Achieving lowest carry-over with Agilent 1290 Infinity LC and LC/MS systems

Edgar Naegele, Ph.D.
Application Scientist

Stephan Buckenmaier, Ph.D.
Application Scientist

Michael Frank, Ph.D.
Product Manager

Introduction

•Avoiding any carry-over is of crucial importance for modern high sensitive liquid chromatography (LC) triple quadrupole mass spectrometry (MS) trace analysis.

•It is necessary to develop software and hardware for specialized cleaning procedures to achieve the stringent requirement of near-zero carry-over.

•This Seminar will give you an introduction to identify possible sources of carry-over and how to avoid these by a properly maintained LC.

•This seminar describes and demonstrates the functionality of the near-zero carry-over enablement, the 1290 Infinity Flexible Cube, in combination with an Agilent 1290 Infinity LC system for mass spectrometry.

•As examples, data were created which show a carry-over specification and a methodology to determine carry-over in relation to the instrument´s sensitivity.

What is Carry-over?

"When an analyte **originating from a previously injected sample appears after the injection of buffer as a blank injection**, this is called carryover."

Goran Mitulovic, Anal. Chem. 2009, 81, 5955–5960

"In a high-performance liquid chromatography (HPLC)-based analytical method, carryover denotes one type of **systematic error that is derived from a preceding sample and introduced into the next sample**."

Wei Zeng, Rapid Commun. Mass Spectrom. 2006; 20: 635–640

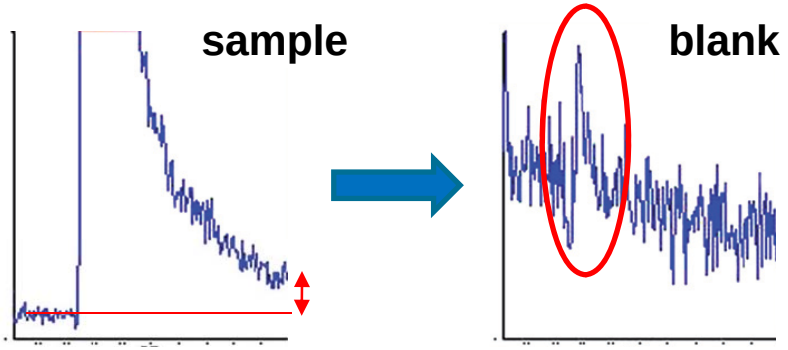
"Carryover in general is **serial in nature** and is caused by residual analyte from a sample analyzed earlier in the run. It does not necessarily involve only the next sample in the sequence and **can affect several samples in a sequence**, if many samples above the calibration ranges are analyzed. Carryover **can also be random**, where carryover from late-eluting residues on chromatographic columns may affect chromatograms several samples later."

Nicola C. Hughes, The AAPS Journal 2007; 9 (3) Article 42

What is Carry-over?

Memory Effect:

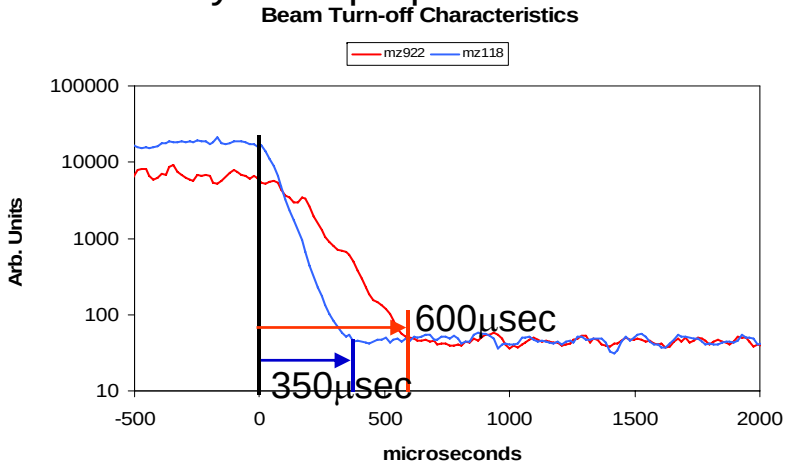
Caused by improper elution of the compound from the column! For example because of insufficient gradient time especially for compounds that show high interactions to silanol groups.



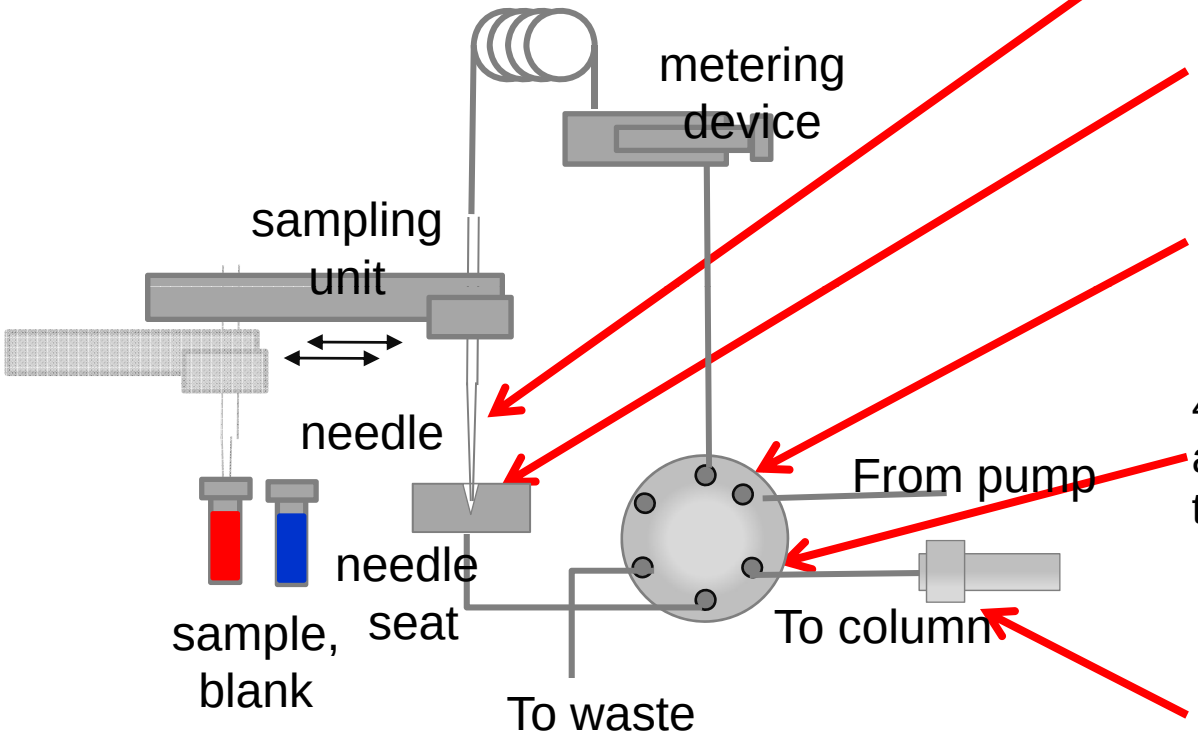
Nicola C. Hughes, The AAPS Journal 2007; 9 (3) Article 42

Cross-Talk:

Cross Talk carry-over is caused by an improper cleaned collision cell in a triple quadrupole mass spectrometer.



Sources of Carry-over in an HPLC system



1. Adsorption to needle and capillary material

2. Needle seat design, improper sealing, worn out

3. Valve Rotor/Stator, design, worn out

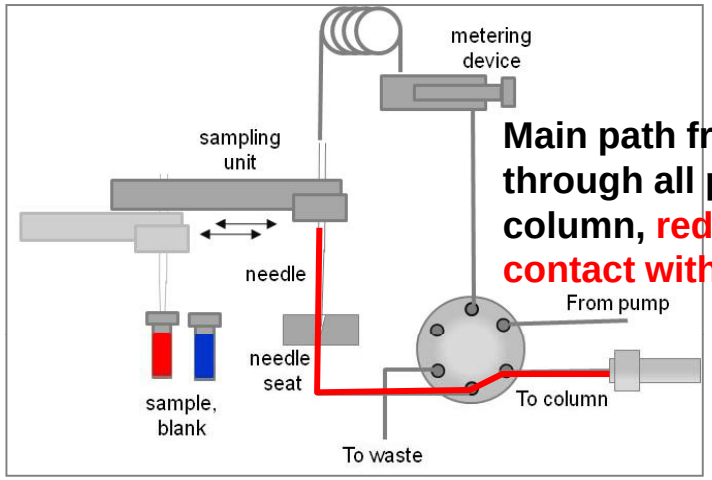
4. Capillary fittings misadjusted (at all connections contaminated with the sample!)

5. Column related carry-over (all fittings and connections of the column, packing, stationary phase-sample interaction)

Don‘t forget initial sampling, sample preparation steps, vials, detection systems, etc.

Sources of Carry-over

1. Needle and capillary material – adsorption effect by metal - ion/dipol interaction

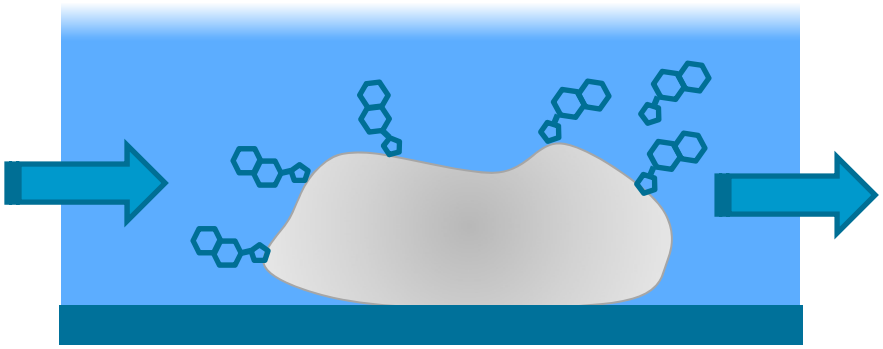


Main path from pump through all parts to column, red parts in contact with sample

In the **main-pass** (!) operation all these parts will be washed at <u>least</u> with the **strong solvent** at the end of the **gradient**.

But **not always the strong solvent of the gradient is sufficient** to remove all adsorbed compounds! Often additional solvents with different solvent properties (polarity, pH, etc.) are required to remove the compounds. Should be part of method development.

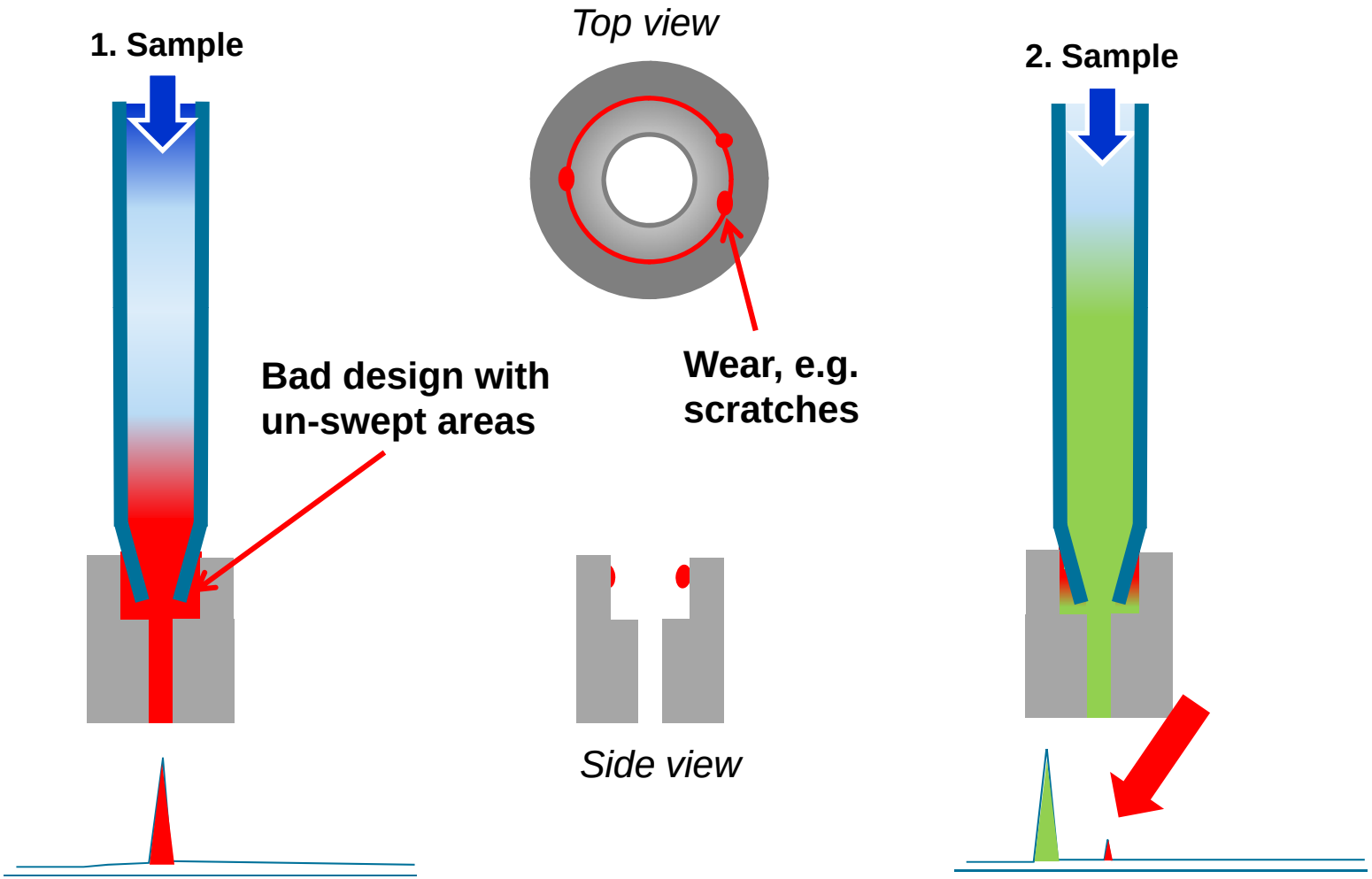
Proteins from a **biological matrix** can act as „glue“:



Unspecific binding of small molecules to the protein surface!

Sources of Carry-over

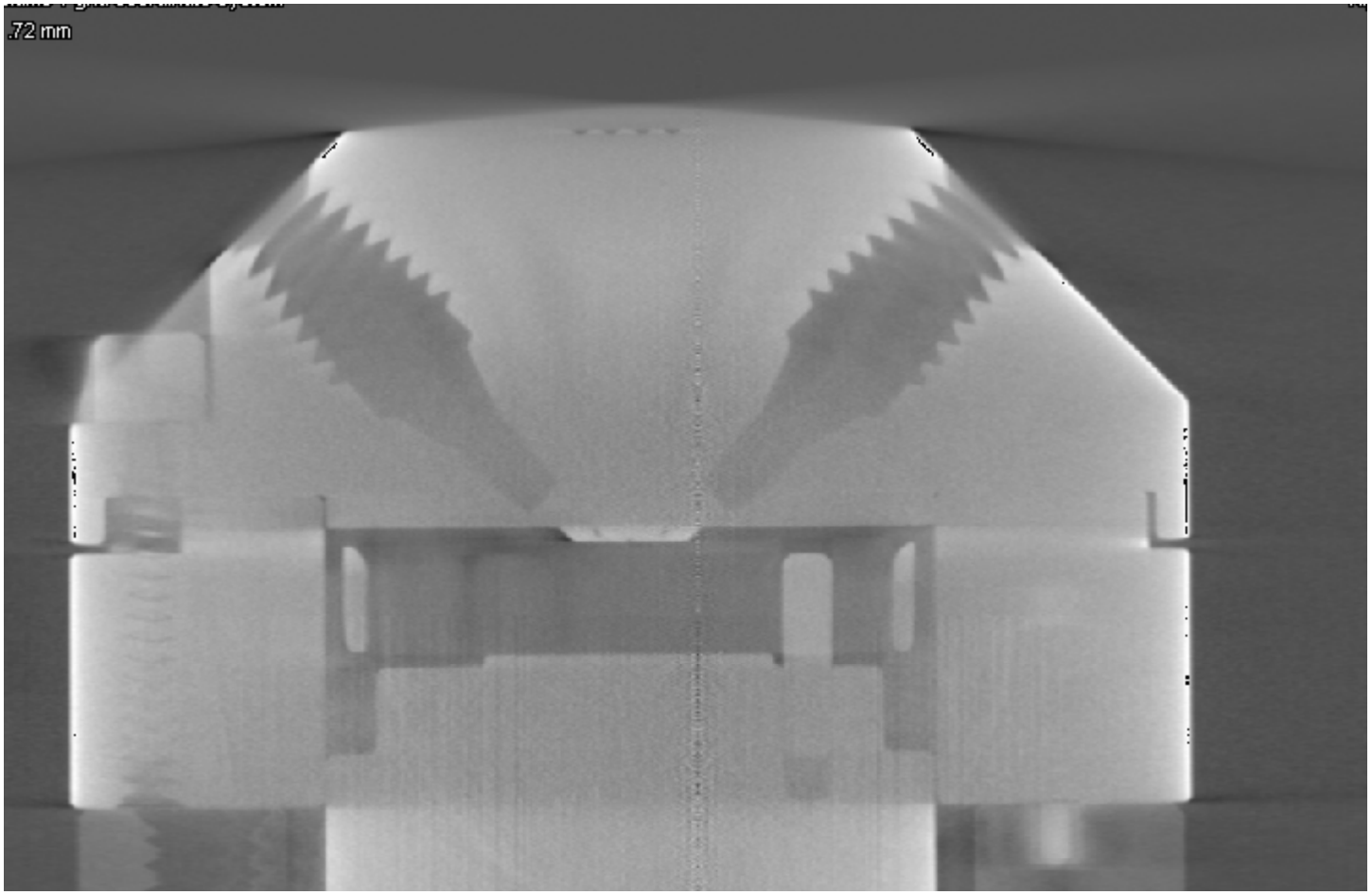
2. Needle seat design, wear



Sources of Carry-over

3. & 4. Valve design and fittings

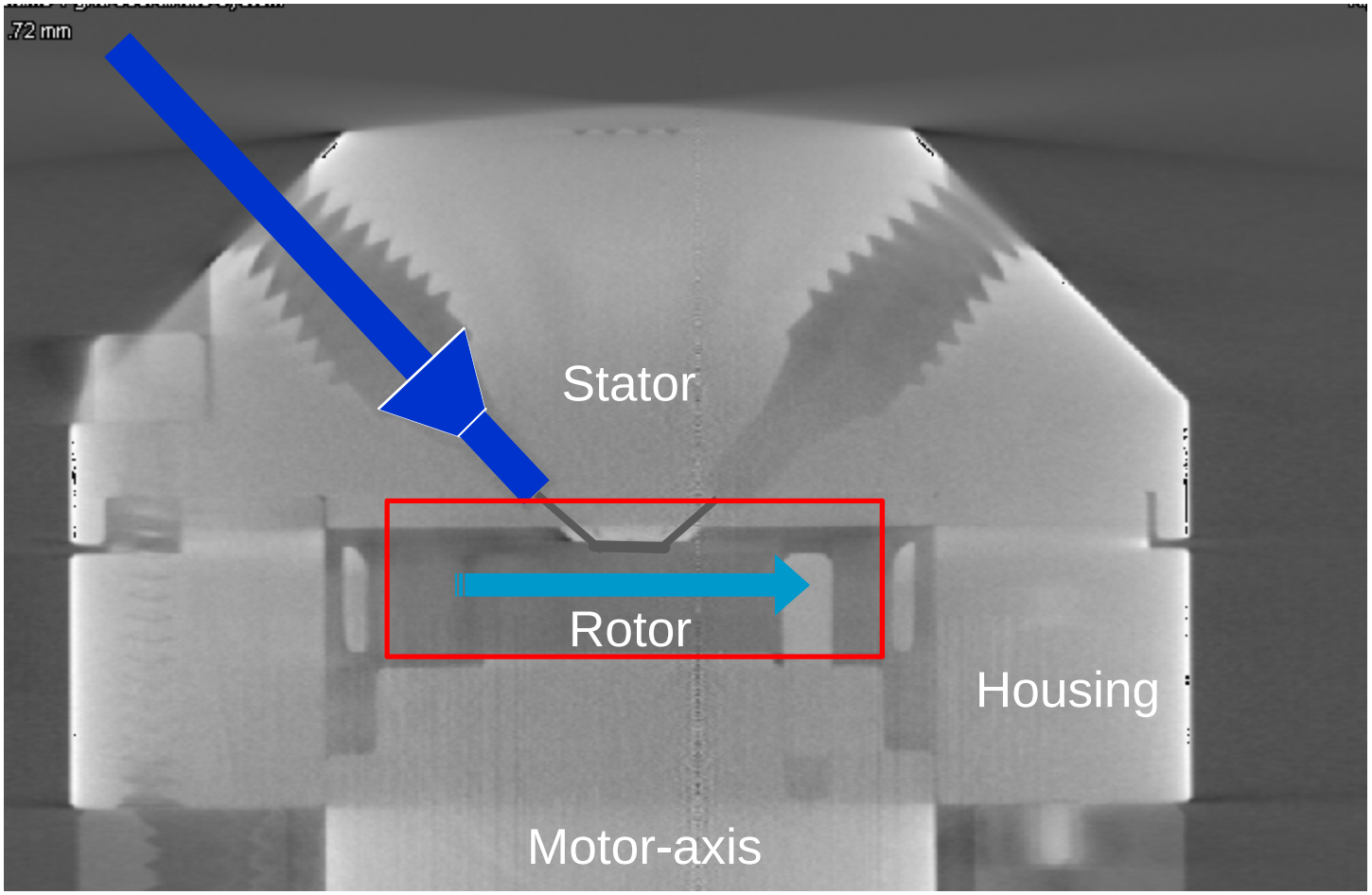
An X-ray of an valve:



Sources of Carry-over

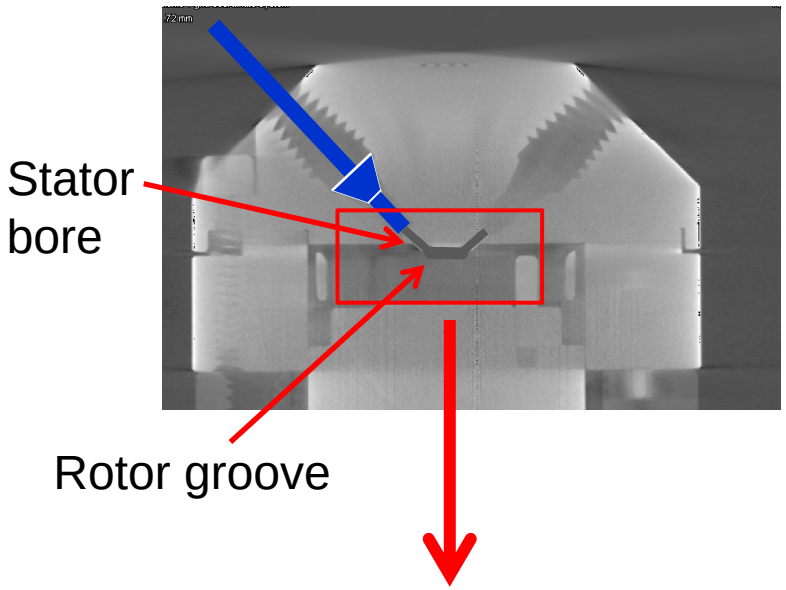
3. & 4. Valve design and fittings

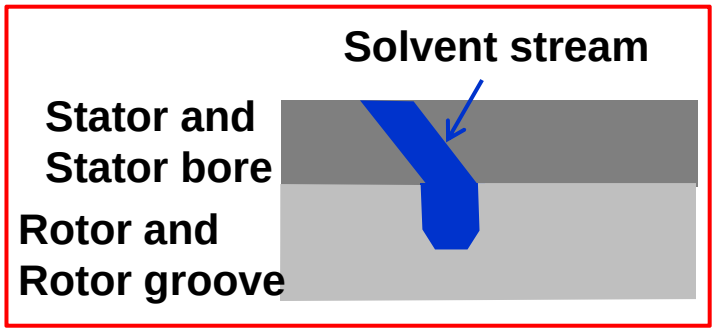
An X-ray of an valve:

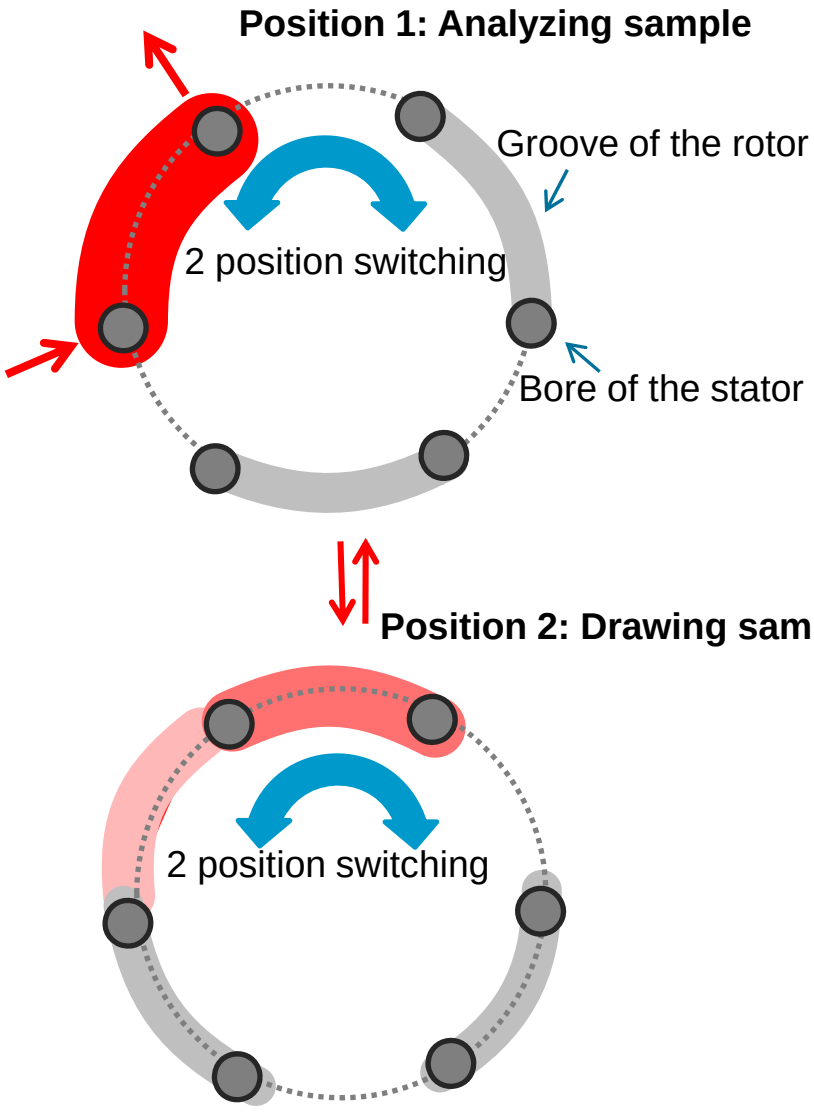


Sources of Carry-over

3. & 4. Valve design and fittings



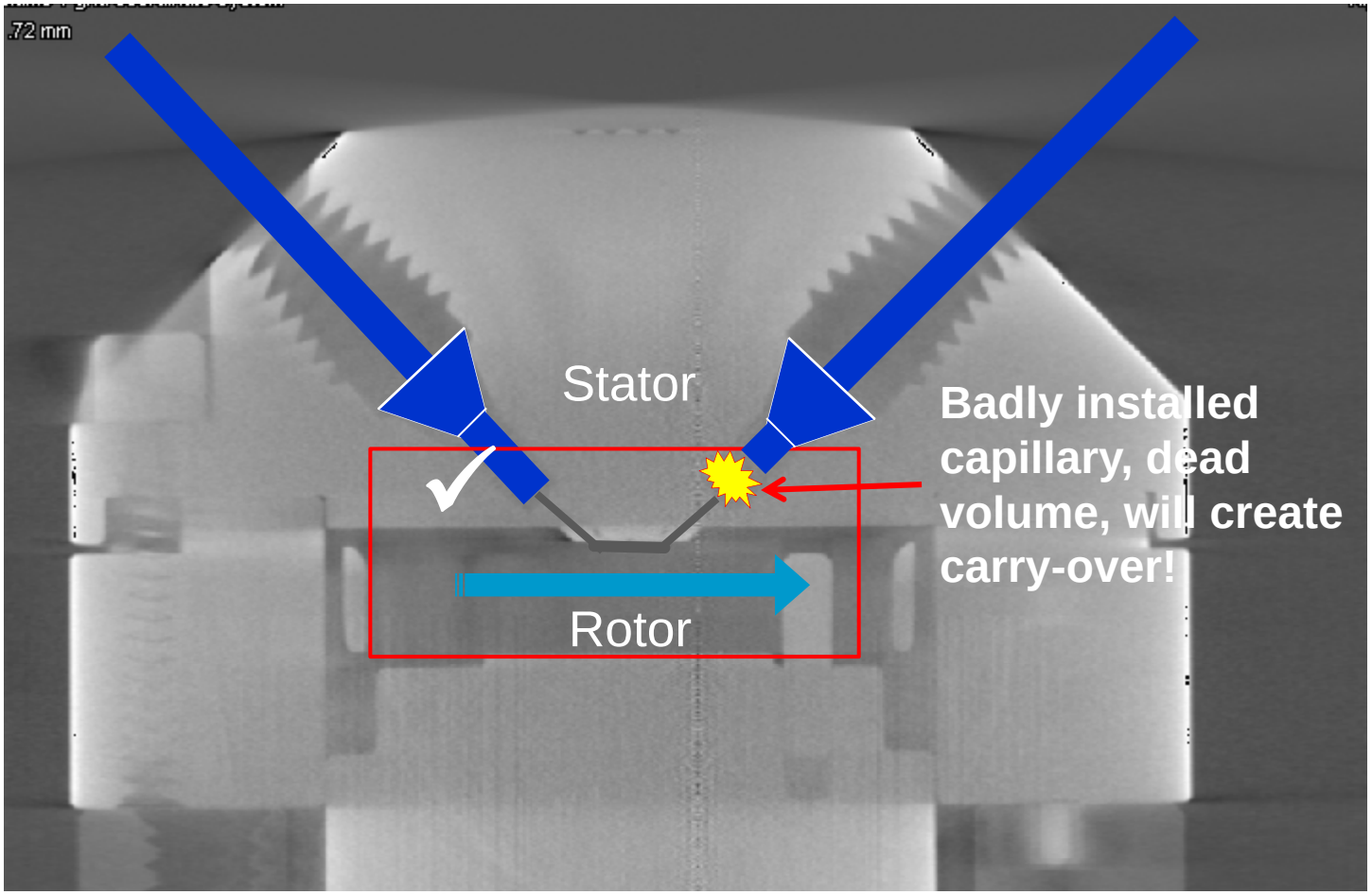




Sources of Carry-over

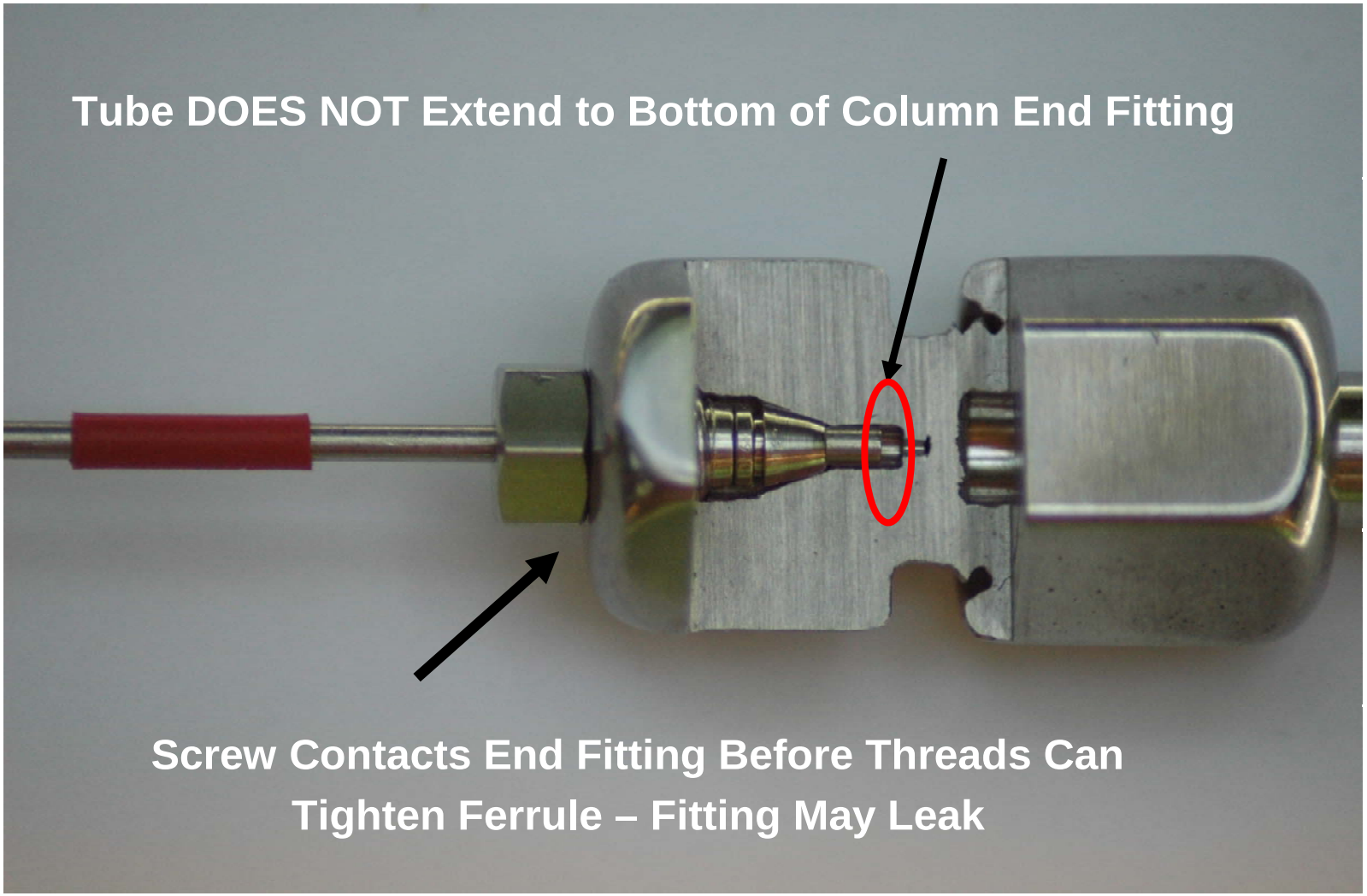
3. & 4. Valve design and fittings

Badly attached fitting – a big source of carry-over!



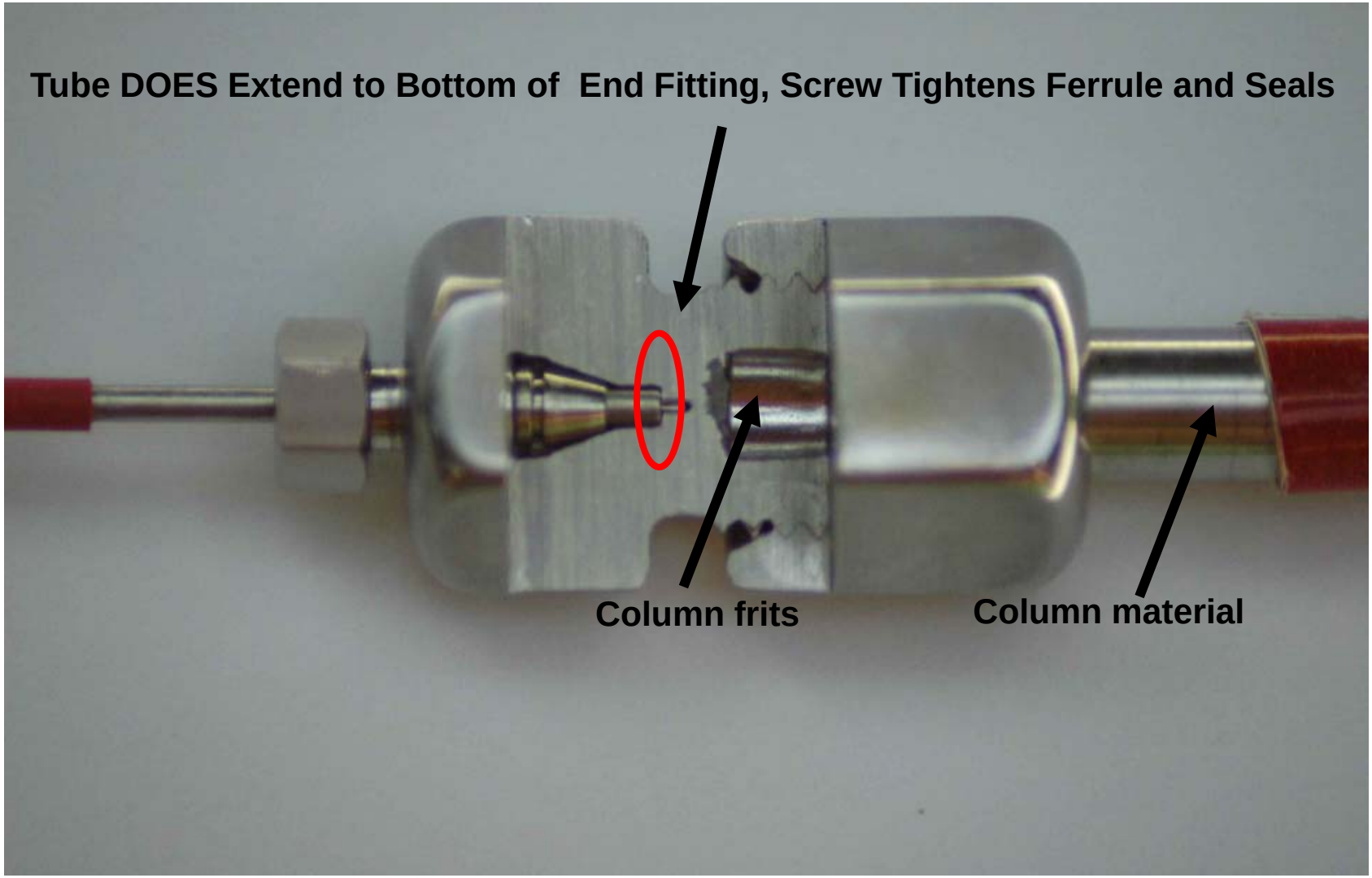
Sources of Carry-over

5. Column carry over – misaligned capillaries



Sources of Carry-over

5. Column carry over – misaligned column



Removing Carry-over with the 1290 Infinity Sampler & 1290 Infinity Flexible Cube

1. **Needle and capillary material – adsorption effect by metal - ion/dipol interaction**
   - → **Surface optimized electropolished needle reduces adsorptive carry-over significantly because of massively reduced surface. Use of two additional optimized solvents to remove adsorbed compounds in valve–needle seat path**

2. **Needle seat design, wear**
   - → **The Needle seat is already optimized in an 1290 Infinity Sampler. By back-flushing this part also long-term carry-over reduction is ensured!**

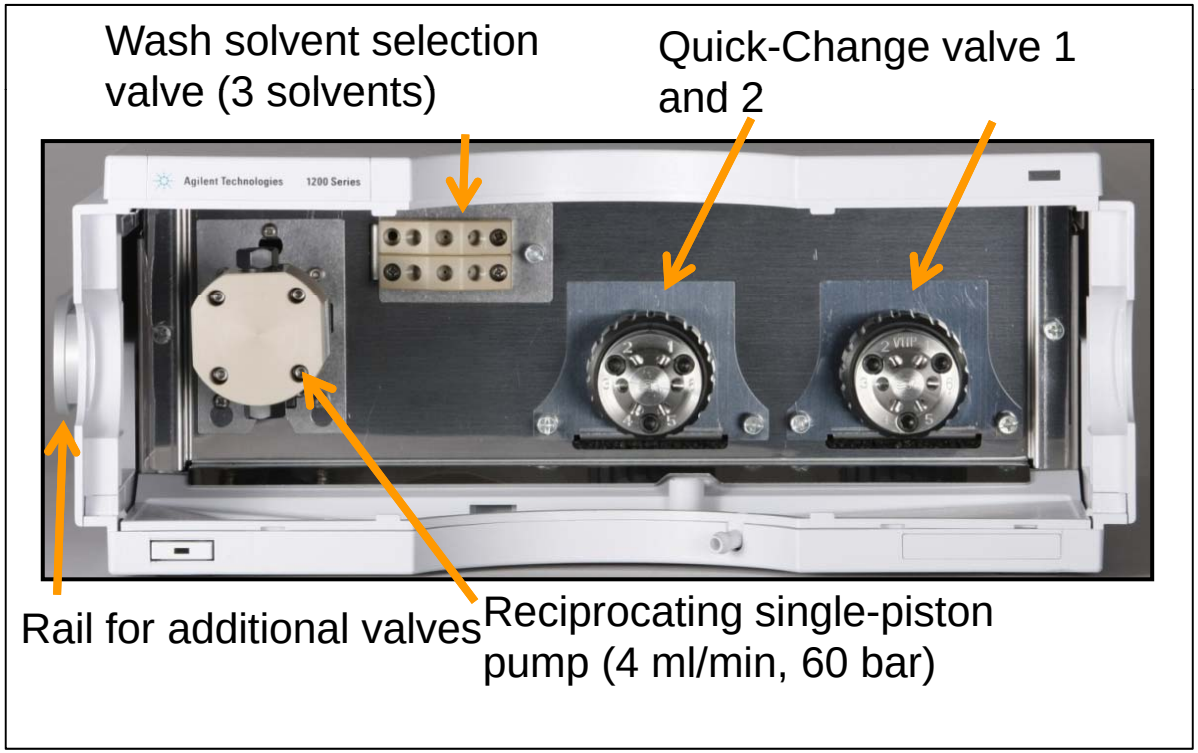
3. & 4. **Valve design and fittings**
   - → **The valve is optimized to have exactly positioned grooves without unswept dead volumes. The FlexCube additionally flushes these areas with up to two optimized solvents.**

5. **Column related carry-over**
   - → **Can be much larger than the instrument related carry-over – check with restriction capillaries first and if the column is identified as the source try different vendors!**

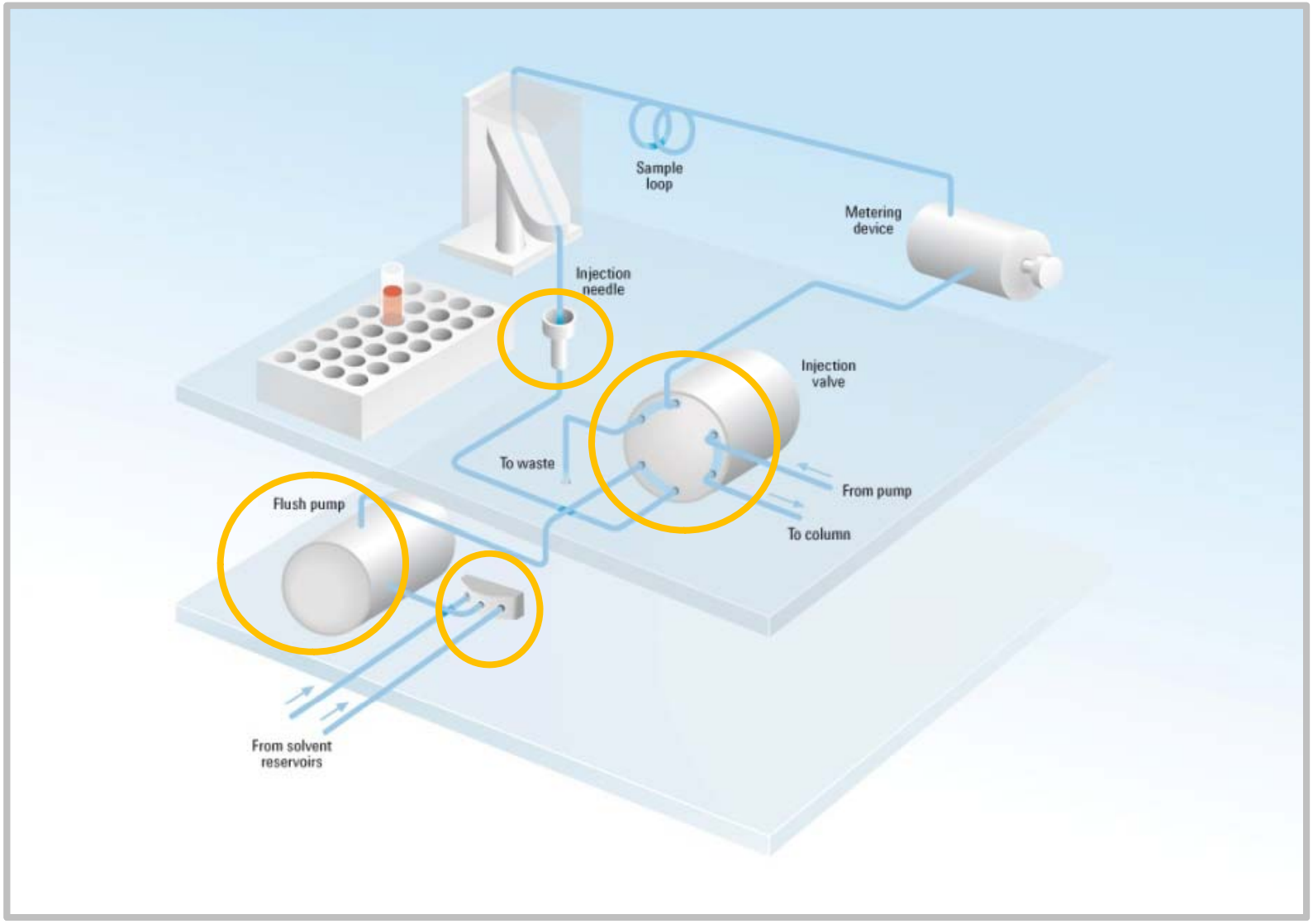
What is a „Flexible Cube“?

The Agilent 1290 Infinity Flexible Cube is an additional module to your 1290 Infinity LC system. It offers several additional functions:

- Reduction of instrument related carry over by needle seat back-flushing
- Long-term reduction of instrument related carry over, even if the sampler parts start to wear
- Any generic valve, due to its flexible design also other configurations and applications would be possible in the future.

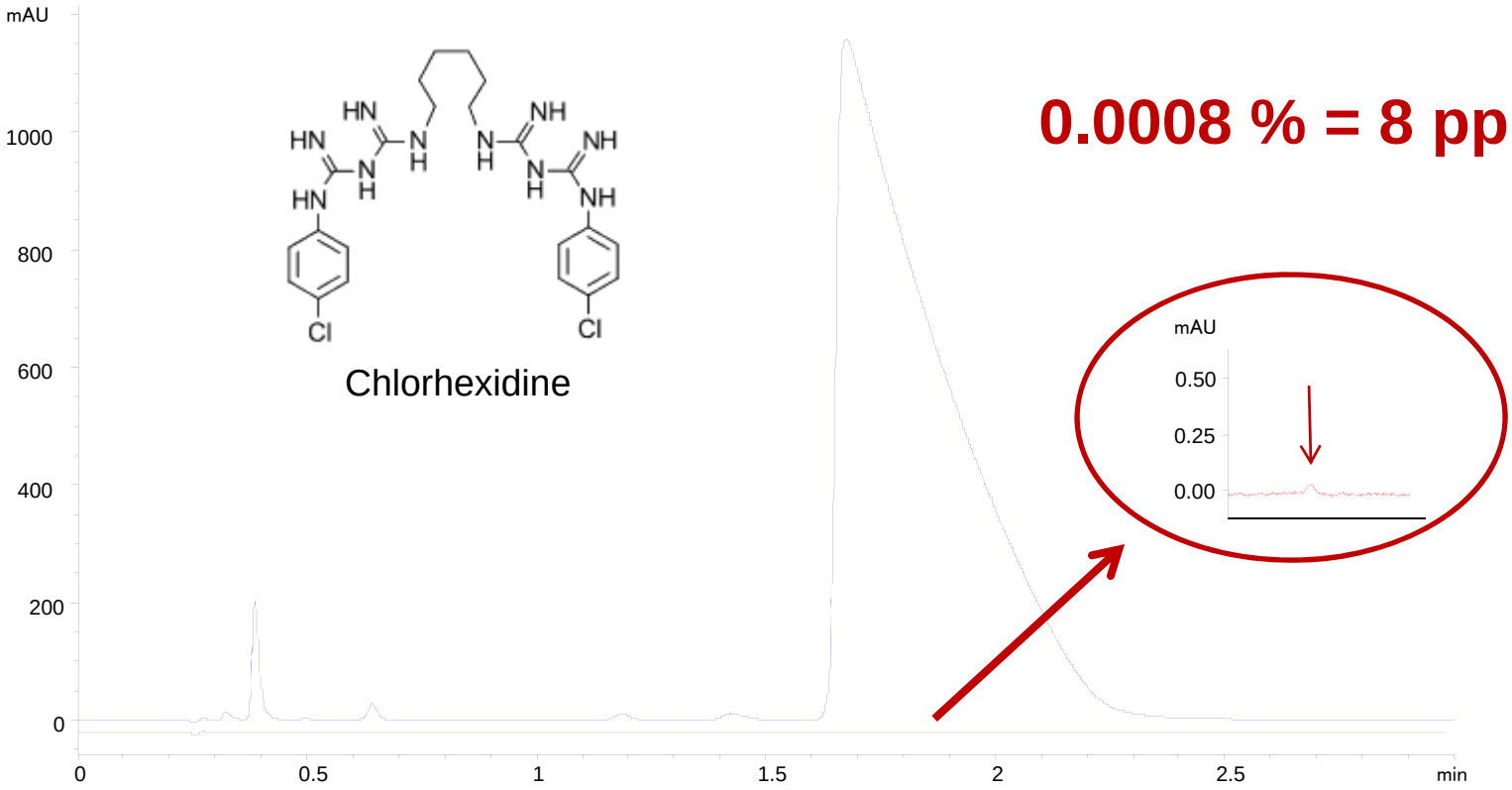


What is a „Flexible Cube“?



Experimental Results with the 1290 Infinity Flexible Cube

1 µL inj. of 3 mg/mL (3 µg on-column) chlorhexidine followed by blank.
Detection: Agilent 1290 Infinity DAD, carry-over:



Experimental Results with the 1290 Infinity Flexible Cube

500 ng/mL Conc.-Max

Typical linear range
= 3 decades

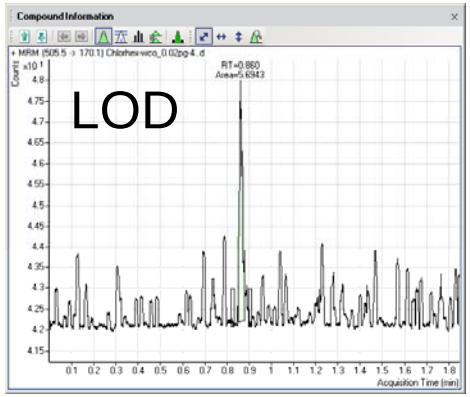
0.5 ng/mL LLOQ

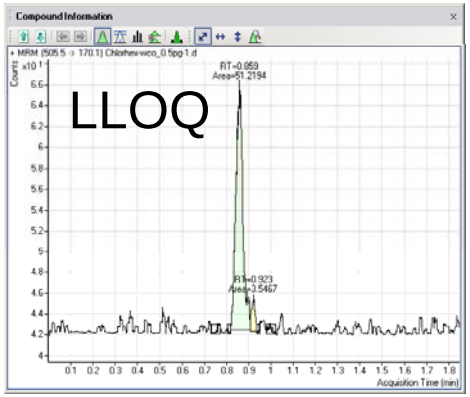
LLOQ = reliable quantification
= Area precision (%RSD) < 20%
= Mean accuracy within 20% range

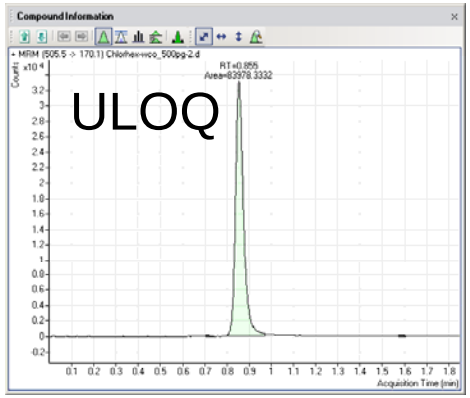
20% of LLOQ

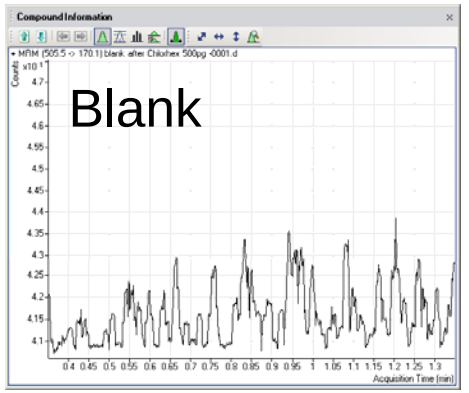
0.02 ng/mL LOD (S/N = 3)

Noise





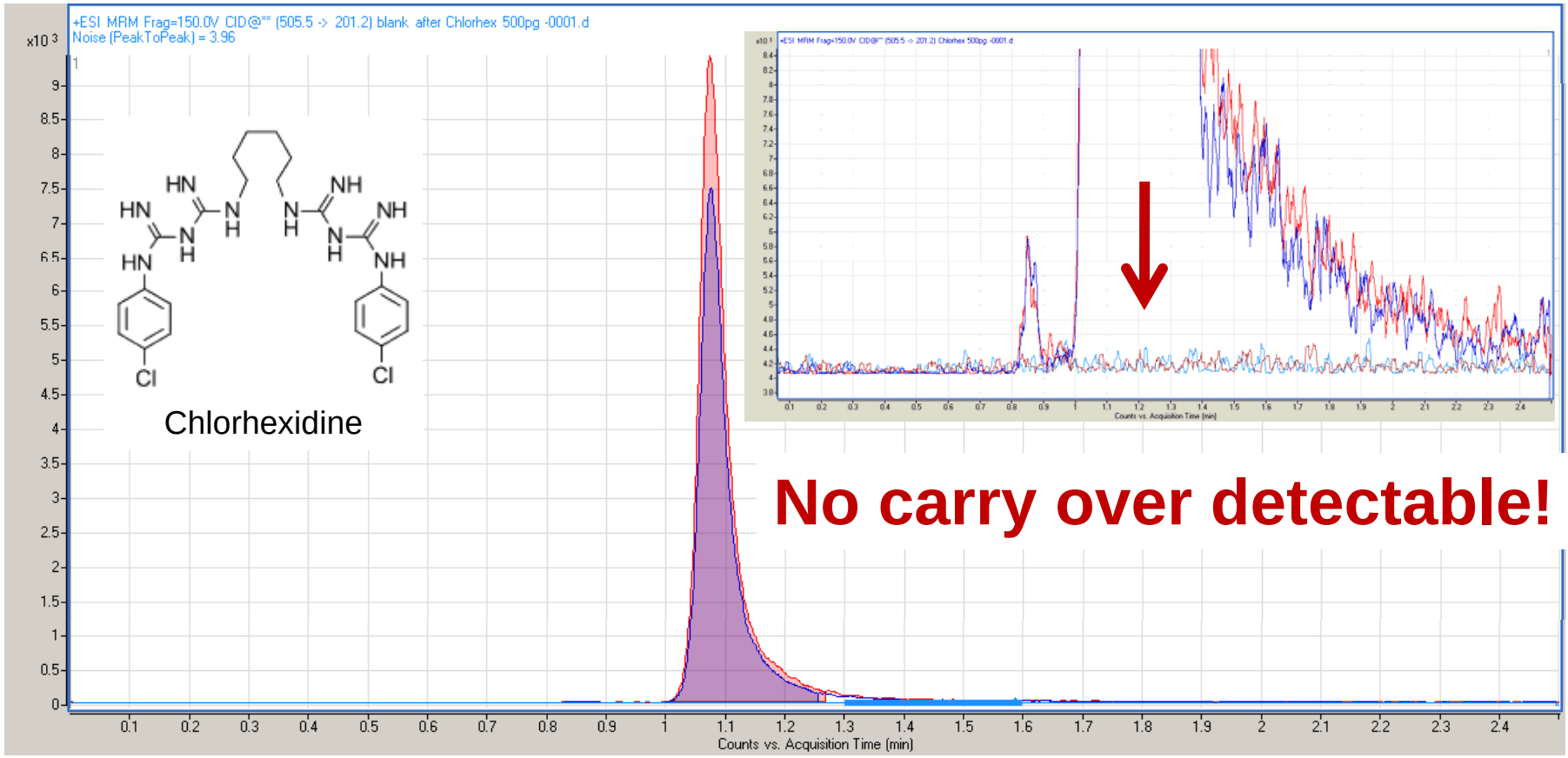




- LOD=20fg (S/N=2.6), LLOQ=500fg (S/N=10), ULOQ=500pg (S/N=109) Chlorhexidine on column
- Determine LLOQ and linearity (R^2=0.9990) as stated to the left
- Area in blanks injected after C-Max should not reach 20% of area found for LLOQ! (20% below the 3 decades)
- Chlorhexidine in first blank after Cmax=below LOD

Experimental Results with the 1290 Infinity Flexible Cube

500 pg Chlorhexidine on-column – detection Agilent 6460 Triple Quad:



Experimental Results with the 1290 Infinity Flexible Cube

Increasing sample concentration 100-fold →

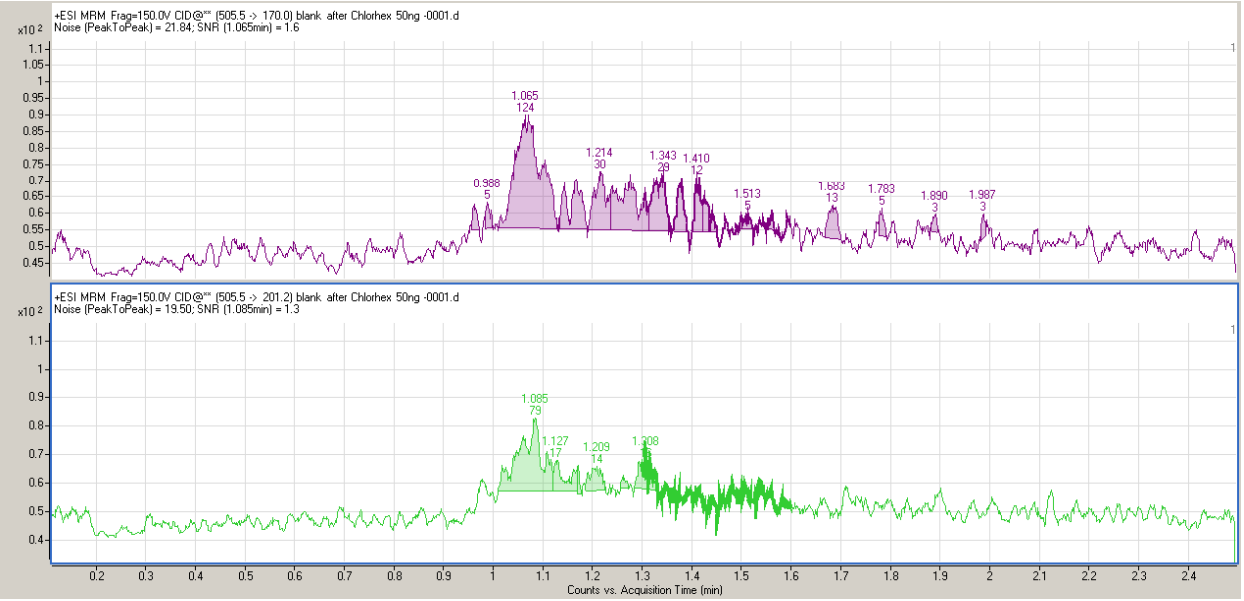
Sample was NOT introduced into the QQQ anymore! Too concentrated.

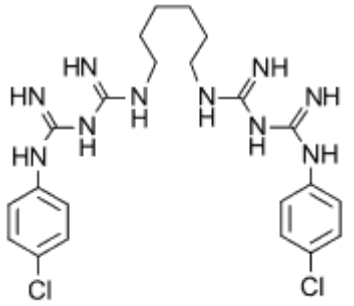
Experimental Results with the 1290 Infinity Flexible Cube

Carry-over including column

50 000 pg Chlorhexidine on-column – detection 6460 Triple Quad:

Carry-over << 10 ppm





Chlorhexidine

Chromatographic Conditions: Column: Agilent Zorbax SB-C18, 2.1x50 mm 1.8 µm (P/N 827700-902); Mobile Phase: A: 0.1% TFA in water, B: 0.1% TFA in Acetonitrile; Isocratic : %B=35%; Flow rate: 0.5 ml/min; Temperature: 30 °C

Cleaning Procedure:

Wash solution: 1 min with Water + 0.1% TFA at 4 ml/min; Needle wash with solvent A 15 sec.

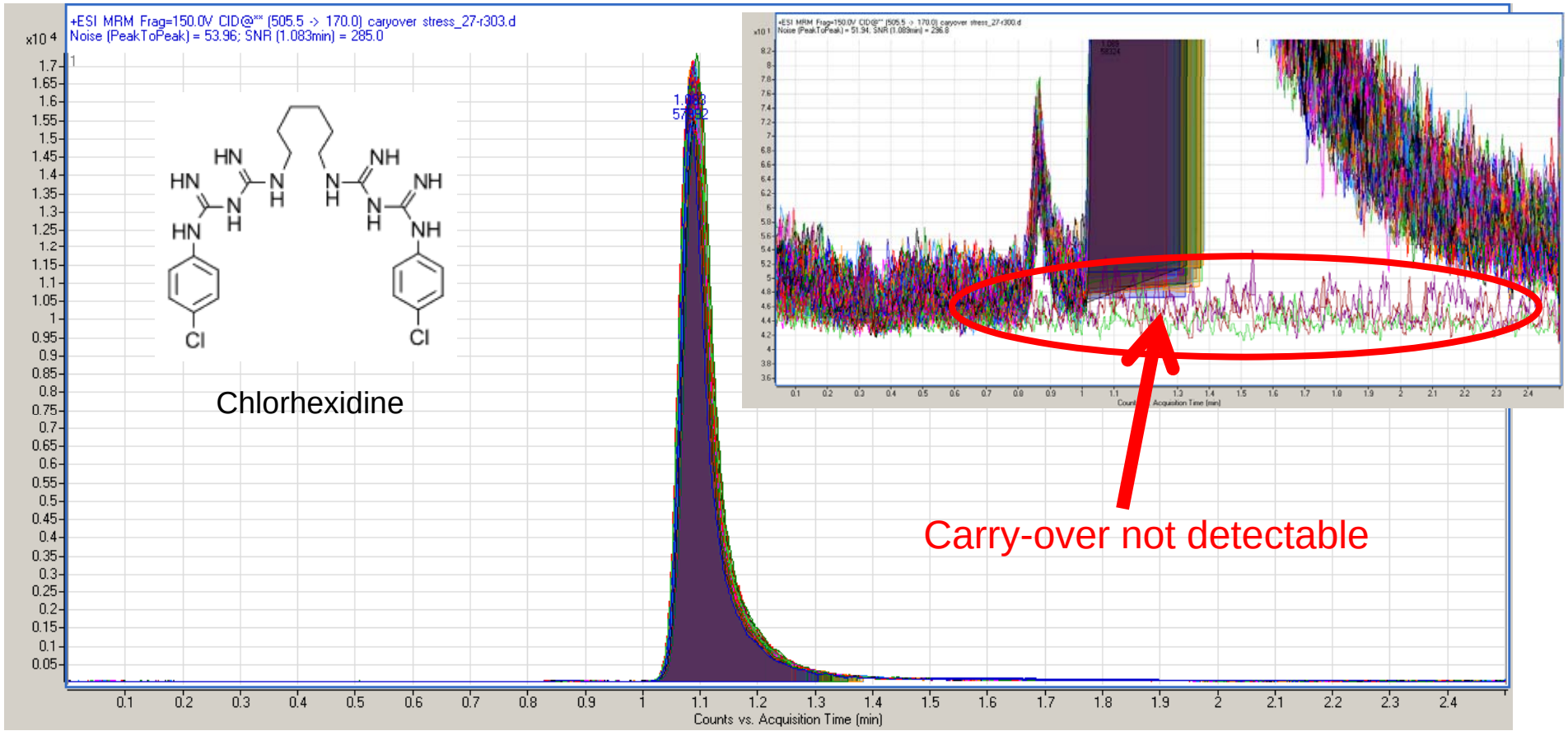
MS-detection:

Sample : 50 ng/µl Chlorhexidine (dissolved in mobile phase A), 1 µl injected and measure on Agilent 6460 QQQ (in specified conditions)

MRM 1: 505.5 →170 (CE: 36 V); MRM 3: 505.5 →201.2 (CE: 20 V); Fragmentor: 150V, Delta EMV(+): 200V

Experimental Results with the 1290 Infinity Flexible Cube

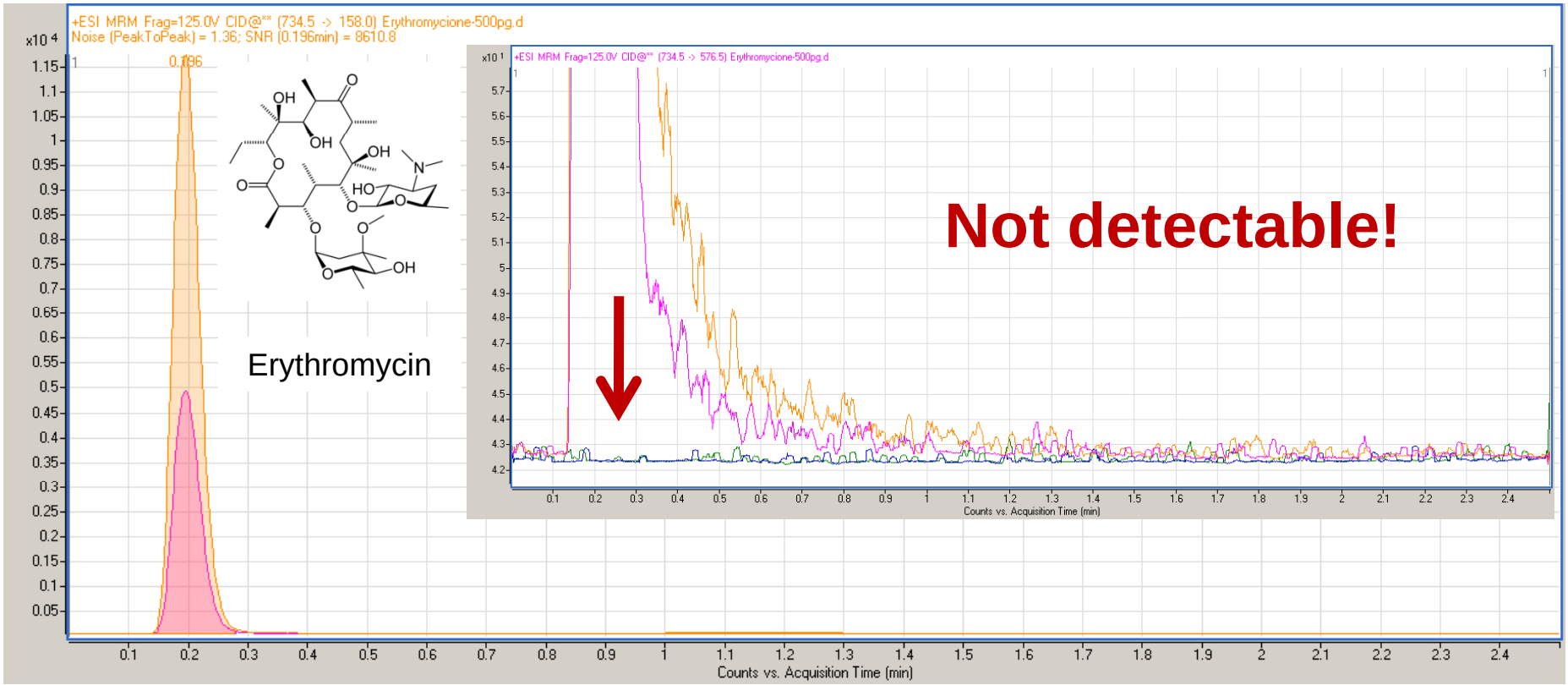
Carry-over robustness - 600 injections of 1ng Chlorhexidine on column and then blanks



Experimental Results with the 1290 Infinity Flexible Cube

500 pg Erythromycin, (without column, with restriction cap.) detection Agilent 6460 QQQ.

Carry-over:



Experimental Results with the 1290 Infinity Flexible Cube

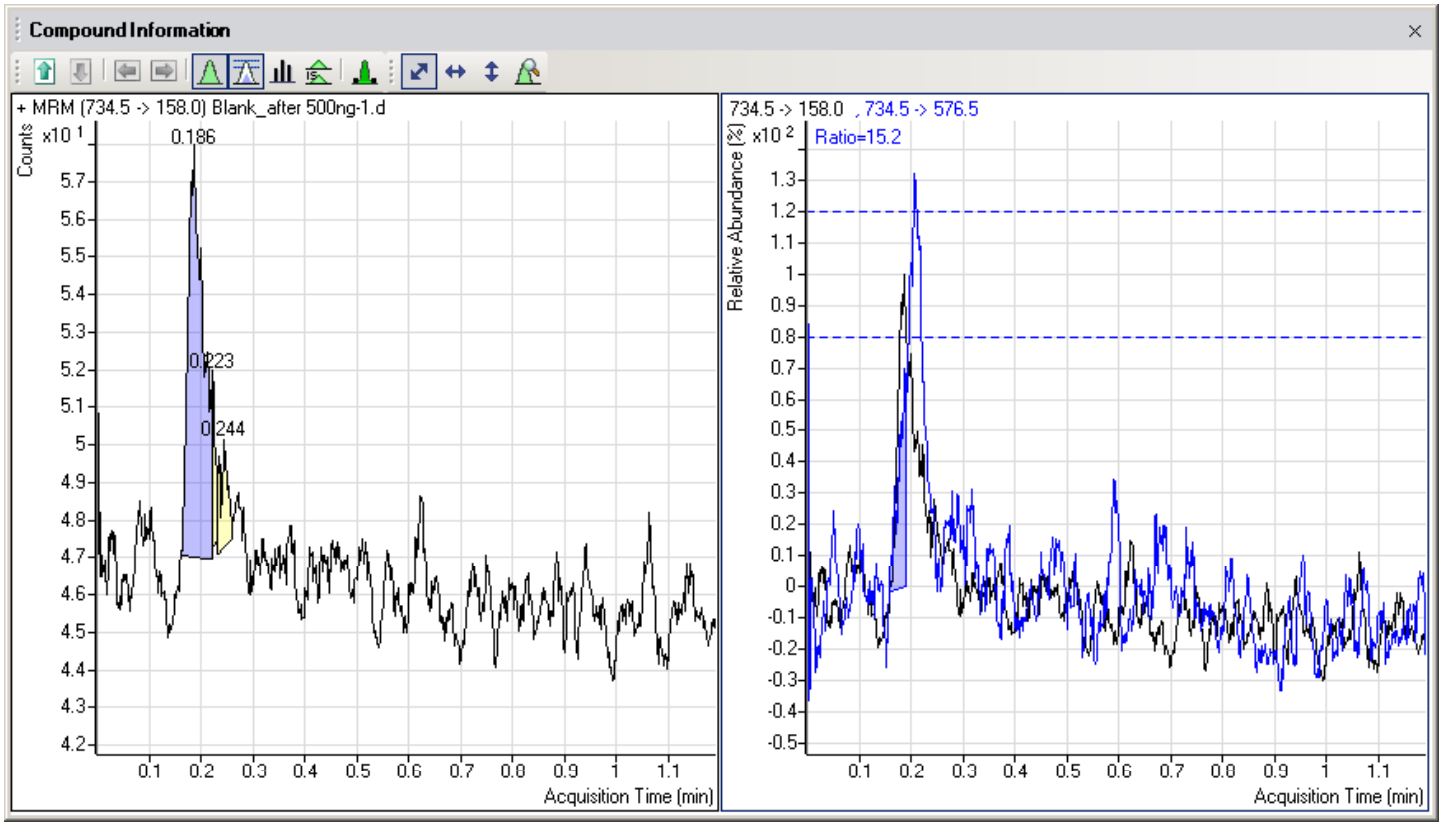
Increasing sample concentration 1 000-fold →

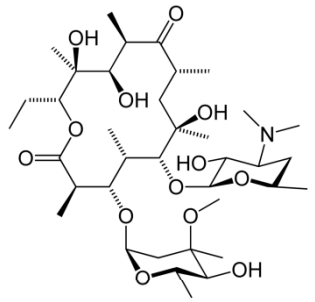
Sample was NOT introduced into the QQQ anymore! Too concentrated.

Experimental Results with the 1290 Infinity Flexible Cube

500 000 pg Erythromycin , (without column, with restriction cap.) , detection Agilent 6460 QQQ.

Carry-over: 0.00024 % = 2.4 ppm





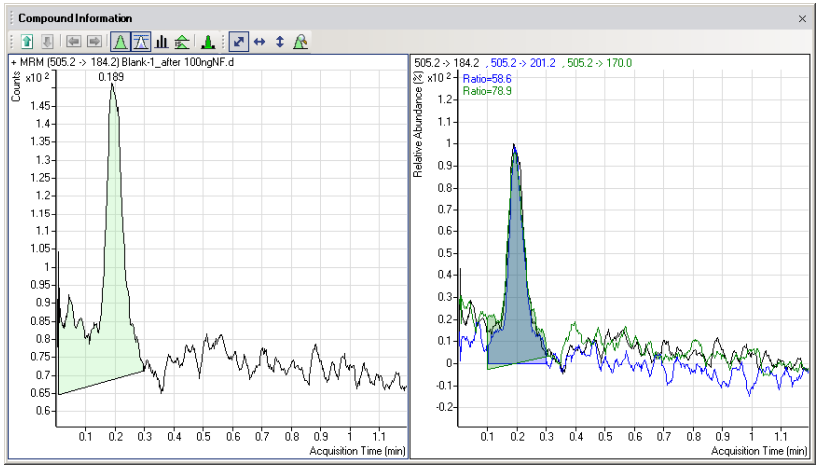
Erythromycin

Experimental Results with the 1290 Infinity Flexible Cube

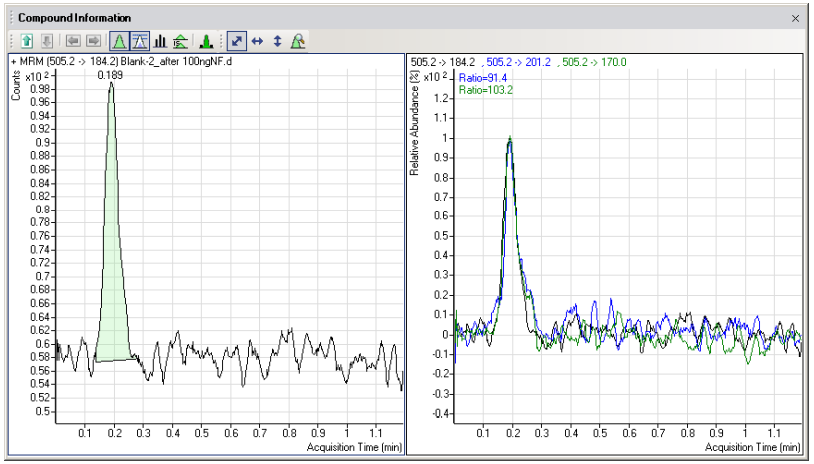
WITHOUT THE FLEXCUBE: Pure instrument carry-over at 100 ng
without column: 0.006 %, (55 ppm, 5.5 pg) – 1st Blank.
0.0015 %, (6 ppm, 1.5 pg) – 2nd Blank.

Column replaced by restriction capillary!

1st Blank



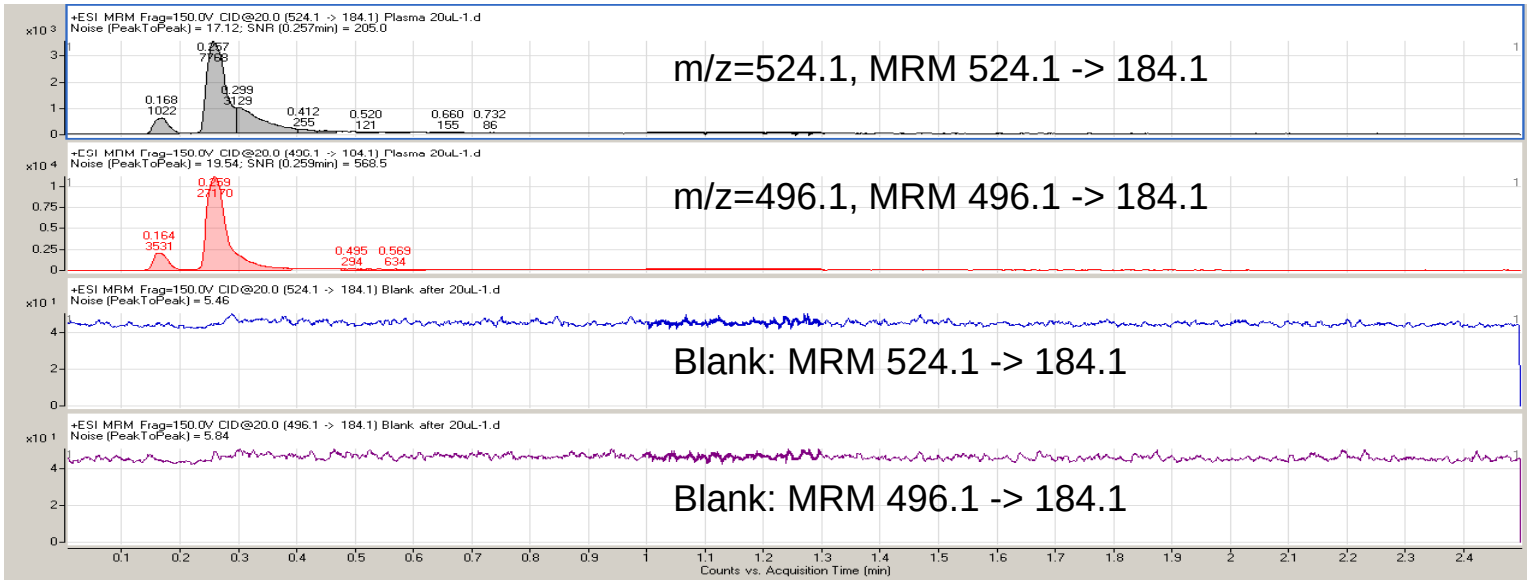
2nd Blank



With FlexCube no carry-over at all is detectable within the second blank!

Experimental Results with the 1290 Infinity Flexible Cube

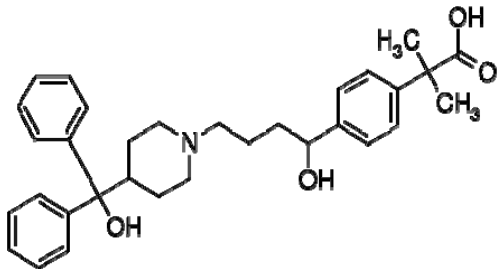
Injection of 20µL precipitated plasma sample, lysophospholipids at m/z 496.1 and m/z 524.1, followed by first blank injection.



Experimental Results with the 1290 Infinity Flexible Cube

Application Example - *Determination of microdose levels of Fexofenadine in human plasma*

- The concept of first-in-human trials (microdosing) accounts for often misleading extrapolation of pharmacokinetic data obtained from in-vitro or animal trials to human dosing.



- This sets stringent demands on bioanalytical sensitivity, since the quantity of drug used for microdosing humans has to be less than a 1/100th of the therapeutic dose that is predicted from animal and in-vitro models.
- For the detection of such low levels, sample carry-over must be avoided!

Sample Preparation & Instrumentation

- Protein precipitation → dilute & shoot → 1290 Infinity / 6460A QQQ

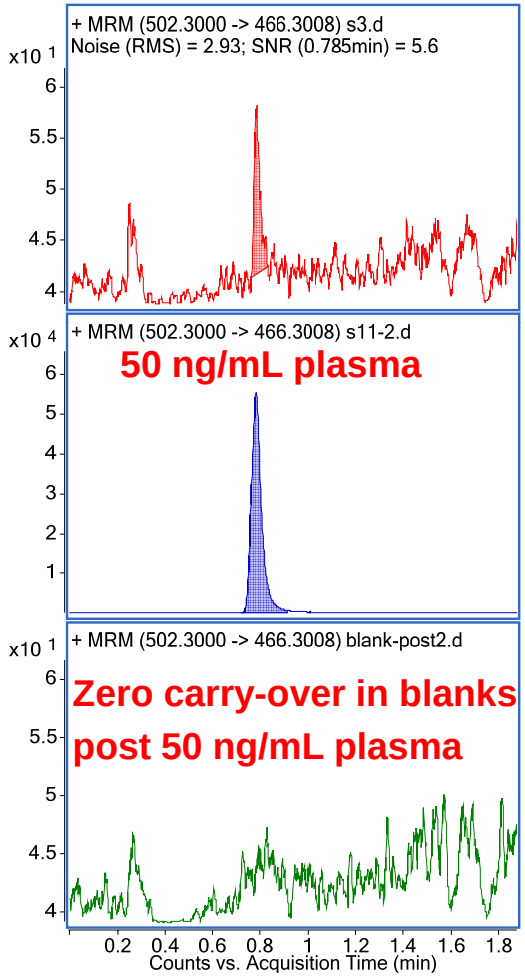
PP
- 100 µL plasma sample
- + 300 µL ACN
- Vortex / spin

Dilution
- + 600 µL Water

Experimental Results with the 1290 Infinity Flexible Cube

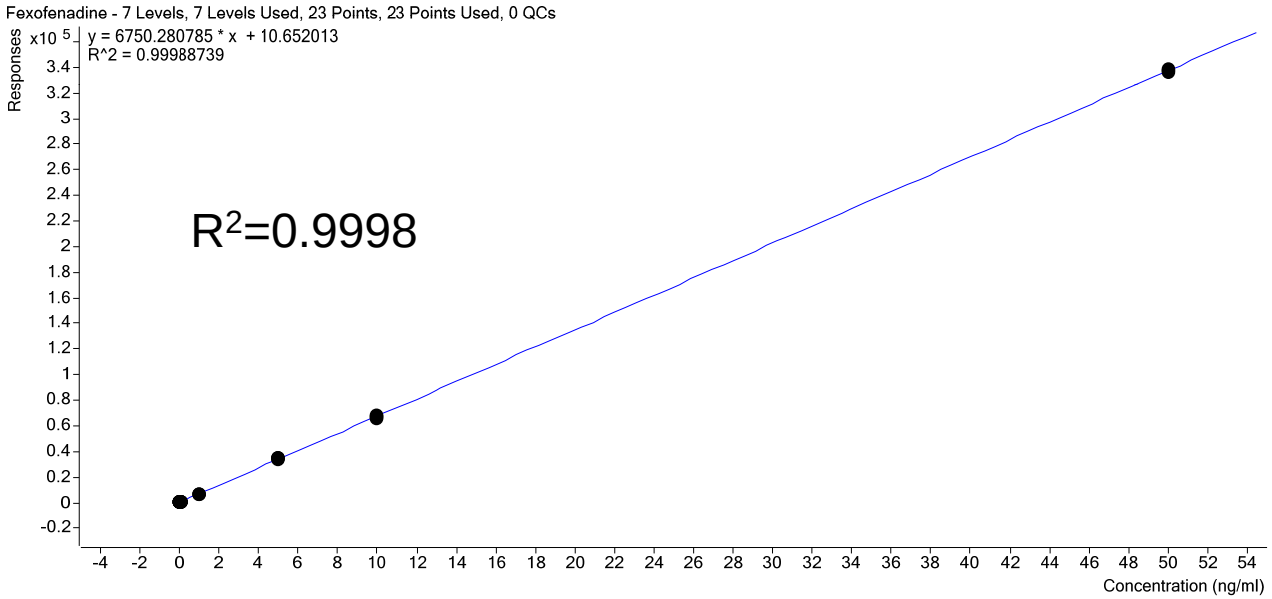
Fexofenadine

- Concentration range: 0.005 – 50 ng/mL in human plasma



LOD 0.005 ng/mL plasma

S/N > 5 (N = 5 x RMS: 0.6-0.7 & 0.9-1.0)



Conclusion

- This presentation demonstrated the use of the Agilent 1290 Infinity LC system in combination with the 1290 Infinity Flexible Cube module for carry-over reduction in high sensitive LC/MS QQQ applications.
- A method for the determination of carry-over independent from the instrument sensitivity was developed.
- It was demonstrated that the measured instrument carry-over within the normally used concentration range is Zero.
- It was also demonstrated that even at a 50 - 100-fold excess concentration the forced carry-over is near zero and can be neglected.
- The Agilent 1290 Infinity Flexible Cube LC solution is ready to use for highly demanding LC/MS applications.