Column troubleshooting guide

The world leader in serving science

Column troubleshooting guide – Reversed phase

Select option

General information on how to start the column troubleshooting process

Choose one of the topics

This guide is designed to aid the troubleshooting of silica based reversed phase columns. While the content may apply in other applications, this is not the intent of the guide.

The guide is best navigated by using the buttons; links to the previous page or to the start can be found in the bottom right of the page.

Column troubleshooting guide – Reversed phase

General approach to troubleshooting

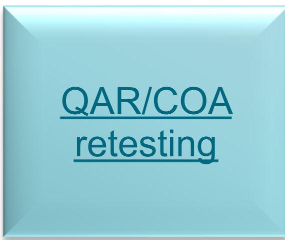
What led us to question the column performance:

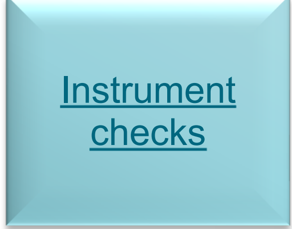
- Instrument reported an error (red lights, instrument alarm, Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) popup on screen, etc.)
- Sample queue failed to run to completion
- Physical observation (wet floor, rusting, liquid leak, etc.)
- Analysis of samples does not produce the expected result (calibration curve is not linear, SST results fail, etc.)
- Chromatography shows poor quality (peak shape not symmetrical, resolution issue, baseline issues, etc.)

Steps to solve the issue:

- Separate instrument issues/chromatography issues/application issues
- Determine if instrument is functioning properly?
- Determine if sample is valid (model with a standard)?
- Does the column need to be replaced?
- Contact technical support when more ideas are needed

Select option





How to contact us

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General approach – Instrument reported an error

Is the error obvious?

- Physical leak
- Sample in the wrong location
- Mobile phase bottles ran empty

Can the audit trail identify the problem?

- Overpressure
- Module was not connected or disconnected unexpectedly
- Detector was not turned on properly
- Issues with the instrument method (lower maximum pressure, flow rate changes etc.)

Can the chromatography data system (CDS) or modules be restarted?

Can the queue be restarted?

Can the problem be identified and fixed?

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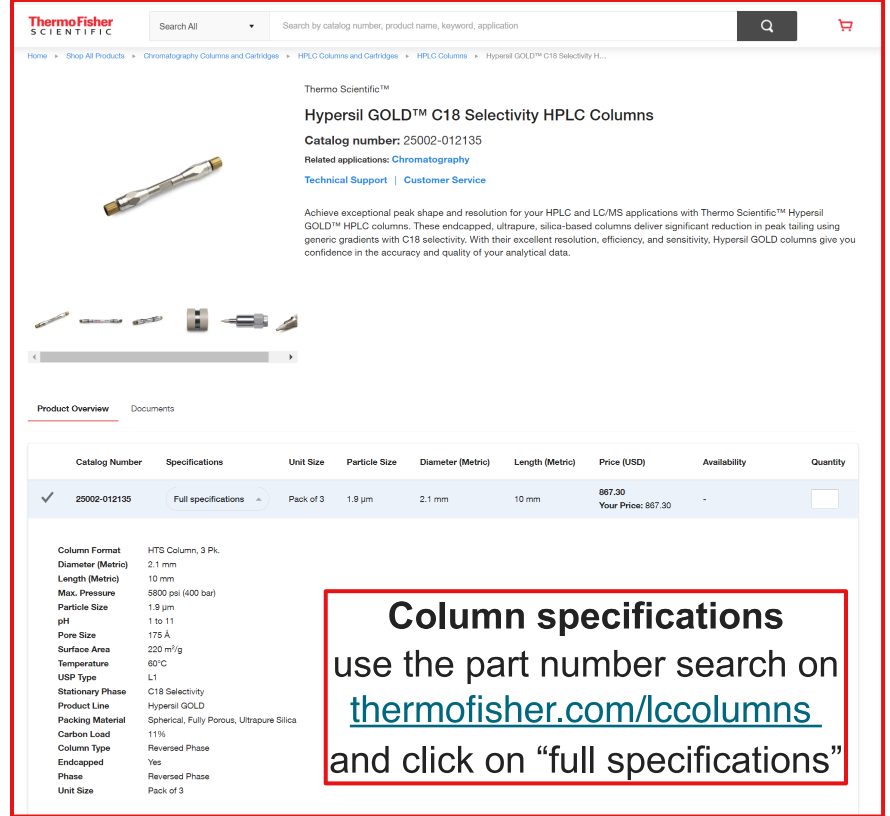
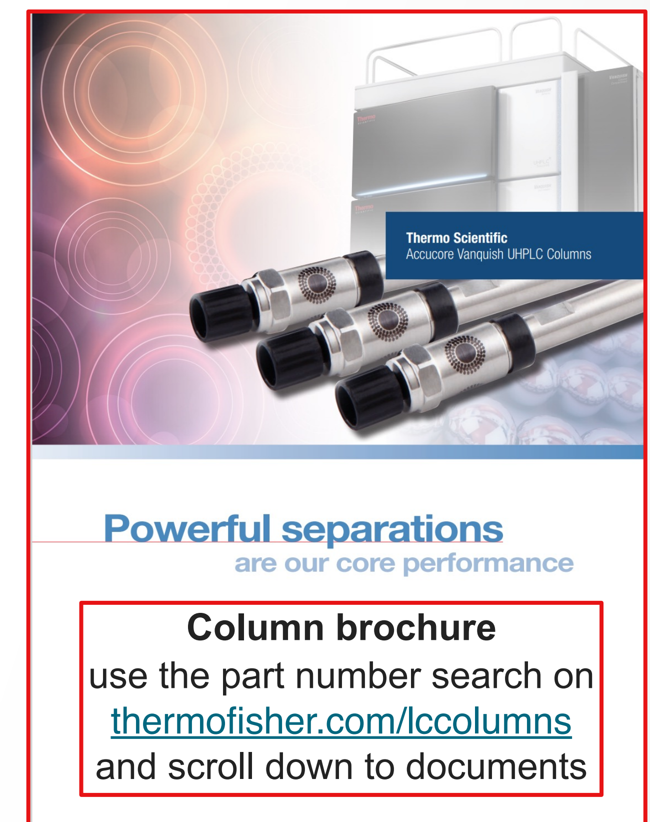
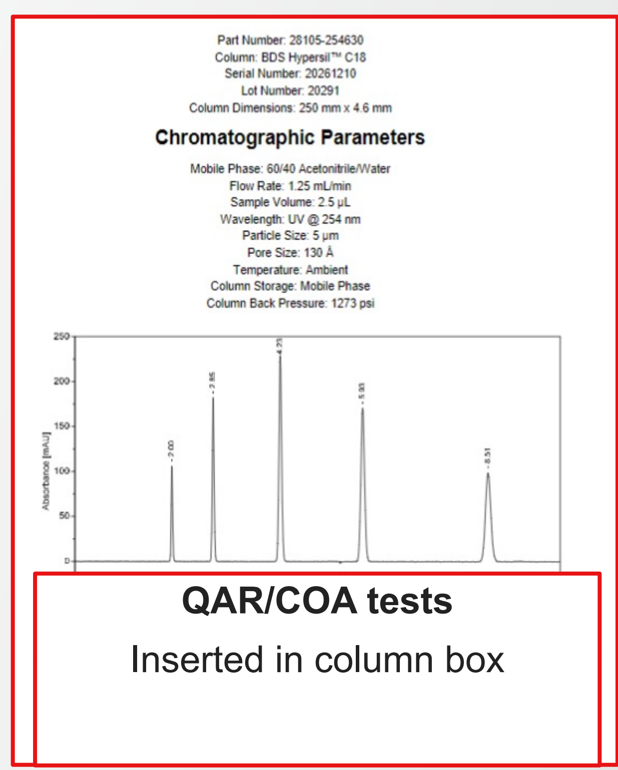
General approach – Product specifications



Column specifications
use the part number search on thermofisher.com/lccolumns and click on "full specifications"



Column brochure
use the part number search on thermofisher.com/lccolumns and scroll down to documents



QAR/COA tests
Inserted in column box

Review the column specifications to determine if it has been used correctly.

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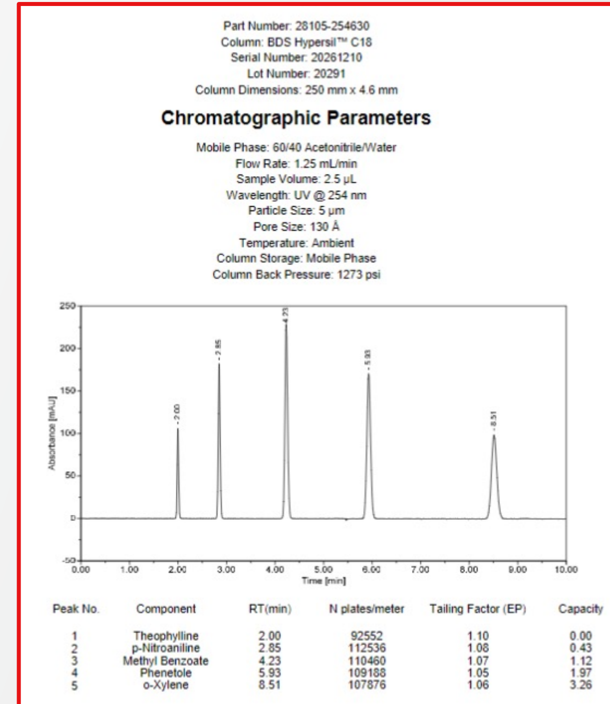
General approach – Certificate of Analysis (CoA)

Content:

- A certificate of analysis (CoA or QAR) is column specific

This provides details of the column specifications and performance, such as:

- Column name, dimensions, serial number and lot number
- Method and sample that the column was tested with
- Performance specifications, which the column must meet to be shipped to a customer
- Actual performance numbers of your column are listed as well as the chromatogram that gave these results. Users may see some slightly different performance numbers due to instrumentation
- You can use this method to verify that your column is meeting our shipping criteria as it left the factory. This may be useful in evaluating whether you need to purchase a new column


Part Number: 28105-254630
Column: BDS Hypersil™ C18
Serial Number: 20261210
Lot Number: 20291
Column Dimensions: 250 mm x 4.6 mm

Chromatographic Parameters

Mobile Phase: 60/40 Acetonitrile/Water
Flow Rate: 1.25 mL/min
Sample Volume: 2.5 µL
Wavelength: UV @ 254 nm
Particle Size: 5 µm
Pore Size: 130 Å
Temperature: Ambient
Column Storage: Mobile Phase
Column Back Pressure: 1273 psi

Peak No.	Component	RT(min)	N plates/meter	Tailing Factor (EP)	Capacity
1	Theophylline	2.00	92552	1.10	0.00
2	p-Nitroaniline	2.85	112536	1.08	0.43
3	Methyl Benzoate	4.23	110460	1.07	1.12
4	Phenetole	5.93	109188	1.05	1.97
5	o-Xylene	8.51	107876	1.06	3.26

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General approach – Instrument checks

Pump

- Have you checked the eluent bottles are filled correctly and there is no bubbles in the draw lines?
- Is the backpressure within the expected range and stable?
- If a gradient is in use, has it been changed?

Autosampler

- Is the autosampler injecting reliably?
- Has it been tested with a standard?
- Is there leaking at the needle seat?
- Is wash liquid available?
- Is the volume being picked up correct?

Detector

- Have you confirmed the detection method is suitable for your analyte? This could include things such as a chromophore for UV detection.
- Can the response of a standard be verified?
- Is the detector warmed up sufficiently?

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Contact us for additional help

- We hope that this troubleshooting guide was able to solve your issue and includes the valuable information you were looking for
- If the issue could not be solved using this troubleshooting guide, please do not hesitate to contact us using the information below

Email

- Americas: usa.techsupport.ccs@thermofisher.com
- EMEA, APAC, and ROW: techsupport.ccs@thermofisher.com
- Describe the issue
- Share part no., serial no., and lot no. of your column
- Include method and sample information
- Send pictures or results showing the issue and what has been done to date

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Select topic

- Baseline
- Pressure
- Resolution
- Peak shape
- Leaking
- Retention
- Contamination
- Column lifetime
- Recovery
- Peak missing

How to contact us

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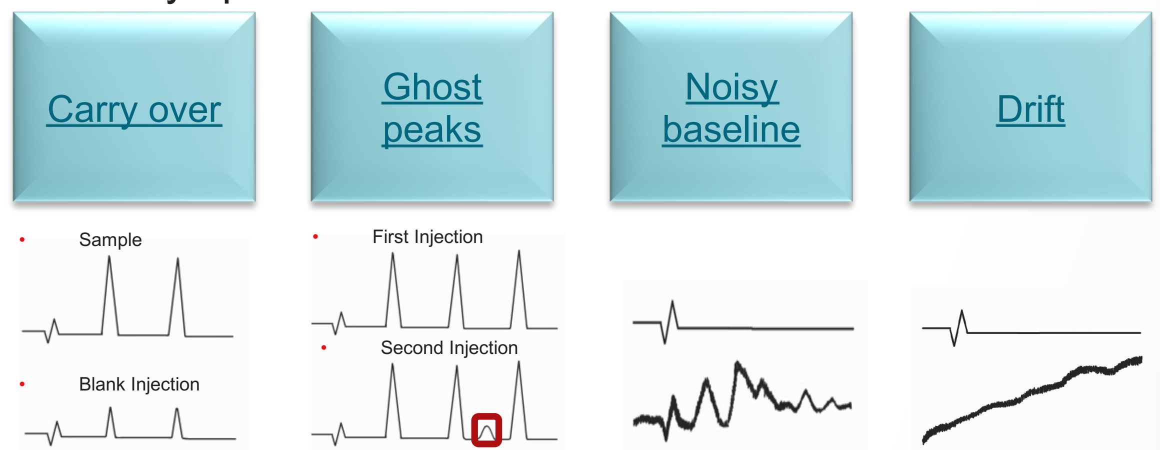
Baseline – General

- **Instrument** – Make sure that your instrument is
  - Free of possible contamination
  - The detectors are sufficiently warmed up
  - Column is sufficiently equilibrated
- **Blank** – Perform a blank run. If the issue can be seen or even reproduced in the blank, a baseline issue could be considered
- **Mobile phase** – A common and general cause of baseline issues are contaminations of the mobile phase. The mobile phase can be responsible for an increased background, spikes, and noise. Make sure to use highly pure chemicals and at least HPLC grade eluents as mobile phase and for sample dilution (including salts, water, buffers, and any modifiers or other components added to the mobile phase) Mobile phases should be prepared fresh on a regular basis due to potential bacterial growth and should be filtered in order to avoid column contamination as well as an unstable baseline

Select symptom



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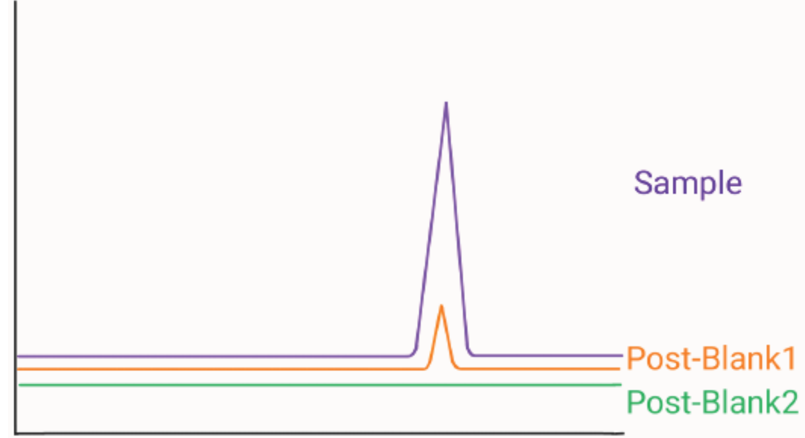
Baseline – Carry over



- Carry Over is a special type of contamination and is described as the blank showing one or more peak(s) at the same retention time as the sample, which was injected before the blank. It can be caused by:
  - **Lack of maintenance and cleaning** – Undesired interactions within the system can cause carry over. Regular maintenance and cleaning of the system is advised.
  - **Overloading** – This can cause sample to remain in the system and cause fouling of the column. It can be avoided by one reducing the injection volume and/or the concentration of the sample
  - **Improper elution** – Your sample might not be properly eluted from the column. In order to properly elute your sample, increase the amount of organic solvent at the end of your gradient, elongate the time of flushing at the end of the run or choose a stronger solvent. We also recommend implementing a column wash on a regular basis (See seesaw gradient wash)
  - **Poor injection needle wash** – The injection needle or wash vial might be contaminated with sample. Make sure to choose a solvent that properly rinses of residual sample of the needle
  - **Vial contamination** – Contaminations in the vial can lead to carry over. This can be caused e.g., by the injection needle or blank preparation. Improve your needle wash procedure and reprepare your blank.

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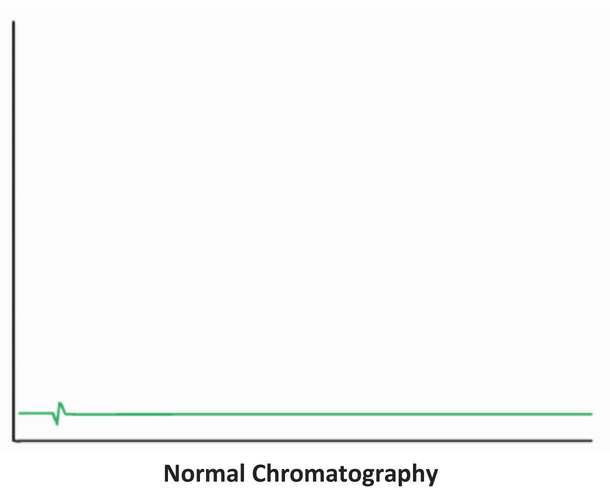
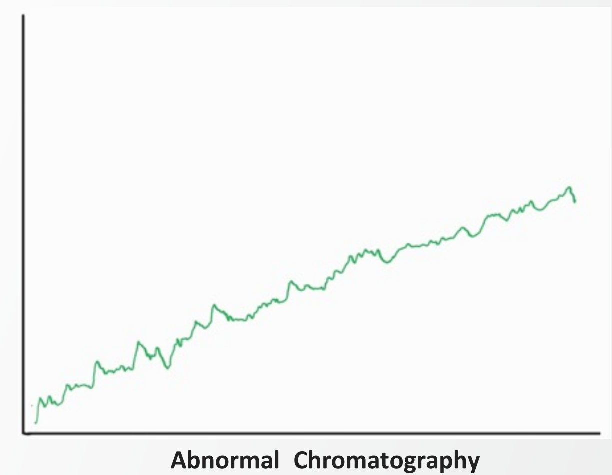
Baseline – Drift



Normal Chromatography



Abnormal Chromatography

- Baseline drifts can be normal when the mobile phase composition, flow rate, column, and temperature are changed. However, baseline drifts are not expected under isocratic conditions
- **Baseline drift** – In case of a decreasing or increasing baseline: ensure that your instrument and detectors are properly warmed up, the column is sufficiently equilibrated, the column has had time for the temperature to stabilize, and your eluent and system are free of contamination. If the above conditions have been met, then review the UV cutoff of your mobile phase and ensure other environmental factors, such as ambient temperature and humidity, have been suitably controlled
- **Mobile phase composition** – Changes to the mobile phase composition can also contribute to a drift which is seen via the detection method. Check if the baseline is also drifting under isocratic conditions. Under isocratic conditions, we recommend premixing the mobile phase

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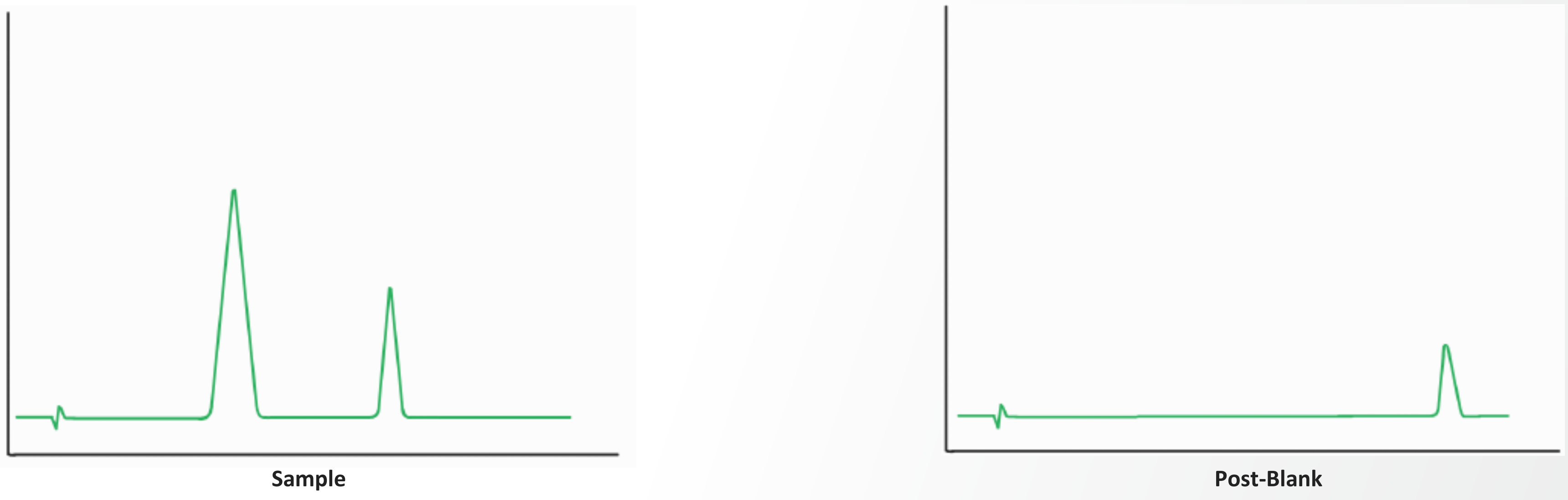
Baseline – Ghost peaks



- **Air** – Air present in the eluent or system might cause sharp peaks with a very small width. Degas your eluents and purge the instruments with eluent before connecting the column
- **Late eluting peak** – If a blank or a following run includes a broader, unexpected peak, it could be that the gradient is too weak or too short to elute all sample components properly. Then, a peak might be eluting in the next run, this is known as wrap-around peaks. Choose a longer gradient, improve the organic solvent strength or add a washing step at end of each run to avoid this
- **Column bleed** – Column bleed can lead to an elevated baseline and continuous elution of background components. If an unexpected shark-fin shaped “peak” occurs during gradient elution, this may be column bleed. This can occur if a column is used out of its specifications, e.g. out of the recommended pH range or with incompatible eluents. Thus, ensure to operate the column within the recommended specifications

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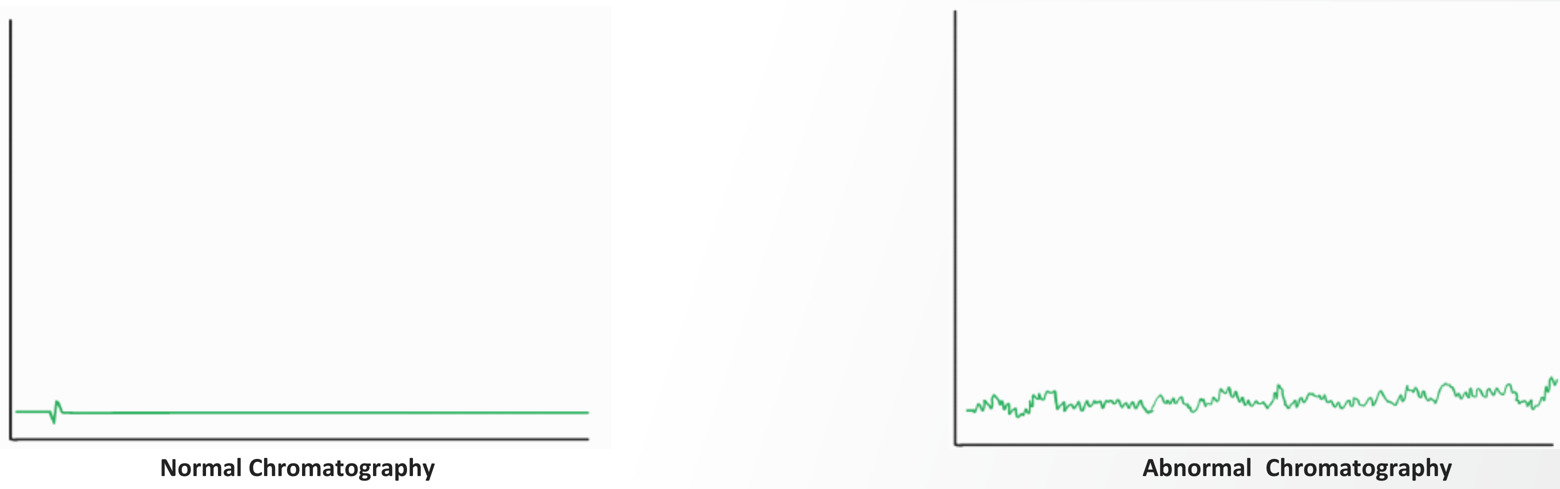
Baseline – Noisy baseline



- **Pump pulsations** – Repetitive baseline noise can be caused by pump pulsations. Make sure the instrument is working properly and regular maintenance is done
- **Proportioning** – Issues with eluent proportioning can contribute to a noisy baseline. In case of isocratic conditions, we recommend premixing the mobile phase instead of using the proportioning valve of the instrument
- **Leak** – Check for leaks and if the pressure is below your regular level. Tighten all connections and see if this solves the issue
- **Contaminated mobile phase** – This can also cause baseline fluctuations. Use only high-quality chemicals for your mobile phase and reprepare it on a regular basis
- **Temperature effect** – If a column is heated to a high temperature and the detector is unheated, baseline noise can occur. Use a post-column cooler to reduce the temperature difference

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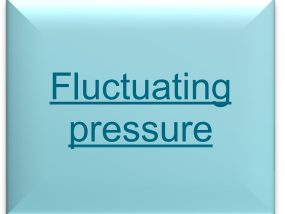
Pressure – General

- Pressure is an important indicator for the state of your instrument and column. Thus, monitoring your pressure and comparing it regularly to the pressure of a well working system is recommended
- Pressure changes can be normal when the mobile phase composition, flow rate, column, and temperature are changed
- However, a lower, higher or fluctuating pressure compared to your regular pressure for the working conditions is often a sign of an issue. This can be sudden or gradually occurring. The speed of its occurrence can hint to the possible reason for the pressure change

Select Symptom



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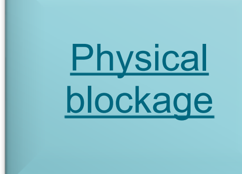
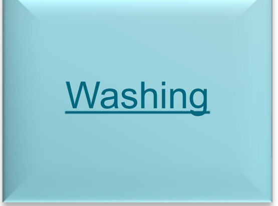
Overpressure

An increase or over pressuring often comes from these common sources:

- Insufficient sample preparation leading to column contamination



- Physical Blockage caused e.g. by larger particulates

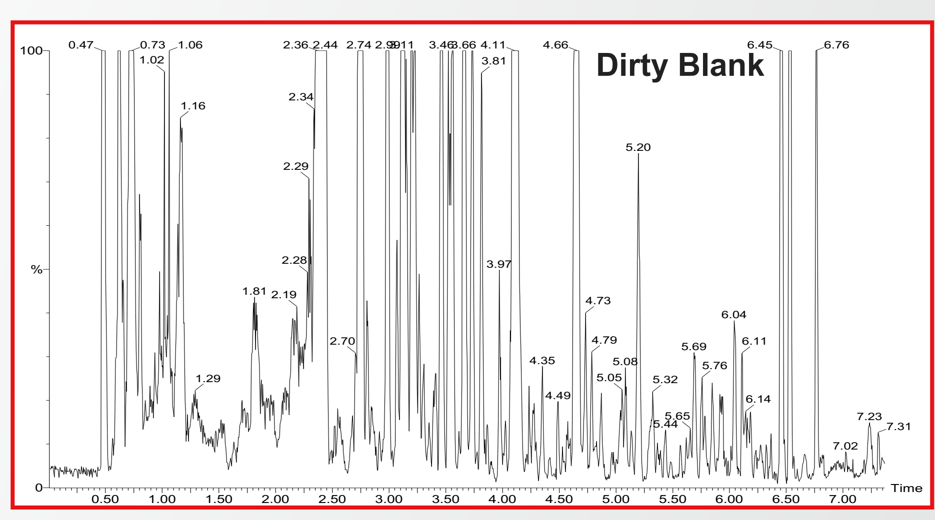


- Lack of prevention (washing, no guard or trap column)

Column troubleshooting guide – Pressure

Sample preparation

- Pressure increases due to insufficient sample preparation can occur suddenly or gradually. The speed of the pressure increase can hint to its cause
- **Gradual pressure increase** – Gradual pressure increase can be caused by the build up of hydrophobic substances from the samples on the column. Include blanks and washing steps during the run to avoid this and make sure blanks are showing no peaks or increased baseline. If something is seen in the blank, increase washing and/or switch to a stronger wash procedure. In addition, investigate a more efficient sample preparation, such as solid phase extraction (SPE) over liquid-liquid extraction. Preventative measures such as using a trap or guard column to keep samples from fouling the column is strongly advised
- **Sudden pressure increase** – A sudden pressure increase is usually due to particulates. Make sure samples are at least centrifuged or filtered. If the pressure increase continues, using SPE during sample preparation might be an option. We also recommend using inline filter, guard, or trap column to prevent large particulates from entering the column



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Column troubleshooting guide – Pressure

Physical blockage

- A typical sign of physical blockage is a sudden pressure increase
- Physical blockage is often caused by larger particles stuck in the system or at the head of the column.
- Incrementally go from mobile phase introduction to the column and break connections to see where the increase in pressure is coming from
  - If the tubing is causing a pressure change, replace the tubing
  - If the guard/column is the source of the pressure change, wash the guard/column (Lifetime-> Washing)
  - If this should not fix the issue, replace guard/column
- Filter your samples, perform thorough sample preparation, and use a trap/guard column to prevent physical blockage
- Backflushing – Backflushing a column while washing can avoid contaminants to be pushed further down the column. It is important to ensure that washing is done to waste, this ensures that any post column fluidics does not become contaminated. Please note that sub-2 µm particle size columns cannot be backflushed

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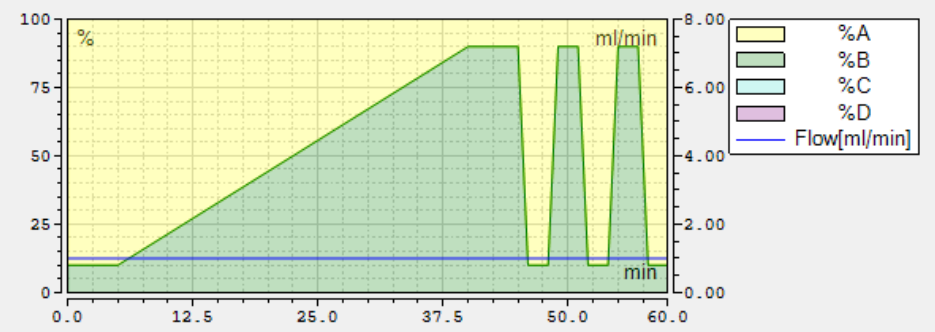
Washing

- Regular washing of your column and trap/guard will elongate its lifetime
- Washing in between samples, such as at the end of your separation gradient, is a good way to prevent build up of materials on the column that lead to an increase in pressure
- Types of washing:
  - For salts, you will need a high aqueous wash
  - For lipids/fats, you will need a high organic wash (IPA wash)
  - May need "seesaw" gradient of high and low organic washes
  - For large particulates, prevention of them entering the column is the key – centrifugation or filtration prior to analysis



No	Time	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	Run				
2	0.000	1.000	10.0	0.0	0.0	5
3	5.000	1.000	10.0	0.0	0.0	5
4	40.000	1.000	90.0	0.0	0.0	5
5	45.000	1.000	90.0	0.0	0.0	5
6	46.000	1.000	10.0	0.0	0.0	5
7	48.000	1.000	10.0	0.0	0.0	5
8	49.000	1.000	90.0	0.0	0.0	5
9	51.000	1.000	90.0	0.0	0.0	5
10	52.000	1.000	10.0	0.0	0.0	5
11	54.000	1.000	10.0	0.0	0.0	5
12	55.000	1.000	90.0	0.0	0.0	5
13	57.000	1.000	90.0	0.0	0.0	5
14	58.000	1.000	10.0	0.0	0.0	5

Example of seesaw column washing

Wash procedures

Column troubleshooting guide – Pressure

No/Low pressure

- **Purge valve** – An open purge valve leads to no pressure. Make sure that your purge valve is closed
- **Eluent lines** – Check that your eluent lines and reservoir are filled with eluent; followed by repurging the system to ensure no air is trapped inside the pumps
- **In-line filter** – Check that solvent is flowing out of the purge valve when opened. If not, clean or exchange the in-line filters.
- **Leaking** – A leak in your system can also cause a lower pressure than expected. See Leaking

Column troubleshooting guide – Pressure

Fluctuating pressure

- **Solvent lines** – Make sure the solvent lines are properly filled with eluent and no air bubbles are present
- **Gas in eluent** – a not fully degased eluent can cause pressure fluctuations. Use a degaser and degas your eluent by using e.g. an ultrasonic bath, vacuum filtration or bubble with inert gas. Care should be taken not to sonicate unnecessarily long as to avoid the risk of partial evaporation of volatile mobile phase components
- **Pump** – Pressure fluctuations are more commonly a pump issue and can be e.g. caused by the check valves. Exchange the column for a different column

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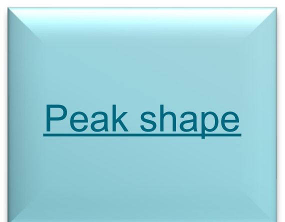
Resolution – General

- Chromatographic resolution is mainly affected by selectivity. Please reprepare the mobile phase to check if the issue is not a result of a change in retention and/or selectivity
- Resolution issues can also be related to peak shape. For example, peak tailing may cause the resolution of two peaks to be decreased
- Please do not exceed the pH range of the column, this avoids damage that can cause a decrease of retention times and loss of resolution

Select symptom

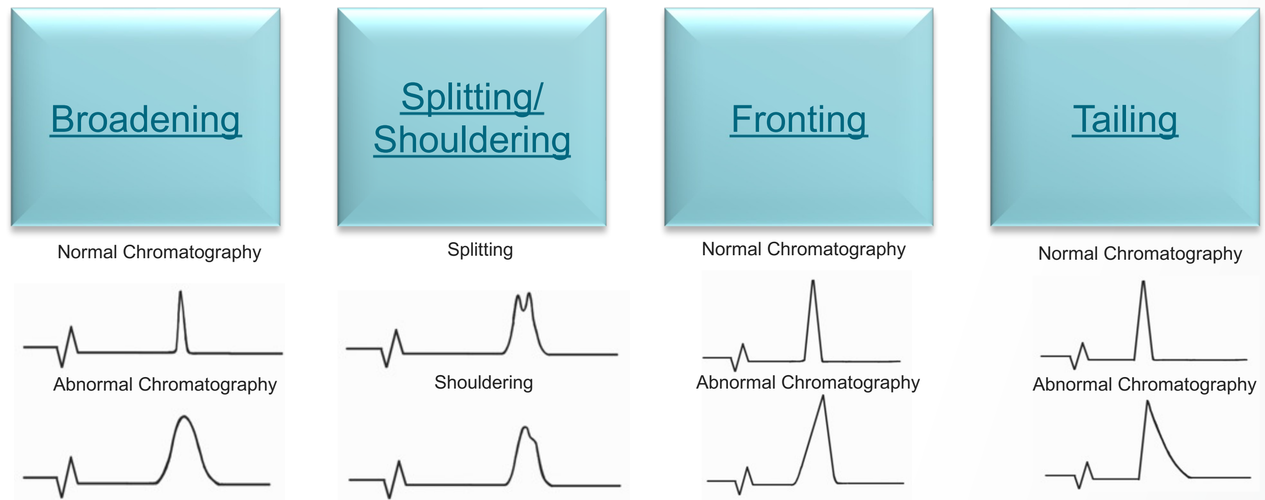


Column troubleshooting guide – Reversed phase

Peak shape – General

- **Coelution of peaks** – Coelution can have the same appearance as a peak shape issue and look like a peak is broadening, shouldering, fronting or tailing. Thus, it is important to confirm, that you are observing a peak shape issue rather than two peaks coeluting, such as two distinct compounds or the target analyte in and its degradation product. Injecting a standard of only one compound is a good way to confirm a peak shape issue
- **Check the connections to the column** – Changes to the tubing, such as poor connections, changes in length or internal diameter can cause performance effects

Select symptom



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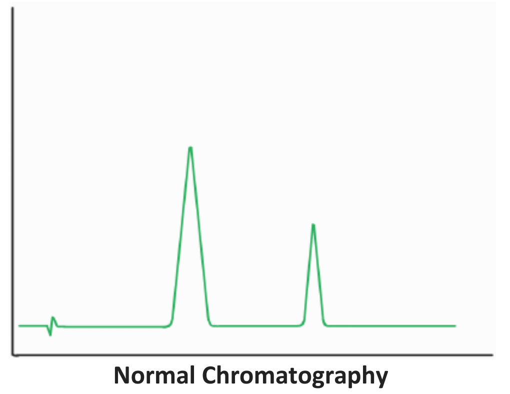
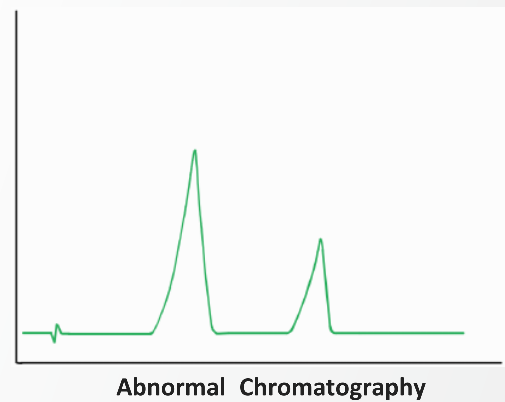
Peak shape – Fronting


Normal Chromatography


Abnormal Chromatography

- **Column overloading** – Injection volume overloading or mass overloading can result in fronting. To eliminate this, try lowering your injection volume or sample concentration to see if the peak shape improves
- **Phase collapse** – Generally accompanied with a loss of retention. This is caused by using the column in a higher aqueous mobile phase than is compatible with the column. Flushing the column with 100% acetone or acetonitrile should reverse this effect
- **Solvent compatibility** – If the analytes has a higher affinity to the injection solvent than the mobile phase, peak fronting can occur. To avoid this, match your sample diluent to the starting mobile phase conditions
- **Physical damage** – It is possible that the column could be damaged, if you have attempted all above mentioned troubleshooting without success, please contact us

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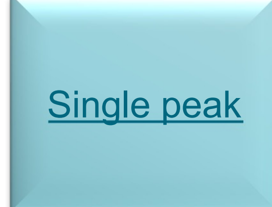
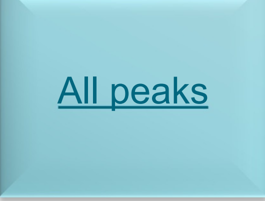
Peak shape – Tailing



Only a single peak in the chromatogram shows tailing issues



All peaks in the chromatogram show tailing issues

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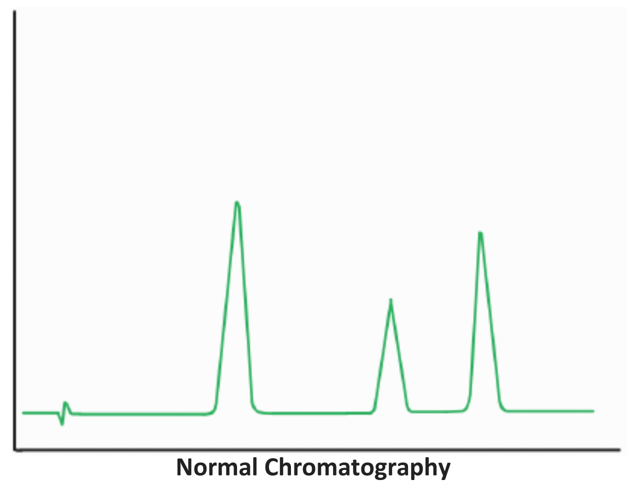
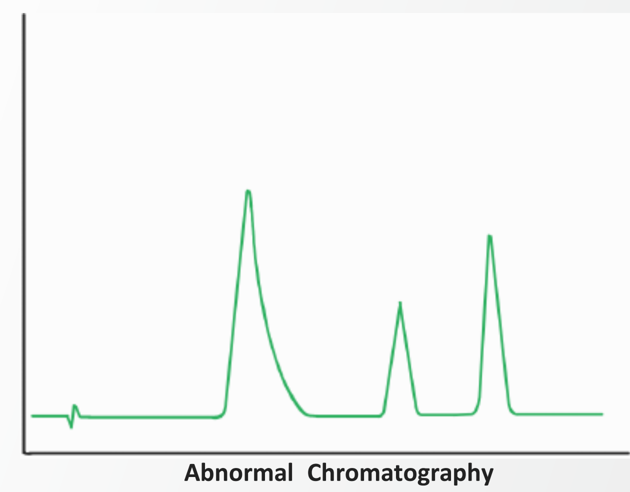
Peak shape – Tailing – Single peak

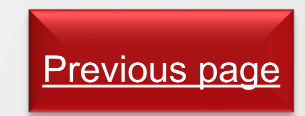


Normal Chromatography



Abnormal Chromatography

- **Basic analytes** – If the issue is related to basic analytes, ensure that you are using an end capped phase
- **pH related issues** – Tailing can be caused by secondary interactions of the stationary phase. Reducing silanol activity can be done by pH adjustment or buffering of the mobile phase
- **Physical damage** – It is possible that the column could be damaged, if you have attempted all above mentioned troubleshooting without success, please contact us



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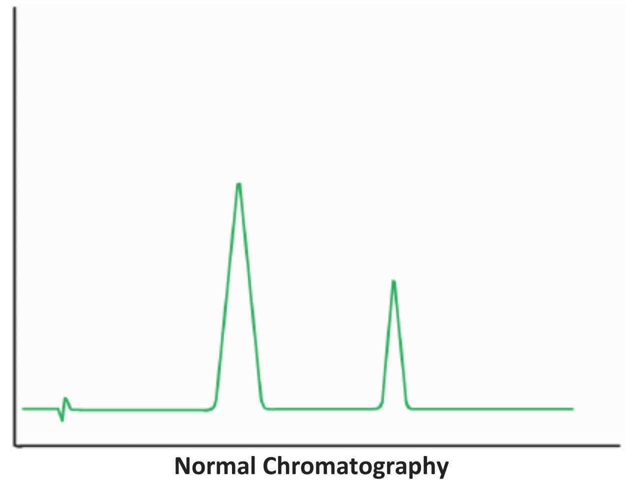
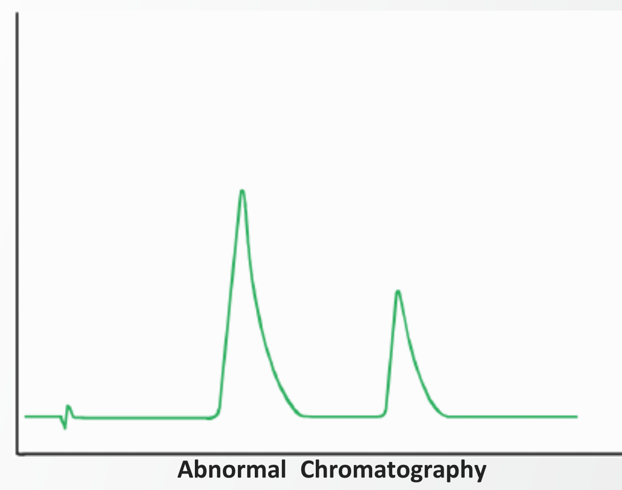
Peak shape – Tailing – All peaks

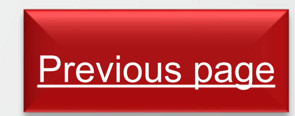


Normal Chromatography



Abnormal Chromatography

- **Physical obstructions** – A blocked or contaminated guard column or inlet frit can cause tailing, refer to our Column care guides to perform a suitable washing procedure and replace the guard cartridge
- **pH related issues** – Tailing can be caused by secondary interactions of the stationary phase. Reducing silanol activity can be done by pH adjustment or buffering of the mobile phase
- **Basic analytes** – If the issue is related to basic analytes, ensure that you are using an end capped phase
- **Connections** – Increases in peak tailing can also be caused by damage caused to fittings or connections, ensure these have been tightened or replaced
- **Physical damage** – It is possible that the column could be damaged, if you have attempted all above mentioned troubleshooting without success, please contact us



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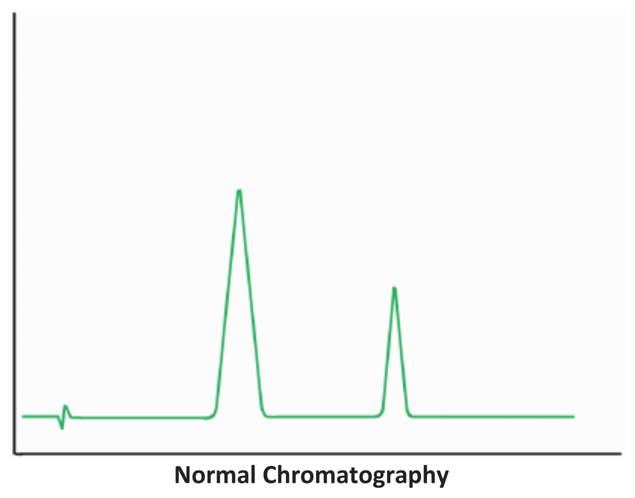
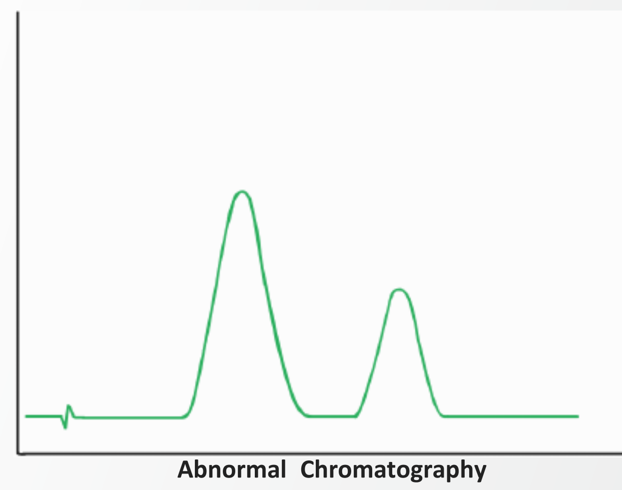
Peak shape – Broadening

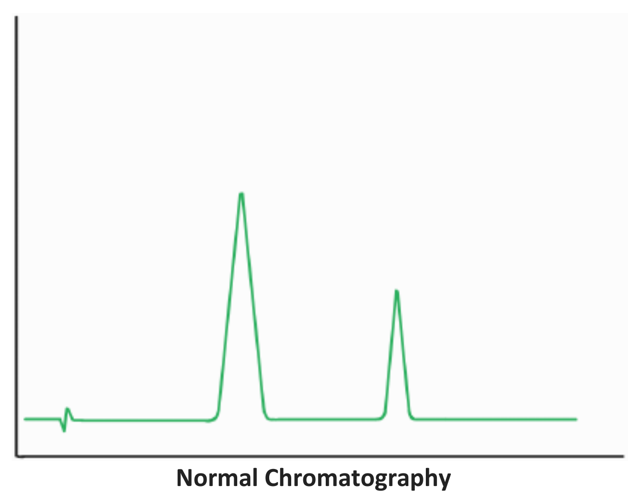

Normal Chromatography

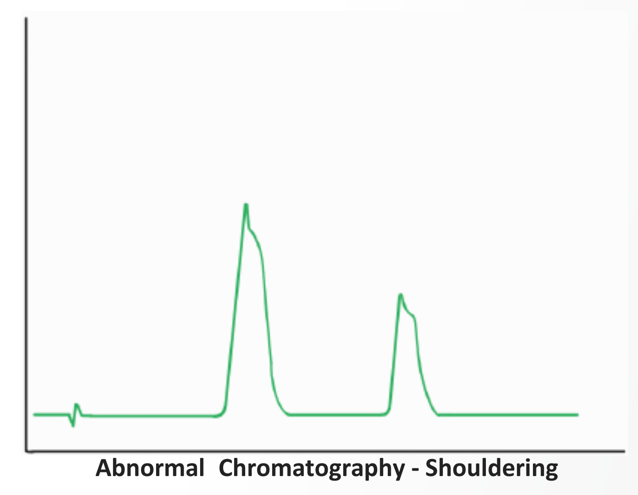

Abnormal Chromatography

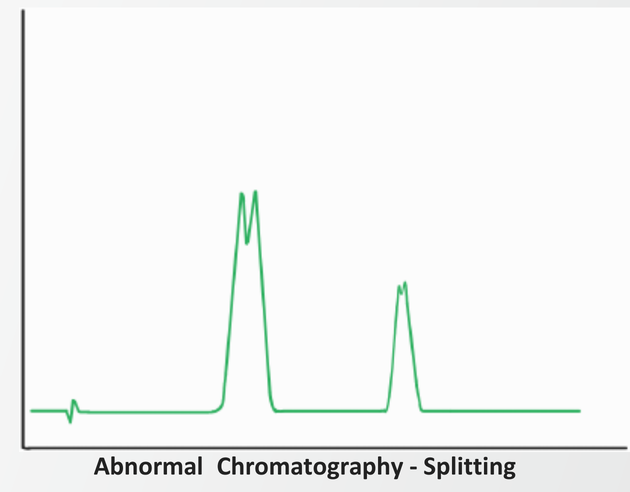
- **Column age** - Loss of efficiency is common in columns as they age; below are some different routes to take if the column is showing peak broadening prematurely:
  - Follow our column care guide to ensure you are extending your column lifetime as much as possible
  - If broadening is accompanied by retention issues, then please check you have the correct temperature and flow rate. Please see Retention
  - If peak broadening occurs on a new column, ensure that you are following the best practices to enable narrow peaks. This includes reducing system dead volume by ensuring you have the correct length and diameter capillaries and are using zero dead volume connections where possible

Column troubleshooting guide – Reversed phase

Peak shape – Shouldering/Splitting


Normal Chromatography


Abnormal Chromatography - Shouldering


Abnormal Chromatography - Splitting

- **Physical blockages** – Splitting or shouldering peaks can be caused by a physical blockage at the column inlet, try backflushing the column if you are using a column with a particle size larger than 2 µm. Unfortunately, columns with particle size smaller than 2 µm cannot be backflushed
- **Extreme pH** – If you are using the column at or close to the pH limit of the column, you will cause degradation of the silica at the head of the column. If you are using elevated temperature the effect will be even more pronounced
- **Physical damage** – It is possible that the column could be damaged. If you have attempted all above mentioned troubleshooting, please contact us

Column troubleshooting guide – Reversed phase

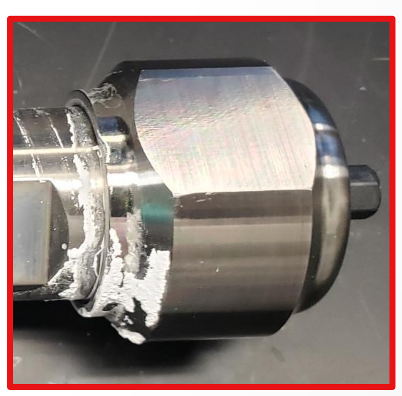
Leaking – General

- Compression and finger tight fittings have a finite lifetime. Eventually, they will have to be replaced with new ones.
- The ferrule of the worn fitting will be swaged to the tubing; i.e. it will be irremovable (stainless steel) or will be removable but will leave an indentation around the tubing (PEEK).This section of tubing cannot be used again, as it would not seal properly and would leak.
- Make sure that connections have been retightened
- Check that the system, connections, and column are used within their pressure limits and specifications

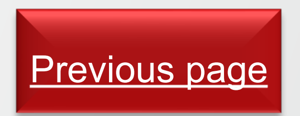
Select symptom

Column troubleshooting guide – Leaking

Leaking – Column



- **Position** – Visual inspection of the column body to determine if mobile phase is coming out. Check the exact position (inlet/outlet)
- **Pressure** – Make sure the column is used under pressure, which is within the column specifications. A pressure above this limit can cause the column to start leaking. The pressure limits of each column can be found on thermofisher.com/lccolumns
- If the column is leaking from the body and has been used at a pressure within the column specifications, please contact us



Column troubleshooting guide – Leaking

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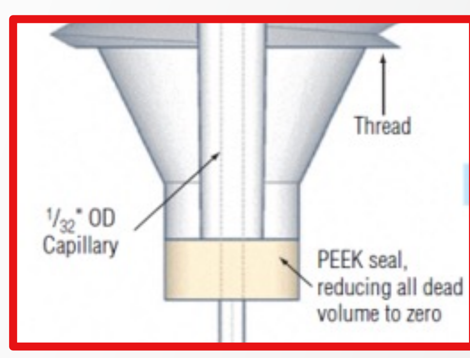
Leaking – Connections



- As a symptom of leaking, the pressure is often not as expected or there is a lack of signal
- Follow connections to determine where the pressure drop is occurring
- Tighten connections at each step and see if pressure increases to the expected level
- Unscrew and inspect Thermo Scientific™ Viper™ capillaries visually to make sure that the PEEK tip is intact
- Replace unsuitable Viper capillaries

Column troubleshooting guide – Reversed phase

Retention – General

- Small differences in retention time from column to column can be expected
- When larger retention time difference between analyses or a previous column are detected, make sure your system and column are fully equilibrated to the starting conditions of your gradient before the analysis. As a rule of thumb, 5 column volumes should be sufficient. It is important to ensure that both the system pressure and the baseline are steady prior to injection
- Retention time issues can occur to a single peak of an analysis, while the other peaks show reproducible retention times, or to multiple or even all peaks in a chromatogram

Select symptom

Column troubleshooting guide – Reversed phase

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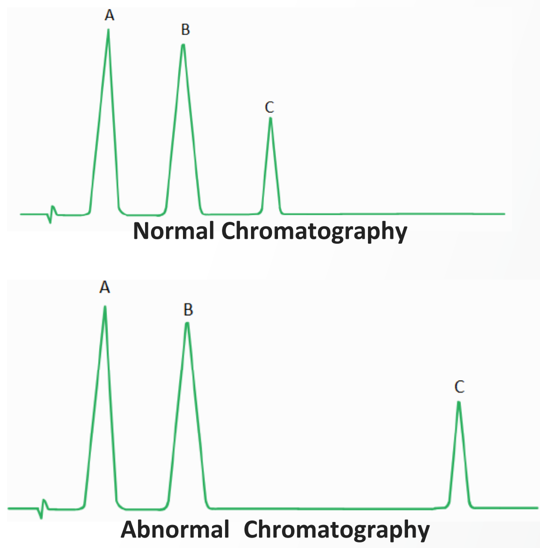
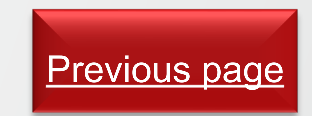
Retention – Single peak



If you find that only certain peaks are changing retention or that certain peaks are shifting by different degrees, then please investigate the following:

- **Contamination** – If this issue is accompanied with other effect, such as high pressure, poor peak shape or other effects, then it is possible that the column may have become contaminated (Contamination)
- **Sample matrix effects** – Retention time differences could be due to differences in the sample matrix composition or the pH. Try injecting a standard mixed in pH matched solution of the starting mobile phase conditions. If this resolves the issue, then additional sample preparation may be required



Column troubleshooting guide – Reversed phase

Retention – Multiple peaks

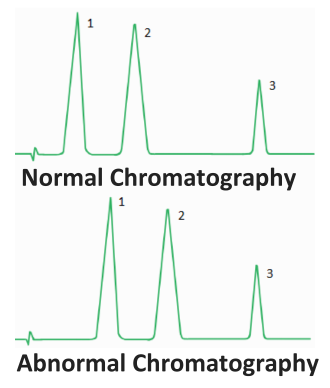
All of your analytes elute earlier or later compared to previous analyses

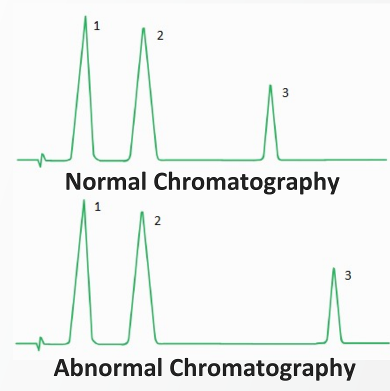
You are not able to obtain the same retention time for consecutive injections of the same sample

All of your analytes have lost retention and are not resolved anymore

Column troubleshooting guide – Reversed phase

Retention – Early/Late eluting peaks

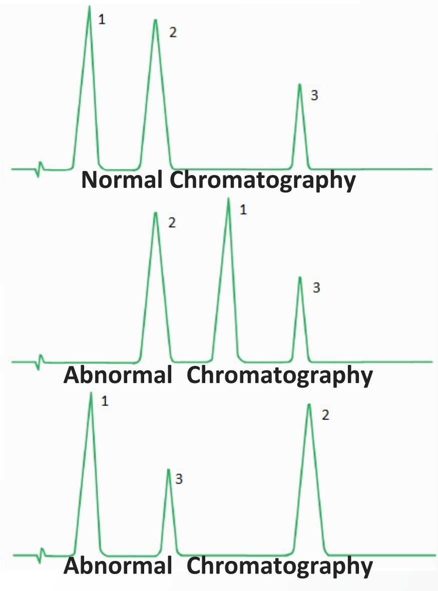




- **Injection peak** – If the "injection peak" does not match previous analyses, then a difference in flow rate is likely the cause
- **Peak shape** – Visually check if the peak shape is suitable. If it is not, refer to Peak shape
- **Temperature** – A higher or lower column temperature can lead to early or late eluting peaks. Make sure to use the temperature control in the column oven. When using elevated temperatures, a pre-column heater is advised
- **Mobile phase** – Incorrect preparation of your mobile phase, e.g., a higher/lower organic content, changes in the buffer concentration or pH can lead to early or late eluting peaks. Reprepare your mobile phase
- **Conditioning** – Insufficient conditioning of the column to your mobile phase might result in shorter or longer retention times. Make sure that your column has been conditioned properly and run a blank injection before your first standard or sample
- **Leaks** – Depending on the location of a leak, leaks can also cause your analytes to be detected at a faster or slower retention time. See the Leaking section
- **Equilibration** – Insufficient equilibration to the starting conditions of your analysis can lead to mostly early eluting peaks
- **Contamination** – Contamination in the column can cause peaks to elute early. See Contamination to find out more about column contamination
- **Modification** – Has the stationary phase of your previously used column been permanently modified, e.g., by using an ion pair reagent or has the phase collapsed due to using a 100 % aqueous phase? If so, a new column will not be able to match the retention times of the old column

Column troubleshooting guide – Reversed phase

Retention – Elution order



- If the peaks vary with some having shorter and some longer retention times, then investigate the pH of the eluent or its composition
- Buffer choice – If using a buffer, ensure that it has the capacity to buffer at your desired pH
- Ensure you are following the “2 pKa rule”, meaning that the pH of your mobile phase is >2 pH above or below the pKa of your analytes, as this should ensure the complete ionization of >99% of your analytes

Column troubleshooting guide – Reversed phase

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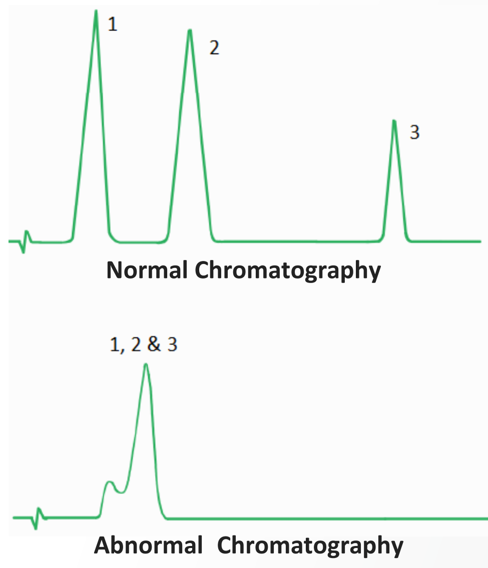
Retention – Loss of retention



- If you observe a complete loss of retention of your peaks in the chromatogram with all eluting at or close to the dead volume, the first step is to check the mobile phase composition
- If you are utilizing highly aqueous mobile phase, it is possible the stationary phase has collapsed and should be regenerated with 100% organic phase. This is generally done using either 100% acetone or acetonitrile

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Column troubleshooting guide – Reversed phase

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Contamination – General

- **Blank runs** – Comparing the blank run of your column to the blank run of a new column can help you identify if your column or your system/mobile phase is contaminated. Consider the advices on this page and refer to Wash procedures for column washes.
- **Particles** – Particles and larger substances can contaminate your column and even create physical blockage increasing the column pressure. Use a syringe filter to remove larger particles from your sample in order to avoid clogging your column. A guard column can prevent contamination of your analytical column and elongate its lifetime. Make sure to replace the guard regularly. See Physical blockage
- **Mobile phase** – A too low quality of your solvents, water, and additives (e.g. buffer, salts) in your mobile can lead to column contamination over time. Symptoms include a higher background signal and pressure. Use high quality mobile phases to avoid this. See Baseline -> General
- **Samples** – Matrix molecules present in your sample can cause column contamination as these might not be properly eluted from your column, increase your column pressure, cause a higher background signal or interfere with your analyte. Use thorough sample preparation e.g., SPE, liquid extraction or filtration to remove as much matrix from your sample as possible. See Sample preparation
- **Sample concentration** – A too high sample concentration can cause overloading effects and contamination. A reduction of the injected sample concentration is advised. See Baseline -> Carry over
- **System contamination** – System contamination can originate from e.g., non-biocompatible systems, the solvent filters, the injection needle or the dead volume of connections. Implementing regular system maintenance and cleaning as well as a needle wash procedure and using Vipers can significantly reduce the risk for system contamination

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Column troubleshooting guide – Reversed phase

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Lifetime – General

- **Clean system** – A clean system contributes to a long column lifetime
- **Eluents** – Make sure to use fresh solvents and buffers in order to avoid particles and bacterial growth on your column
- **Filters** – Blocked or saturated filters as well as bacterial growth on filters can decrease the column lifetime significantly. Make sure to exchange your inline filters, pre-columns, guard columns, and guard cartridges on a regular basis. We recommend exchanging the pre-columns, guard columns, and guard cartridges at least every six months or when exchanging the analytical column
- **Column specifications** – Only use the column within its specifications to avoid irreversible column damage, such as column bleed caused by dissolution of the column material or surface modification
- **Sample load** – Overloading a column can result in a shorter column lifetime. Avoid overloading by reducing the injection volume and choosing the right detector for your analysis
- **Guard column** – The use of a guard column can extend your column lifetime significantly. More information can be found here: Guard column

Select symptom

Column troubleshooting guide – Reversed phase

Lifetime – Column storage

- **Short term storage** – For short-term (<3 days), it is acceptable to leave the column installed on the LC and filled with mobile phase at room temperature
- When using phosphate buffers or other harsh eluents, it is important to remove these and replace them with unbuffered/less harse phases
- **Long term storage** – During long term storage, buffer/salt might precipitate in the column and bacterial growth could occur. Thus, we recommend to thoroughly flush the column with unbuffered mobile phase to remove any non-volatile salts, acids and ion-pairing agents. In a next step, flush the column with 70% or more organic phase (e.g., acetonitrile). The column can be stored in 70% or more organic phase
- See the Column care guide

Column troubleshooting guide – Reversed phase

Lifetime – Wash procedures

- **Backflushing** – Backflushing a column while washing can avoid contaminants to be pushed further down the column. Please note that sub-2 µm particle size columns cannot be backflushed.
- **Seesaw gradient wash** – Due to its particular shape, the seesaw gradient wash is very efficient and our most recommended wash (See seesaw gradient wash)
- **RP Isopropanol (IPA) gradient** – If the seesaw gradient wash failed in removing contaminants from your column, the stronger RP IPA wash is recommended (RP IPA wash)
- **Biological wash** – If the suspected contamination is related to proteinaceous sample and matrices (See biological wash)
- More information on washes for specific issues can be found in the Column care guide

Column troubleshooting guide – Reversed phase

Lifetime – Seesaw gradient wash

Name	Water/ACN Gradient
Time Taken	Minimum: 30 minutes — Recommended: 2 hours
Solvents Required	A: Deionised water B: Acetonitrile
Description	This is a standard procedure for a water ACN gradient designed for both routine and non routine column washing.
Suitable for:	Reverse phase columns
Procedure	1) Starting at 90% A / 10% B 2) Over 5 minutes raise the gradient to 10% A / 90% B 3) At 5 minutes return to 90% A / 10% B 4) Repeat for atleast 30 minutes

Flow Rate:	A flow rate of approximately 50-100% method flow rate.
Notes:	Please ensure that all buffers have been carefully removed. Make sure that all washes the linear velocity is started lower and slowly moved up as to monitor back pressure

No	Time	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	Run				
2	0.000	0.500	10.0	0.0	0.0	5
3	5.000	0.500	90.0	0.0	0.0	5
4	5.000	0.500	10.0	0.0	0.0	5
5	10.000	0.500	90.0	0.0	0.0	5
6	10.000	0.500	10.0	0.0	0.0	5
7	15.000	0.500	90.0	0.0	0.0	5
8	15.000	0.500	10.0	0.0	0.0	5
9	20.000	0.500	90.0	0.0	0.0	5
10	20.000	0.500	10.0	0.0	0.0	5
11	25.000	0.500	90.0	0.0	0.0	5
12	25.000	0.500	10.0	0.0	0.0	5
13	30.000	0.500	90.0	0.0	0.0	5
14	30.000	0.500	10.0	0.0	0.0	5
15	*New Row*					
16	30.000	Stop Run				

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Column troubleshooting guide – Reversed phase

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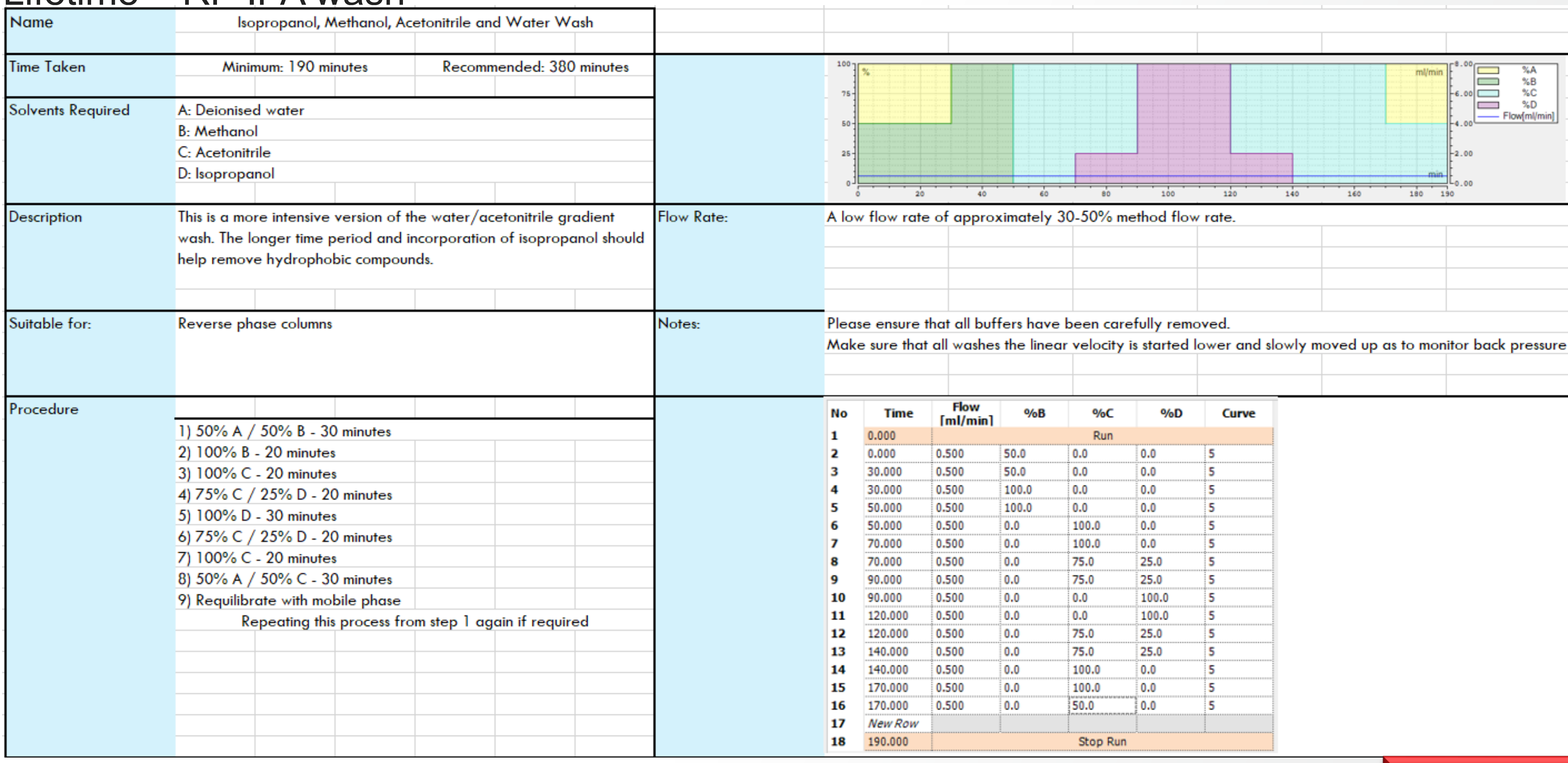
Lifetime – RP IPA wash



Field	Value
Name	Isopropanol, Methanol, Acetonitrile and Water Wash
Time Taken	Minimum: 190 minutes; Recommended: 380 minutes
Solvents Required	A: Deionised water B: Methanol C: Acetonitrile D: Isopropanol
Description	This is a more intensive version of the water/acetonitrile gradient wash. The longer time period and incorporation of isopropanol should help remove hydrophobic compounds.
Flow Rate:	A low flow rate of approximately 30-50% method flow rate.
Suitable for:	Reverse phase columns
Notes:	Please ensure that all buffers have been carefully removed. Make sure that all washes the linear velocity is started lower and slowly moved up as to monitor back pressure

Procedure

1) 50% A / 50% B - 30 minutes
2) 100% B - 20 minutes
3) 100% C - 20 minutes
4) 75% C / 25% D - 20 minutes
5) 100% D - 30 minutes
6) 75% C / 25% D - 20 minutes
7) 100% C - 20 minutes
8) 50% A / 50% C - 30 minutes
9) Requilibrate with mobile phase

Repeating this process from step 1 again if required

No	Time	Flow [ml/min]	%B	%C	%D	Curve
1	0.000	Run				
2	0.000	0.500	50.0	0.0	0.0	5
3	30.000	0.500	50.0	0.0	0.0	5
4	30.000	0.500	100.0	0.0	0.0	5
5	50.000	0.500	100.0	0.0	0.0	5
6	50.000	0.500	0.0	100.0	0.0	5
7	70.000	0.500	0.0	100.0	0.0	5
8	70.000	0.500	0.0	75.0	25.0	5
9	90.000	0.500	0.0	75.0	25.0	5
10	90.000	0.500	0.0	0.0	100.0	5
11	120.000	0.500	0.0	0.0	100.0	5
12	120.000	0.500	0.0	75.0	25.0	5
13	140.000	0.500	0.0	75.0	25.0	5
14	140.000	0.500	0.0	100.0	0.0	5
15	170.000	0.500	0.0	100.0	0.0	5
16	170.000	0.500	0.0	50.0	0.0	5
17	New Row					
18	190.000	Stop Run				

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Column troubleshooting guide – Reversed phase

Lifetime – Biological wash

Name	Wash for Biological Matrices
Time Taken	Minimum: 1 hours — Recommended: 2 hours
Solvents Required	A: 0.1% trifluoroacetic acid in deionised water B: Propan-2-ol C: D:
Description	This wash procedure is designed for the cleaning of suspected peptide and protein contamination on a reverse phase column. Equilibrating with mobile phase after is imperative.
Suitable for:	Bonded silica phases which have been used in RP protein, peptide and other biological separations.
Procedure	This method is ran isocratically at a 40:60 Ratio. 1) 40% A / 60% B - 1 Hour
Alternative Procedures:	1) Repeated injections of DMSO 2) Flush column with 7 M guanidine hydrochloride or 7 M urea. (Adjusted to acceptable pH)

Flow: A low flow rate of approximately 30-50% method flow rate.
Care should be taken not to overpressure the system due to the high visocity of the solvents used.

Notes: Please ensure that all buffers have been carefully removed.
Make sure that all washes the linear velocity is started lower and slowly moved up as to monitor back pressure

No	Time	Flow [ml/min]	%B	Curve
1	0.000	Run		
2	0.000	0.300	60.0	5
3	60.000	0.300	60.0	5
4	*New Row*			
5	60.000	Stop Run		

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Column troubleshooting guide – Reversed phase

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Lifetime – Sample preparation

- Sample preparation is the most important factor to increase the lifetime of your column. While a thorough sample preparation can be time consuming, it has the biggest influence on any column issues, especially column lifetime
- **Matrix** – Sample preparation steps, which remove large parts of the sample matrix increase the column lifetime. These include e.g. SPE, liquid-liquid extraction, and solid-liquid-extraction (SLE)
- **Filtration** – Using syringe filters to filter your sample before the analysis removes particles, which can decrease the column lifetime.
- We have linked some useful documentation for sample preparation – SPE Method development guide, Application notebook, and Sample preparation catalog

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Column troubleshooting guide – Reversed phase

Recovery – General

- **Sample and standard preparation** – Errors during the sample preparation can lead to both too high and too low recovery rates. Reprepare your sample and evaluate again
- **Matrix effects** – Matrix present in complex samples might influence your recovery significantly. Improve your sample preparation in order to avoid injecting too much matrix into your LC system. If this should not be possible or not successful, try to match the matrix of the sample in the standards. Alternatively, use standard addition or isotope dilution as calibration methods instead of an external calibration
- **Standards** – Use certified reference standards if possible or determine the amount of analyte present in your standard. Be aware that standards might degrade over time and should be replaced on a regular basis

Select Symptom

Column troubleshooting guide – Reversed phase

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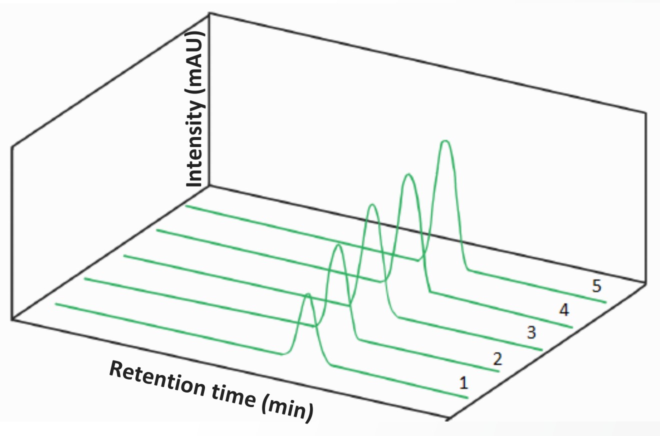
Recovery – High recovery



- **Carry over** – Carry over can lead to higher recovery rates than expected. To read more about it and find out how to avoid this, see Baseline –> Carry over
- **Evaporation of the solvent** – If the solvent/diluent of the sample has partially evaporated, higher recovery rates might be detected. Make sure to analyze your sample right after the sample preparation, do not reuse already pierced caps, cool the autosampler if this should be compatible with the sample e.g., there is no precipitation risk, and switch to a less volatile sample diluent, if possible.

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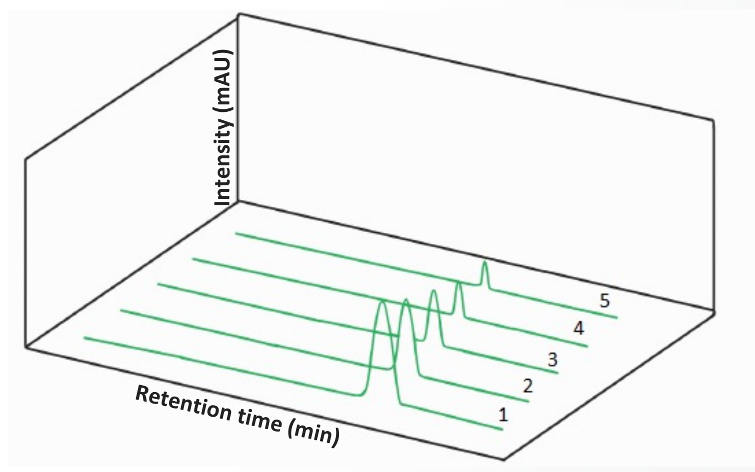
Column troubleshooting guide – Reversed phase

Recovery – Low recovery



- **Insufficient elution** – If the solvent is not strong enough to completely elute the analyte, low recovery might occur. Make sure to fully elute your sample
- **Conditioning of new columns** – Some methods require some repeated injections of sample to saturate highly active adsorption sites. This can be identified by the recovery increasing over multiple injections on a new column. This process can be expedited by using high concentration sample injections during conditioning
- **Column contamination** – If the sample is not completely eluted from the column, it might modify the column surface and cause further sample components such as the analyte to bond to the column, which might not be properly elute from the column. Make sure to perform a washing step on a regular basis or even at the end of each run-in order to avoid column contamination
- Please see -> Carry over and Contamination for more information

Column troubleshooting guide – Reversed phase

Peak missing – General

- **Standard** – If one of your peaks is missing in your chromatogram, inject a standard of this analyte, dissolved in the corresponding diluent, on its own and check if the peak appears. If yes, reprepare your sample and have a closer look at your sample preparation method. If it does not appear, check your method and see Retention
- **Coelution** – If one of your analyte peaks has a larger peak area than usual and maybe even a peak shoulder, analyze standards of your missing analyte and the analyte with the larger peak area on their own. If they are eluting at the same or very similar retention times, they will be coeluting in your sample analysis. See Contamination and Retention. We also recommend washing your column (-> See Column washing)
- **Detection window** – Broaden your MS detection window in case the retention time of your analyte has increased or decreased. If this applies, see Retention
- **Wavelength** – Review that your UV detector is set to the correct wavelength under which your analyte can be detected.
- **Dead volume** – Check if your analyte elutes in the dead volume. If so, see Retention
- **Run Time** – Double the length of your run time to see if your analyte elutes later. Refer to Retention if this should be the case
- **Baseline** – If you should detect a higher baseline as usual and expect a small analyte peak, your analyte might be hidden underneath the baseline. Inject a higher concentration of your analyte in order to confirm this. See Chapter Baseline
- **Leaking** – A leak in your system can avoid your analytes to reach the detector. Refer to chapter Leaking

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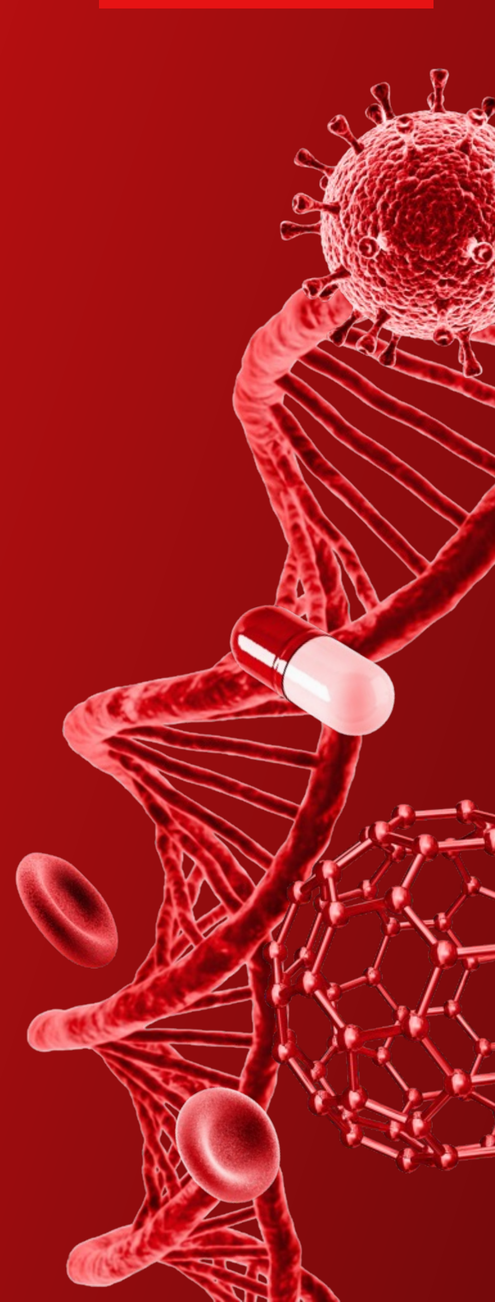

General Laboratory Equipment – Not For Diagnostic Procedures

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