A.: Hmm… This is more complicated. It all depends how the comparison is made. We need to discuss this step-by-step to see under what circumstances each advantages can best be achieved. A lot of this may seem quite counterintuitive, and we need to look at the details more closely.

Let us start with the simplest case: same column length packed with different particle sizes. I believe that this is the comparison that you are referring to anyway.

Q.: Yes, indeed. Same column length and column diameter, and different particle sizes.

A.: Under these circumstances, you will get a higher plate count, i.e. more resolution, for the 3 µm packing in most situations. However, you will also get a higher backpressure. If you have been driving your analysis time to the fastest possible with a 5 µm column, and if the limitation was the column backpressure, than it makes no sense whatsoever to use a 3 µm column of the same length. The higher backpressure of the 3 µm column will slow down the analysis.

What you really need to do to get the speed advantage of the smaller particle size, is to use a shorter column with the 3 µm packing. The best thing to do is to reduce the particle size and the column length in the same proportion. If you

have used a 5 cm long column with the 5 µm packing, you want to use a 3 cm long column with the 3 µm packing.

If you now use the same flow rate on both columns, the backpressure on the 3 µm column will still be higher, but also, since you are using a shorter column, the run time will be shorter. In addition, if you reduce the flow rate in proportion to the reduction in column length, your backpressure on the 5 cm 5 µm column and on the 3 cm 3 µm column will be about the same. If you do this, then your analysis time will also be about the same.

Q.: This is a bit complicated. Let me repeat! I have started with a 5 cm column packed with 5 µm particles at a flow rate of 1 mL/min. What you recommend is to use a 3 cm column packed with 3 µm particles at a flow rate of 3/5 of the previous flow rate, i.e., at a flow rate of 0.6 mL/min. Then the analysis time and the backpressure of the 3 µm column and the 5 µm column are the same. Then why would I consider using the 3 µm column, if there is no benefit whatsoever?

A.: What you have not yet considered is the fact that under these conditions the 3 µm column will give you a better performance than the 5 µm column. So, if the speed of your separation was limited by the backpressure of the column, you will still have a superior separation on the 3 µm column under conditions that give equivalent backpressure and analysis time.

Let us assume for the moment that you can tolerate a somewhat higher backpressure and can run at 1 mL/min on the 3 µm column. Then, if the speed of your separation is limited by the resolution of a pair of peaks, the 3 µm column will allow you to run the separation faster than it was possible with the 5 µm column. The shorter run time allows you to increase throughput. This is the primary benefit of the smaller particle size. In today's world, where there is a lot of pressure on everybody to increase the output of a lab, a gain in analysis time by 50% or so is a large improvement. For this reason, more and more people are using columns packed with smaller particles.

If there is plenty of resolution in your chromatogram, then the first thing to do is to explore how fast you can go with your 5 µm column, or even better, how short a 5 µm column you can use to do the analysis. In many simple QC applications, the column length is much larger than needed for the assay. Often, all that is needed is adequate separation of the analyte from the internal standard, and there is a giant gap between these peaks. Under these circumstances, you should simply explore a shorter 5 µm column at higher flow rates.

For example, an assay for content uniformity is run at 1 mL/min on a 15 cm 5 µm column, and the backpressure is about 1000 PSI. The assay takes about 12 minutes. There are two peaks in the assay, the compound of interest and the internal standard, and they are well separated from each other. The first thing to do is to decrease the column length, maybe to about 5 cm. Using the same flow rate as on the long column, your run time is reduced to about 4 minutes, and the peaks are still well separated. But your backpressure is only about 350 PSI now. You can take advantage of this by increasing the flow rate. 3 mL/min will give you about the same backpressure as you had on the 15 cm column, but now your run time is under 1.5 minutes. Your peaks will not be as well separated as they were in the initial assay, but you are still able to resolve them cleanly at these fast conditions. Following this logic, the time needed for many simple assays can be drastically reduced.

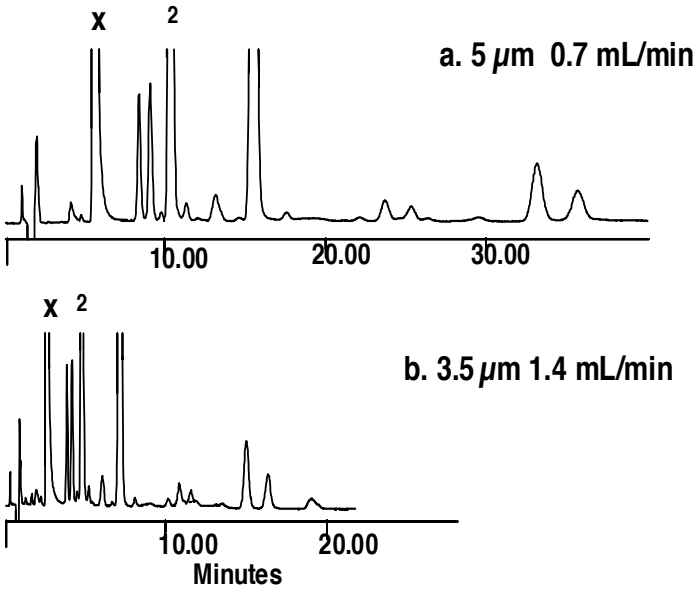

Figure 1: *Separation of chlordiazepoxide degradation products using a 150 mm x 3.9 mm 5 µm Symmetry® C_{18} column (a) or a 100 mm x 4.6 mm 3.5 µm Symmetry® C_{18} column. The plate count of peak 2 is practically identical for both chromatograms.*

Of course, many application examples do not tolerate such a radical reduction in run time. In many cases, there are several peak pairs in the chromatogram that do not tolerate a significant change in plate count. Under such circumstances, a switch to a shorter column packed with a smaller particle size results primarily in an improvement in the resolution between the peak pairs. Scaling the column and the flow rate according to the rules mentioned above can get you into a spot of equal resolution but reduced run time. An example for such a case is shown in figure 1. A 150 mm x 3.9 mm column packed with 5 µm particles is compared to a 100 mm 4.6 mm column packed with 3.5 µm particles for the analysis of degradation products of chlordiazepoxide. The shorter 3.5 µm column was run at a higher flow rate, allowing a reduction in analysis time by a factor of 2 at equal resolution of the critical peak pairs. An important consideration in this comparison is the fact that both columns have the identical

column volume, which makes extra-column effects and sensitivity independent of the choice of the column.

This is an example of the scaling of a separation to the same resolving power. In such a case the shorter column with the smaller particle results in a shorter run time. The backpressure will be higher, but as long as it is below the instrument limitations, this is not a problem. In many typical analyses, the pressure limitation of the instrumentation is still far away from the actual running conditions of an assay.

As you can see, the best solution to faster analysis depends on the assay. If there is a large separation between the different peaks and the chromatogram contains only a few peaks, the easiest way to reduce the run time is to use a shorter column with the same particle size. If you have a complicated chromatogram, the better choice is to reduce the column length and the particle size at the same time. If you only reduce the particle size without changing the column length, you will get a larger plate count at a higher backpressure, but you will miss out on the real benefits of smaller particles: shorter analysis time at equal resolution.

40. Column Backflushing

Q.: Column backflushing is a common practice in our lab. When a column shows a deterioration in peak shape, we connect it to an HPLC that is not used at the time and flow through it in the direction opposite to the normal flow direction. Sometimes, we use different solvents for this backflushing. While I have seen some improvements, it does not work reliably; actually, most of the time the improvements are small, and after about another hundred injections we throw the column away anyway. What can we do to improve this procedure?

A.: Actually, I am not an advocate of column backflushing. Most of the time it does not work, and when indeed it does work, I think that the improvement can be accomplished in more rational ways. In addition, I believe that there are more effective solutions to improve column life. Let us discuss the issues in detail!

What would be an event that would get us to try a backflushing of the column? There could be two reasons; one is an increase in backpressure, and the other one a significant deterioration of column performance. Both are believed to be caused by the accumulation of sample ingredients or debris from the mobile phase or HPLC instrumentation at the head of the column.

The first question is, where is this debris accumulating? If it is large enough, it will collect on the external face of the frit. Under these circumstances, a replacement of the frit would do the same good as backflushing. A replacement frit costs maybe $ 10. If you have a replacement frit on hand, you can substitute the old frit with a new one and you are up and running again. Then you can check, if the old frit can be rejuvenated, maybe by putting it into an ultrasonic bath for a while or by just plainly backflushing the frit. You can do this using the empty barrel of an old column. This is a fairly rapid procedure.

However, replacing the frit will only work if the frit is the problem. In my experience, this is rarely the case. Most of the time, something has accumulated on the head of the column. The mere fact that the accumulation occurs indicates that the material is strongly adsorbed at the column top, in a relatively narrow band. If there is a simultaneous increase in backpressure, this indicates that the contaminant has a reasonably large molecular weight or low solubility and is clogging the channels between the particles. As the backpressure increases, this means that a significant force is applied to the first layers of particles in the column, and that these layers will rearrange and ultimately cause a deterioration of the peak shape. To repair this kind of damage to the packed bed structure is very difficult or even impossible. This is the reason why column backflushing is a gamble at best.

Q.: At least one can get a little bit more life out of the column. Backflushing may not be a very good solution, but it does help.

A.: Of course it helps, but I would prefer a more reliable and more permanent solution. Have you ever used guard columns? They protect the column from all different types of debris, from the sample or from the instrumention. If your sample is relatively clean, then a guard column can last for a thousand injections. The analytical column is often as good as new afterwards. If your sample is fairly dirty, then a guard column will last only for maybe 50 injections. You will know what to expect, since the lifetime of a guard column is about the same as the lifetime of your analytical column today.

Of course, guard columns are not free. However, the price that you pay for the guard column is relatively low, compared to the cost of an analytical column. Often, a holder is needed, since most guard columns are in the form of a cartridge column. But this one-time cost is soon recovered by the longer lifetime that you get from your analytical columns.

What guard column should you use? The entire discussion assumed that you use the same packing in your guard column as in your analytical column. Most of the time, this is the best solution, for several reasons. The most important one is that the addition of the guard column does not effect your separation to any appreciable degree if you use the identical packing as in the main column. At the same time, this approach is generally also the best way to protect the analytical column. The use of different packings from different manufacturers is generally not recommended. The bonded phases from different manufacturers are quite different, and a guard column made from a different packing can not guarantee as good a protection as a guard column made from the same packing. In addition, the differences between the packings may cause some additional bandspreading of your analytes. As you can see, there are

several good reasons to keep the packing in the guard column and in the analytical column identical.

There are a few rare occasions, where the contaminant that is causing reduced column life is known and the use of a different guard column would capture this contaminant more effectively. You need to think this through quite carefully. An example might be the case where the samples, which are analyzed using a C_{18} packing, are contaminated with a polyamine. This polyamine can be adsorbed much better on a cation exchanger than on a C_{18} guard column. If you are using a different packing in the guard column, you need to make sure that it does not interfere with your analysis. In the example given here, all the analytes of interest are acidic or neutral compounds. Therefore, the addition of a cation exchanger to capture the polyamine is quite feasible. If the analytes of interest would contain basic compounds, the use of a cation exchanger as a precolumn is more problematic and requires a thorough investigation. However, cases as the one described here are fairly rare, and most of the time the best guard column contains the identical packing as the analytical column.

Q.: Many different guard column designs are commercially available. Which one should I use?

A.: The important thing is to use a guard column that contains the identical packing as your analytical column. Therefore, you should talk to the supplier of your analytical column, as to which guard column he recommends. Sometimes, several types are available, with different prices and different functions. Practically all guard columns that I am familiar with are of the cartridge column type. This reduces the cost of the guard column as the endfittings can be reused. Some are designed to be connected to your analytical column with an additional piece of tubing, some connect directly to your analytical column. While some guard column designs may be better than others, the important point is to use a guard column with the identical packing as in the packing in your analytical column. With this approach, column life can be increased much more effectively than with column backflushing.

41. Selectivity Shift

Q.: We are determining the impurity profile of a drug. For the parent drug and all impurity peaks but one the retention time is constant. For one of the impurity peaks the retention is decreasing slowly, day by day. The key difference between the compound with the shifting retention and all the other compounds is the fact that it contains a carboxylic acid group. The column used is a reversed-phase column with an embedded polar group. The mobile phase consists of methanol and an ammonium acetate buffer adjusted to pH 4.9. What is going on? Why is this peak shifting, while all the other ones have a constant retention?

A.: The observation that the retention remains rock solid for most of the peaks tells us that the overall properties of the column and the mobile phase are not changing. General shifts in retention could be explained for example by impurities collecting on the column. Since this is not the case, we need to look specifically into the properties of the analyte, for which this shift occurs.

You describe the analyte as the only one that contains a carboxylic acid group. This indicates that the reason for the shift in retention is specifically related to this group. This is not impossible as you have described that you are using a reversed-phase column with an embedded polar group. This bonded ligand could be the explanation of the phenomenon observed.

There exist several types of bonded phases with an incorporated polar group. They contain different embedded polar groups, and the preparation procedure can be different. The SymmetryShield™ and XTerra™ RP columns (1,2,3) manufactured by Waters Corp. contain a carbamate group, the Prism® and Spectrum columns (4) from Keystone an urea function, and the Discovery™ RPAmideC16 from Supelco an amide group (Fig. 1). All of these columns are prepared in a single-step bonding reaction: the ligands are assembled first, and then attached to the surface in a single step, or at least there is no evidence for a multi-step surface reaction.

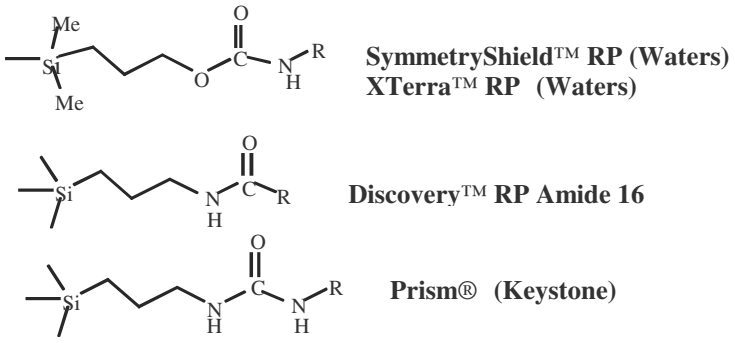

Figure 1. *Commercial bonded phases with incorporated carbamate group*

Some of the older packings (5) with an incorporated amide group, and unfortunately also some of the newer ones (6), are prepared in a multi-step surface reaction. In this case, an aminosilane is attached to the surface first. Then the amino bonded phase is reacted with a long-chain acid chloride or something similar to create the reversed-phase character of the phase. Unfortunately, due to steric effects, such a surface reaction is always incomplete and leaves many residual amines on the surface. Attempts are made then to remove the residual amines with something like acetic anhydride or some other activated short-chain acid, but even such attempts have not been successful. The surface of such a packing is shown in figure 2. As it turns out, it is as difficult to remove the residual amino groups on such a packing as it is to remove residual silanols on a classical bonded phase.

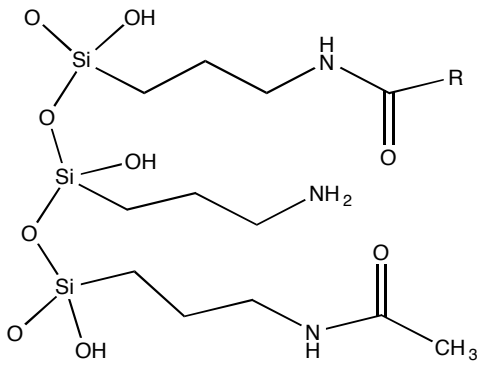

Figure 2. *Amide phase prepared via a two-step reaction. The phase contains measureable amounts of residual amines.*

Q.: Indeed, the phase that I am using is an amide phase. But why does the retention of my acidic analyte change with time?

A.: If your phase has such residual amino groups, one would expect the acidic analyte to interact with these groups via ion-exchange. This would result in increased retention, just as the interaction of basic analytes with silanol groups results in increased retention on classical packings. It is also known from the preparation of amino phases that the stability of the amino group in an aqueous mobile phase is not very good. It appears as if your column is slowly loosing the residual amino groups, and that this is the cause of the reduced retention of your acidic analyte from one day to the next. All the other analytes do not interact with the amino group, therefore their retention times remain stable.

Q.: This is a possible explanation of the problem, but I do not know anything about the preparation procedure of this particular packing. How can I solve my problem? I have to say that I do like these phases with the embedded polar groups. They give good peak shapes for practically everything.

A.: First, I would call up the manufacturer and discuss the problem with him. If a manufacturer is aware of a problem, he can address it in a next generation product. Or, you can choose one of the products that is free from this problem.

Generally, I have to agree with you: the bonded phases with embedded polar groups indeed give a superior peak shape for even the most difficult samples. Unless one makes an error in the choice of the mobile phase, no tailing is observed for practically all analytes. Another good feature of these packings is the fact that they can be used without difficulty with mobile phases containing 100% water. This means that even fairly polar compounds can be analyzed without difficulty using packings with an embedded polar group. Many of the better reversed-phase packings do not tolerate highly aqueous mobile phases well due to hydrophobic collapse, but this is not a problem for bonded phases with embedded polar groups. The retention mechanism is no different than the standard reversed-phase mechanism, and the bothersome interactions with residual silanols are largely eliminated. More and more people are discovering the advantages of these phases, and they are used in more and more applications. Good phases are available from more than one manufacturer, but you should inquire about the bonding process. Single step processes are preferred; they are free from the complications that you encountered in your application.

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42. New Method

Q.: In my department, we are constantly developing new HPLC methods, versions of which will be used for multiple purposes. Most of the time, the primary goal is to establish an impurity profile for new drug substances. Versions of these methods will then be used for dissolution testing or stability testing, and ultimately simplified versions will be used to test content uniformity. Currently, the methods often get reinvented as they move from department to department. Is there a way to streamline this process?

A.: This is a difficult question which has both a technical component as well as a managerial component. Let us just discuss the technical component here!

The tasks you describe suggest to me that you are working in the pharmaceutical industry. Some of the answers may be specific to your industry, but I am sure that similar issues exist in other industries as well. Nevertheless, I'll address your question in the context of the pharmaceutical industry.
An impurity profile a method often requires the resolution of a large number of compounds. Analysis time is often not a factor, since such an analysis is only done once in a while. In dissolution testing, on the other hand, you are only interested in a single peak, and you want to have a fast method with good column lifetime. The differences between the needs of the two types of analysis are great, and in principle there are good reasons for a "reinvention" of the method, as you called it.

Some components of this process can be streamlined, and actually optimized. First of all, you need to decide on a column family where the same packing is available in many different column configurations. In principle, this should not be difficult, since nearly all column suppliers have a large number of column configurations. For example, the XTerra™ family of columns (1) from Waters Corp. comprises over 270 part numbers. A large number of column configurations should allow you to select the correct column for the different needs of the different departments.

Next, you should select a column configuration that is suitable for generating an impurity profile. This is often is the most complex type of analysis. Fortunately, it is also the analysis which is needed earliest in a project. Therefore, other methods can be derived from the method developed for the impurity profile.

Method development for an impurity profile is frequently a most demanding task. Often, columns with the best resolving power (long columns packed with small particles) are used. Gradient elution is most of the time the only way a reasonable chromatogram of all possible impurities can be obtained. A method with a complex elution profile also needs to be rugged: you need to demonstrate that the results from the method can be achieved using different columns and different instruments in different departments. The good news is that the person who has developed the method for the impurity profile has done a large amount of homework useful for the development of subsequent methods. This knowledge can be used for the simpler methods either in the same department or in other departments.

How does one go from this complex impurity profiling method to a simpler one? In some cases, people just use the same column and develop an isocratic separation for the other needs, for example, for content uniformity testing. On one hand, the use of the same packing material has its merits. After all, the studies for the impurity profile just established that the method is reproducible using this packing. On the other hand, for a simple two-peak-chromatogram one does not need a high-resolution column. One can use the same packing in a shorter column to speed up the separation. Or one can use a shorter column packed with a larger particle size of the same packing to improve the ruggedness of the method.

The path from a complex gradient method to a simple isocratic method can be very rapid and straightforward, if you have the right information. You need to determine, at which solvent composition the parent peak elutes in the gradient method. In other words, you need to know the exact solvent composition at the column exit at the point of elution of the parent peak. This requires a good knowledge of your instrument, since there is a delay between the time that the gradient is formed and the time it reaches the column exit (2). You need to know the gradient delay volume of the instrument, and the dead volume of the column. The dead volume of the column is easy to obtain: you just inject an unretained peak, such as dihydroxyacetone for a reversed-phase column. The gradient delay volume of the instrument is obtained by running a gradient with a UV absorber. The delay volume is calculated from the difference between the programmed start of the gradient and the time that the UV absorber shows up in the detector.

The development of the isocratic method should start with the same packing material and the solvent composition at which the compound elutes in the gradient. If you do this, you should get a retention factor around 2 and no less than 1. You now have the start of a simple and fast isocratic method for the other assays. It may be entirely sufficient to have such a low retention factor. On the other hand, people prefer a slightly higher retention factor, maybe between 2 and 5. To obtain this, you simply need to adjust the solvent composition slightly.

For content uniformity, stability testing and dissolution testing, you do not need such a high-powered column as for the impurity profile. You should select a shorter column. Often, even a 5 cm 5 micron column gives satisfactory results for these tests, the run time is much shorter and the backpressure is much lower than with a 25 cm 5 micron column. For the dissolution testing, you may consider a guard column to protect the analytical column from the acidic sample. However, no major redevelopment of the methods is needed. As a matter of fact, the same simple isocratic method can be used for content uniformity, stability testing and dissolution testing. This should also streamline the method validation process (3).

Of course, in many places the different tasks are executed in different departments. Therefore, this requires a coordination of the efforts in the different departments. In today's world, with the large workload for everybody, such a streamlining can only be beneficial.

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43. Negative Peaks

Q.: I am running a reversed-phase separation with a phosphate buffer at pH 2.5 and 20% THF on a C18 column. The detector is a PDA, and the wavelength is 225 nm. I am getting a negative peak in the middle of the chromatogram, between the second and the third peak. I am accustomed to seeing negative peaks at the beginning of the chromatogram, around V_0, but I have never seen one in the middle of the chromatogram. Can you explain what is happening?

A.: Maybe the best thing to do is to start with a simple philosophy: a peak indicates a difference in the composition of the sample and the mobile phase. It matters not, whether the peak is positive or negative or whether it is retained or not. Therefore, what we need to do is to figure out, what the source of the extra peak in the chromatogram is. The fact that it is negative is additional helpful information.

You state that you are used to negative peaks early in the chromatogram. These peaks are mostly due to differences in the composition of the sample matrix and the mobile phase. This can be the solvent composition used to make up the sample, or the pH. Unless one is extremely careful in the preparation of the sample in mobile phase, extra peaks can stem from this difference. For most of us, this is not a concern. We realize that there are differences between the sample solvent and the mobile phase. Sometimes, we also create such differences on purpose, for example for an enrichment of the sample on the column.

Q.: This is indeed correct. Often, my samples contain extraneous ingredients such as excipients. However, they typically elute with the solvent front. This is the case here as well. Also, the sample is not exactly dissolved in the mobile phase, but is it diluted with mobile phase.

A.: To sort out, if the extra peak is coming from excipients or from somewhere else, all you need to do is inject standards. If this is an excipient peak, it should disappear.

Q.: I thought of this already. The extra peak is still present, when I inject standards or even mobile phase.

A.: OK. Now, this is an important piece of information. If you get the same phenomenon independent of the nature of the sample, we can exclude the sample as the cause of the problem. Therefore, we should focus on instrument components or the mobile phase itself.

If your extra peak were positive, one could consider a contamination in the injector, the syringe used, seals in the sample vials or related things associated with the injection process. Since this is not the case, I would reject the injection process as a source of the problem.

The mobile phase itself and all the components that it is in touch with are the most likely source of the problem. Imagine an ingredient in the mobile phase that is present at very low concentration and has some retention on the column. If you inject mobile phase that does not contain this ingredient, you will get a negative peak with the same retention factor as the ingredient would have, if you would inject it as a sample. This is called vacancy chromatography. To solve your problem, all we need to do is to find this ingredient. The unfortunate part is that you are working at a very low wavelength. You will see more compounds at a higher sensitivity in the low UV region. I am sure that this is the reason why you selected this wavelength to start with.

Q.: This sounds like a good description of what my problem might be. What can I do to narrow it down further so that I can eliminate it?

The first thing to do is to check the mobile phase itself. What is the quality of the THF? What is the quality of the water? You can simply inject a sample of THF and a sample of water and see if you encounter a peak at the same retention time as your negative peak. If you can create a peak, you have found the source of the problem, and then you can go to the next step. If it is the THF, you can investigate the quality and purity of the THF. THF is prone to form peroxides, and some forms of the THF contain components that are designed to inhibit the formation of peroxides. You should be able to get this information from the label on your solvent bottle. THF is also an excellent solvent for plastic parts and for extracting additives from these plastics. Anything in touch with your THF solvent line is suspect: filters, the tubing, seals, ferrules, anything…

The water is a possible source as well. In most places, water purification devices such as a Milli-Q™ system from Millipore Corp are used. The filters in these devices have a limited capacity and should be replaced after some period of time.

Another possible source of contamination is the buffer or the buffer preparation itself. What did you use to stir the flask when preparing the buffer? Was the flask clean? If you measured the pH with a pH meter, did you clean the electrode before you used it?

After you checked all the components of the mobile phase and the devices in touch with the mobile phase and the problem still has not yet been resolved, I would check the HPLC instrument in detail. Are there any components that are incompatible with your mobile phase, especially with THF? Are there any pockets in the instrument that have not been purged properly? Is there a possibility for bacterial growth in your solvent path? I would place my bets on the solvent, the water and the buffer preparation and look very closely at these steps.

Q.: This sounds good, but it also is a large amount of work. Is there a way to narrow it further?

A.: On first glance, I do not see how. On the other hand, you may want to ask yourself the question, why do you want to eliminate the negative peak. From the description of the problem it appears that the negative peak does not interfere with the analysis. Therefore, you may just declare that this is a fact of life and ignore this. Of course, if you are in a regulated environment, this should be thought through carefully.

44. Tricky, Tedious, Time Consuming

Q.: We are doing a very large number of routine assays of samples from clinical trials. The samples are plasma samples from patients, and we are determining the metabolic fate of the parent drug and its metabolites. This is very important work, and the samples are precious, but the work is also very laborious. The sample preparation is the major problem. We are using C18 cartridges to remove plasma constituents, and the entire sample preparation procedure has to be carried out with a high precision. If we don't do that, the results vary a lot. The actual analytical method is a HPLC method. Is there a better way to do the sample preparation?

A.: Indeed, this type of work is tricky, tedious and very time consuming. This applies both to the development of the methods and to the handling of the samples themselves. However, in recent years a range of developments has occurred that makes this task easier (1).

The first problem encountered with classical C_{18}-type packings is the difficulty encountered with the wettability of such packings with aqueous samples. The typical preconditioning of the SPE cartridge requires a wetting with methanol, then a brief conditioning with water or buffer before the loading of the sample. If the SPE cartridge dries between the preconditioning step and the loading of the sample, low recoveries of the analytes of interest are encountered. This requires to pay close attention so that this does not happen. This is not such a large problem, when one is dealing with individual samples. But it does create difficulties when one needs to deal with many samples at the same time such as in a 96-well plate. Therefore, manufacturers have introduced improved SPE products that do not suffer from this problem. An example is the Oasis® HLB SPE product line from Waters Corp. This packing material contains a mixture of both hydrophobic and hydrophilic groups. The hydrophobic groups impart exceptional retention, higher than most C18 packings, while the hydrophilic groups provide good wetting with aqueous samples. The consequence is that these packing materials can dry out during the sample preparation step without loosing capacity. This makes the sample preparation step much less tedious. Now you don't have to sit there any more and carefully watch the fluid level in your SPE device to make sure that it does not dry out.

Q.: This sounds interesting. What is the secret behind this?

A.: It is very simple. The reason for the loss in capacity when a C18 cartridge dries out is the fact that C18 is not wetted by water. What prevents the water from penetrating the pores is the poor wetting angle between water and the hydrophobic surface of the C18. If one provides hydrophilic groups on the surface, the wetting is improved and water stays in the pores or penetrates the pores easily. For example, the Oasis® HLB packing is prepared from a mixture of divinylbenzene as the hydrophobic component that provides retention and N-vinyl pyrrolidone, which provides the wettability with water. The control of the amount of both components provides the right balance between hydrophobicity for solute retention and hydrophilicity or water wettability.

Q.: OK. I understand the improvement in wettability. However, how does it behave as an adsorbent? I like the way the reversed-phase packings are acting. It is easy to understand, and not difficult to make them work.

A.: There are a lot of similarities between these hydrophobic sorbents and a C18 packing. Generally, the retention mechanism is similar: whatever is retained strongly on a C18 packing due to a reversed-phase mechanism is retained strongly on these packings as well. On the other hand, they are not based on silica, which means that the retention mechanism is not complicated by silanols. Plus, for the same reason, they can be used without difficulty with alkaline solutions, which opens new doors in the sample preparation process.

Q.: The fact that there are no silanols is a mixed blessing. I can see how this can make the elution protocol simpler. But we also use the silanol interaction to improve retention, if we need it….

A.: On classical C18 packings, residual silanols are a side product. The control of their activity is generally not as good as the control of the hydrophobic activity of these packings. While residual silanols indeed can help in retaining basic analytes, the reproducibility of such a procedure is more tricky than that of a packing that exhibits only reversed-phase character.

On the other hand, these polymeric packings are stable to higher pH-values than silica-based packings. This allows you to use pH as a more powerful tool in SPE than was possible with silica-based packings. You can selectively improve the retention of basic analytes by carrying out the washing protocol at basic pH values. Or you can achieve selective elution by changes of the pH. Generic procedures for this have been worked out (2,3). The manipulation of the ionic interaction is now entirely under your control, and does not depend any more on the concentration of "residual" silanol groups.

The good wettability of these polymeric packings also opens a new avenue: they can be used without difficulty in parallel processing schemes such as 96-well plates (2). The fact that you do not have to worry about the drying of every single well just makes the whole sample preparation process simpler. In addition, the simultaneous processing of many plates eliminates many sources of error. For example, standards can be processed together with the samples to control the process or to correct the results. Also, significant time savings are realized by parallel processing of many samples.

To summarize:
The favorable wettability of the second generation SPE sorbents makes sample preparation less tedious. The absence of strong secondary interactions such as the silanol interactions of C18 packings makes sample preparation less tricky. The use of 96-well plates for parallel processing of samples makes sample preparation much less time consuming than it used to be. There is progress…

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45. Fast Separations

Q.: I recently heard a lot about ultra-fast separations, with a complete analysis within less than 5 minutes. Another subject that interests me is the subject of "ballistic" gradients. I would be interested to hear more about this, and what one can do today.

A.: This is a very interesting subject indeed. While it is not a troubleshooting subject, I think that it is worth a discussion.
The development of these very fast separation techniques is driven by the ability to generate new chemical entities very rapidly through combinatorial chemistry. One would like to get good information on the success of these fast synthetic techniques. Mass spectrometry or NMR can help you to determined whether you have synthesized the chemical entity that you wanted to create, but only a separation technique such as HPLC can get you information on the quality or the yield of your synthesis and the possible side products. The best approach is therefore a combination of LC with MS. If you can synthesize 1000 new entities per day, your analysis time can not be much longer than 1 minute to keep up with the demands.

Until a short time ago, the difficulty has been that HPLC was considered to be too slow to get such a short analysis time (1, 2). However, with some rethinking of the details of the process, faster and faster analyses have become possible (3).

There are two elements that have contributed to this. One is a reconsideration of the parameters underlying the gradient separation. The other element is the commercial availability of very short columns packed with very small particle sizes (4). The combination of both elements is the secret to ultra-fast separations.

In order to maximize the number of peaks that can be separated in a gradient, one needs to consider two separate contributions. The first one is the width of the peaks stemming from the manipulation of flow rate, or better linear velocity, the second one is the interplay between the gradient volume and the width of the peaks as the gradient volume expands. Both parameters together determine, how many peaks can be crammed into a limited analysis time.
The maximum number of the peaks that can be generated in a gradient is called the peak capacity. Since the peaks in gradients are for the most part of the same width, it can be calculated easily by dividing the gradient duration t_g by the peak width w:

$$P = 1 + \frac{t_g}{w} \qquad (1)$$

The theory of chromatography allows us to calculate the peak capacity as a function of the operating conditions (3).

$$P = 1 + \frac{\sqrt{N}}{4} \cdot \frac{B \cdot \Delta c}{B \cdot \Delta c \cdot \frac{t_0}{t_g} + 1} \qquad (2)$$

N is the plate count of the column under the operating conditions, $B \cdot \Delta c$ is a parameter that relates to the type of samples to be separated and the solvent span Δc over which the gradient is executed, t_0 is the column dead time, and t_g is the gradient duration, as above. We can take the factor $B \cdot \Delta c$ to be a constant that depends only on the type of analysis that is run. There are two reasons for this: one is the fact that the types of compounds to be analyzed in a combinatorial library are all similar to each other with a similar structure and a similar molecular weight. The other is the fact that we are always running the same gradient, mostly from 5% organic to 95% organic. The column plate count is a function of the linear velocity, which in turn depends on the column dead time t_0, and the gradient duration determines the total analysis time.

From equation 2, we can generate a three-dimensional graph showing peak capacity as a function of the linear velocity - or better flow rate - and the gradient duration (Figure 1). It can be seen as a graph which measures the resolution capability of a column. For every column length

and particle size, different graphs can be generated. The example in Figure 1 shows the performance of a 5 cm 5 µm column with an internal diameter of 4.6 mm. For every gradient duration, the graph exhibits a performance optimum at a different flow rate. Also, longer gradients give a higher peak capacity than shorter gradients. The maximum flow rate that can be used with every column depends on the pressure limitation of the instrument.

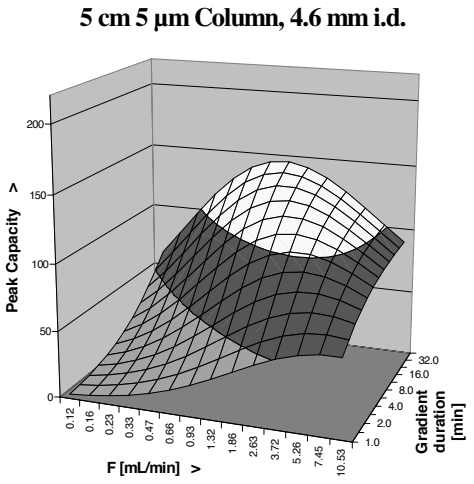

Figure 1. *Peak capacity as a function of the flow rate and the gradient duration for a 5 cm 5 µm column*

How does the picture change if we use another column? Figure 2 shows the same graph for a 2 cm 2.5 µm column with the same internal diameter.

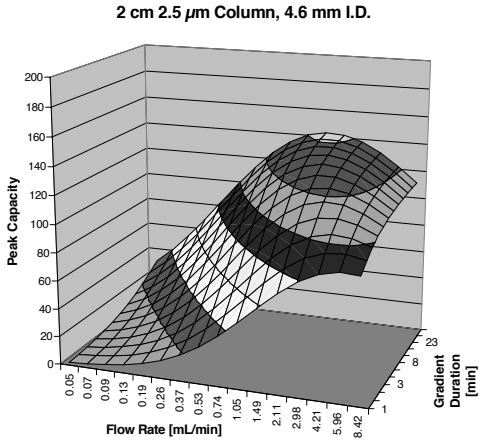

Figure 2. *Peak capacity as a function of the flow rate and the gradient duration for a 2 cm 2.5 µm column*

It can be seen that the performance maximum occurs at a fairly high flow rate for very fast gradients. This is the result of the interplay between the expansion of the gradient as expressed by the ratio t_0/t_g in equation 2 and the dependence of the plate count on the linear velocity or flow rate. For the column shown in figure 1, the best flow rate is around 7 mL/min for a 1 minute gradient. This is a higher flow rate than what most people consider to be best.

We see immediately that the optimum performance is higher at fast gradient times. Also we see that the flow rate at which the optimum performance occurs is similar to the 5 cm 5 µm column. One can see that the same one-minute "ballistic" gradient can still be executed on these very short and very fast columns with better resolving power than with the more traditional 5 cm 5 µm column shown in Figure 1. The faster mass transfer of the 2.5 µm particles shifts the optimum of the curve up to a higher resolution value. This means that quite powerful separations can be carried out in timeframes compatible with the needs of high-throughput combinatorial chemistry – at least for the moment. If combinatorial chemistry output speeds up still further, the analytical chemists will need to think again how to speed up chromatography still further.

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46. Buffers for LC/MS

Q.: What mobile phase additives can be used with reversed-phase columns and LC/MS detection?

A.: Since hyphenated LC/MS instrumentation has become more and more popular during the last few years, the standard buffers of former times, for example phosphate, have dropped out of favor. The issue is that one would like to use volatile mobile phase additives. Phosphates are not volatile, and - with time – will clog the LC/MS interface. This results in significant downtime, and more importantly, in a large amount of work to clean up the interface again. Some new source designs can accommodate now up to 10 mM of a phosphate buffer for an extended period of time, but regular cleaning is still required.

Therefore, standard HPLC methods used with MS detection use mobile phase additives that are volatile. The

simplest additives are simple acids, such as formic acid or acetic acid. They are often used in a concentration of 0.1% up to 1%. This provides an acidic environment, enough to fully protonate basic analytes. In many cases, this is sufficient to provide good control over the retention of both acidic and basic analytes.

However, there are exceptions, where such an approach has its limitations. One of the disadvantages of a strongly acidic mobile phase environment is the fact that the ionization of acidic analytes is suppressed. Therefore, a better condition for MS detection is to use a slightly higher pH in conjunction with detection in negative ion mode. In such a case, ammonium acetate and ammonium formate buffers are used, and pH control is important to control retention. The pK_a of formic acid is 3.75, and the pK_a of acetic acid is 4.75. Consequently, these are the optimal pH values for these buffers. The typical concentrations are around 20 mM or lower, even as low as 5 mM are possible. At such a low buffer concentration, it is definitely best to use the buffers at a pH very close to the pK_a of the buffering ion. A reasonable rule of thumb for such low concentrations is to use a buffer at +/- 1 pH units around the pK_a of the buffer.

Another difficulty that can be avoided with a correct choice of the pH is the suppression of the ionization of analytes due to matrix interferences. This is a common problem in the analysis of plasma samples by LC/MS. However, most matrix interferences are ionic or ionizable in nature, and the analytes of interest often are ionic as well. Therefore the coelution pattern can be changed by changing the pH of the mobile phase. With an appropriate change in the elution pattern of the analytes and the interferences, ion suppression can be avoided.

Occasionally, it might be desirable to run the chromatography at alkaline pH. The reason for this can be the avoidance of ion suppression, or a desire to improve sensitivity by improving the ionization of the analytes of interest. Acidic analytes give the best MS response in the alkaline pH range. On the other hand, we found that even basic analytes still respond surprisingly well under alkaline conditions. The mobile phase additive that we use for weakly alkaline mobile phases is ammonium bicarbonate. The pK_a values of the buffer constituents are 9.2 and 10.2. Therefore, ammonium bicarbonate can be used over a broad alkaline pH range, with the most preferred value at pH 10. This buffer is completely MS compatible. Upon heating, ammonium bicarbonate decomposes into only volatile components: ammonia, water, and carbon dioxide.

To summarize: we have some good solutions for MS compatible buffers in the acidic pH range from pH 3 to pH 5.5 and in the alkaline pH range from pH 8.5 to pH 10.

Q.: How about the salt ammonium acetate? I have seen it being used at neutral pH.

A.: Yes, I have seen this as well. However, we must realize that it is not a buffer at all. At pH 7, ammonium acetate has no buffering capacity whatsoever. One should consider it to be just a salt additive. It may help in the MS ionization process, but it has little function in the separation process. For the neutral pH range, the best solution is to start with ammonium bicarbonate and add formic acid. The actual buffering is provided by the first dissociation of the carbonate ion. At high concentrations, carbonic acid dissociates to form carbon dioxide and water. But at the low concentrations of the buffers used in LC-MS, this is a functional approach and no degassing has been found.

47. Alkaline Buffers for RPLC

Q.: In the last few years, reversed-phase packings have become available that are stable in the alkaline pH range. I would like to explore some of these new capabilities. What buffers can be used with reversed-phase columns in the alkaline pH range?

A.: Indeed, the exploration of a broader pH range opens up new capabilities that were not accessible in the past. Some of the newer packings are stable to pH 10, and others even to pH 12. Even classical packings can often be used in the alkaline pH range, if less aggressive buffers are chosen.

Q.: What are the more aggressive buffers that should be avoided?

A.: Clearly, phosphate has been found to be more aggressive in the alkaline pH range than other buffers. Therefore, I do not recommend to use alkaline phosphate buffers with silica-based packings or related packings based on inorganic-organic hybrids. Phosphate has a limited pH range anyway. Ammonia is also fairly aggressive, but it can be substituted without difficulty with organic amines. In addition, organic amines cover a broad pH range, with pK_a values ranging from 9 to 11.5.

Q.: OK, then which buffers are recommended?

A.: We have assembled three tables (1) of buffers suitable for the alkaline pH range. Table 1 contains inorganic buffers, table 2 organic buffers, and table 3 zwitterionic buffers, as they are commonly used in biochemical applications. Some of the buffers can be used as mobile phase additives as well, just as formic acid and acetic acid are used in the acidic pH range. Mobile phase additives may be useful for the control of the ionization of analytes outside +/- 2 pH units around the pK_a of the analyte. However, if the pK_a of the analyte and the pK_a of the mobile phase additive are in the same pH range, a control of retention or peak shape may not work as well as with a true buffer.

Among the inorganic buffers, ammonium bicarbonate is a very good choice for a multitude of reasons. For one, it covers a broad pH range due to the combined buffering capabilities of the carbonate ion and the ammonium ion. In

addition, it is compatible with MS detection, since it decomposes into volatile components. While it has a small background in the low UV-range, the absorbance is about the same as the background of formic or acetic acid or TFA in the acidic range. This is not perfect, but it can be used even in gradients in the low UV with a good match in the composition of the mobile phase.

Table 1, Inorganic Buffers

Buffer	pKa-Value	pH-Range
Phosphate 2	7.21	6.2 – 8.2
Borate 1	9.14	8.1 – 10.1
Ammonia	9.25	8.2 – 10.3
Carbonate 2	10.25	9.2 – 11.3
Phosphate 3	12.33	11.3 – 13.3
Ammonium Bicarbonate	9.25 and 10.25	8.2 – 10.5

Table 2, Organic Buffers

Buffer	pKa-Value	pH-Range
Glycine	9.8	8.8 – 10.8
Trimethylamine	9.74	8.7 – 10.7
1-Methylpiperidine	10.3	9.3 – 11.3
Triethylamine	10.75	9.7 – 11.8
Piperidine	11.2	10.2 – 12.2
Pyrrolidine	11.3	10.3 – 12.3

Table 3, Zwitterionic Buffers

Buffer	pKa-Value	pH-Range
MES	6.2	5.2 – 7.2
MOPSO	7.0	6.0 – 8.0
MOPS	7.3	6.3 – 8.3
TAPS	8.5	7.5 – 9.5
CAPSO	9.7	8.7 – 10.7
CAPS	10.5	9.5 – 11.5

Among organic bases (table 2), several options are available that cover a broad range of pK_a-values, from 9.7 for trimethylamine to 11.3 for pyrrolidine. Trimethylamine has a very low boiling point and is compatible with MS detection. Of course, you need to use a MS compatible counterion such as formate or you can use trimethylamine just as a mobile phase additive, as you would use formic acid or acetic acid in the acidic pH range. We have used pyrrolidine buffers at pH 11.5 successfully for extended periods of time (2) with XTerra® reversed-phase columns. Triethylamine is the traditional additive to reversed-phase buffers used to suppress tailing of basic analytes. People try to avoid using it due to its unpleasant odor.

The odor is a general disadvantage of the bases. However, there is a solution to this as well: zwitterionic buffers, shown in table 3. They are not volatile, therefore there is no smell. On the other hand, this makes them not compatible with MS detection any more. However, if your method will never see a mass spectrometer, zwitterionic buffers may be a very good choice. They are used in many applications in biochemistry, and are available in good purity. The pK_a-values in table 3 range from 6 to 10.5. There is a suitable buffer for all the pH ranges of interest in this discussion.

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48. Post-Column Derivatization

Q.: I am considering post-column derivatization to improve the specificity and the detection limits of a method. What do I need to take into account to be successful?

A.: A lot of things! First of all, you need to find a fast reaction scheme for the compound(s) of interest. To be compatible with an HPLC method, the reaction needs to give you a good response in less than 5 minutes, preferentially in less than 2 minutes. Second, the reaction needs to be compatible with the HPLC mobile phase. Specifically, if you are using a reversed-phase method for your separation, the reaction needs to be compatible with an aqueous medium. This may not always be the case. Third, the reaction product(s) need to be easily detectable with common HPLC detectors. To achieve low detection limits, the reaction product(s) should be detectable under conditions where there is little native interference from other compounds. Examples of this condition is absorbance detection in the visible wave-length range or fluorescence detection. Fourth, the detection should be sufficiently specific for the goals of your analysis. For example, a generic reaction scheme for amines will enhance the detection of all amines in the world, but if your sample contains only those amines that you want to detect, it may be the best solution to your problem.

Q.: Indeed, there are a lot of requirements. I would like to understand them a little bit better. Why do I need such a short reaction time?

A.: I just gave you a rule of thumb. This means that there is some flexibility around this. Let us consider, what is required! The HPLC separation requires a constant stream of mobile phase. This stream of mobile phase is mixed post-column with the derivatization reagent. Then the combined streams need to be stored somewhere until the reaction has developed the desired signals. The common way for doing this is in a flowing stream. The mobile phase may be flowing at 1 mL/min. To this, you add reagent at the same flow rate. If your reaction takes 5 minutes, you need a storage volume of 10 mL between the column and the detector. This is about 250 m of standard 9/1000 capillary tubing. Even if you are using tubing with an i.d. of 0.5 mm, you still need about 50

m of tubing. This is a lot of tubing. You need to worry about the bandspreading in the tubing as well. If you would use a run-of-the-mill tubing, the bandspreading in it would be in the order of a milliliter or more. The peak width of a peak eluting at a retention factor of 2 from a 4.6 mm x 150 mm column is only about 0.2 mL. Peaks that are well resolved in the column would be diluted and mixed with each other. Therefore the extra-column bandspreading in our example would destroy the separation that you have achieved in your high-performance HPLC column. Now you see, why one wants to have short reaction times.

Q.: OK, I see. You said that this applies when one is using a "run-of-the-mill"tubing. What does this mean, and what other options do I have?

A.: What I was referring to was a straight piece of tubing or simple coils. If you are using teflon tubing, there is something that you can do about the bandspreading. You can "knit" it.

Q.: What's that? Knitting a piece of teflon tubing???

A.: You heard correctly. This refers to a technique of applying a controlled deformation pattern to the tubing (1, 2, 3). This controlled pattern creates a secondary flow inside the tubing that provides radial mixing. The secondary flow reduces the bandspreading in the tubing quite drastically. The HETP may be 100 or even 1000 fold smaller in a well designed geometrically deformed tubing than in a straight tubing. The effect costs a bit in pressure, but this is not a problem under typical operating conditions.

The important part about the knitting technique is the pattern of the deformation of the flow path (1). One wants to achieve a constant shift of the direction of the centrifugal force that acts on the flow in the tubing and creates the radial mixing. Something like a three-dimensional roller coaster. The best configurations have an HETP around 0.5 cm, resulting in about 10 000 plates in 50 m of tubing. Such high-performance configurations make post-column derivatization compatible with HPLC separations and largely eliminate the bandspreading problem described above.

Teflon tubing is also a good choice for other reasons (3). It is inert to practically all reaction media and mobile phases that one can think of, and it has a good temperature stability. In order to speed up a reaction, you may want to work at elevated temperature. Other plastic tubing may become soft under the conditions that you may want to use.

Steel or copper tubing are other choices for your post-column reactor. They may be sufficiently inert to the reaction medium of your derivatization reaction. However, to deform them into the optimum forms of the "knitted" pieces of tubing is not a simple task. You will compromise bandspreading performance compared to what can be done with teflon tubing.

Q.: Let us discuss the reaction conditions! What do I need to think about?

A.: First of all, the reaction should satisfy your needs. Is it specific enough, and is the detection sensitive enough. A large number of reactions have been explored already, and a book has been compiled on the subject (4). This should help you to get the basic information that is needed. You need to explore the differences between the analytes of interest and the matrix. The more specific your reaction and your detection schemes are, the more matrix interferences you can tolerate. Also the response of the compounds of inerest should be significantly different from the non-reacting background. This is the reason why most published reaction schemes result in the formation of color or fluorescence.

Next, you want to have a reasonably short reaction time. Slow reactions and short analysis times are not compatible. For most practical purposes, the reaction time should not exceed 5 minutes. You can speed up most reactions by increasing the temperature or by increasing the concentration of the reagent. The reaction does not need to go to completion. Due to the combined effect of the progress of the reaction and the bandspreading in the tubing, you will get the maximum detector response before completion of the reaction. However, you need to find conditions, where small variations in the reagent concentration or the temperature do not affect the response to an appreciable degree. It is usually not difficult to find a plateau in the response pattern. Of course, this can be analyzed mathematically as well (1), but for most practical purposes a few experiments will provide the correct answer.

You are planning to get involved in an exciting technology, and I wish you much success.

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49. Gradient Dwell Volume

Q.: What is the gradient dwell volume of an HPLC system, and how does it affect the analysis?

A.: The gradient dwell volume is the volume between the point where the gradient is mixed and the column inlet. This volume delays the onset of the gradient, and is therefore also called the gradient delay volume.

Most modern gradient HPLC systems are single-pump low-pressure gradient systems. This means that the gradient is mixed upstream of the pump. The first component of the gradient delay volume is the volume of the gradient mixer. To this you need to add the connections between the gradient mixer and the pump heads. Next is the volume of the pump heads. What follows is the connection tubing to the injector. Often, this volume is increased to provide a mixing of the gradient components to smoothen the gradient. The volume of the injector and the connection between the injector and the column represent the next part of the gradient dwell volume. As you can see, with a typical low-pressure gradient system, there is a lot of volume where the gradient can dwell…

It is also possible to generate the gradient on the high-pressure side. This requires at least two pumps, but enables us to eliminate all of the gradient delay volume. However, high-pressure gradient systems are not commonly set up this way. Normally, the connection between both pumps is made upstream of the injector. This has the advantage that the sample is always transported to the column, and does not depend on the flow in either pump. A better approach, albeit one where a bit more care is needed, is to inject the sample into the flow of the pump that delivers all or most of the starting mobile phase. This means that the gradient is mixed behind the injector, and the gradient delay volume can be made completely negligible. A small drawback of this technique is that the sample is diluted a bit with the second component of the mobile phase. This is only a minor issue if the gradient starts with 90% of the A-component of the gradient. Then the sample is diluted only by 10%. The other thing is that in general most peaks in a gradient are focussed on the column. Therefore this small drawback may affect to a small degree only a few peaks that elute in the isocratic portion of the chromatogram before the gradient starts. Under these circumstances, we need to make sure that the volume between the injector and the column is negligibly small. High-pressure gradient systems allow us to completely avoid any gradient delay.

Now let us discuss how the gradient delay volume affects the separation! Since it takes time for the gradient to reach the column, the onset of the gradient is delayed. Therefore there is a time period where the column is operated isocratically in the mobile phase used at the beginning of the gradient. The first effect may be that early eluting peaks are affected by the gradient delay volume and may elute differently on another instrument with a different gradient delay volume. If the gradient delay volume is a substantial part of the chromatographic run, one can observe quite significant differences in the elution profile early in the run. Late in the gradient, the elution pattern remains the same independent of the delay volume. However, the elution time will shift in direct proportion to the change in the gradient delay volume. Both of these conditions are rather annoying. They are the reasons why gradient methods are often avoided in a QC laboratory. After all, analytes are commonly identified using the retention time, and if the retention times change from instrument to instrument, one has trouble with the identification. Of course, there are ways around this. For example, one can specify the retention pattern in the form of differences from the retention times of a standard. However, this complicates things…

Q.: I agree, this is a bit more complicated, but it is not an insurmountable obstacle. Are there any other issues to consider?

A.: If there is a substantial mixing volume in a low-pressure gradient system, one will also observe that the gradient is not sharp. This usually does not play a big role in standard linear gradients, but it may affect the elution pattern if steps are used in the gradient. Generally, it is preferred to use gradient systems with a small delay volume. Many modern HPLC systems are designed for a minimal gradient delay.

For very rapid gradients with minimal cycle time, even a very small gradient delay volume may not be sufficient. Therefore other solutions have been implemented. For very rapid routine analyses, the delayed injection technique can be used. In principle, the gradient itself is executed just like under normal operating conditions. However, the first injection is delayed until the gradient reaches the injector. Right at that moment, the sample is injected. The sample is now essentially separated by the gradient as if no gradient delay volume were present. Of course, there is a small delay volume comprising the volume of the injection loop and the tubing between the injector and the column. But this can be made negligibly small.

After the gradient has been executed, the column is reequilibrated with the starting mobile phase. Of course, the reequilibration will be delayed by the same time as the gradient itself. Therefore we don't have to wait until the column is reequilibrated to start the next gradient run. We just have to remember that what we program into the instrument and what the pump is executing is different from what is actually happening at the column inlet. Gradient execution and column reequilibration are just offset by the time needed to purge the gradient delay volume. This modern solution to the problem of the gradient delay allows us to execute fast gradients without delay on single-pump instruments. In addition, if we execute the same program with different instruments, the possible differences in the gradient delay volume can be made negligibly small. This should enable us to execute true gradients reproducibly on different instruments. Of course, all of this assumes that the gradient generator works reliably and reproducibly and indeed generates the gradient that we would like to see.

Q.: OK, this sounds good. How can I measure the gradient delay volume and how can I make sure that the system accurately delivers the gradient that I want?

A.: There is a standard test that is quite useful. You can run your gradient without a column in place with a small amount of an UV absorber in the mobile phase B. This allows you to observe the actual execution of the gradient. From the

difference between the program and the actual execution of the gradient, you can determine the gradient delay volume of your system. This is something that you may want to know anyway, if you want to take full advantage of the trick with the delayed injection. The more you know about your system, the more successful you will be eliminating the wasted time in gradient separations.

50. Buffer Capacity

Q.: What is buffer capacity, and why is it important?

A.: Buffer capacity is a measure of the strength of the buffer. Mathematically, it is the reciprocal value of the slope of the titration curve of a buffer. It specifies the amount of hydronium ions or hydroxy ions that are needed to change the pH of the buffer by a certain value. The larger the buffer capacity, the larger is the amount of acid or base that can be added to the solution without a change in pH.

This also shows why the buffer capacity is important in chromatography. We know that the retention of ionic or ionizable analytes may depend on the pH of the mobile phase. If we are in a situation like this, it is important to have good control over the pH of the mobile phase. If we do not have good control, the retention of the analytes and even the selectivity of the separation may vary from day to day with the mobile phase preparation. This of course is not desirable. Let me give you a few examples to clarify this. Let us assume that we are separating two acids with a similar pK_a between 4 and 5 using a reversed-phase column. We have prepared the mobile phase with KH_2PO_4, which gives us a pH of around 4.5. This is about the worst situation that one can encounter. In reversed-phase chromatography, the retention of both of our sample acids will depend strongly on the degree, with which the acids are ionized. The ionic form of an analyte is always much less retained than the non-ionic form. A good rule of thumb is that the retention of both forms is different by about a factor of 30. Therefore the retention of both of our analytes can change a lot, if the pH varies just a little. In addition, the change in retention may be different for both acids, especially considering that both have a similar pK_a, but not the same pK_a. Consequently, the resolution between both compounds will change, as there are small changes in the pH of the mobile phase. Of course, if the peaks are separated by a mile, this is not relevant, but if they elute close to each other, a small shift in pH may mean that sometimes we obtain perfect resolution and another time the peaks overlap. The consequence of this is that we need good control over the pH of the mobile phase to obtain a good reproducibility of retention and resolution.

However, this is not achievable with the mobile phase that we are using. We have prepared the mobile phase with KH_2PO_4, which has no buffering capacity whatsoever at pH 4.5. The pK_a values of phosphate are at 2 and 7 If only a small amount of acid or base are added to this mobile phase, the pH changes drastically. Since the pH changes, the retention of both of my analytes will change and the resolution will change as well. With this set-up, we have maximized the irreproducibility of the separation. Of course, other problems are possible as well. It is highly likely that the peak shape of one or even both analytes suffers as well, especially if the amount of sample injected onto the column is high.

Q.: OK, I agree that we have a problem. What can we do to solve it?

A.: The simplest thing is to use a buffer with a good buffering capacity. If the pH can be controlled such that it is the same from day to day with every preparation of the mobile phase, we have a much better chance of achieving reproducible chromatography. We want to maintain the pH to maintain the peak spacing. Therefore we need a buffer that has a good buffer capacity around pH 4.5. A buffer has always the best buffer capacity around its pK_a. Acetic acid has a pK_a of 4.75. It is ideally suited for the separation problem under discussion. It has a very good buffer capacity at pH 4.5. If indeed the best separation and the best retention is obtained at pH 4.5, we can use an acetate buffer at pH 4.5. On the other hand, if the separation is still satisfactory at pH 4.75, it is even better to select this pH since the buffer capacity of a buffer is always highest at the pK_a of the buffer.

Q.: This is interesting. Can you provide a bit more background on the buffer capacity?

A.: Yes, let me describe the theory behind this! The buffer capacity β is defined as follows:

$$\beta = 2.303 \cdot C \cdot \frac{K_a \cdot [H^+]}{(K_a + [H^+])^2} \quad (1)$$

C is the total concentration of the buffer, K_a is the dissociation constant of the buffer, and $[H^+]$ is the hydrogen ion concentration. In our common nomenclature of pK_a and pH, this can be rewritten to read:

$$\beta = 2.303 \cdot C \cdot \frac{10^{-pK_a - pH}}{(10^{-pK_a} + 10^{-pH})^2} \quad (2)$$

With this equation, we can calculate the buffer capacity of any buffer. The buffer capacity curve for an acetate buffer is shown in figure 1. The curve looks similar to a chromatographic peak. One can see that the buffer capacity is highest around the pK_a of the buffer, at pH 4.75. It slowly drops in both directions, and at pH 3.15 and 6.35 it is only at 1/10 of the value it had at the maximum. This is the reason for the rule of thumb that says that a buffer should never be used outside 1.5 pH units around its pK_a, since it looses its buffering capacity outside this range. We are commonly using buffers with a concentration of 50 mM, and the rule has been created for buffers of this concentration. However, in LC/MS applications, the buffer concentration is often only around 10 mM. This means that at such a low buffer

concentration one may want to limit the rule of buffer usefulness to approximately +/- 1 pH unit around the pK_a of the buffer.

You can use the equations shown here to calculate the buffer capacity of other buffers, such as phosphate buffers or citrate buffers. For buffers with multiple dissociation constants, the buffer capacities of the different species can simply be added up. However, the important point of this discussion is the fact that we should choose buffers with a good buffering capacity at the pH that we need to use to optimize a separation.

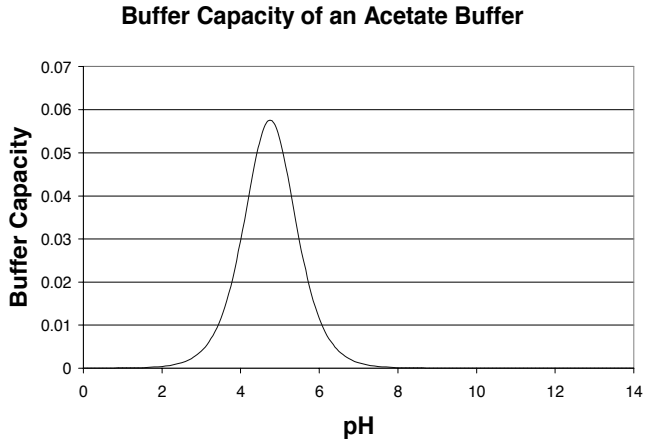

Figure 1: *Buffer capacity of a 0.1 M acetate buffer.*

51. Flow Rate Changes and Quantitation

Uwe D. Neue and Tony Gilby

Q.: My injector is doing something very peculiar that I do not understand. It injects double the amount of sample at 0.5 mL/min than at 1 mL/min, and it does it reproducibly. What's happening?

A.: How do you come to the conclusion that this is the case?

Q.: Well, I put into the sample injection table an injection volume of 5 µL of sample. I run the method at flow rates of 1 mL/min and 0.5 mL/min, using a UV detector. The peak areas that I obtain at 0.5 mL/min are nearly exactly double the peak areas that I get at 1 mL/min.

A.: OK, now I understand. Here is what is happening: your injector is working just fine, but the integration of the peak area depends on the flow rate. Let me explain!

Nearly all classical HPLC detectors are designed to give an output signal which measures the amount of sample in the flowcell at a given moment in time - the number of analyte molecules if you like. Since the cell volume is constant, this is equivalent to measuring the average concentration in the cell. A typical example of this is the UV absorbance detector. If I let a sample sit in the detector cell of a concentration sensitive detector, the signal will remain constant, at least within reasonable times.

Peak area is determined by integration of the signal over time. This means in simple terms that if I let the same signal be output by the detector for double the time, I get also double the peak area. This is exactly what is happening when you change the flow rate from 1 mL/min to 0.5 mL/min.

Here is the math. The peak area A is the signal S multiplied by the time t:

$$A = S \cdot t \qquad (1)$$

The signal S is proportional to the concentration c of the analyte:

$$S = p \cdot c \qquad (2)$$

$$A = p \cdot c \cdot t \qquad (3)$$

The concentration is mass m divided by volume V:

$$A = p \cdot \frac{m}{V} \cdot t \qquad (4)$$

And finally, volume divided by time is nothing but the flow rate F:

$$A = p \cdot m \cdot \frac{1}{F} \qquad (5)$$

As we can see, the peak area is proportional to the mass injected divided by the flow rate. This is the reason why you get double the integrated signal at half the flow rate.

Q.: OK. I understand. You said that this holds for concentration sensitive detectors. What does this mean? Aren't all detectors concentration sensitive?

A.: No, not all detectors are. In the case discussed above, the signal remains the same, even if I change the flow rate. All the classical LC detectors are of this type: photometric detectors such as UV and UV-Vis absorbance or photodiodearray detectors, fluorescence detectors, refractometers or detectors measuring the dielectric constant, or conductivity detectors as used in ion chromatography. Other rarely used detectors fall into the same category. Examples are detectors that measure the optical rotation of a sample.

A simple test can be carried out to show if one is dealing with a true concentration sensitive detector or not: one pumps an analyte carrying liquid into the detector, and then

stops the flow. If the signal remains constant, one is dealing with a true concentration sensitive detector.

Q.: I once made use of a radioactivity detector, and noticed that if the flow stopped, the detector output kept increasing. Is this different from the concentration sensitive detectors you describe above?

A.: No, this is still a concentration sensitive detector. The counts per second are a measure of the number of radioactive isotope atoms in the cell. What is confusing is that the detector output, total counts, is actually the integral of the count rate over time. The detector output is giving you directly the integrated signal over the peak. At slower flow rates, you will get a higher signal from the same peak, and therefore a higher sensitivity. You can optimize the signal to the needs of the analysis by stopping the flow at the time the sample enters the detector.

Detection via post-column derivatization can be complicated as well. The actual detectors used are concentration sensitive detectors such as absorbance detectors or fluorescence detectors. However, only for very fast reactions, the reaction is driven to the endpoint. Most of the time, the reaction is incomplete. Under these circumstances, the yield will depend on the flow rate, with slower flow rates resulting in a longer reaction time and consequently a higher yield and a higher sensitivity.

An interesting case is the use of electrochemical detectors such as amperometric detectors and coulometric detectors (1). For amperometric detectors, the electrolysis of the detected species is not complete. Only 1% to 10% of the analyte are consumed. Therefore, the amperometric detector works as a concentration sensitive detector. In coulometric detection, all of the analyte is consumed in the detection process. Thus, the total integrated signal is independent of the flow rate. This is the opposite of what we had discussed above for a UV detector. Coulometric detection therefore is a mass-proportional detection technique.

The main characteristic of a true mass-proportional detector is the fact that the integrated signal does not depend on the time it took to acquire the signal. This is the most important thing to remember about mass-proportional detection. Here is an example. A simple mass proportional detector has been reported in the early times of HPLC (2). The column effluent was collected on a balance, the solvent was evaporated and the residue was weighed. While this was interesting, it did not prove to be practical. However, it clearly demonstrates how a mass proportional detector works. In this case, the height of the signal is the mass that has accumulated on the balance. Thus, the signal is always an integrated signal, and its height does not depend, if I transport the sample to the detector quickly or slowly. If I inject the same mass into the HPLC system at high flow rate or slow flow rate, it always remains the same mass, and my balance gives me the same signal.

Other mass proportional detectors are some GC detectors such as the flame ionization detector (FID), which results in complete destruction of the analyte molecules. The FID has been used in LC through the use of specific interfaces, such as the moving belt or moving wire interface. The use of GC detectors in LC has remained rare though.

Today, mass spectrometers are often used in combination with LC. Whether a mass spectrometer is a mass or concentration proportional detector depends on the interface. Current electrospray interfaces behave like concentration sensitive detectors over the flow-rate range commonly used. One might expect that the number of analyte ions measured by the mass spectrometer per second (its signal, an ion current) would increase if the number of analyte molecules reaching the electrospray tip per second were increased - i.e. by increasing the flow rate. This is not found in practice, because of competition in the interface to ionize solvent molecules, which are also arriving at a faster rate. Without frequent injection of mass standards, mass spectrometers and the associated HPLC interface, make unreliable quantitative detectors.

I hope that this small excursion into detector response has clarified the issues in quantification. The most important thing to remember for the user of liquid chromatography is the fact that most LC detectors are concentration sensitive detectors, and that the integrated signal of concentration sensitive detectors depends on the flow rate.

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52. Analysis of polar compounds

Q.: I have a few very polar compounds that I need to analyze. I have trouble with retention on standard C18 columns. What can I do?

A.: You are not alone. This question is appearing in ever increasing frequency on bulletin boards discussing HPLC problems. The good news is that there are a few solutions available to this problem, and one or the other is likely to work for your specific problem.

Q.: Oh good. I am glad to hear this. What are these solutions?

A.: Overall, there are three possibilities that can be used to get good retention for very polar compounds. The first one is to switch from reversed-phase columns to HILIC…

Q.: HILIC? What is that?

A.: HILIC stands for hydrophilic interaction chromatography, a term first used by Andy Alpert (1). It works with polar stationary phases such as silica and mobile phases with a high content of organic solvent and a smaller content of water. The retention mechanism is the opposite of reversed-phase. Polar compounds are retained more strongly, and retention decreases when the water content of the mobile phase increases. The best known example is the separation of sugars on amino columns, a technique first published by Fred Rabel and Art Caputo (2). In this case, the mobile phase consists of about 75% acetonitrile and 25% water. The underlying principle of this technique is the partitioning of the analyte into a surface layer highly enriched with water (3). Therefore, the more soluble the analyte is in water, the more retention is observed. Conversely, the less soluble the analyte is in mobile phase, the more retention is observed. This technique can be enhanced further via other retention mechanisms, such as ionic interactions or ion exchange (4).

One of the difficulties of this technique is the low solubility of very polar samples in a mobile phase with a high concentration of acetonitrile. However, it is not as bad you would think. Among other things, you can dissolve the sample in a solvent composition with a higher water content than the mobile phase, or even in pure water. If you do that, you just need to reduce the injection volume so that you do not get peak distortions.

In general, the important thing to remember is that HILIC works best for very polar samples with a good solubility in water.

Q.: OK, sounds good. What are the other options?

A.: The second possibility is the use of reversed-phase columns that were specially designed for the retention of very polar compounds. The issue with some of the best standard reversed-phase columns is that they cannot be used with mobile phases containing 100% water.

Q.: Exactly. When I tried to use a mobile phase with a very high water content, I could not get reproducible retention, and sometimes I got less retention than I thought I should get.

A.: Yes, this is the problem that I was referring to. It is sometimes called “hydrophobic collapse”, but a better expression is “dewetting of the pores”. The modern very hydrophobic and very deactivated phases loose retention in highly aqueous mobile phases. The underlying issue is the wettability of the stationary phase with water. Since water does not wet the surface, it is driven out of the all or part of the pores of the packing. The surface becomes “unwetted”, and retention is lost.

There are two ways in which a reversed-phase packing can be made more compatible with an aqueous mobile phase. The first one is the incorporation of a polar group into the stationary phase, either built into the ligand or as a second reaction. Typical functional groups for the phases with incorporated polar groups are amide or carbamate groups (5). These polar functional groups prevent the hydrophobic collapse, but they also reduce somewhat the hydrophobicity of the stationary phase as well. Therefore they give an improvement of the retention properties of a packing, but there is a still better solution.

This second solution is simply a reduction of the hydrophobic effect that causes the hydrophobic collapse. The hydrophobic collapse is a wetting phenomenon. If I reduce the ligand density of the C18 ligand, I can make a reversed-phase packing that is still very hydrophobic, but is also water wettable. This requires a good understanding of the underlying properties of the packing, and the influence of the different parameters on wettability and retention. Packings have been prepared that deliberately balance hydrophobicity with water wettability (6). A carefully designed reversed-phase packing like this still gets good retention in fully aqueous mobile phases without undergoing the hydrophobic collapse.

Q.: OK, and what is the third option?

A.: The last option depends on the type of analytes that you are using. If they are ionic compounds such as amines or acids, they can be converted into a neutral form by changing the pH of the mobile phase. Amines specifically can be converted to a neutral uncharged form in the alkaline pH, around pH 10. Under these circumstances, the chromatographic retention increases by a large factor. Often a 10- to 30-fold increase in retention is found by changing the pH and changing the analyte from the ionic form to the non-ionic form. Of course, you need a packing that has been designed for use at high pH. Fortunately, packings of this type are available today (7), and this procedure can be used very effectively and without difficulty.

Q.: Thank you for your description of the different options! My compounds are very polar compounds with acidic and basic groups. It appears that either one of these solutions might work for me. Which one do you recommend?

A.: Without experimental details, this may be hard to predict. I mentioned already the difficulty with the solubility of the analyte in the mobile phase with HILIC. Among the reversed-phase solutions, I would go with the solution that has been optimized specifically for the purpose of achieving high retention for the type of polar compounds that you need

to separate. However, you also need to consider that any one of these solutions can give you the improvement that you need. In addition, the selectivity of the separation is likely to be different between the different solutions. In most cases, it might therefore be worthwhile to explore more than one tool.

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