YMC EUROPE GMBH

(U)HPLC *TROUBLE* SHOOTING

Contents

1. GENERAL (U)HPLC TROUBLE SHOOTING APPROACH

Who hasn't experienced the following?

Suddenly the chromatographic results have changed.
For example, peak deformation such as splitting or tailing occur, retention time shifts or nothing is detected at all.

Then it is time to find the cause of the anomaly and fix it. Because the final chromatogram is influenced by various factors, all the various components and variables should be examined in a systematically manner.

A systematic review of the following parameters is recommended; each procedure will be described in more detail later in this document:

System

- **Pumps** *(constant flow? Pressure fluctuation?)*
- **Autosampler** *(Contamination of injection needle? Correct injection?)*
- **Detector** *(UV: Lamp o.k.?)*
- **Column oven** *(Temperature correct?)*
- **Capillaries** *(Correct connection? Fittings correct? Dead volumes?)*

Eluents

- **Solvents and buffer salts used**
  (manufacturer changed? Different batch?)
- **pH value, pH value adjustment**
  (Changes of pH? pH adjusted? How adjusted?)
- **Age/Storage** *(Buffer storability? Precipitation? pH change?)*

Sample

- **Sample changes**
  (Production changed? Composition? Concentration?)
- **Sample solvent**
  (Elution strength? Influence on chromatography?)

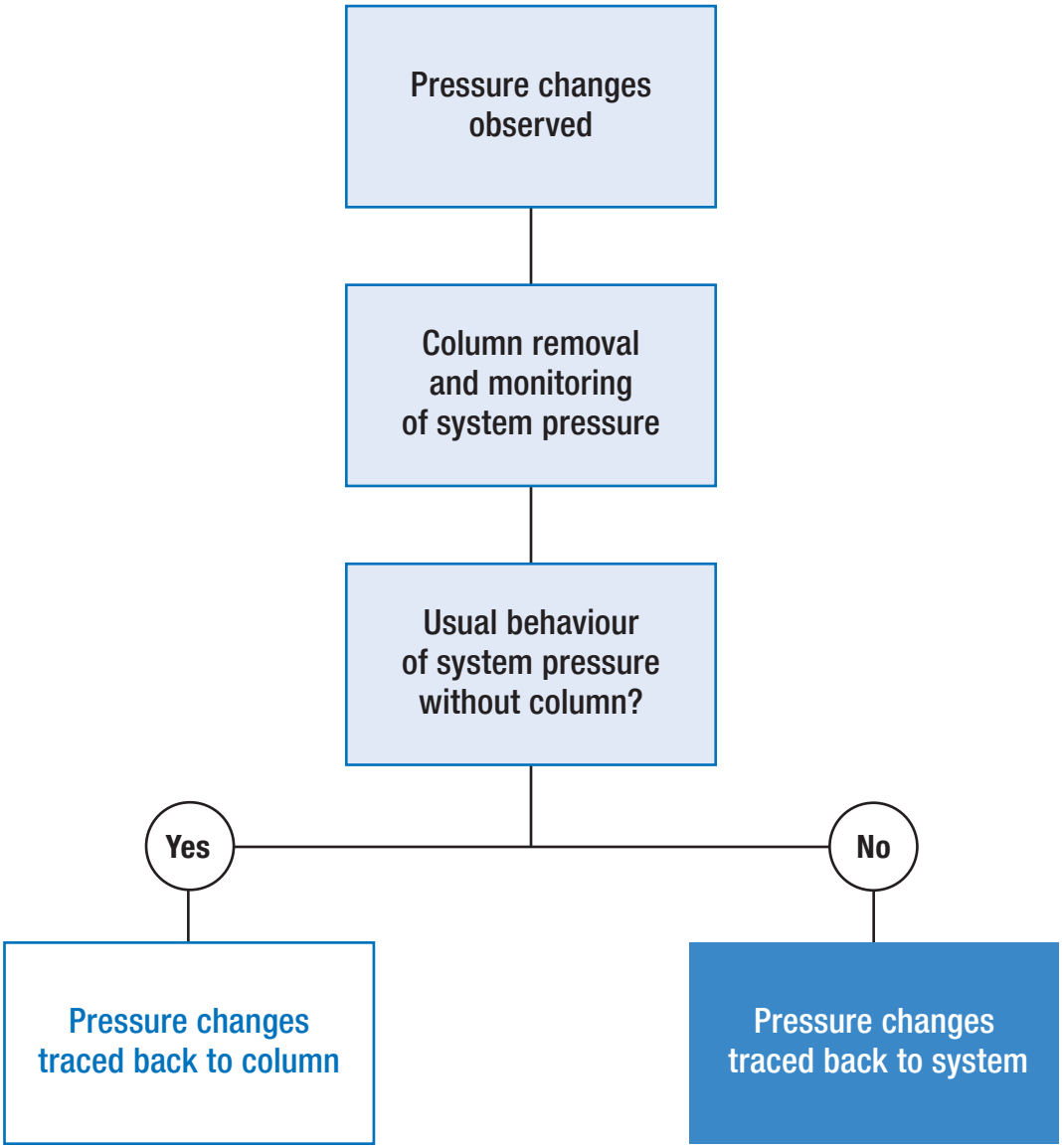
Column & Guard

- **Guard column** *(Age? Replacement necessary?)*
- **Column suitability for method** *(pH? Temperature? Eluents?)*
- **Column connection** *(Inlet and outlet correctly connected? Ferrule correctly fitted?)*
- **Column history** *(Injections? Pre-conditioning of the column?)*

2. PROBLEMS IN (U)HPLC AND THEIR CAUSE/CORRECTION

2.1. Back pressure changes

The root cause of back pressure changes can often be traced back to incorrect handling of samples and solvents which finally results in a blockage or contamination of system components and the analytical column itself.


Pressure changes observed
Column removal and monitoring of system pressure
Usual behaviour of system pressure without column?
Yes
No
Pressure changes traced back to column
Pressure changes traced back to system

No pressure /pressure too low

Potential Causes	Solution
Formation of negative pressure in the eluent reservoir	• Avoid formation of negative pressure, utilisation of equalising valves in the caps of the eluent flask, prepare fresh buffer
Leakage at piston seal or valves of the pump	• Check tightness, if necessary, replace seals or valves
Air or particulates in head of pump or valves of the pump	• Purge the pumps with water or IPA, flush water or IPA with the aid of a syringe through the pump while turned off
Leakage in transmission lines	• Check and if necessary, exchange screw connections and capillaries

Increase in pressure / pressure too high

Potential Causes	Solution
Blocked injector or capillaries	• Clean injector and blocked capillaries or if necessary, replace them
Contamination of water/buffer due to algae / bacteria	• Prepare fresh buffer (daily)
Blocked guard column or column inlet frit	• Backflush the column (if allowed) • Replace the column
Contamination of stationary phase	• Wash column with strong solvent • Consult the manufacturer's suggested column regeneration procedure • Backflush the column (if allowed) • Replace the column

Pressure changes

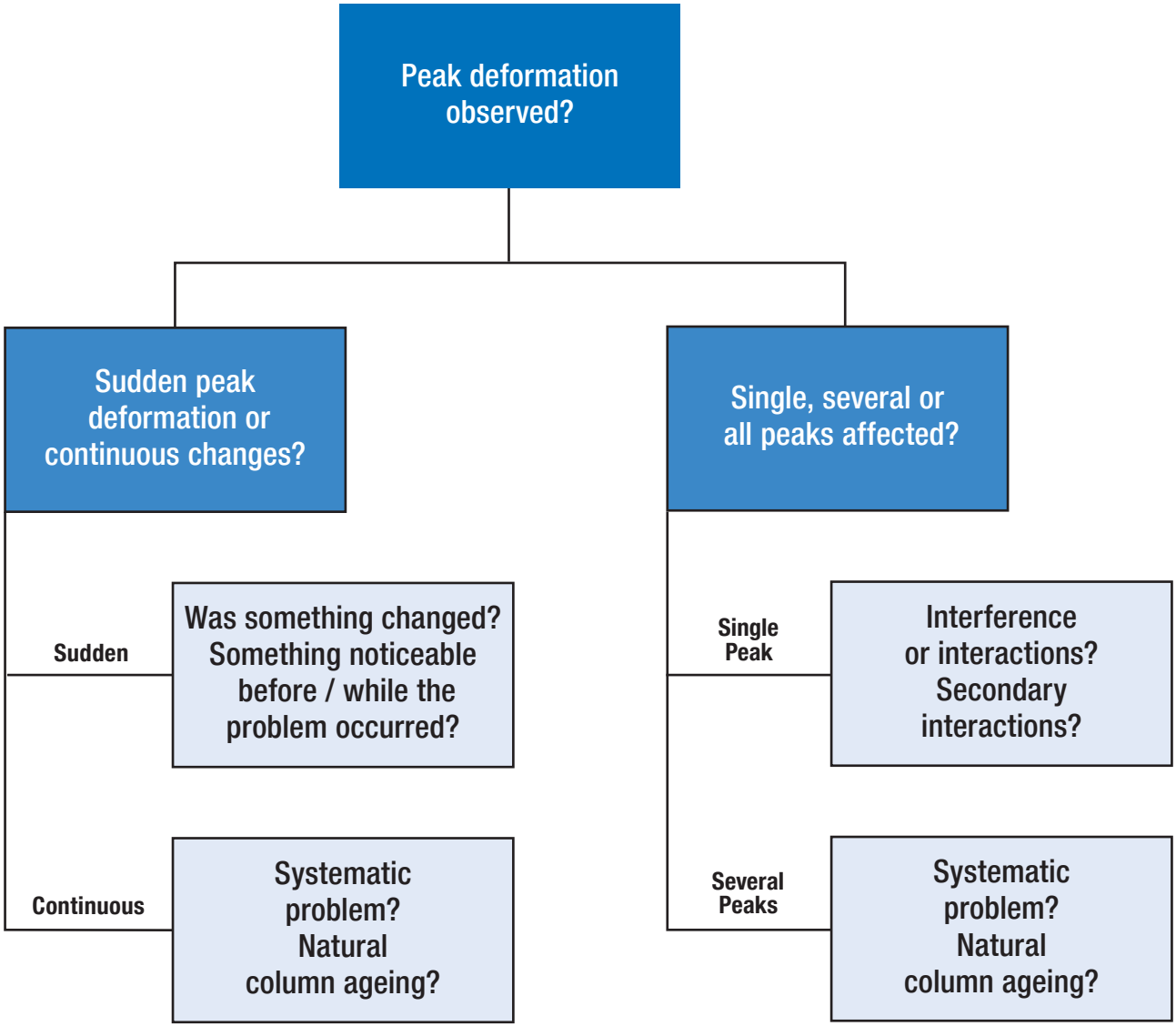
Potential Causes	Solution
Leakage at piston seal or valves in the pump	• Check tightness, if necessary, replace seals or valves
Air or particulates in head of pump or valves of the pump	• Purge the pump with water or IPA, flush water or IPA with the aid of a syringe through the pump when turned off

2. PROBLEMS IN (U)HPLC AND THEIR CAUSE/CORRECTION

2.2. Peak deformation

In most cases systematic errors in the method conditions, inappropriate choice of column or solvent or natural column ageing are the root causes for peak deformation. By far the most common cause is the use of a too strong or otherwise inappropriate injection solvent, as well as an injection volume that's too large.

Typical symptoms as well as potential causes and solutions are listed in the following tables. These symptoms often appear in combination with other abnormalities in the chromatographic results (e.g., pressure changes or retention time shifts).


Peak deformation observed?
Sudden peak deformation or continuous changes?
Single, several or all peaks affected?
Sudden
Was something changed? Something noticeable before / while the problem occurred?
Continuous
Systematic problem? Natural column ageing?
Single Peak
Interference or interactions? Secondary interactions?
Several Peaks
Systematic problem? Natural column ageing?

Peak broadening

Potential Causes	Solution
Injection volume too large	• inject smaller volume
Detection rate too low	• Increase detection rate
Retention time too long	• Gradient elution • Use of another eluent with higher elution strength
Viscosity of the eluent too high	• Use a different eluent • Increase temperature, use solvent pre-heater
Contamination of the stationary phase	• Increase column temperature • Wash column with strong solvent • Consult the manufacturer's suggested column regeneration procedure • Backflush the column (if allowed) • Replace the column

Fronting

Potential Causes	Solution
Overloading of column	• Decrease injection volume or sample concentration • Use a column with larger ID • Use a stationary phase with higher surface area
Viscosity of sample or mobile phase too high	• Increase temperature • Change to different mobile phase • Decrease injection volume or sample concentration
Contamination of the stationary phase	• Wash column with strong solvent • Increase column temperature • Consult the manufacturer's suggested column regeneration procedure • Backflush the column (if allowed) • Replace the column

Tailing

Potential Causes	Solution
Interactions of basic analytes with silanol groups	• Reduce pH value of mobile phase (pH 2–3) • Increase ion strength of eluents • Switch to a more inert stationary phase • Use ion-pairing agents
Dead volume	• Re-pack column, if possible • Replace column
Ageing of packing material due to high temperatures	• Replace the column
Blocked guard column or column inlet frit	• Backflush the column (if allowed) • Replace the column
Wrong pH value of mobile phase	• Adjust pH value respectively for stationary phase and analytes
Elution of a second sample component	• Optimise sample preparation • Optimise method parameters • Optimise mobile or stationary phase • Use a longer column
Contamination of stationary phase	• Wash column with strong solvent • Consult the manufacturer's suggested column regeneration procedure • Backflush the column (if allowed) • Replace the column

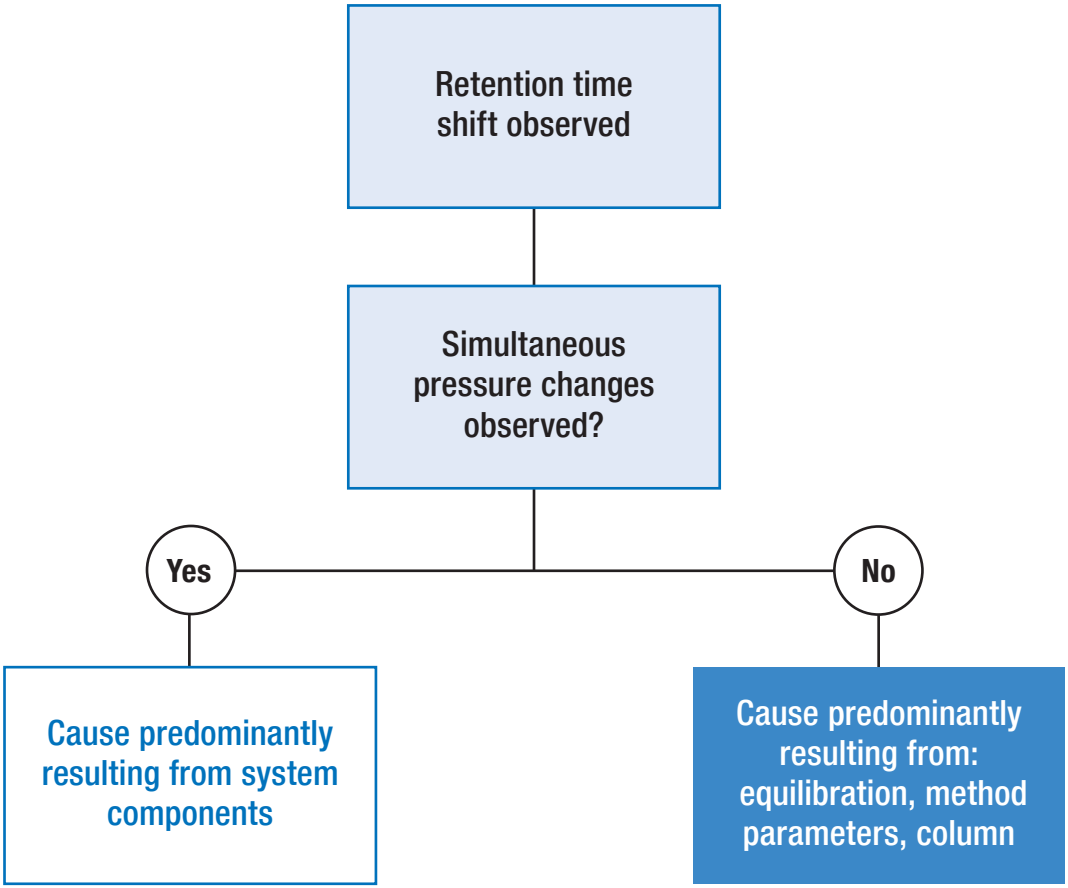
Split peaks

Potential Causes	Solution
Blocked guard column or column inlet frit	• Backflush the column (if allowed) • Replace the column
Overloading of column	• Reduce injection volume or sample concentration • Use a column with larger ID
Inappropriate injection solvent	• Injection in weaker solvent • Adjust pH value of injection solvent to pH of eluents • Use a stronger eluent
Dead volume	• Re-pack column, if possible • Replace the column
Elution of a second sample component	• Optimise sample preparation • Optimise method parameters • Optimise mobile or stationary phase • Utilisation of a longer column
Carry-over of last analysis	• Clean the column and system • Use a more inert stationary phase and column hardware

2. PROBLEMS IN (U)HPLC AND THEIR CAUSE/CORRECTION

2.3. Retention time and resolution

A change of retention time and resolution often is accompanied by a change in peak symmetry and efficiency. These are symptoms of a usual column ageing, but can also be caused by leakage in the system, problems with the injector, unstable temperature, or by contamination or damage to the stationary phase due to inappropriate method parameters.


Retention time shift observed
Simultaneous pressure changes observed?
Yes
No
Cause predominantly resulting from system components
Cause predominantly resulting from: equilibration, method parameters, column

Retention time is shortened

Potential Causes	Solution
Overloading of the column (accompanied by too high /broad signal)	• Reduce injection volume or sample concentration • Use a column with larger ID • Use a stationary phase with higher surface area
Ageing of the phase/ phase damaged by harsh conditions	• Work within the described specifications of the column • Replace the column • Prolonged work at the limit of the column specifications will shorten its life time
Contaminated column	• Backflush column (if allowed)
Elevated flow rate	• Control flow rate
For phases not stable in 100% aqueous conditions: dewetting of the stationary phase	• Use special aqueous stable phases • Long equilibration of conventional phases

Longer retention time

Potential Causes	Solution
Eluent composition has changed	• Check mixing system • Cover storage bottles to avoid a vaporisation of components
Reduced flow rate	• Control flow rate

Retention time shift in one direction

Potential Causes	Solution
Leakage of pump seal	• Replace pump seal
Overloading of the column	• Reduce injection volume or sample concentration
Insufficient equilibration	• Equilibration/ flush with 10 column volumes

Retention time fluctuation / random shift

Potential Causes	Solution
Temperature fluctuation	• Ensure constant temperature • For optimum results preheat the mobile phase
Insufficient solvent mixing	• Evaluate gradient system • Where necessary use preheater (mixing chamber)
Wrong buffer concentration or pH value	• Check eluent pH • If necessary, prepare fresh buffer
Insufficient equilibration	• Equilibration/ flush with 10 column volumes
From one system to another: Difference in dwell volume	• Repeat method with initial system and original conditions
Leakages	• Search for leakages and seal them
Defective pump valve	• Check valves and if necessary, exchange them
Air in the pump, air in the mobile phase	• Degas the mobile phase • Inspect system degasser unit • If necessary, replace seals
Gradient valve/proportioning valve/ MCGV leaks (with low-pressure mixing units, ternary and quaternary systems)	• flush all mobile phase lines with IPA for 1h • if necessary, replace seals

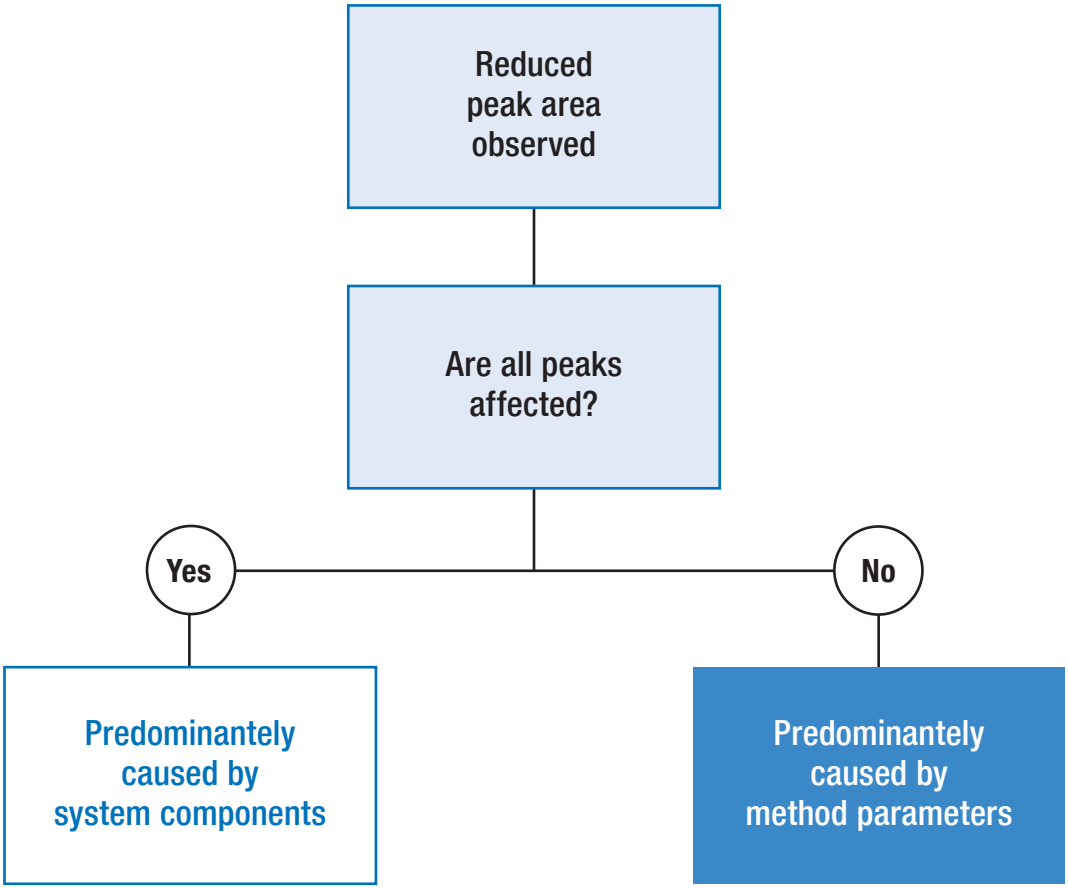
Loss of resolution

Potential Causes	Solution
Contamination of mobile phase	• Prepare fresh mobile phase (and replace frequently to avoid contamination)
Blocked pre-column	• Replace pre-column (in case of change in retention / resolution replace pre-column in time to avoid contamination of main column)
Ageing of stationary phase	• Flush column regularly and work inside the phase specifications to prevent early ageing of stationary phase. • Replace column

2. PROBLEMS IN (U)HPLC AND THEIR CAUSE/CORRECTION

2.4. Changes in baseline and peak recovery

A drifting or noisy baseline can complicate integration of peaks, reduce signal-to-noise ratio and provide decreased sensitivity of the method.
The most common cause for this can be found in the components and settings of the detector used or in a change of eluent composition and can be rectified relatively easily. Further causes might be an inappropriate stationary phase or column hardware, which can lead to signal loss, ghost peaks or carry-over.


Reduced peak area observed
Are all peaks affected?
Yes
No
Predominantely caused by system components
Predominantely caused by method parameters

Spikes

Potential Causes	Solution
Air bubbles in mobile phase	• Degas mobile phase • Inspect system degasser unit • Verify leak tightness of all connections
Air bubbles in column	• Always store columns tightly sealed

Drift to higher signal

Potential Causes	Solution
Build-up and elution of contaminants	• Optimise sample preparation • Use solvents in (U)HPLC grade • Follow column cleaning procedure
Mobile phase viscosity is too high	• Use solvent composition with lower viscosity • Increase column temperature
In gradient runs: increasing component B exhibits strong UV-absorption	• Use solvent with lower or no UV-absorption

Drift to lower signal

Potential Causes	Solution
In gradient runs: decreasing component A exhibits strong UV-absorption	• Use solvent with lower or no UV-absorption

Noise

Potential Causes	Solution
Wave-like noise: Temperature fluctuation in the environment	• Ensure constant ambient temperature (column oven, isolation of column)
Continuous noise: detector lamp defect or detection cell contaminated	• Exchange lamp • Clean detection cell
Periodic noise: air in pumps	• Clean pumps • Purge air from pumps
Random noise: contamination	• Optimise sample preparation • Clean column • Use solvents in (U)HPLC grade
Spikes: air bubbles in detector, eluents or pumps	• Avoid air bubbles • Purge air from components

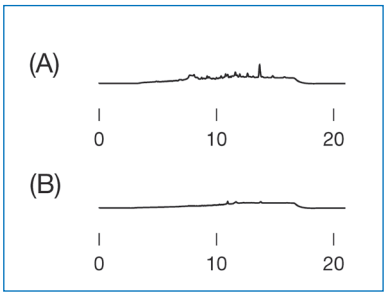
Negative peaks

Potential Causes	Solution
RI-detector: refractive index of analytes lower than that of mobile phase	• Switch detector polarity
UV-detector: absorption of mobile phase higher than absorption of analytes	• Adjust mobile phase

Reduced peak area

Potential Causes	Solution
Loss of sample due to leakage in injector, capillaries or connections	• Inspect injector components (valve and needle), connections and capillaries and replace leaky parts
Reduced signal intensity due to contaminated or damaged flow cell/ old UV-lamp	• Clean flow cell • Inspect and replace UV-lamp
Injection volume too low	• Increase injection volume
High UV-absorption of eluent	• Use solvents in (U)HPLC grade
Contamination of detector	• Clean detector

Ghost peaks



Potential Causes	Solution
Strong adsorbing component in sample	• Optimise sample preparation • Clean column
Poor quality of water or organic solvent	• Use (U)HPLC grade water/solvent • Clean column
Oxidation of trifluoroacetic acid	• Prepare eluents daily
Microbiological growth in eluent bottles or system	• Prepare eluents daily • Clean the system and column
Additional analytes in sample	
Carry-over from last analyses	• Clean the system and column • Use a more inert stationary phase and/or inert column hardware
Air bubbles in mobile phase	• Degas mobile phase

3. COLUMN REGENERATION

Accumulation of contaminants on the stationary phase is a common reason for adverse effects on column performance. This usually manifests itself in:

- **Higher back pressure**
- **Shift in retention time**
- **Deformed peaks**

To avoid accumulation on the column, the use of pre-columns and appropriate sample preparation such as filtration or extraction is advised.

Cleaning procedures are an effective measure for column regeneration in case of adsorption which has already occurred on the material of the stationary phase or to prevent accumulation of contaminants in the first place. Because contaminants usually accumulate and remain at the column head, cleaning procedures should always flush the column in the opposite direction to the usual flow to remove the contaminants more easily and not spread them throughout the whole stationary phase. Sufficient cleaning can be accomplished with a minimum of 20 column volumes of appropriate solvent.

When choosing the appropriate solvent, properties of the contaminants as well as stability of the column used should be considered. Examples for suitable solvents depending on the contamination present are listed in the following table. Higher efficiency of the cleaning procedure can be achieved by increasing the column temperature. Depending on the solvent and the stability of the stationary phase 40°C – 90°C is an appropriate temperature range for this.

For further recommendations follow the instructions of the column manufacturer, usually found in the **“Care and Use Instructions”**.

Removal of contamination due to different substances

Salts

- Water and aqueous organic solvents

Non-polar substances

- Acetonitrile
- Isopropanol
- Tetrahydrofuran
- Dichloromethane
- Chloroform

Polar substances

- Water and aqueous organic solvents
- Methanol
- Tetrahydrofuran

Proteins

- Injection of dimethyl sulfoxide
- Gradient starting at 10% to 90% B with A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile

4. PREVENTATIVE MEASURES

General considerations:

- **Frequent cleaning of system, column and injector**
- **Regular replacement of worn and damaged parts such as filters, frits and seals**

to avoid contamination.

Additionally, troubleshooting can be simplified immensely by frequently recording a column journal. We recommend you record the identity of the column, number of injections, resulting backpressure, analytes and method used and regularly verify the quality of the column with the results of a standard test method. Switching of eluent bottles, error messages or other abnormalities should also be recorded.
A column journal can reduce the duration of troubleshooting from days to minutes and can additionally provide a way to track your productivity gain after the optimization of your method parameters.

Use of guard columns

A guard column or guard cartridge systems protects the main column from a build-up of contaminants by absorbing them before they can reach the column.

Therefore, guards prevent:

- **Fast and early ageing of the column**
- **Shift of retention times**
- **Peak deformation**
- **Void formation in stationary phase due to pressure pulses**

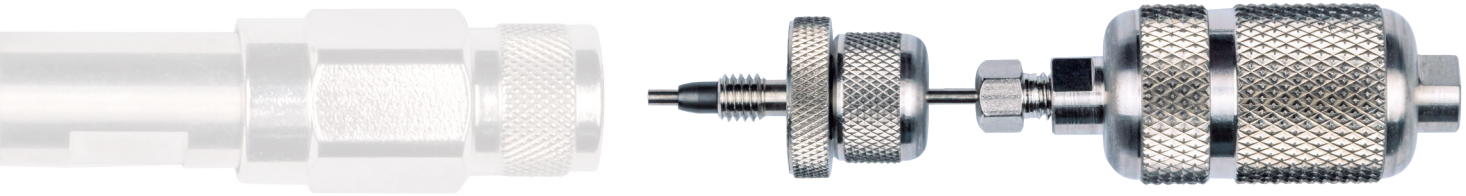


FIGURE 1: UNIVERSAL GUARD COLUMN HOLDER WITH GUARD CARTRIDGE.

Guards should be replaced regularly e.g., when back pressure increases or retention times shift. With a general test method, you can record resolution, peak symmetry and back pressure under known conditions to evaluate the status of your column and guard.

Zero dead volume connections

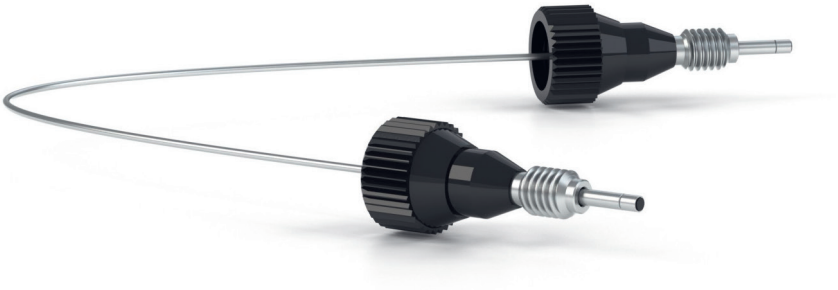


FIGURE 2: MARVELXACT ZERO DEAD VOLUME UNIVERSAL (U)HPLC CONNECTOR.

Especially in UHPLC applications additional column dead volume leads to band broadening. To prevent broad peaks due to non-optimal connections the use of zero dead volume universal connectors such as MarvelXACT is recommended. Additionally, all capillaries in the chromatographic system should be as short as possible.

Filtration of sample and eluents

Before use the sample and eluents should be filtered with a filter pore size of 0.45 µm (for > 3 µm particle phases) or 0.2 µm (for ≤ 3 µm particle phases) depending of particle size of the stationary phase.
Protection against:

- **Blockage of the inlet frit**
- **Particulate contamination of the stationary phase**
- **Peak splitting**
- **Fronting**
- **Tailing**
- **Increased back pressure**

Frequent replacement of eluents

Due to ageing of the eluents (by biological growth in aqueous buffers, polymerisation of pure acetonitrile, etc) the chromatographic system can become contaminated.
Replacement of aqueous mobile phase is recommended:

HPLC: at least once a week

UHPLC: at least every three days

Degassing of eluents

Eluents can dissolve air and contain air bubbles, which can lead to variations in retention times.
Always degas all eluents after preparation and filtration by:

- Purging the eluent with helium (daily)
- Use online vacuum degassing

Adjust sample solvent to mobile phase conditions

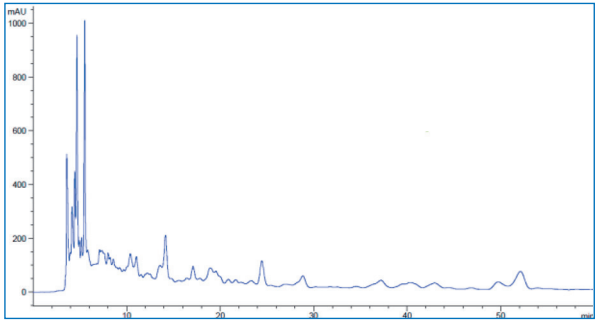
The sample solvent can have a strong influence on the quality of the separation and a suitable composition should be considered:

- Use a weak solvent composition for the sample **➔ or fronting of early eluting peaks**
- Avoid large differences in the pH of sample and eluents **➔ or peak splitting**

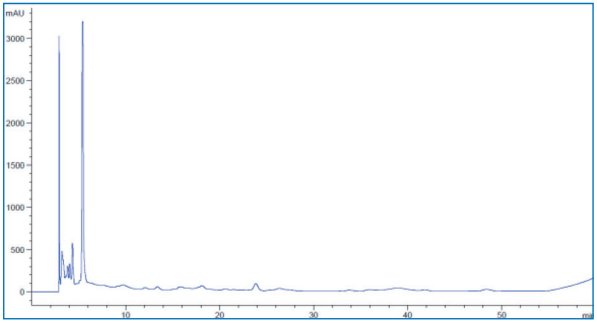
Sufficient equilibration

When equilibrating and cleaning a column there's usually the question:
How long does the column have to be flushed?
If the equilibration time is too long you lose productivity and increase cost. If it is too short, there is a risk of non-reproducible results i.e., because of retention time shifts. So in case of doubt, to ensure correct results a longer duration is more favourable.

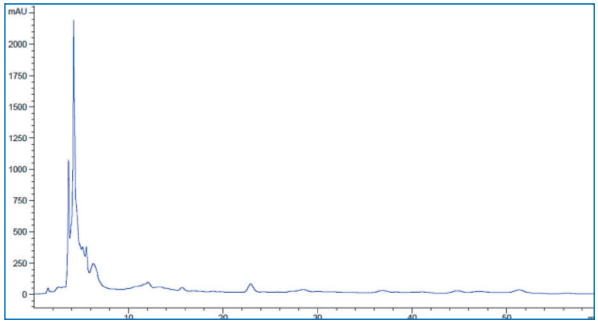
Example:
Different test conditions were chosen for the analysis of a plant extract to optimise the duration of the equilibration of a YMC-Triart C8 column (column volume 2.5 mL): a usual result (5 CV equilibration), without equilibration, equilibration with 1 column volume, equilibration with 10 column volumes.
The column was flushed with 50 mL (= 20 CV) of 100 % acetonitrile after each test cycle.
The equilibration and isocratic separation were achieved in a water/acetonitrile (80/20) mixture.
The dwell volume of the system was 130 µL.



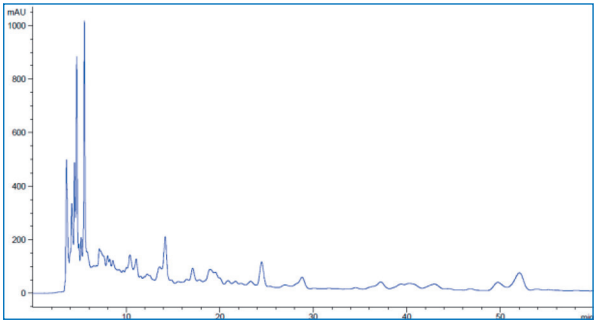
1. Usual result (5 CV).



2. Without equilibration.



3. After insufficient equilibration (1 CV).



4. After sufficient equilibration (10 CV).

It is shown that without sufficient equilibration, retention times shorten significantly, whereas equilibration with 5 or 10 CV leads to a reproducible application.

Estimation of equilibration duration

How can the optimal equilibration duration be estimated?

➔ by means of the geometrical column volume!

The geometrical column volume is a useful parameter to estimate the necessary solvent volume for cleaning and equilibration steps in HPLC – a requirement for reproducible and valid results.

$$\text{Geometric column volume [mL]} = \text{length [cm]} \times (\text{radius [cm]})^2 \times \pi$$

Calculation of column volume

Example:

column:	**YMC-Triart C18**
column dimensions:	250 x 4.6 mm ID
column volume[mL]	= 25 cm × (0,23 cm)^2 × 3,14
	= 4.2 cm^3

Table 1: overview of geometrical column volumes [mL] for selected column dimensions

L [mm] / Ø [mm]	50	75	100	150	250	300
2.0	0.2	0.2	0.3	0.5	0.8	0.9
3.0	0.4	0.5	0.7	1.1	1.8	2.1
4.6	0.8	1.2	1.7	2.5	4.2	5.0
6.0	1.4	2.1	2.8	4.2	7.1	8.5
8.0	2.5	3.8	5.0	7.5	12.6	15.1
10.0	3.9	5.9	7.9	11.8	19.6	23.6
20.0	15.7	23.6	31.4	47.1	78.5	94.2
30.0	35.3	53.0	70.7	106.0	176.7	212.1
50.0	98.2	147.3	196.3	294.5	490.9	589.0

Additional considerations

Dwell Volume

- Volume of the system before solvent reaches the column.
- Dependant on system (ask the manufacturer)

Volume of the stationary phase

- Include correction factor (0.6–0.8)
- Dependant of stationary phase and packing density

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