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HPLC Troubleshooting

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HPLC MOBILE PHASES - 10 BAD HABITS TO AVOID

1. **Measuring the pH of the mobile phase after the organic has been added**
   pH meters are calibrated to give the correct pH readback in aqueous solution – the buffers you verify this with are aqueous. If you measure the pH with the organic added, the pH will be different to that of measuring before organic addition. However, the most important point is to be consistent. If you do always measure pH after the organic is added, make sure you state this in the method so that everyone does it the same way. It won't be 100% accurate, but at least it will be consistent. This is probably more important than having the exact pH.

2. **Not using a buffer**
   Buffers are present to control pH and resist a change in pH. Many other parts of method (e.g. sample matrix, CO2 in air, source of water used for your mobile phase) can change the pH of the mobile phase causing shifts in retention, peak shape and peak response. Formic acid, TFA etc. are not buffers.

3. **Not using the buffer in its correct pH range**
   Each buffer salt has a 2 pH unit wide range over which it provides the optimal pH stability. Outside this window the salt is ineffective at resisting change in pH. Either use your buffer within the correct range or pick a buffer whose range covers the pH you require.

4. **Adding buffer to organic**
   Mixing aqueous buffer into the organic phase carries a high risk of the buffer being precipitated – in many cases so finely that it may not be obvious it has happened. ALWAYS add the organic to the aqueous phase, this greatly reduces the risk of buffer precipitation.

5. **Using the pump to mix gradients from 0%**
   Modern pumps are very effective at mixing mobile phases and degassing online, however not everyone who ends up using your method has a high quality pump. Premix your A and B starting mix to a single solution that runs at 100% on line A. e.g. Prepare the starting mixture by mixing 950ml Aqueous with 50ml organic, then filter and degas. This reduces variability between HPLCs, reduces the risk of bubbles and precipitation in the system. Note however that 95:5 mixed on the pump will not give the same retention time as 95:5 premixed in the bottle – you normally need to add a few more percent organic when premixing.

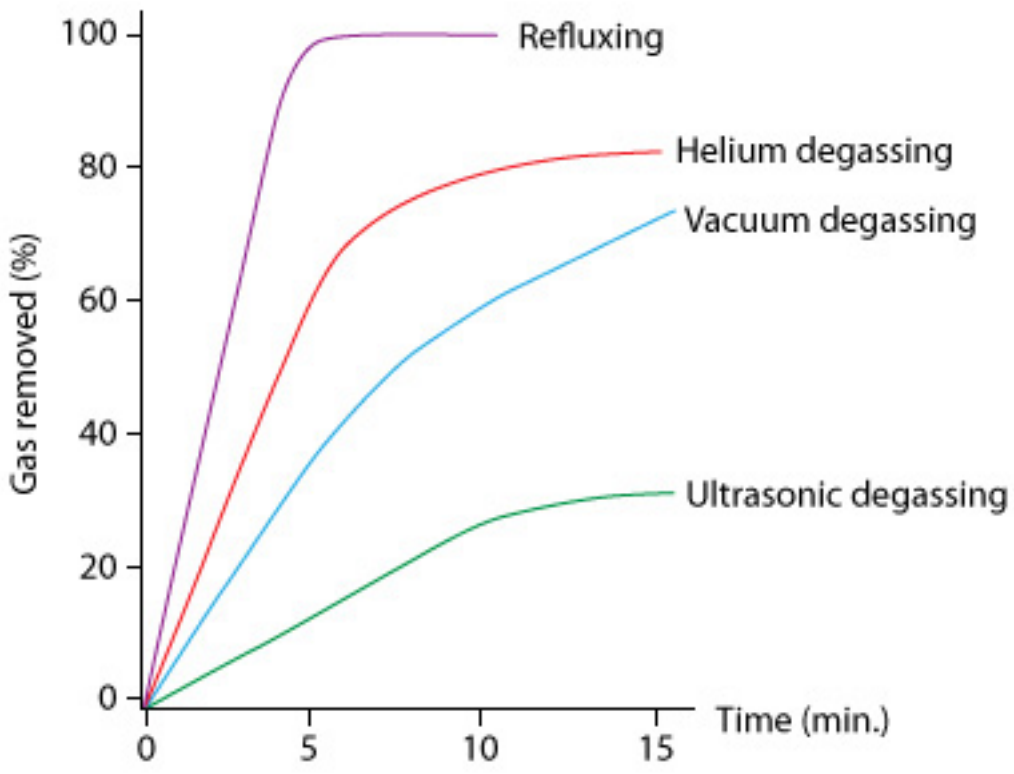
6. **Not using the correct pH modifying acid or base for your buffer**
   Only use the acid or base that forms the buffer salt you are using. E.g. sodium phosphate buffers should be adjusted with only phosphoric acid or sodium hydroxide.

7. **Not stating the full information of your buffer in the method e.g. weigh 5g of sodium phosphate into 1000ml of water**
   The type of buffer (mono, di or tribasic) determines its pH buffering range. The required molarity is what determines the buffer strength. 5g or anhydrous sodium phosphate and 5g of monohydrate sodium phosphate will have different buffer strengths and will affect retention.

8. **Filling lines with organic without checking what was in there before**
   If the previous method used buffer in line B and your method uses organic in line B there's a good chance you will precipitate buffer in your pump tubing / pump head. I did it in my early days and it caused a lot of damage. If in doubt – flush it out (80:20 water : organic).

9. **Propping up bottles to get last drop out**
   It's 5 to 5 and you've barely got enough mobile phase to finish the run – it'll be running on fumes by the last few samples. Apart from the risk or running your pump and column dry, mobile phases evaporate from the surface, so the mobile phase at the top of the bottle will have changed composition from the bulk. This portion from the top is exactly what will be running through the column if you use the last dregs in the bottle.

10. **Using sonication to degas mobile phase**
    It's great for making sure all your buffer salts have dissolved, but it's the least effective method of degassing AND it quickly heats up the mobile phase causing the organic portion to evaporate. Save yourself problems later – take 5mins to vacuum filter your mobile phase – it degasses and filters in a single step.



HPLC COLUMN MAINTENANCE - PREVENTION IS BETTER THAN CURE

Column troubleshooting is more usually performed by reference to the chromatogram and chromatographic peak. System operating parameters may also provide indicative troubleshooting information - the component perspective. It is always advisable to protect the analytical column from damage against particulates and strongly retained sample material. These may eventually block the inlet frit of the column and occlude the packed stationary phase, resulting in increased backpressure and poor chromatographic peak shape respectively. Therefore, installation of an in-line filter and/or a guard column in the system is strongly recommended.

Particulates and strongly adsorbed species can potentially come from:

- Piston seals
- The injector switching valve rotor seal
- Undissolved or precipitated buffer salts
- Contaminated sample matrices

Dedicating Columns

Once a column has been used for the separation of 'real' samples, then the selectivity offered by that column will necessarily differ from that of a new column of the same brand and part number. Columns can become chemically modified in a variety of ways once they have been used experimentally:

- Stripping of a column's endcapping species may occur under low-pH conditions.
- Strongly retained sample material can bind irreversibly to the stationary phase surface, especially at the column inlet, causing a rise in system backpressure and a change in the chemical nature of the stationary phase.
- Analytes that are strongly basic in nature can bind irreversibly to free silanol groups on the column's stationary phase. Performing separations at low pH can reduce the extent of silanol ionization, as they typically possess a pka of 3.8-4.1.
- As each HPLC method operates under slightly different conditions and is used for different sample types, then the column changes that can potentially occur will be unique for that method. Therefore, once a column has been used routinely for one application then it may no longer offer an identical separation to its new column equivalent, and chromatographic differences can then often be observed.

This may also be experienced when using the same column, but switching to a different method and analyte. Then it may be necessary to perform several "priming" injections with the new analyte prior to obtaining reproducible chromatography. Column equilibration times may also have to be extended, to accommodate the selectivity changes.

In order to minimize experiencing such variations it is recommended that a column be dedicated to a specific method and analyte type. The indiscriminate swapping of columns to develop methods may result in an unrepresentative chromatographic separation once a new column, which has never been exposed to the variety of sample types of its predecessor, is subsequently employed. Such variations can be relieved by incorporation of a correct column washing procedure.

In-line Filter

A common indicator of column failure is the gradual development of excessive backpressure. Such pressure increases typically result from the accumulation of particulate material on the inlet frit of the column, and one of the least problematic and inexpensive ways to extend a column's life is to incorporate an in-line high-pressure filter between the autosampler and the column. In this location an in-line filter prevents particulates derived from both the sample and the autosampler injection switching valve rotor seal reaching the head of the column.

It is highly recommended that an in-line filter be incorporated in to each HPLC system, the only caveat being extremely low dead-volume systems, where the increase in extra column volume would contribute more significantly to chromatographic peak dispersion and band broadening. Apart from this scenario no appreciable change in the chromatogram will be observed.

The in-line filter can be either visually examined for contamination build up, or if the particulates are colorless, changed when the system backpressure has risen appreciably (Figure 1). In-line filters contain a frit that is most commonly 0.5 µm in porosity (0.2 µm are used for UHPLC applications). The porosity should be similar to, or smaller than, the frit porosity of your analytical column so that the frit catches any particles that would be likely to block the column frit.

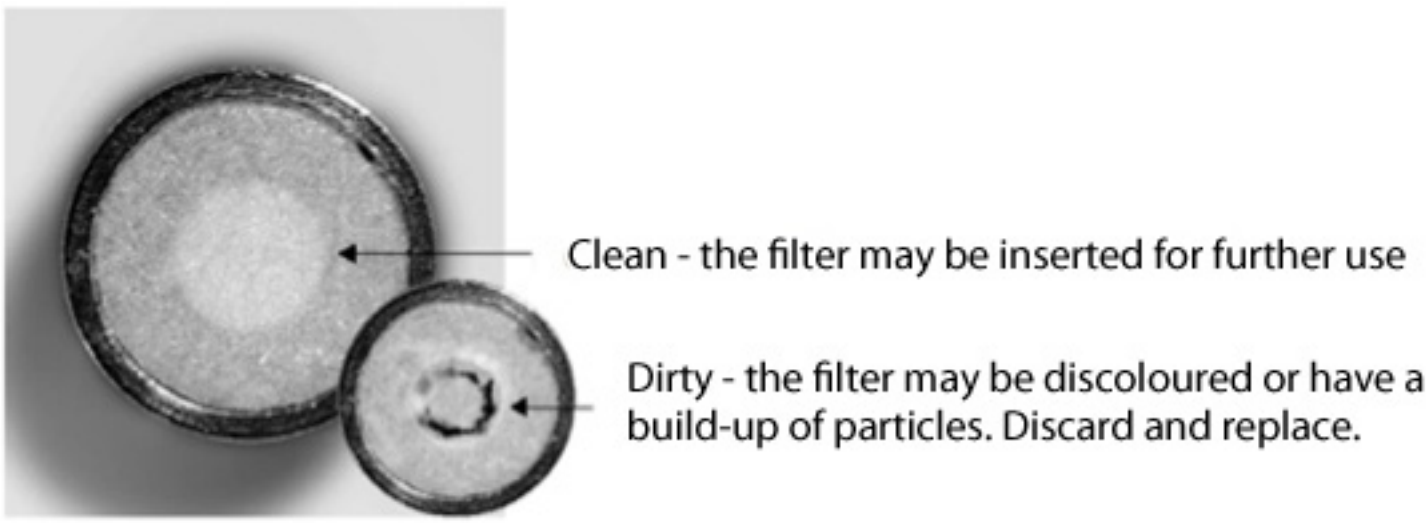


Figure 1: Clean and dirty in-line filters.

Guard Columns/Disks

A guard column is a short column - typically 10-20 mm in length - that is packed with the same stationary phase material as the analytical column. If a cartridge system is being employed then an integral cartridge holder must also be used. Guard disks are small fritted disks, which when fitted in to their holder are screwed directly in to the analytical column. In contrast to the in line filter, a guard column or disk should only be fitted to act as a chemical filter, ensuring the removal of any potentially strongly retained or aggressive material, thereby preventing subsequent fouling of the analytical column.

In some instances a guard column can provide sufficient protection for the analytical column to reduce or eliminate the need for any off-line sample clean-up procedure. However, as the guard column can potentially become contaminated with a variety of strongly retained material, it is an absolute requirement that when being flushed with a strong solvent it is done so off line from the analytical column, thereby preventing any such species being inadvertently washed on to the head of the analytical column.

A guard column should not be used as a silica saturator column, as any void that subsequently develops in the guard column will compromise the chromatography (Figure 2). If a saturator column is felt necessary for a particular application i.e. high pH mobile phase, then it should be placed upstream of the injector with an in-line filter immediately afterwards to retain the resulting small particulate material resulting from the subsequent silica dissolution.

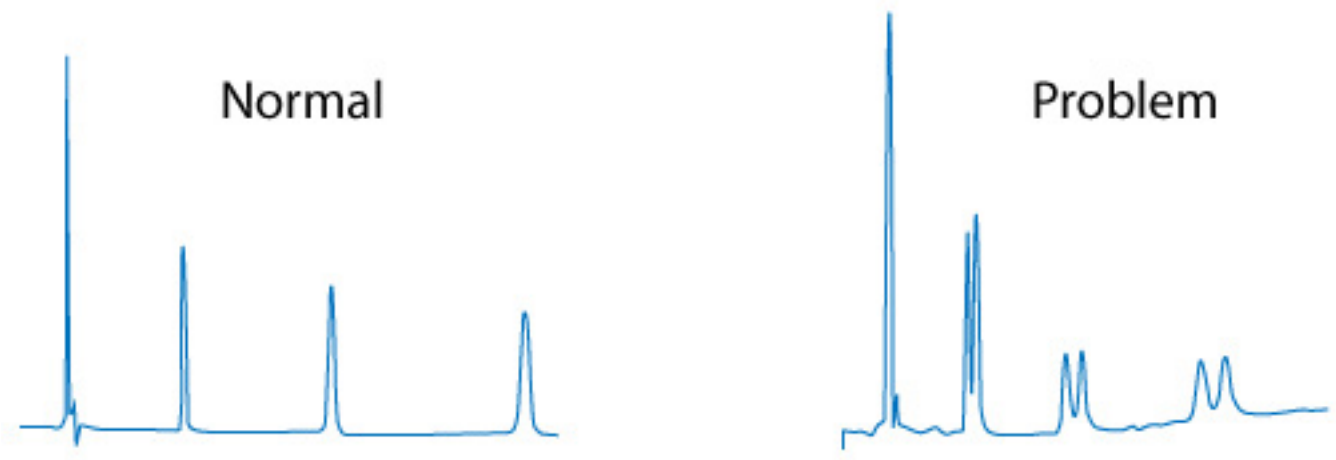
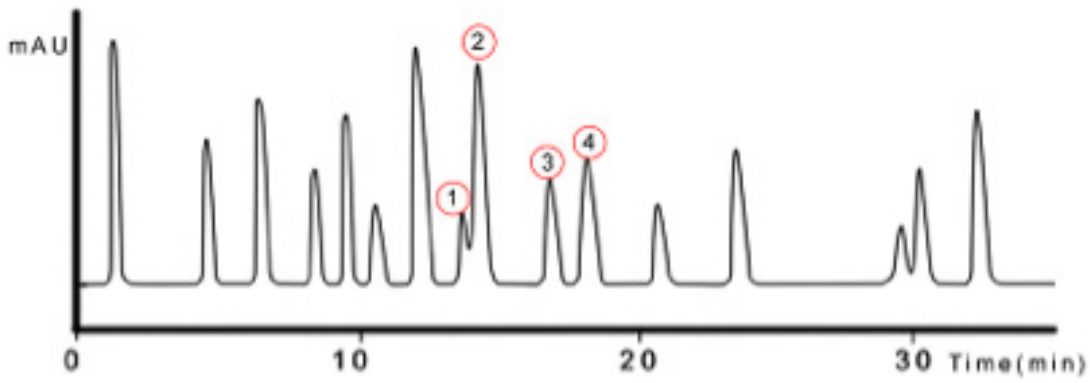


Figure 2: Chromatograms illustrating the detrimental effect of formation of a guard column void on the resulting chromatographic peak shape.

As with the incorporation of an in-line filter into the HPLC system, a guard column will also introduce an increase in extra column volume, which would contribute to chromatographic peak dispersion and band broadening in extremely low dead-volume systems.
When do you replace a guard column? There are three potential approaches that can be used to determine when a guard column should be replaced:

1. Waiting for the overall chromatographic separation to deteriorate is not recommended. It may be possible to monitor a pair of peaks in the chromatogram that are more poorly resolved than the pair of interest, making it possible to correlate a change in their critical separation with that of the peaks of interest. This would allow an informed judgment to be made as to how close the guard column is to potentially failing.

   This is illustrated in the chromatogram below, which shows the separation of a range of herbicide samples. The critical pair, the chromatographic peaks that are least resolved from one another, are labelled as 1 and 2. The chromatographic peaks of interest are labelled as 3 and 4. By monitoring the continual reduction in resolution between peaks 1 and 2 over time, a valued judgment can be made as to when to change the guard column without compromising the analysis with respect to the sample peaks of interest 3 and 4



2. Monitor the number of samples injected with the guard column in place before the system fails to deliver a satisfactory separation. Alter the method's SOP so that the guard column is replaced after approximately 80% of its expected lifetime – be proactive rather than reactive.

3. Monitor the guard column's effective life in terms of the solvent volume used or the calendar time it has been installed.

Sample Clean-up

In the context of sample clean-up, it becomes a financial balancing act of weighing up the cost of such sample clean up against the cost of column replacement. Consider the bioanalytical example of measuring the concentration of a drug substance in plasma. Protein precipitation by the addition of acetonitrile to the plasma, vortexing, centrifuging and injecting the supernatant will still produce a reasonably “dirty” sample, perhaps giving between 100-500 injections.

In contrast a much cleaner sample can be obtained by using a solid phase extraction (SPE) procedure, enabling a column lifetime of between 1000-2000 injections. This whole SPE process and subsequent sample injection using a 96-well plate format could be automated. Whilst the gain in column lifetime would not in itself justify the cost of such an elaborate and expensive clean-up process, the method would benefit from improved reliability and if required for many years to come then this should be given due consideration.

Column Storage

To avoid contamination, store LC columns with end plugs securely fastened and be sure to include information describing the storage solvent.

- For short-term storage, columns should be flushed with a solvent identical in composition to the most recently used mobile phase minus any buffered, acidic, or basic components.
- For long term storage, reversed phase columns should be stored in 50% water/50% organic solvent (i.e. acetonitrile or methanol). Normal phase columns should be stored in a non-polar solvent (i.e. hexane). Ion exchange columns should be stored in methanol following flushing with water. HILIC columns should be stored in 80% acetonitrile/20% water.

Important: All buffers should be washed out of the column (use water) before flushing with acetonitrile as buffer salts are generally insoluble in acetonitrile and will precipitate and cause blockages in the column.

Column Replacement

By incorporating the points outlined above then the effective lifetime of any analytical column can be increased, ultimately however columns should be considered disposable items. How long a column lasts is determined by a number of inter-related factors e.g. buffer strength and pH, sample cleanliness, but typically between 500-2000 injections should be considered an acceptable range.

HPLC AUTOSAMPLER CONTAMINATION

Contamination in any chromatographic method is a complex problem to troubleshoot. Extra peaks, often referred to as ghost peaks, in a blank or sample chromatogram can have a number of sources

- Elution of analytes retained from a previous injection
- Mobile phase contamination
- Sample preparation
- System contamination
- Column contamination

This article will focus on autosampler contamination, how to determine if the autosampler is the source, and how to remedy the problem.

The sample first makes contact with the HPLC system via the autosampler. Contamination is often presented in the form of sample carryover as evidenced when injection of a solvent blank produces a mini-version of the previous sample's chromatogram.

Most carryover occurs in the rotor seal and is due to sample adsorption. Extra peaks which are sharp are often due to sample contamination. The appearance of broad, less efficient peaks within an otherwise good chromatogram may indicate the elution of highly retained species from previous injections (Figure 1).

HPLC AUTOSAMPLER CONTAMINATION

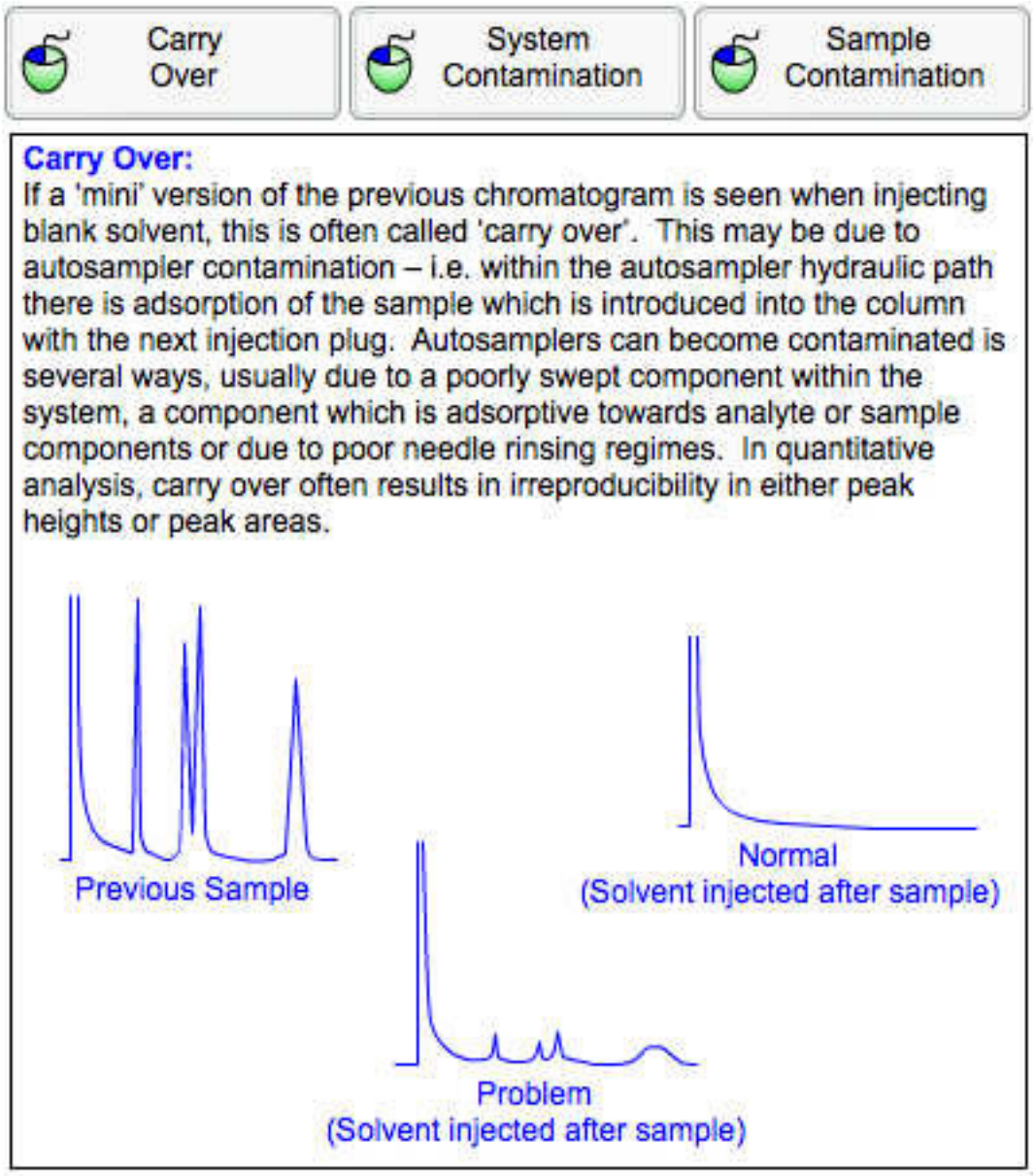


Figure 1: Identifying carryover and contamination.

It can be verified that the autosampler is the source of contamination by removing it from the flow path and carrying out a blank run to see if the ghost peaks disappear. If the contamination peak is not present then the autosampler is the source.

Conversely, if the contamination peak is present without an injection then contamination does not lie with the autosampler and other sources such as the rest of the system, solvents, and column etc. should be considered.

There are common contamination sites in an autosampler.
Figure 2 details these sites and remedial action which can be taken to remove and prevent further contamination.

Autosampler Contamination

Use your mouse and roll over where indicated for further information and remedial action of common contamination sites on an autosampler.

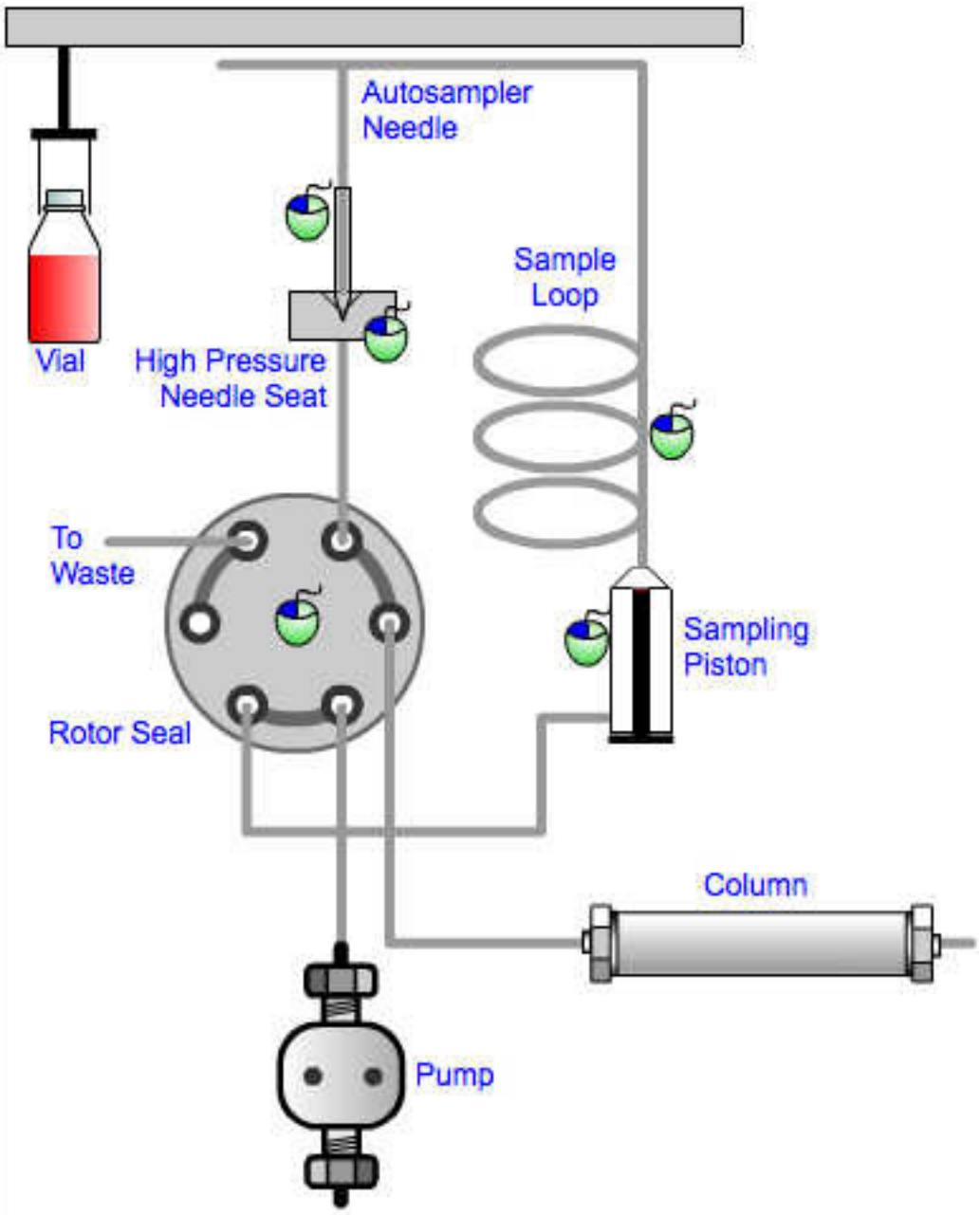


Figure 2: Sites of contamination and remedial action for autosamplers.

RETENTION TIME VARIABILITY IN HPLC

I'm sure we have all experienced it – that sinking feeling when you realize your analyte retention times have drifted outside the software 'window' and you have a pile of chromatograms with no quantitative results. Or you are trying to get that system suitability result to begin your batch of analyses as you really need to get out of the door fast but your retention times just won't settle down. Or you are trying to reproduce Bob from the R&D centers' method and his retention times (or chromatogram..!) look nothing like yours. Or you are trying to validate your method and the three column lots you are trying give different retention behavior to the column that you just developed your method on. Or every time you do an injection the retention time of the analyte changes just a little – doesn't cause anything to fall over, but you just don't understand why.

Yes – there are a whole bunch of retention time issues that cause problems in HPLC. A lot of the underlying causes we can do something about – others we just need to be aware of the cause and put our minds to rest. The remainder of this technical tip will outline how to overcome, or better control, all of the situations outlined above.
Overarching rules on retention time variability:

- If the void (hold-up) time (t0) and analyte retention time (tR) vary together, suspect a flow rate change. In this scenario the analyte capacity factor (k) will remain constant

- If only the analyte retention time varies, with the void (hold-up) time remaining constant, then k will also change. In this scenario suspect a change in the selectivity or retentivity of the separation system

Drifting Retention Times

This is typically due to a change in mobile phase composition, which can be caused when pre-mixed mobile phases lose some of the organic solvent through evaporation as the run progresses. Ever noticed this seems to happen more towards the end of a run? Well of course the organic solvent is being continually lost to the atmosphere – or as the eluent in the sealed bottle depletes there is more headspace for the more volatile component to evaporate into - and of course a small amount of evaporation makes a bigger overall composition change in the ever diminishing volume of liquid. This is typically why we see elution times becoming longer rather than shorter. What to do? Mix eluents (even isocratic ones) online or at the very least ensure the reservoir you are using is capped. We may also experience a change in the pH of the aqueous component of the eluent over time which is caused by ingress of CO_2; lowering the eluent pH and changing the retention and perhaps even the selectivity of the separation....so again, cap your bottle. Do not use lab film to cover eluent reservoirs – especially when using MS detection (watch out for ions at 142 Da as you leach the plasticizer from the film!)

Another related note on eluents here – if we de-gas pre-mixed mobile phases using vacuum, the very act of sucking the mobile phase through the filter under vacuum can cause loss of the more volatile component which will lead to irreproducible changes in eluent composition from batch to batch of eluent. The same is also true when degassing pre-mixed phases using ultrasonic baths; the warming of the eluent in the sonic bath can lead to loss of the organic modifier and, hence, change retention characteristics.

Temperature is another variable that can alter retention time, changing not only the viscosity of the eluent but the kinetics of the retention mechanism. Ionizable compounds tend to be affected by temperature more than non-ionogenic compounds; therefore, selectivity may also change. Most systems come with column heaters / chillers these days, but if yours doesn't, and you get large temperature variations in the lab, this can cause retention time variability (especially when the system is placed directly below your air-con). Even systems which do have column heaters work in different ways – some pre-warm the eluent prior to entry into the column for example and these systems may well give different retention times to those which heat the column only.

Variable Retention Times

Equilibrating or priming a column when beginning an analysis can also show up some strange retention time shifts and variability. Without going into too much detail, this is due to the stationary phase surface being modified by your eluent or sample components. Primarily it's the 'wetting' of the surface (especially with more hydrophobic phases such as C18) as the bonded phase takes on a layer of hydration - a slightly crass description but one which will do for this short tip. Furthermore, the polar or ionized silanol (Si-OH) groups on the silica surface can irreversibly bind with polar analyte components or buffer ions to change the overall surface polarity. What to do – well you can try to inject a 10x more concentrated sample than you normally would to try and achieve the equilibration in a shorter time (fewer injections).

In this category we must also consider the more esoteric issue of the sample diluent. For reasons that are too detailed to enter into here, the eluotropic strength and ionic strength of the sample diluent can sometimes have a big effect on analyte retention time and peak shape – yes that's the sample diluent, the thing you dissolve the sample in – not the HPLC eluent. You should always strive to match the aqueous / organic ratio of the eluent (at the start of the gradient if doing gradient elution) as well as the buffer strength of the eluent. If the diluent is more highly organic than the eluent (for solubility reasons) – try to restrict your injection volume to 10 µL or less.
For more information see this article:

https://www.chromacademy.com/chromatography-Sample-Diluent-HPLC.html

Column to Column Retention Time Irreproducibility

And what of the situation in which the column you used to develop your method doesn't behave like the new shiny ones you just bought to do your validation? This is the same as the previous situation really. Everything you put down the column (eluent and samples) modifies the surface – sometimes irreversibly, and the same goes for all of the stuff your colleague also put down the column before you used it to develop your method. What to do – buy a new column for method development and let it equilibrate properly before using it. If you have an ion pair reagent (and remember TFA is an ion pair reagent) and you remove it, use a different pairing reagent, or switch to a different eluent modifier – you should contact your column supplier before continuing with method development – it may well be that you need a new column.

Retention Time Issues in Method Transfer

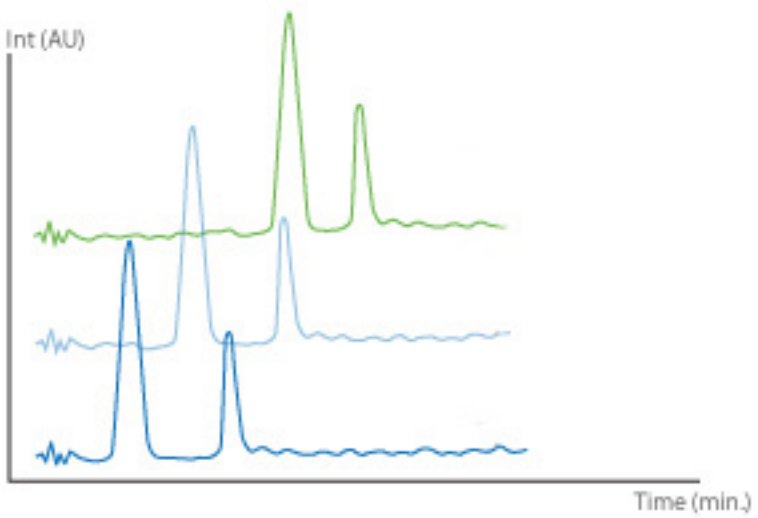
Matching retention times with Bob from R&D's method is another tricky situation. You must make sure that you are preparing the eluent in the same way – including weighing solid buffers, taking care with volumetric work, adding organic to aqueous portions, and adjusting the pH using the same acid or base and doing so with a properly calibrated pH meter. Further, the use of the same buffer is important – and just a note to all users of phosphate buffers – monosodium dihydrogen phosphate is not the same as disodium monohydrogen phosphate and neither of them has real buffering capacity between pH 3 and 6. Crucially, if the method involves gradient elution, you REALLY MUST know the gradient dwell volume for each system and adjust for any differences prior to repeating the method. If Bob's dwell volume is shorter than yours then you need to use an instrument capable of injection delay (injection occurring after the gradient has started) and if his dwell volume is larger than yours, add an isocratic hold at the start of the method equal to the difference in volume x flow rate. If he was smart when he developed the method, then he would have inserted an isocratic portion at the start of the gradient which you can adjust to make sure the gradient dwell volume differences are catered for.

Hardware Related Retention Time Problems

Pumps pump at a fixed flow rate – until they leak or break. In the majority of cases poor pump performance will be accompanied by other symptoms such as low, high, or cycling back pressure – but not always. The easiest way to check your flow rate accuracy is to run the eluent into a 10 mL volumetric cylinder and time it. If you get 10 mL in 10 min when operating at 1 mL/min then all is well – if not – all is bad and you need to get the system checked out.

TROUBLESHOOTING HILIC

Insufficient Retention



Incorrect stationary phase

The analyte log P or log D values should be matched to the stationary phase polarity. For acidic analytes, a stationary phase with anion exchange properties will provide increased retention; conversely, cation exchange phases will exhibit greater retention for basic analytes.

Incorrect mobile phase pH

Analyte retention increases as the polarity of the analyte increases. Ionized analytes are more polar; therefore, adjust the pH to give the ionized analyte - two pH units above analyte pKa for acids and 2 pH units below the analyte pKa for bases (Figure 1).

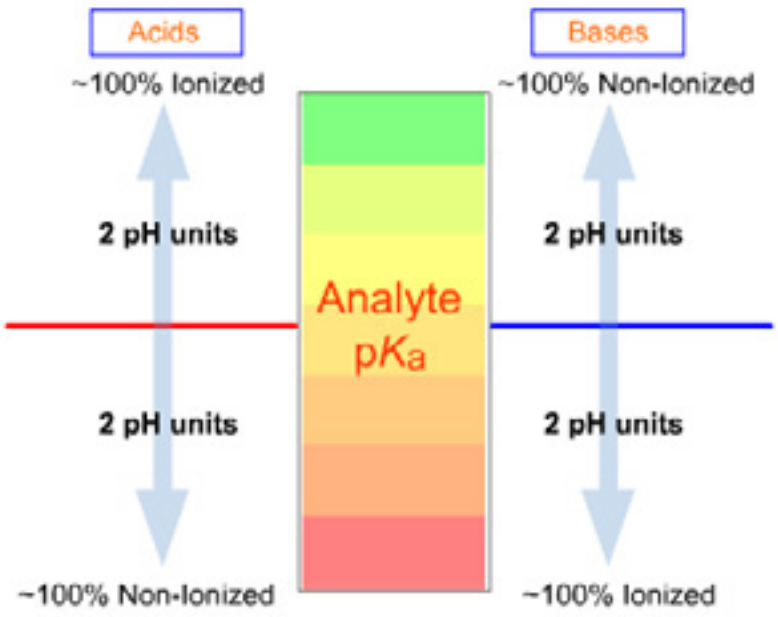
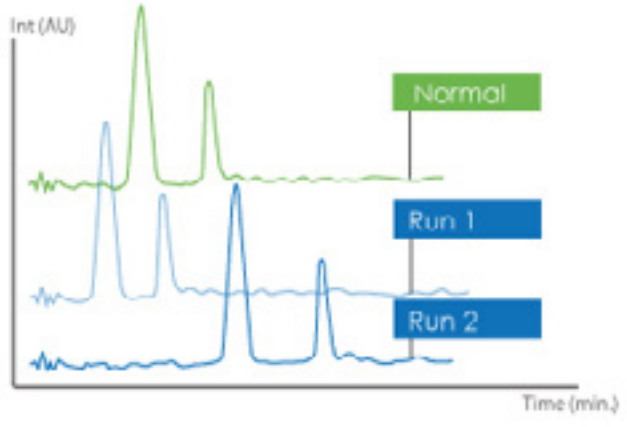


Figure 1: 2 pH rule.

Too much water in the mobile phase

Increase organic content, although ensure that there is a minimum of 3% water to maintain analyte partitioning and stationary phase hydration.

Retention Time Drift



Column insufficiently equilibrated

HILIC columns generally take longer to equilibrate than reversed phase HPLC columns, primarily due to the need to establish ionic strength/ion exchange equilibria on the stationary phase surface, as well as the time required to re-equilibrate the adsorbed aqueous layer.

Most manufacturers will have their own equilibration guidelines, however as a general recommendation, a new column should be flushed with at least 50 column volumes of the mobile phase being used and 20 column volumes daily in routine use. A re-equilibration of 10 EMPTY column volumes (VM, Equation 1) is recommended between each injection for gradients, and an occasional water wash is suggested to remove retained ions when operating in an isocratic mode.

$$V_M = \pi r^2 L \quad (1)$$

Where:
VM = column volume (µL)
r = radius (mm)
l = column length (mm)

Calculation for a 4.6 x 150 mm column

$$V_m = \pi\left(\frac{d_c}{2}\right)^2 L0.68$$

$$= \pi\left(\frac{4.6}{2}\right)^2 \times 150$$

$$= \pi \times 5.29 \times 150 \times 0.68$$

$$= 2492.85\ \mu L \approx 2.5\ mL$$

10 column volumes = 2.5 x 20 = 50 mL

Re-equilibration time at a flow rate of 1.5 mL/min = 50/1.5 = 33.3 minutes.

Column contamination

Shifting retention times or resolution in conjunction with an increase in system pressure indicates column contamination or a blocked inlet frit. The column can be reversed (unconnected from the detector to avoid washing particulates into the flow cell) and flushed with the mobile phase to remove particulates from the inlet frit. To remove strongly retained components from the column, flush the column in the reverse direction with strong solvents such as 50:50 methanol:water. Always consult the column user guide for any specific column washing instructions.

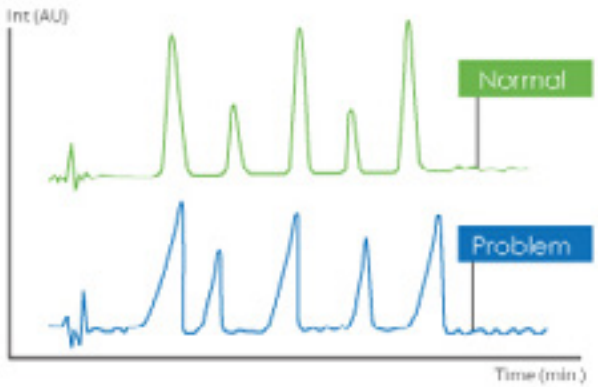
Incorrect mobile phase pH

If the mobile phase pH is close to the analyte pKa this can cause retention time drift. Adjust the pH of the buffer to two pH units above or below the analyte pKa (depending on whether it is an acid or base, Figure 1). Make sure the correct buffer has been selected for the pH range - buffers have optimum buffering capacity ±1 pH unit from their pKa (Table 1).

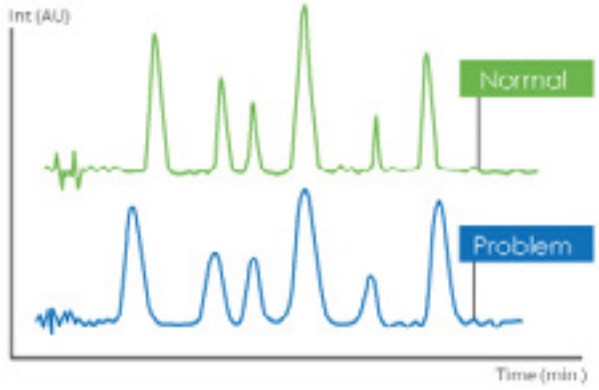
Buffer	pKa	pH Range	UV Cut Off (nm)
Phosphate	2.1 7.2 12.3	1.1-3.1 6.2-8.2 11.3-13.3	<200
Acetate*	4.8	3.8-5.8	210 (10 mM)
Citrate	3.1 4.7 5.4	2.1-4.1 3.7-5.7 4.4-6.4	230
Carbonate	6.1 10.3	5.1-7.1 9.3-11.0	<200
Formate*	3.8	2.4-4.8	210 (10 mM)
Ammonium bicarbonate	7.6	6.6-8.6	230
Borate	9.3	8.3-10.3	N/A

Table 1: Common HPLC buffers. * denotes volatile buffer suitable for use with mass spectrometry.

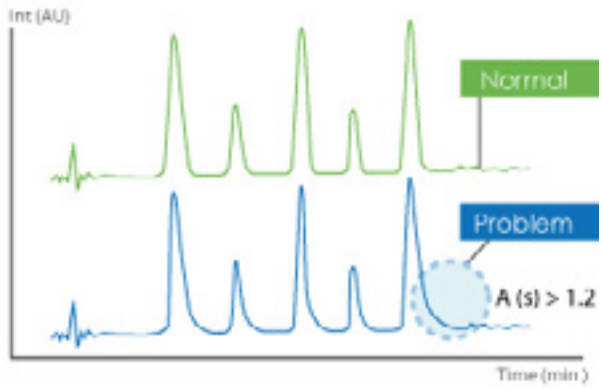
Poor Peak Shapes



Peak fronting



Peak broadening



Peak tailing

Injection solvent/mobile phase mismatch

It is recommended that the injection (sample) solvent composition is as close to the initial mobile phase composition as possible, or at least has an organic content greater than 50%. Using highly aqueous injection solvents with high elution strength leads to peak broadening as it impairs analyte partitioning into the stationary phase. This can also result in column overload, reduced retention, and loss of resolution. For polar analytes which exhibit low solubility in organic solvents, using methanol instead of water is recommended. When there are extreme solubility issues even the aqueous portion of the mobile phase can be replaced by polar non-aqueous solvents; this technique is referred to as non-aqueous HILIC.

Injection volume too high

If the injection volume is too large fronting, broad, tailing, or flattened peaks will be seen.
The recommended injection volumes are 0.5-5 µL for a 2.1 mm i.d. column and 5-50 µL for a 4.6 mm i.d. column.

Needle wash/mobile phase mismatch

Needle wash solutions should match the mobile phase composition, but with the buffer portion replaced by water. If too much water is present in the wash solution broadened peaks will be evident. Pure organic solvents should also be avoided as they will not be polar enough to remove the analytes which will result in contamination, carryover, ghost peaks etc.

Insufficient mobile phase buffering

Peak tailing can be caused by insufficient buffer capacity as analytes will undergo secondary interactions with the stationary phase. Increased buffer concentration promotes hydrogen bonding between the analyte and stationary phase, overcoming other secondary interaction, and improving peak shapes. When increasing buffer concentration, be aware of the potential to suppress ion signal with mass spectrometry. Furthermore, always use volatile buffers for HILIC-MS applications.

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