Agilent 1290 Infinity II 2D-LC Solution Biopharmaceutical Polymer Analysis

WCBP Jan 2017

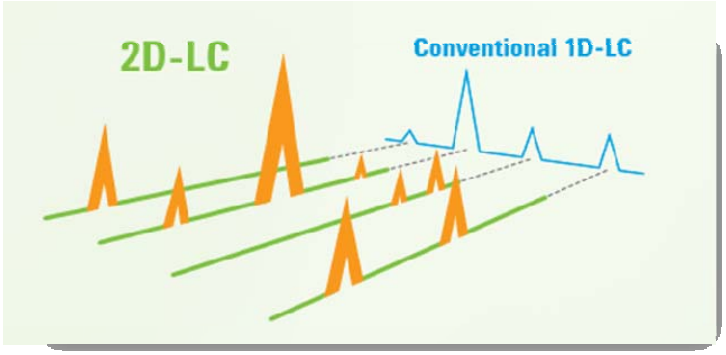
Washington, DC

Overview

- Resolving power and how to measure it
- Why two-dimensional LC?
- Setup of a 2D-LC System
- Different modes of 2D-LC
  - Heart-Cutting 2D-LC
  - Multiple Heart-Cutting 2D-LC
  - High Resolution Sampling 2D-LC
  - Comprehensive 2D-LC
- One software for all 2D-LC modes
- Online 2DLC for Biopharmaceuticals – 4 case studies

Increasing Lab Efficiency
UHPLC and Other Solutions

- Accelerating Analysis
  - UHPLC particles (sub-2µm columns + UHPLC systems)
- Automation
  - Automated sample preparation
  - Valve solutions
- Increasing peak capacity
  - Longer columns
  - Online 2D-LC



Why Two-Dimensional LC?
General Thoughts in Separation Science

ELSEVIER

Journal of Chromatography A, 778 (1997) 3–21

JOURNAL OF CHROMATOGRAPHY A

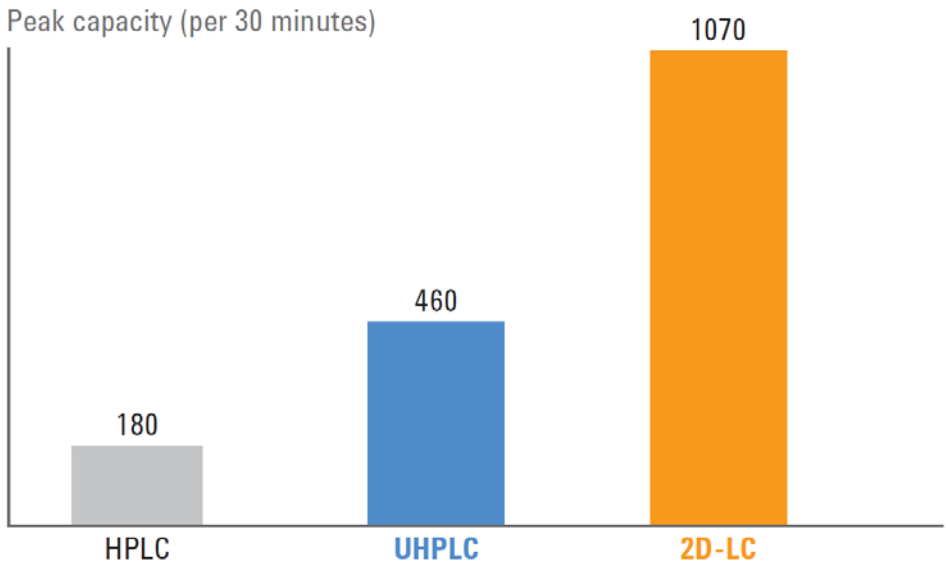
Review

Some reflections on speed and efficiency of modern chromatographic methods

H. Poppe

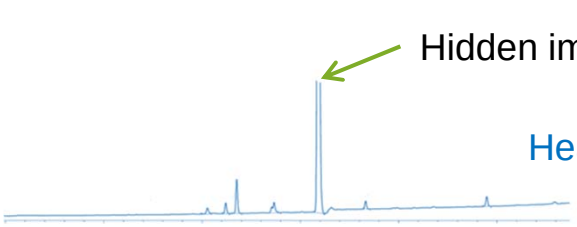
Amsterdam Institute for Molecular Studies (AIMS), Laboratory for Analytical Chemistry, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, Netherlands

"Resolving power is what it is all about in analytical separation science."

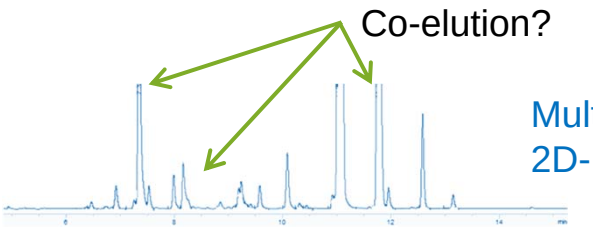


Why Two-Dimensional LC?

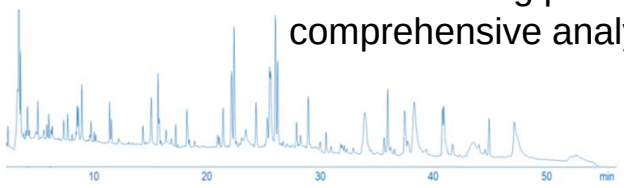
- Increased peak capacity
- Further resolution of a complex mixture that cannot be separated on a single column
- Sample cleanup by removing matrix or interfering compounds
- Increase sample throughput (two separations going on at once)



Heart-cutting 2D-LC

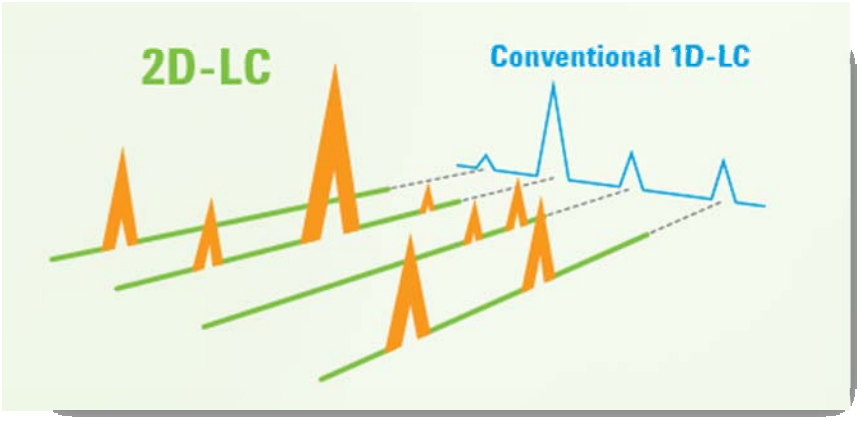


Multiple heart-cutting 2D-LC

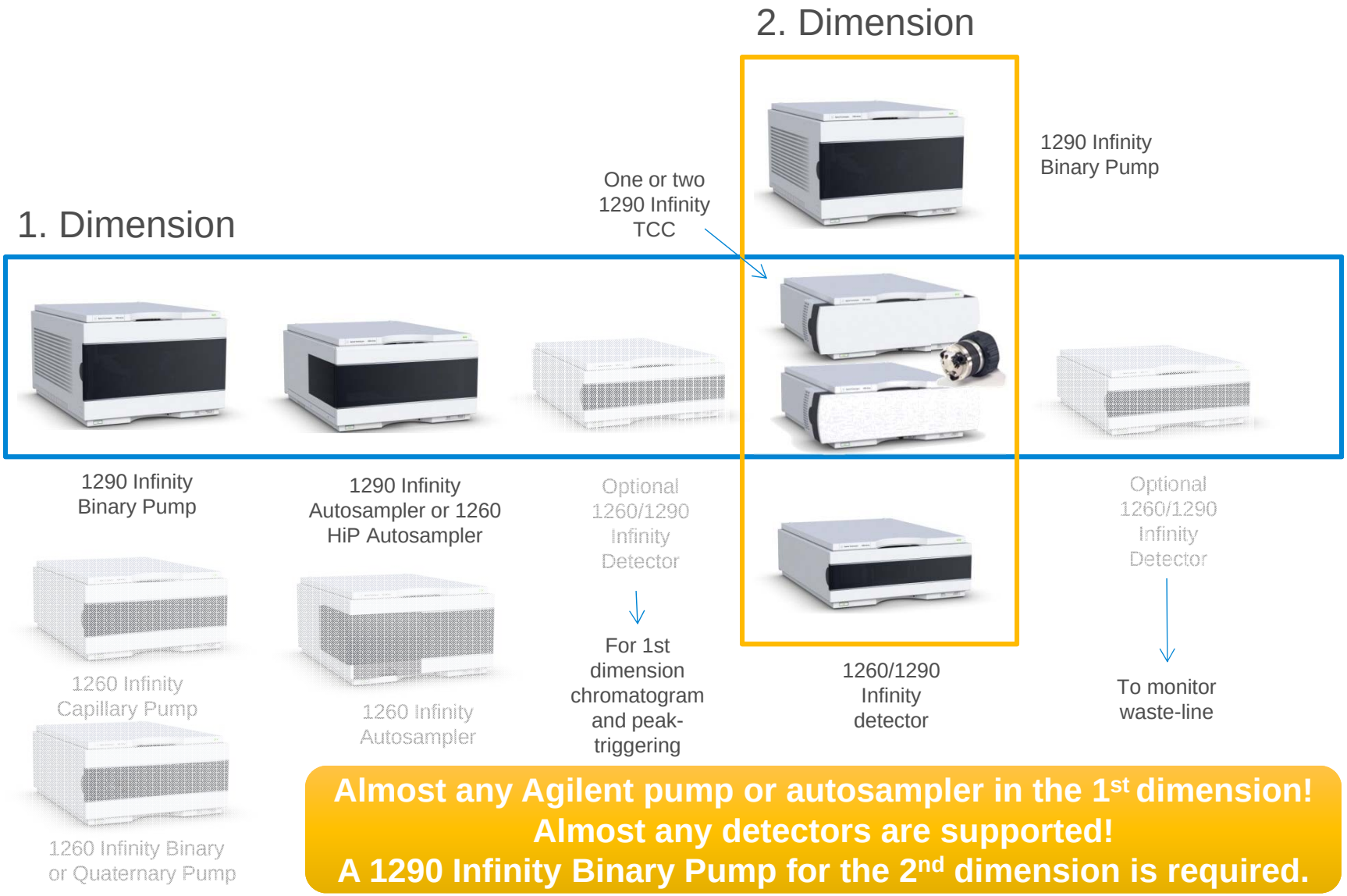


Comprehensive 2D-LC

Agilent's 2D-LC Solution

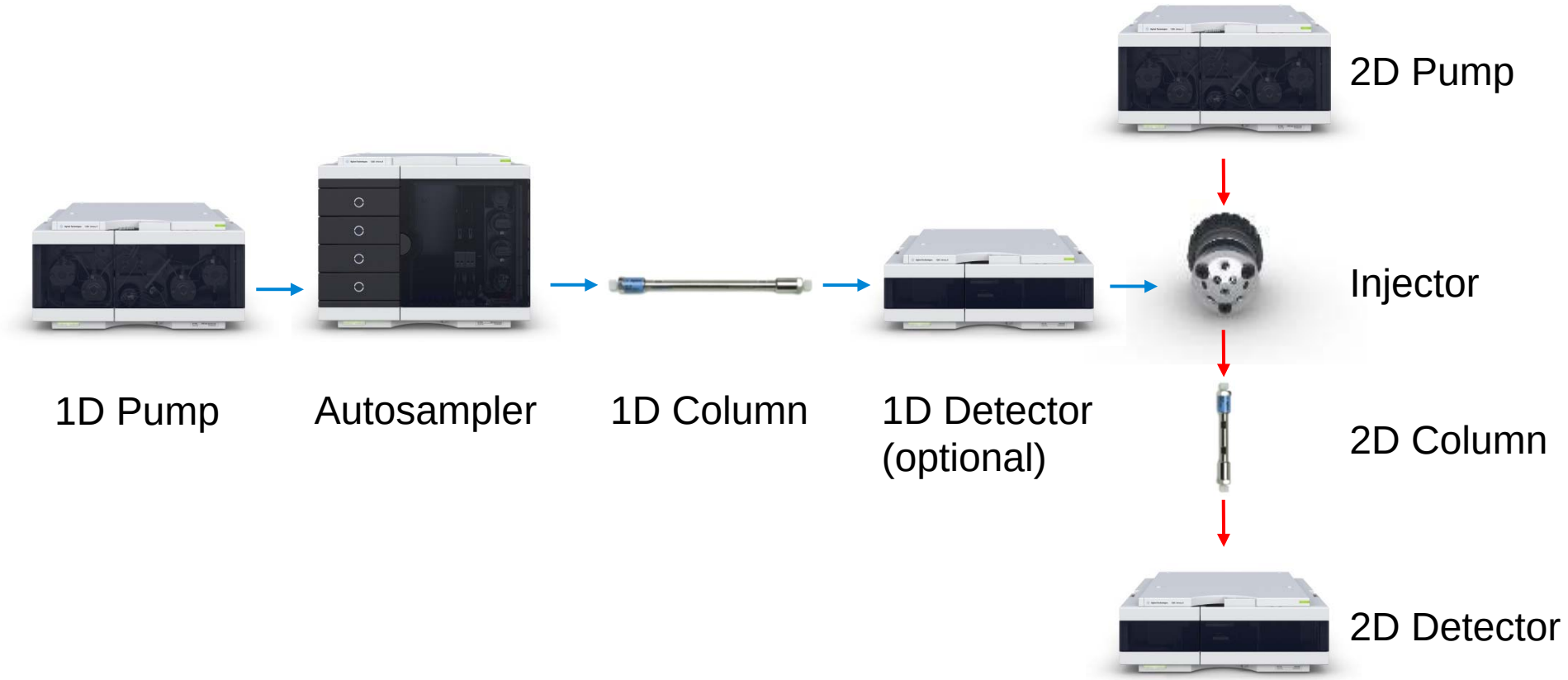


2D LC Modules



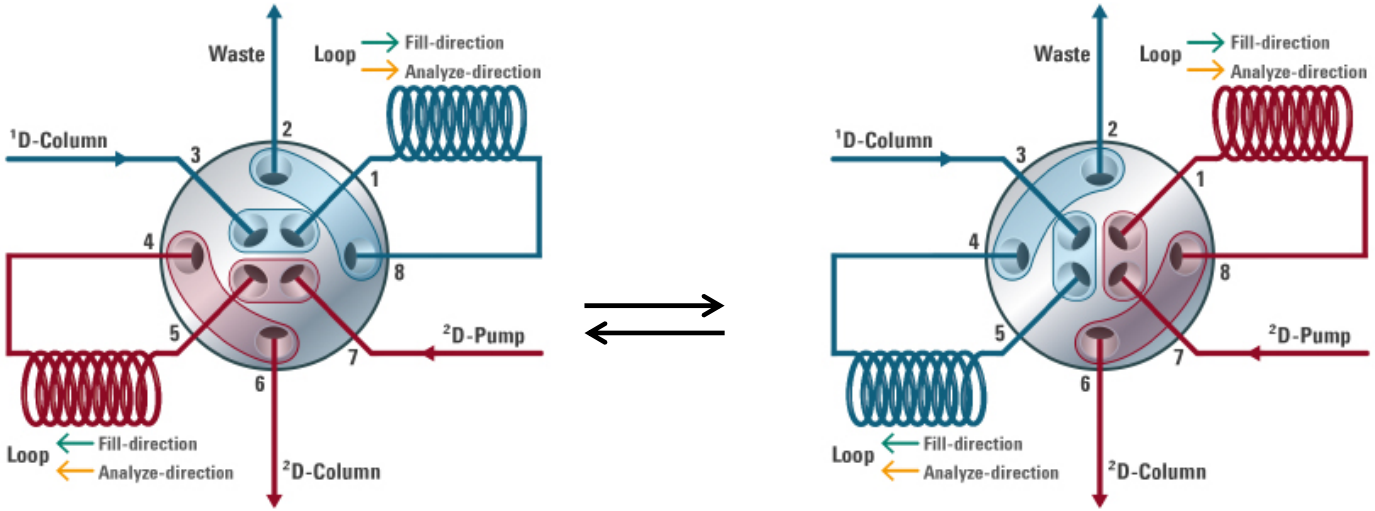
Almost any Agilent pump or autosampler in the 1st dimension!
Almost any detectors are supported!
A 1290 Infinity Binary Pump for the 2nd dimension is required.

2D-LC System Configuration

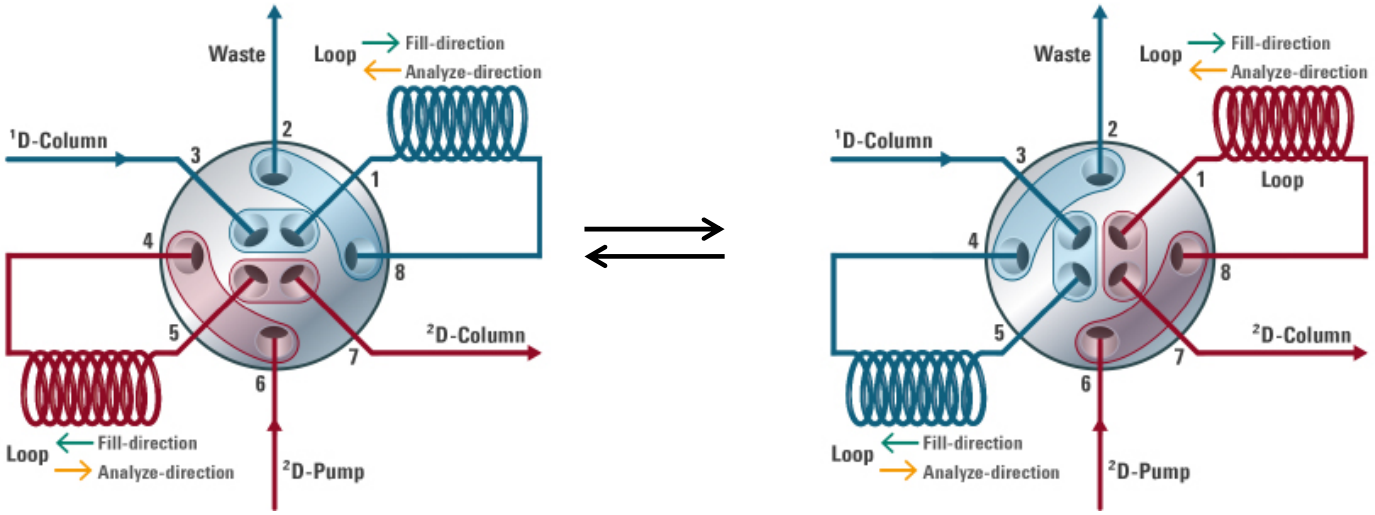


Unique Agilent 2D-LC Valve

Cocurrent



Countercurrent



Consider Dedicated Bio-inert HPLC Protein Analysis for First Dimension

Unspecific surface binding

Low throughput, low resolution

Peak tailing for critical proteins

Corrosion and pH issues with standard LC

Decreased column lifetime and chromatographic performance

Metal-free chromatography needs (e.g. Cr Speciation)

High pH applications

Phosphorylated compounds, oligonucleotides

Agilent 1260 Infinity II Bio-Inert Quaternary LC

The New Standard in Bioanalysis

100% Bio-inert

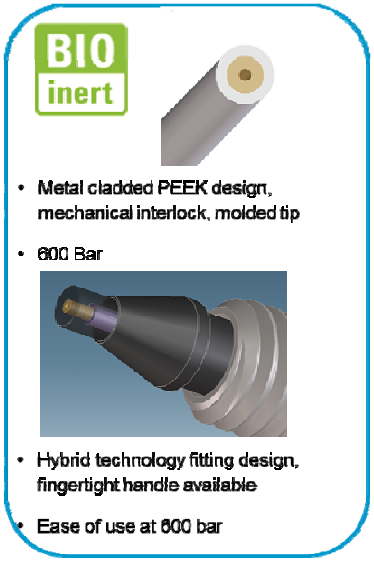
- ✓ Precious sample does **not touch metal surfaces**
- ✓ pH range 1-13 (shortterm 14)
- ✓ 2 M salt, 8 M urea
- ✓ No stainless steel in mobile phase flow path
- ✓ New **capillary technology**

UHPLC capability

- ✓ 600 bar

Ease of Use and Robustness

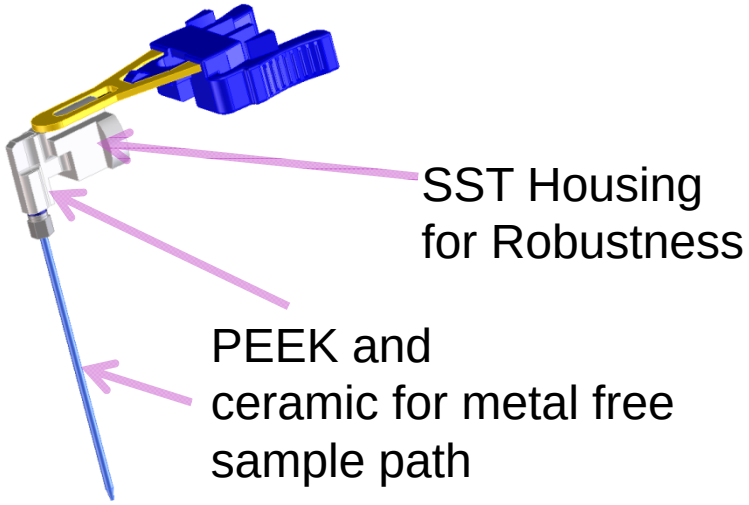
- ✓ Corrosion resistant
- ✓ Active seal wash
- ✓ Quaternary buffer mixing
- ✓ Bio-HPLC columns for biotherapeutic characterization



The choice for both, bioanalytical and biopurification up to 10 ml/min

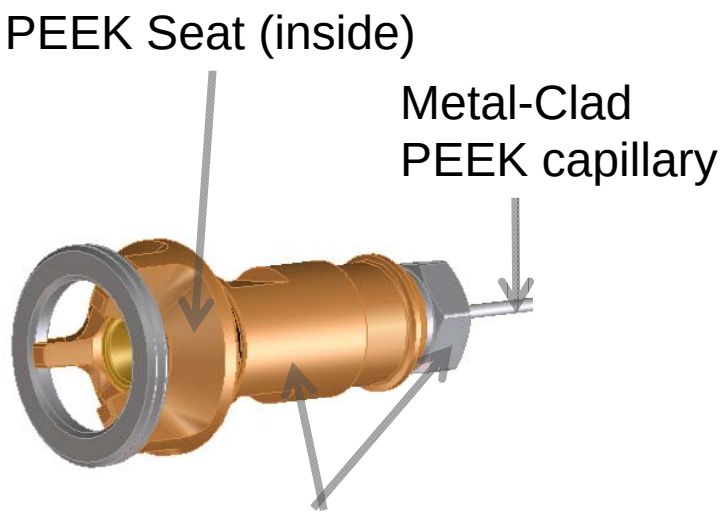
Bio-Inert Components

Inert materials: Titanium (mechanical parts), PEEK, Ceramics, PTFE, FFKM



Needle

Seat

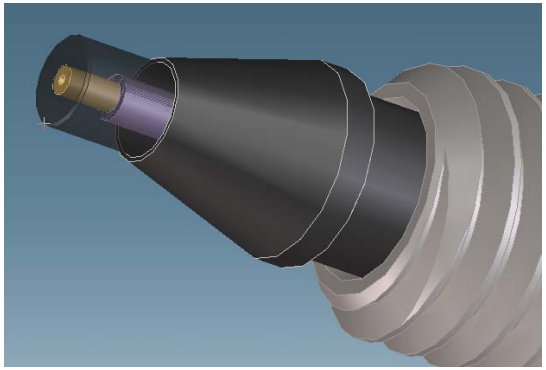


New Capillary Design Ensures Bio-Inertness

Capillaries

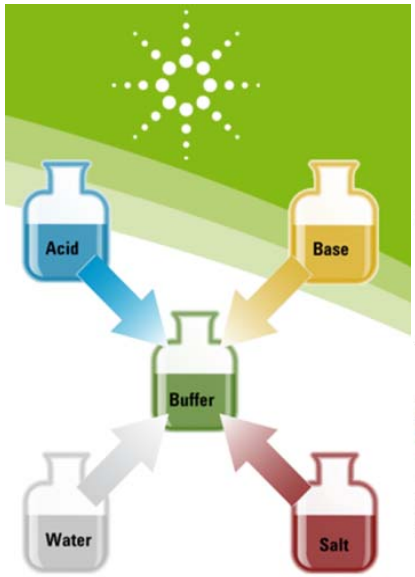
Metal-clad PEEK capillary
(Titanium Capillary also available)

Newest fitting design: Hybrid technique

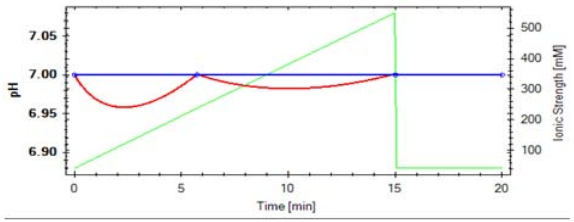


New capillary technolgy
enables **600 bar**
AND is completely metal free !!

Quaternary Buffer Mixing with Buffer Advisor
Use with Ion Exchange Chromatography



Create a salt gradient with constant pH

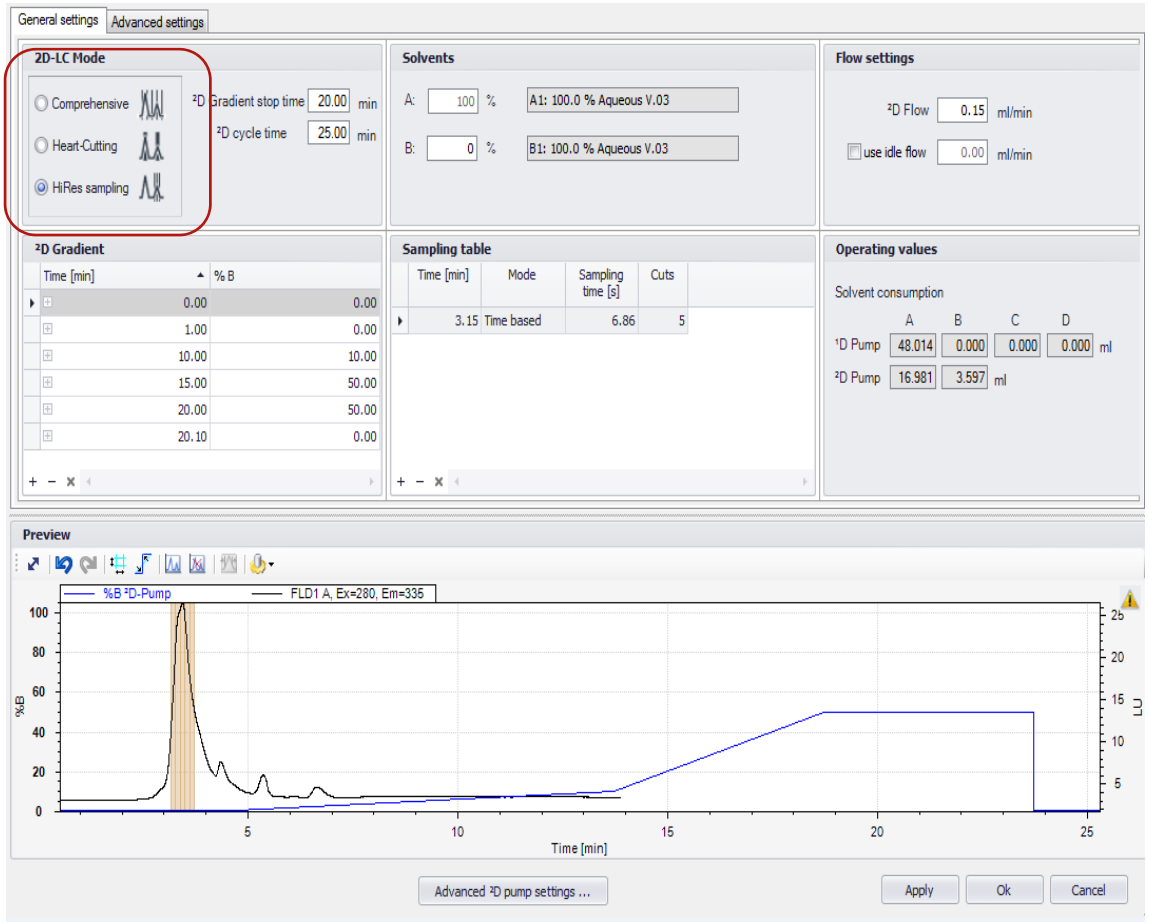


Result Pump Gradient Time Table

Time	% A	% B	% C	% D	Init. pH	Calc. pH	IS	BC	Cond.	Status
0	36.6	0	37.5	25.9	7	7	41.2	12	0.252	Correct
5.73	28.6	12.7	25.5	33.2	7	7	238	10.1	2.15	Correct
15	10.8	33.3	18.2	37.7	7	7	550	8.15	4.95	Correct
15.01	36.6	0	37.5	25.9	7	7	41.2	12	0.252	Correct

2D-LC Acquisition Software

One easy-to-use software for all operation modes

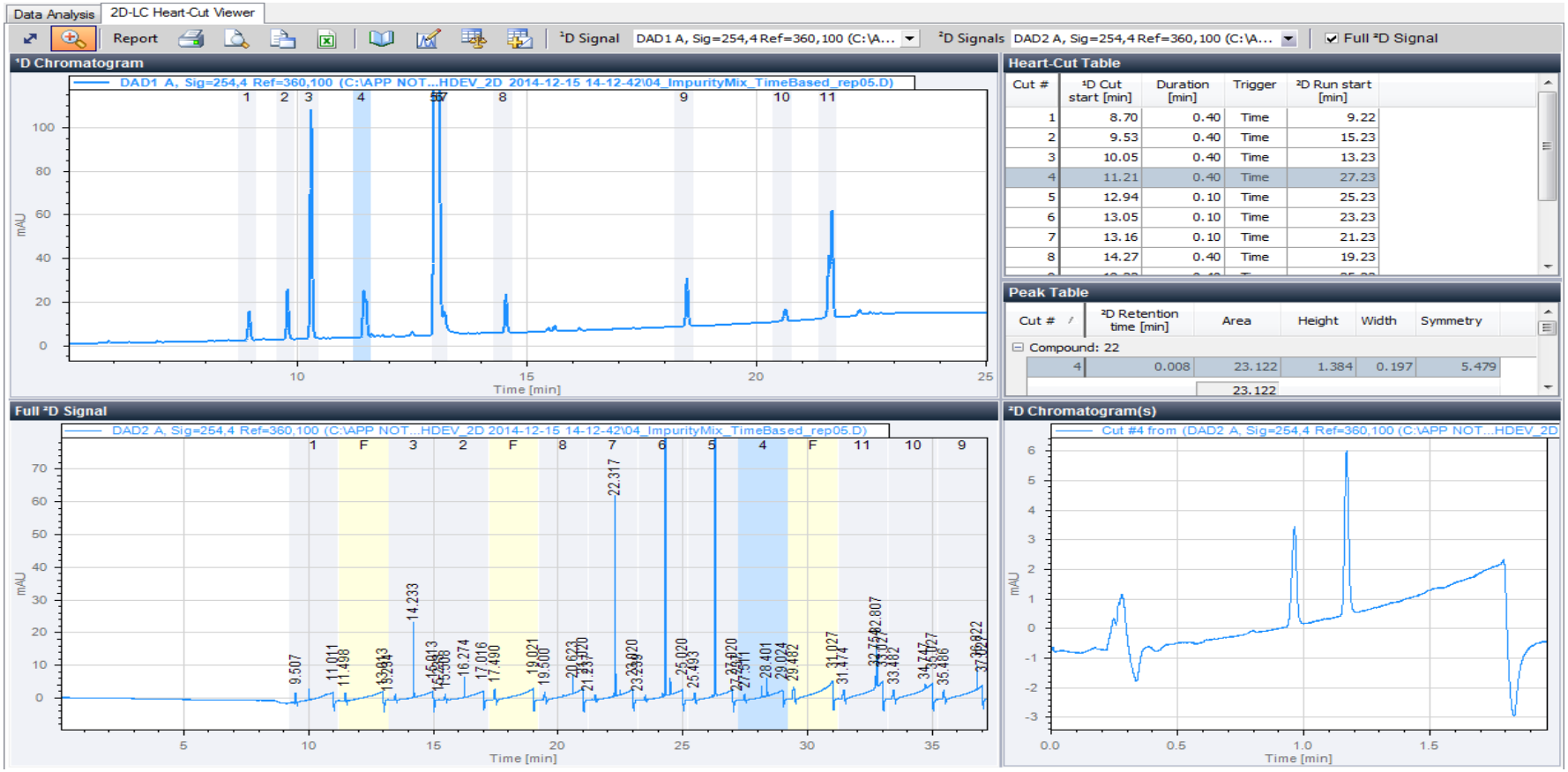


Most intuitive software to set up and edit methods within seconds:

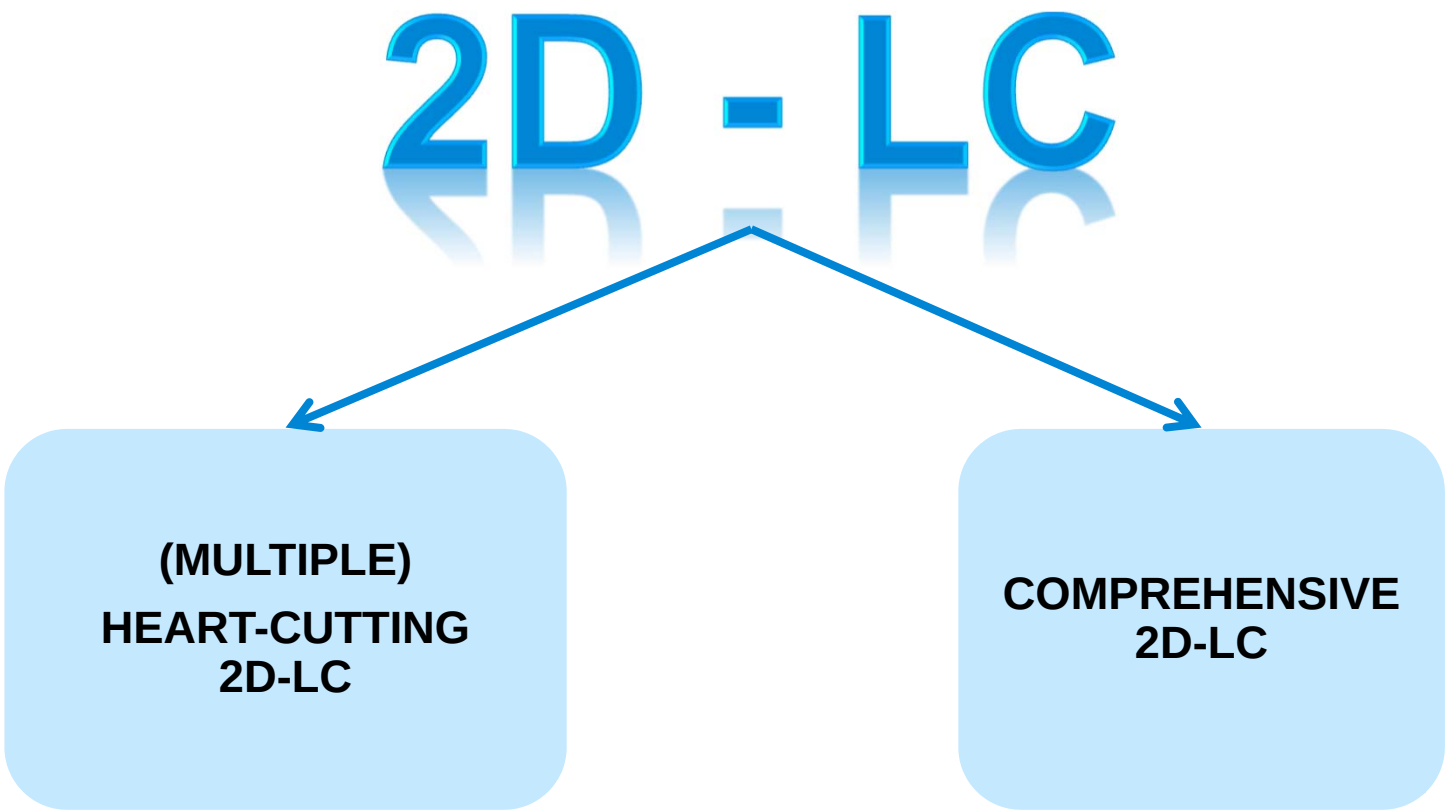
- 1st dimension gradient
- 2nd dimension gradient
- Gradient shift
- Time-segments
- Method parameter
- Method set-up calculator
- Reference chromatogram overlay for heart-cutting
- Time-based or peak-based mode

Data Analysis
(Multiple) Heart-Cutting and High Resolution Sampling

The 2D-LC Heart-Cut Viewer allows straightforward data analysis of (multiple) heart-cutting and High resolution sampling 2D-LC data.

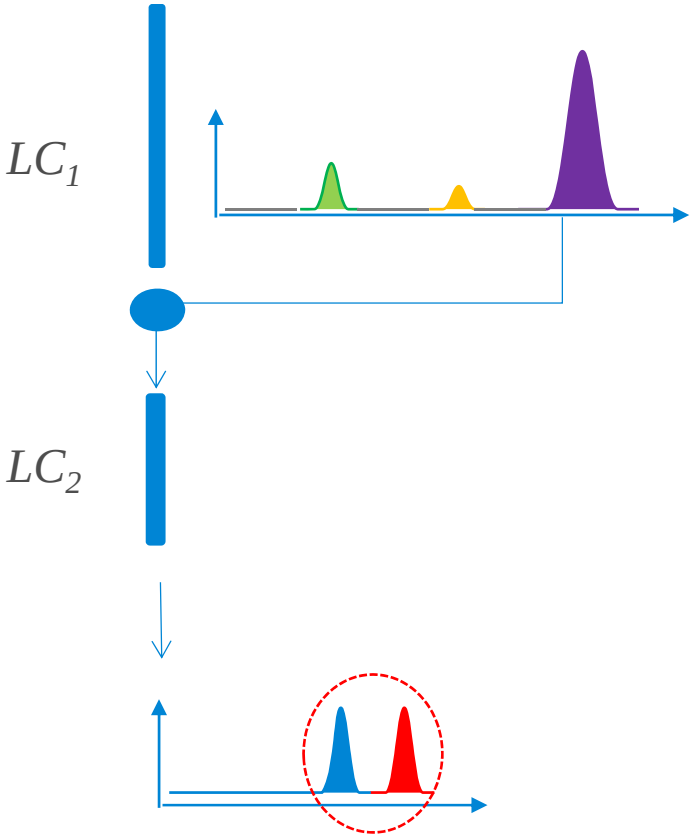


2D-LC Major Types of Operation



2D-LC – Heart-Cutting

Heart-cutting 2D-LC (LC-LC):

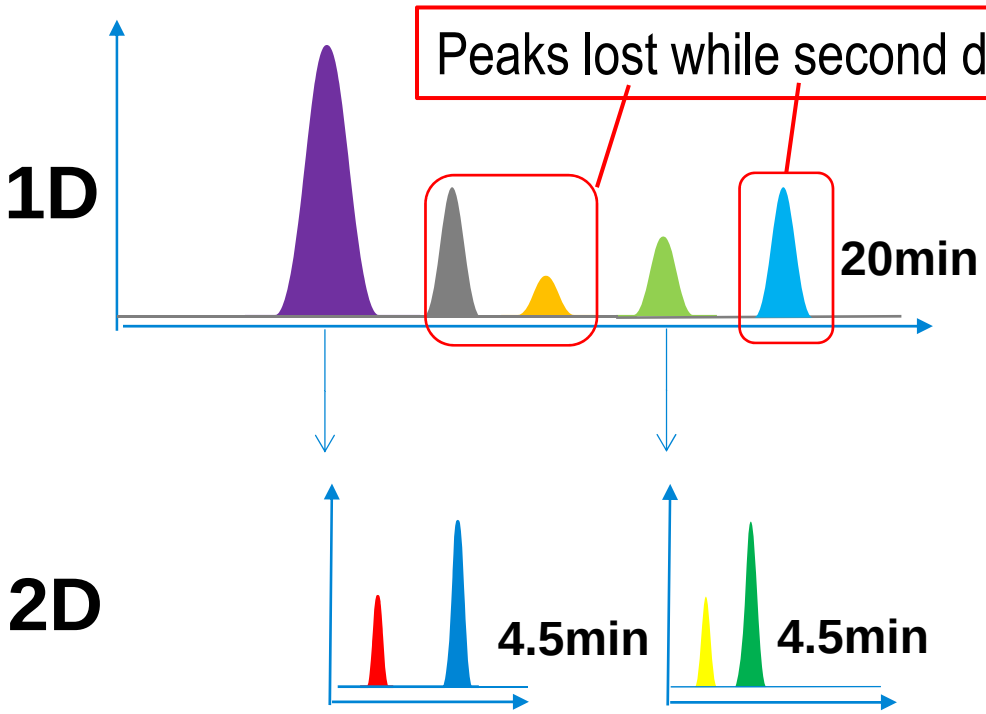


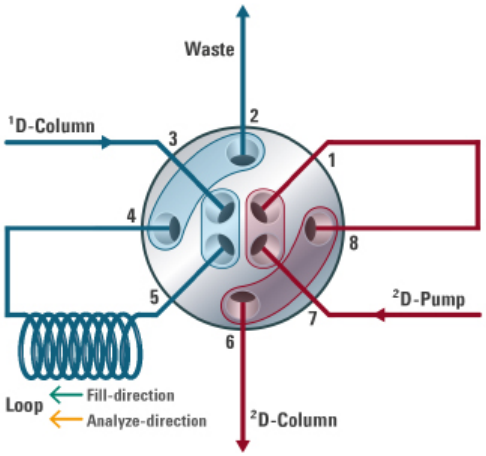
Parts of the 1D effluent are injected onto 2D system

Long 2D gradients possible → good data quality

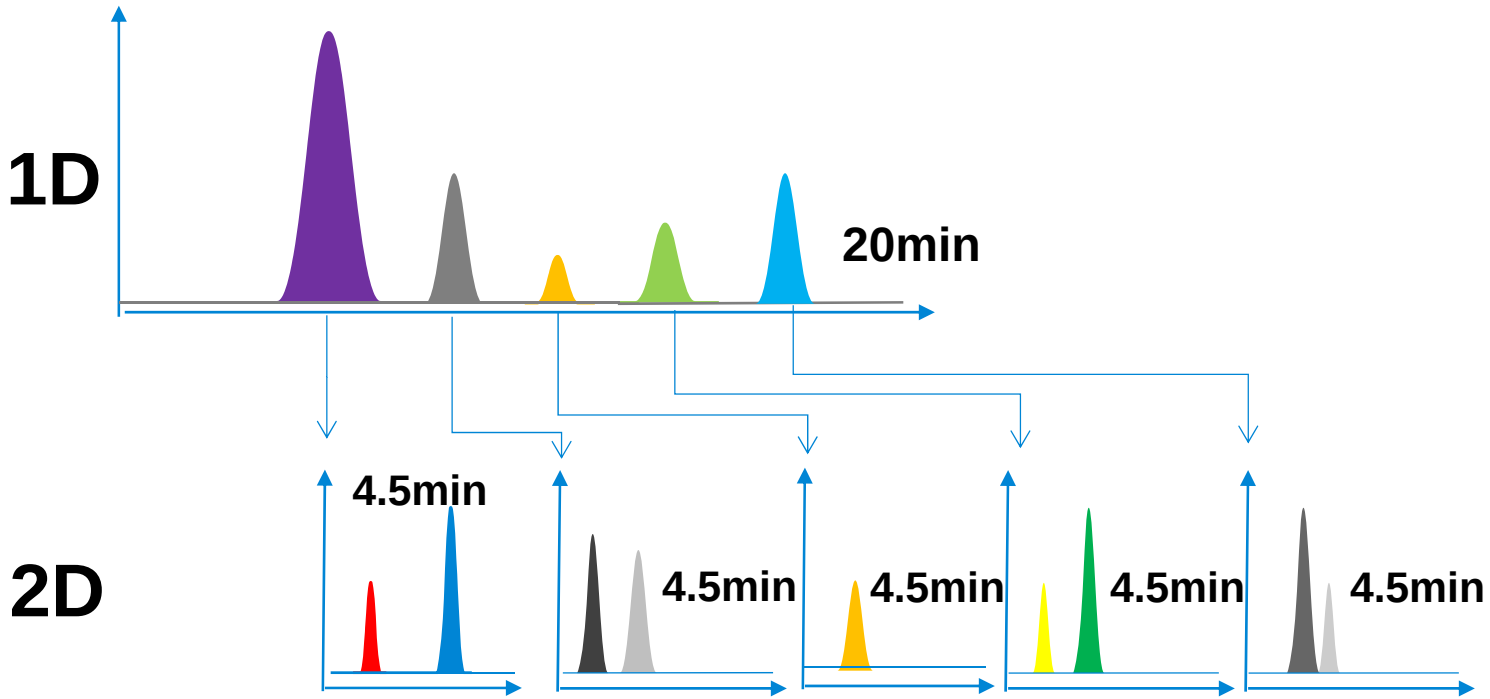
Limited 2D information

Limitation of Heart-Cutting 2D-LC



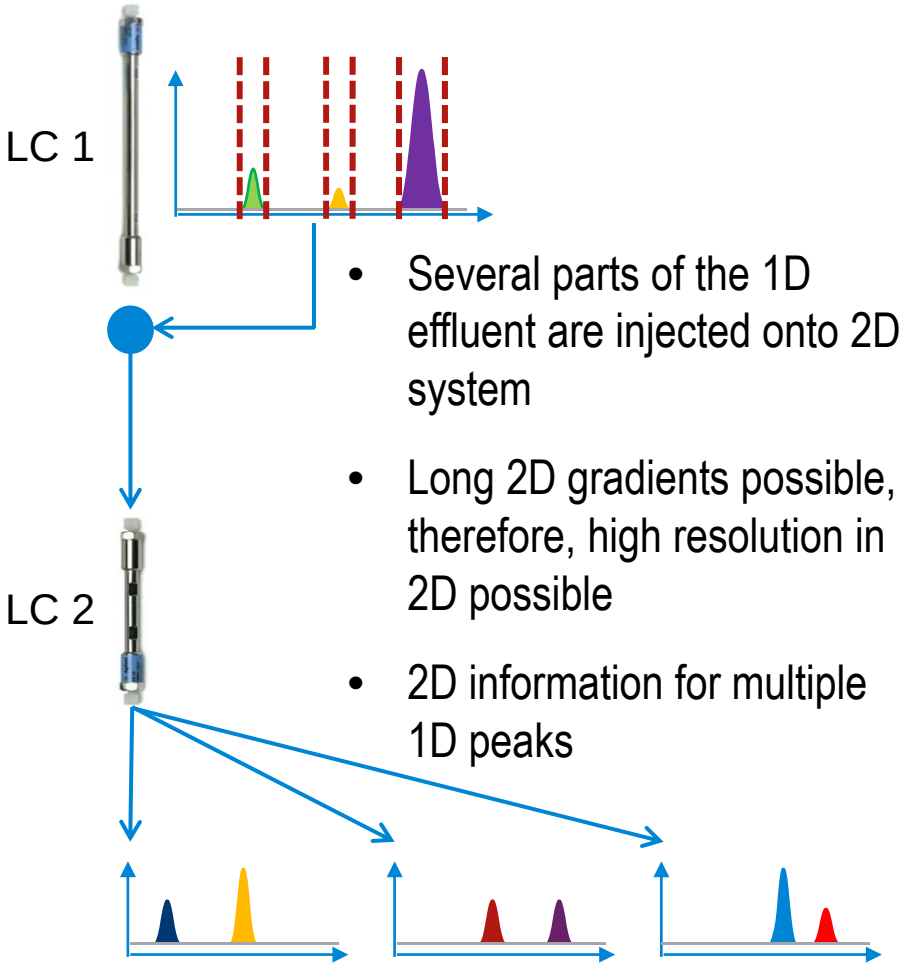


From Single to Multiple Heart-Cutting 2D-LC



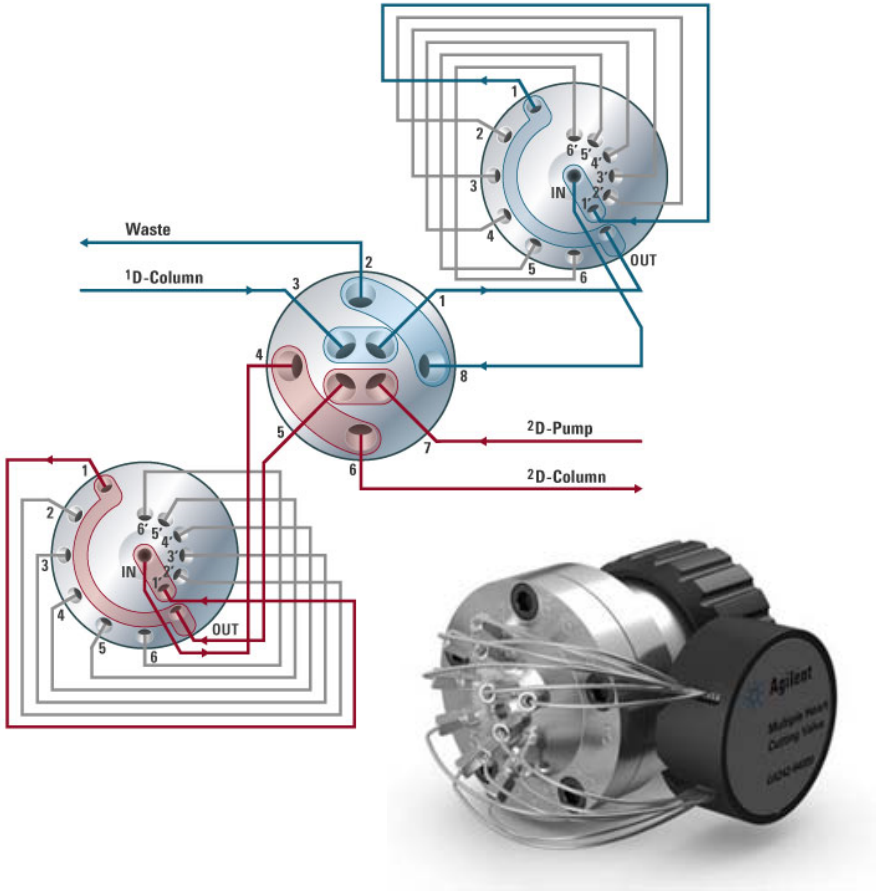
Idea: De-couple the first and second dimensions by intermediately storing peaks eluted from the first dimension for later analysis in the second dimension.

Multiple Heart-Cutting 2D-LC



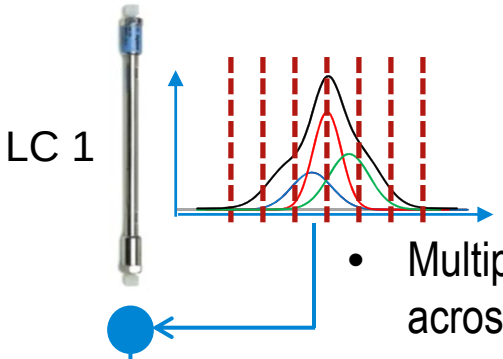
- Several parts of the 1D effluent are injected onto 2D system
- Long 2D gradients possible, therefore, high resolution in 2D possible
- 2D information for multiple 1D peaks

Valve and loop configuration

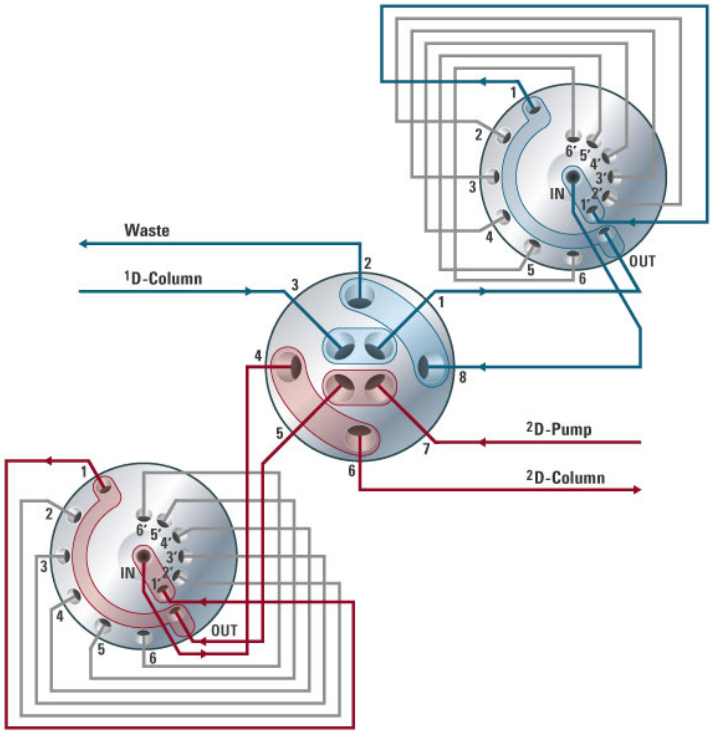


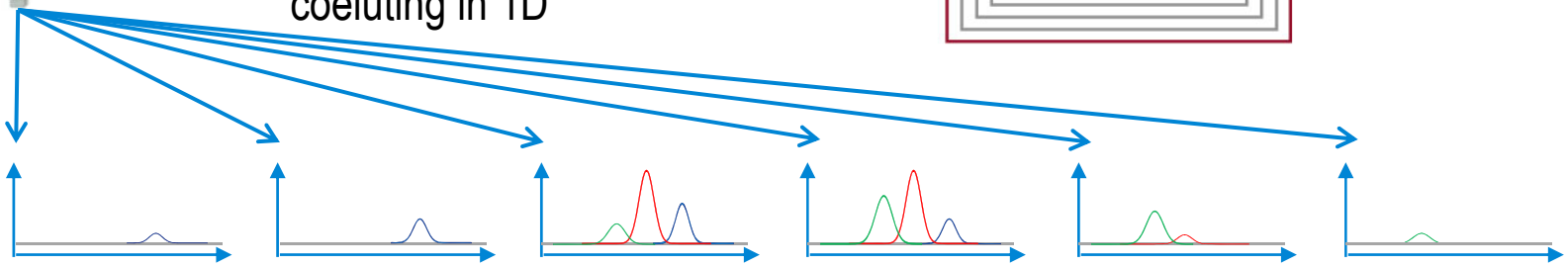
High-Resolution Sampling 2D-LC

Valve and loop configuration

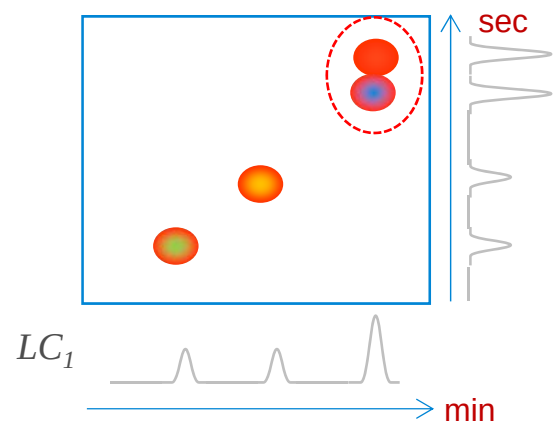


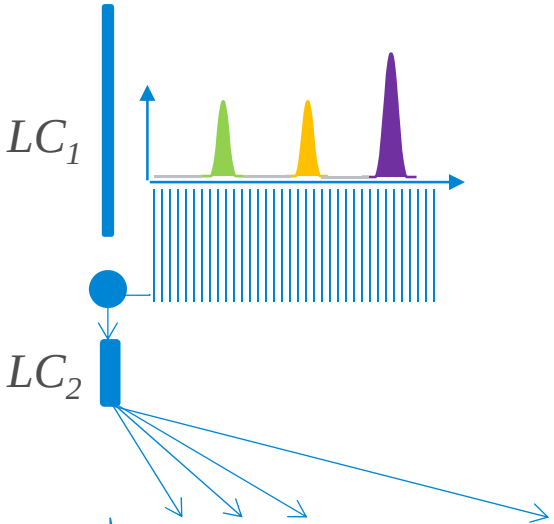
- Multiple consecutive heart-cuts across a 1D peak or region of interest
- Long 2D gradients possible
- Detailed 2D information for a certain 1D peak
- Quantification of compounds coeluting in 1D





Comprehensive 2D-LC

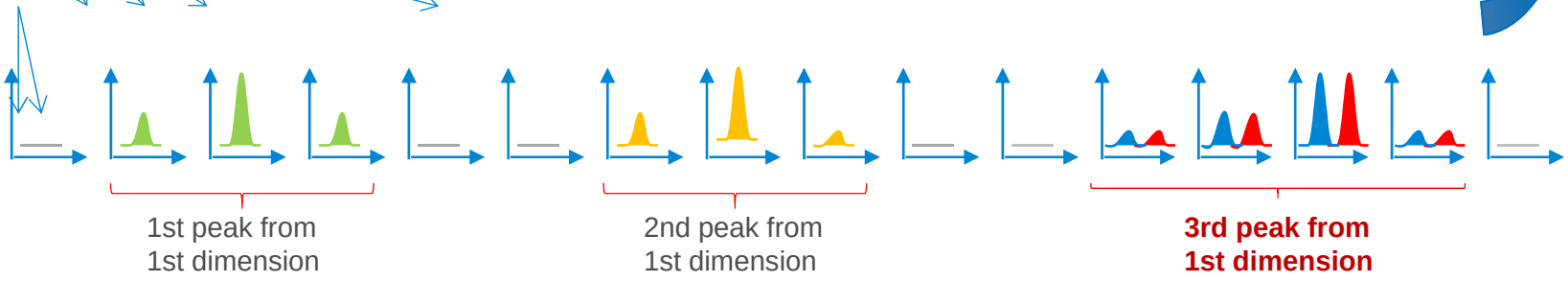




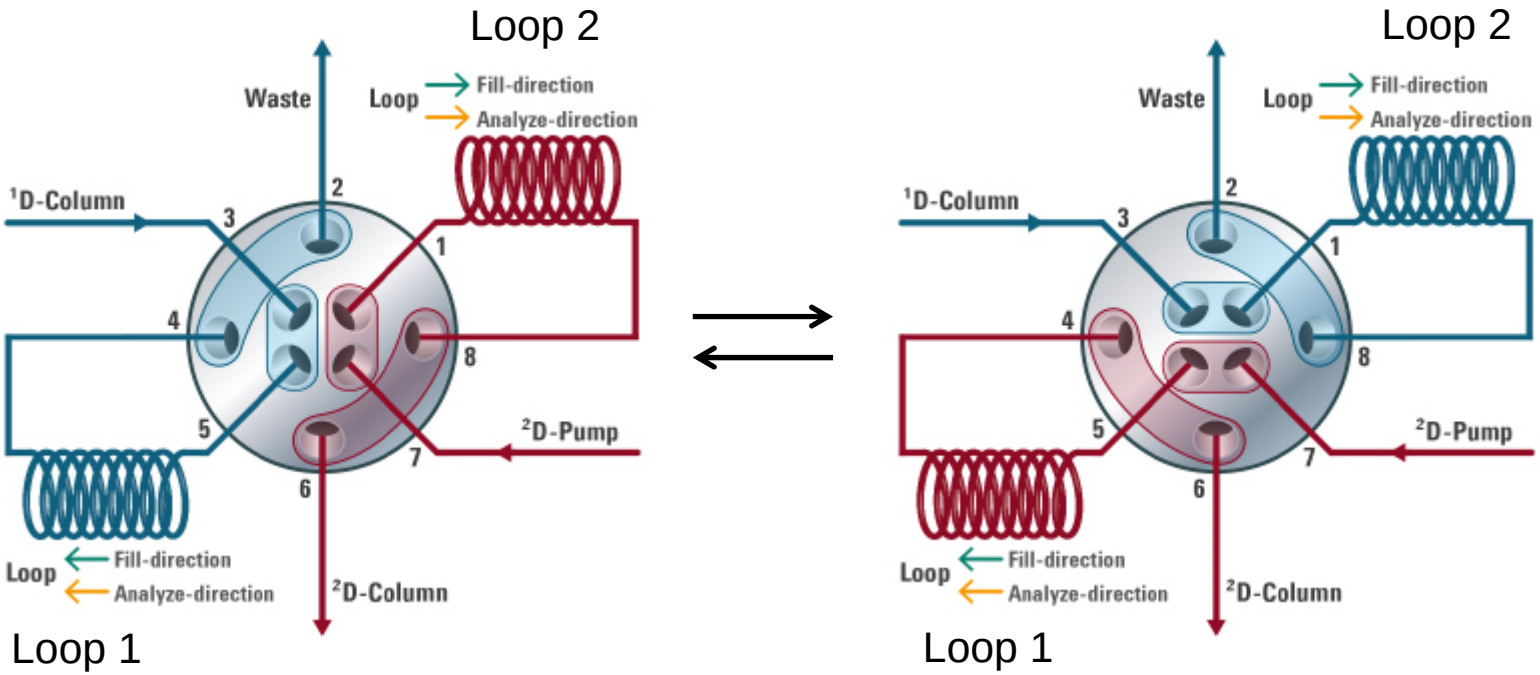
The whole 1D effluent is injected onto 2D system

(Ultra)Short 2D gradients necessary → Good data quality with fast pumps & detector

Full („comprehensive“) 2D information!



Comprehensive 2D-LC
How it works



- **Collection of effluent from the first dimension column in loop 1**
- **Analysis of the content from loop 2**

- **Collection of effluent from the first dimension column in loop 2**
- **Analysis of the content from loop 1**

Applications of Comprehensive 2D-LC

Finger Printing and Profiling Analysis

Analysis of very complex samples

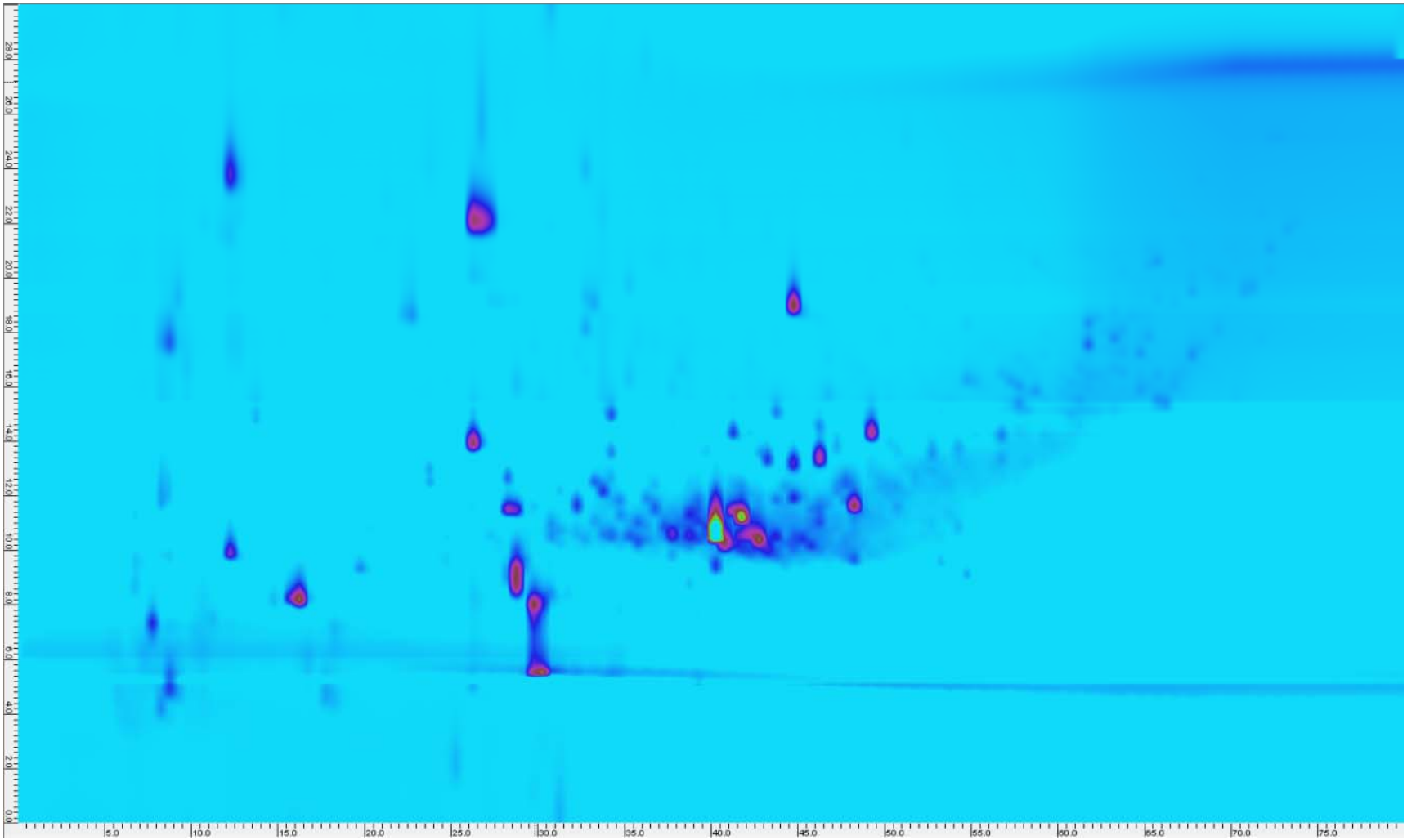
Traditional Chinese Medicine (TCM)/Chinese Herbal Medicine (CHM):

- Holistic healthcare system
- Pharmaceutical efficacy regarded to depend on synergistic effects of multiple components of the plants

Biopharmaceutical analysis:

- Peptide maps
- Complex glycan pattern

Comprehensive 2D-LC –Data View



Case Studies
Online 2DLC of Monoclonal Antibodies

- Monoclonal Antibody Digests HILIC × RPLC-MS
  → Focus: Peak capacity

- Characterization of Monoclonal Antibodies Protein A x WCX
  → Focus: Combination of two different workflows

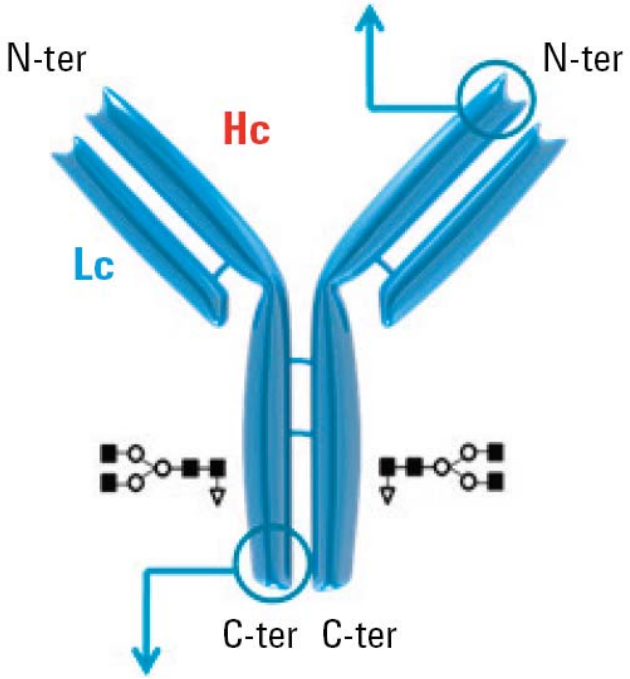
- Characterization of Monoclonal Antibodies SEC x WCX
  → Focus: Combination of two different workflows

- Characterization of Charge Variants WCX x RP
  → Focus: Desalting after IEX prior to MS

Case Study 1 Comprehensive 2D-LC
Orthogonal Separations of mAb Tryptic Digest

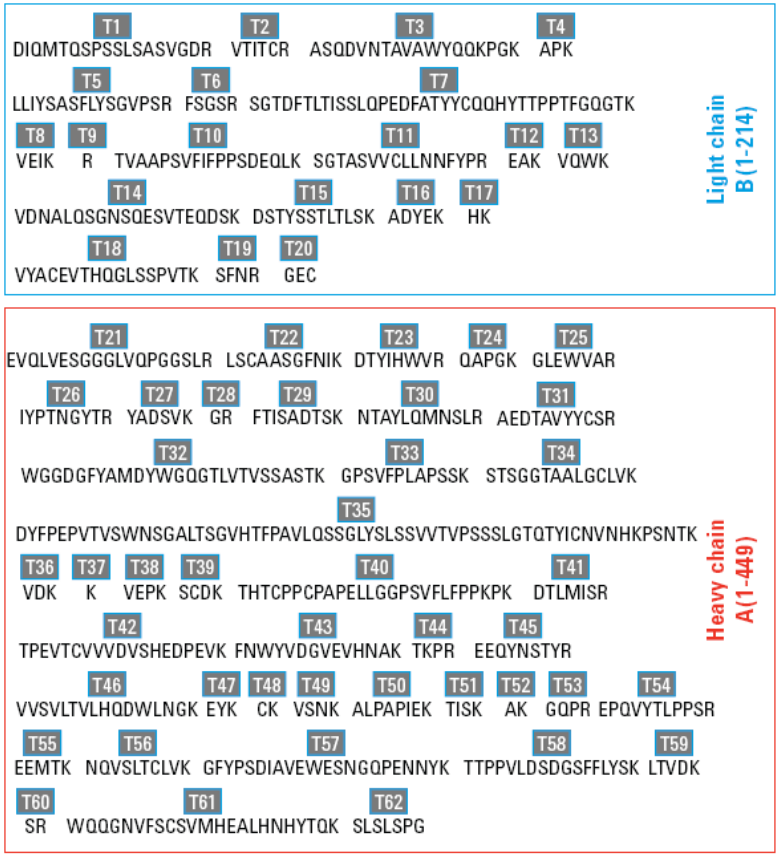
- Aggregation Studies
- Charge Variant Analysis
- Peptide Mapping
- Glycan Profiling
- Titer Analysis
- And others…

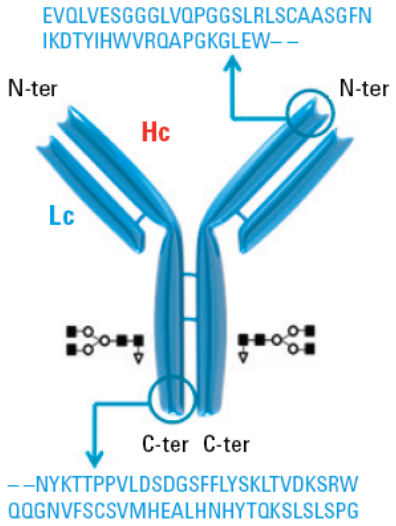
Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution



Focus: High Peak Capacity

Structure and Amino Acid Sequence of Trastuzumab

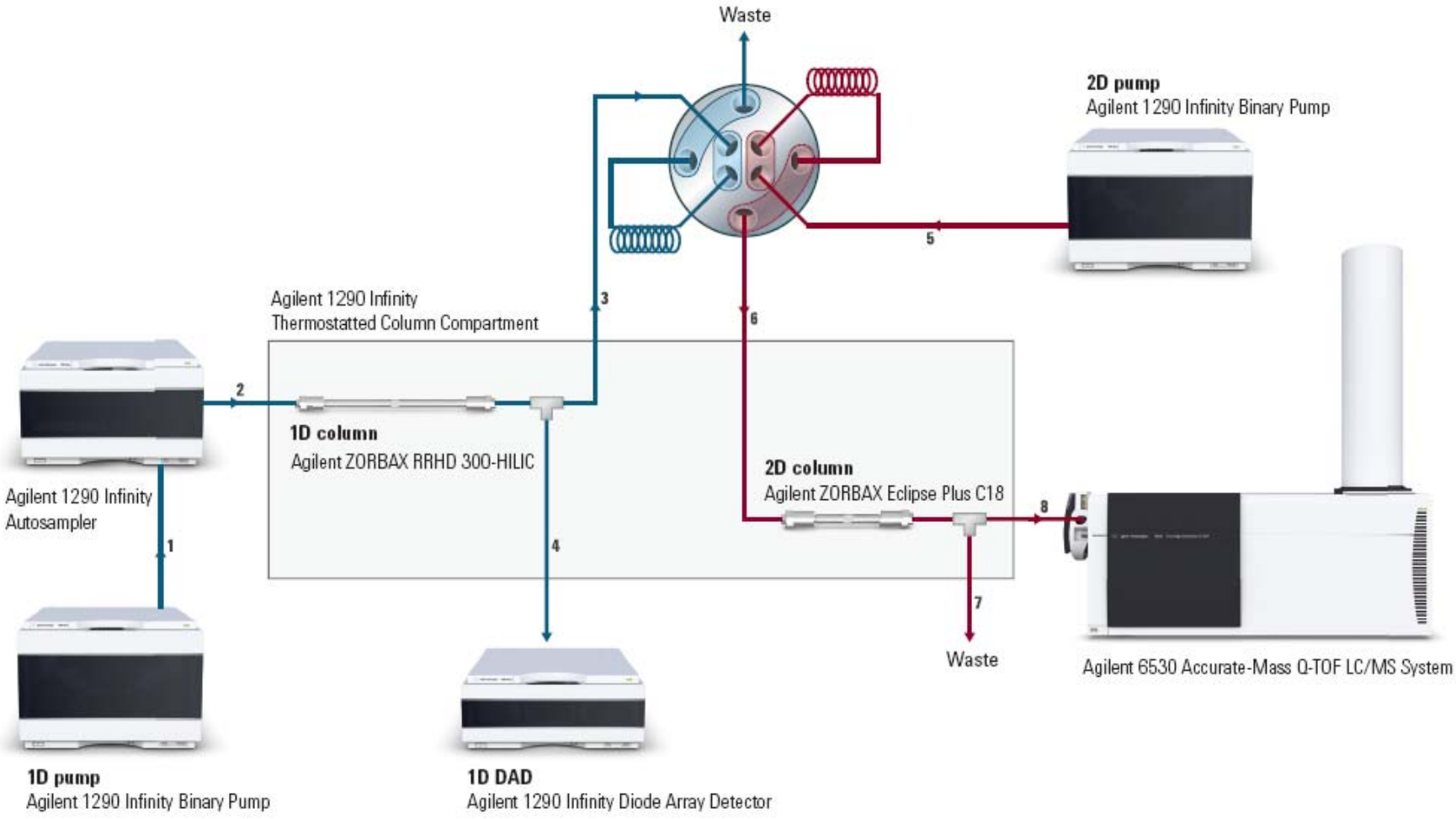




- 62 identity peptides
- Modifications
- Incomplete and aspecific cleavages
- ...

> 100 peptides

Instrument Setup



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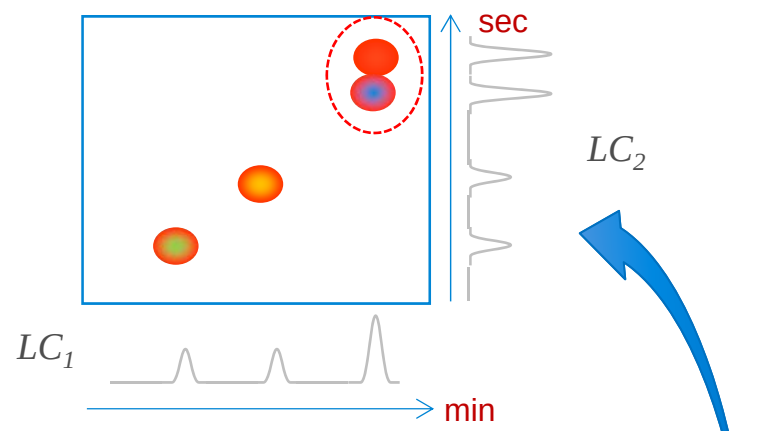
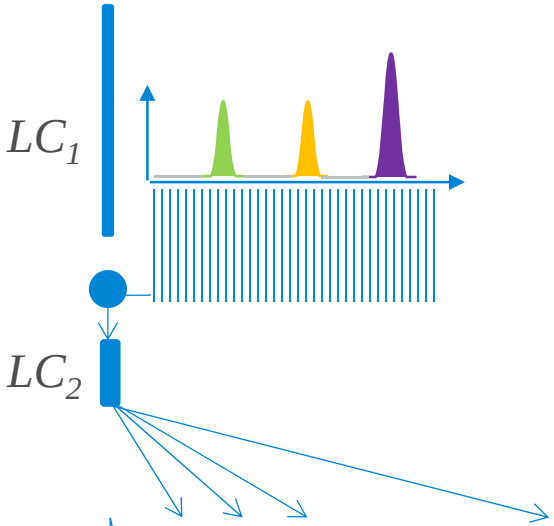
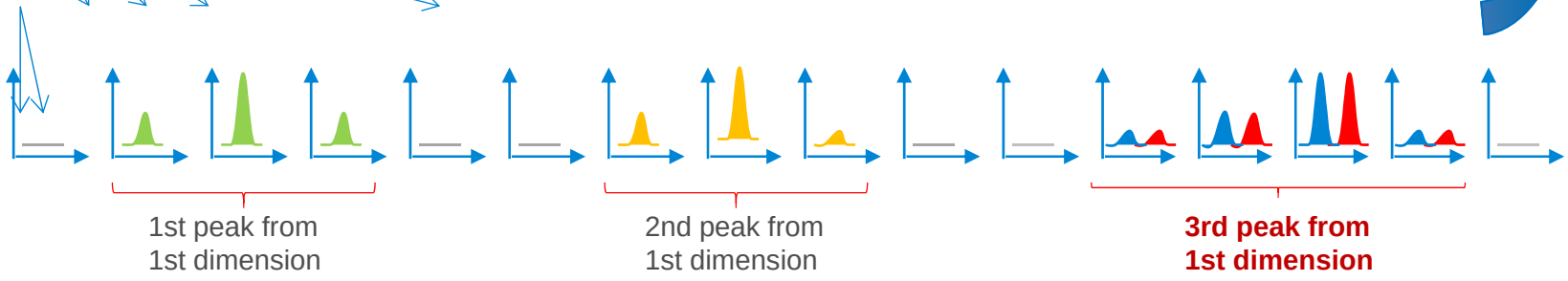




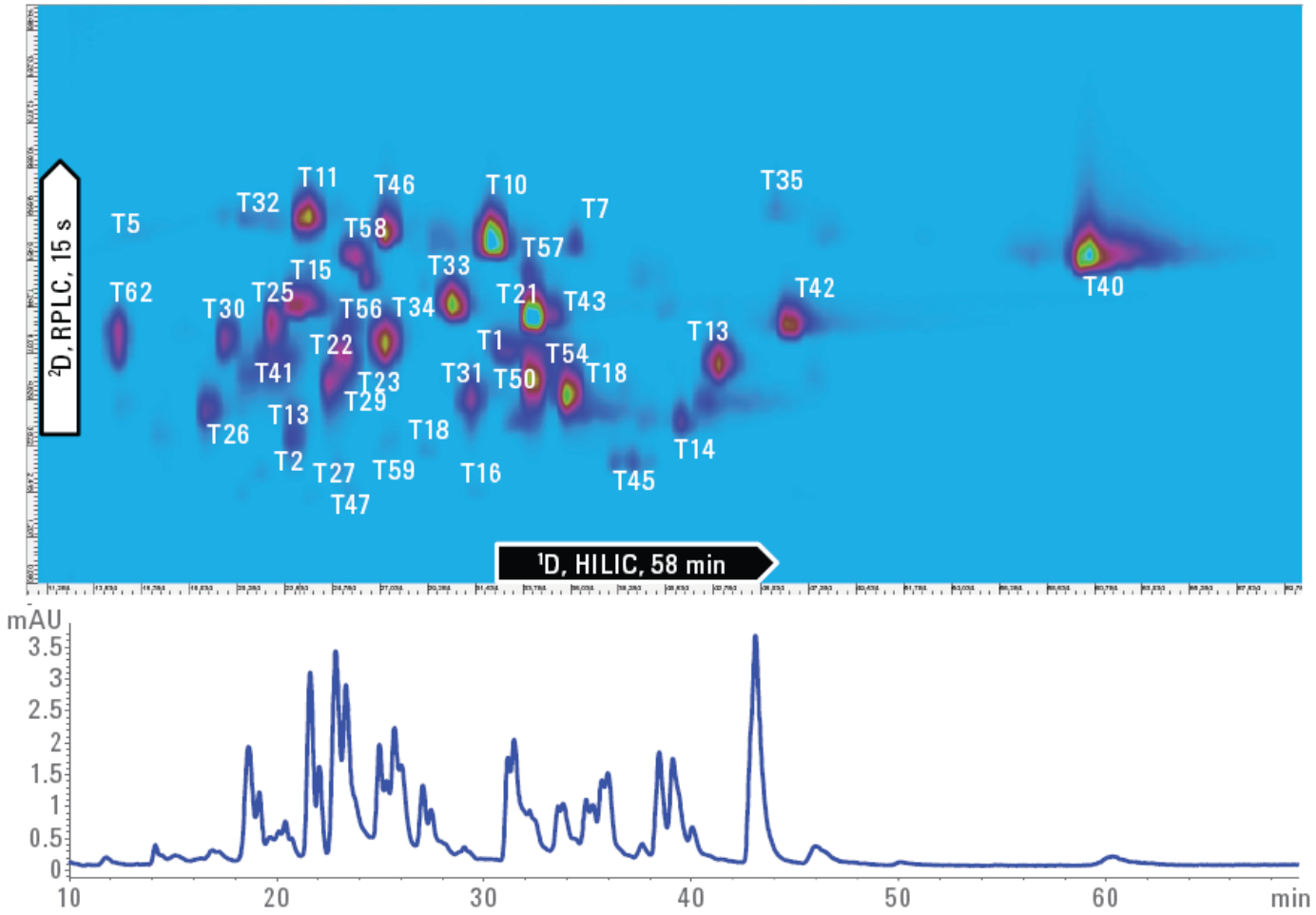
The whole 1D effluent is injected onto 2D system

(Ultra)Short 2D gradients necessary → Good data quality with fast pumps & detector

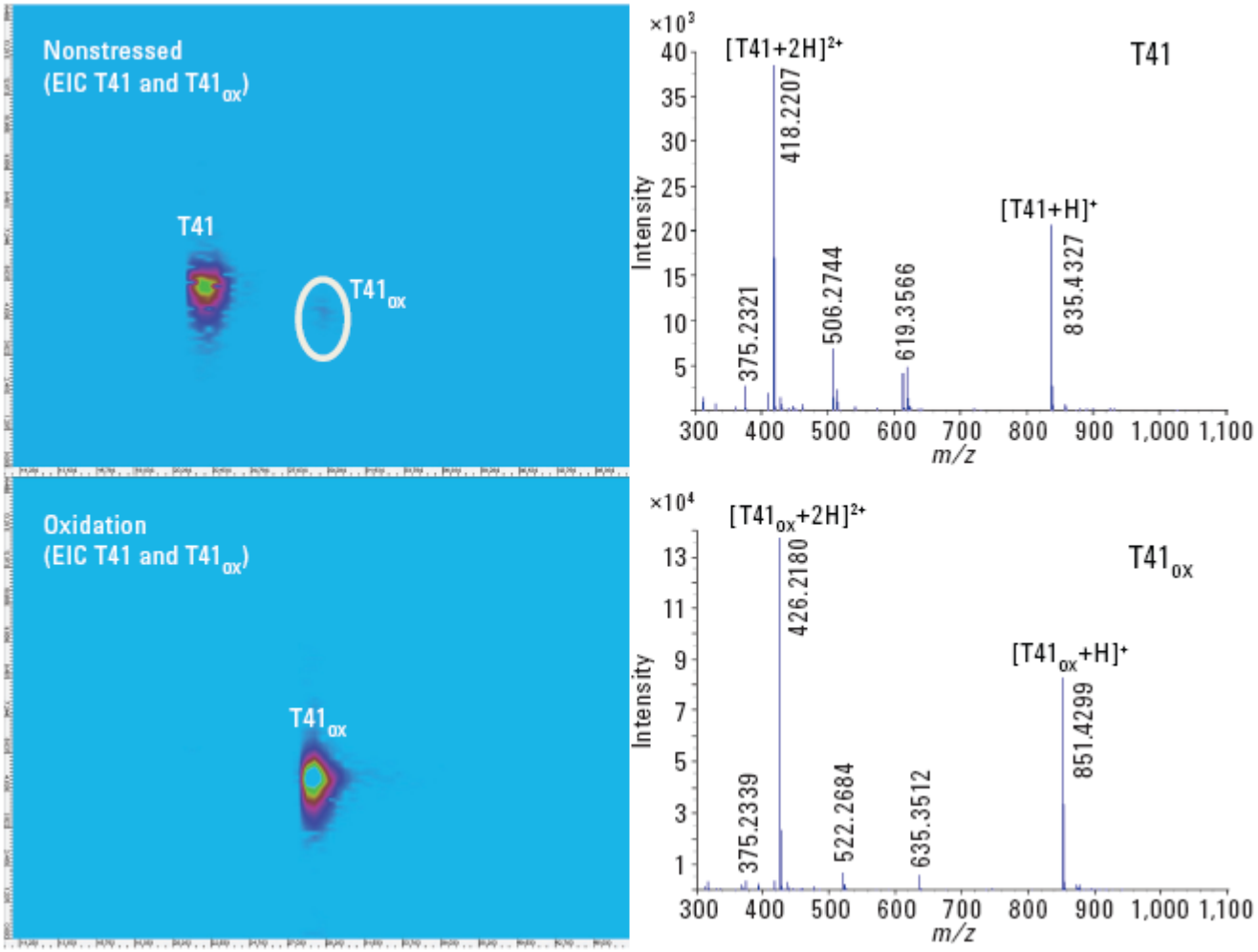
Full („comprehensive“) 2D information!



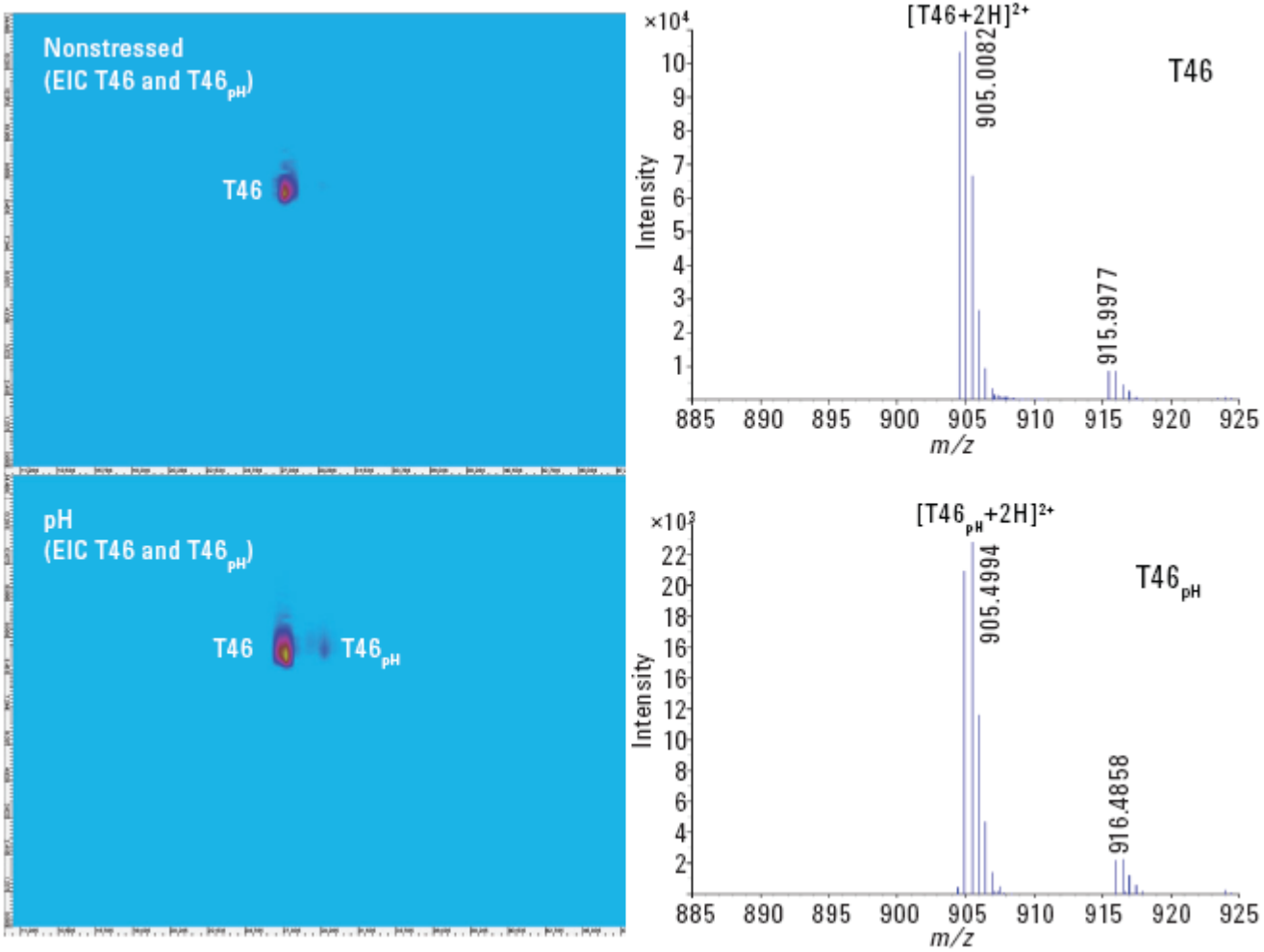
2D-LC Contour Plot



2D-LC Trastuzumab Oxidation



2D-LC Trastuzumab Degradation



Comprehensive 2D-LC mAb Tryptic Digest Summary

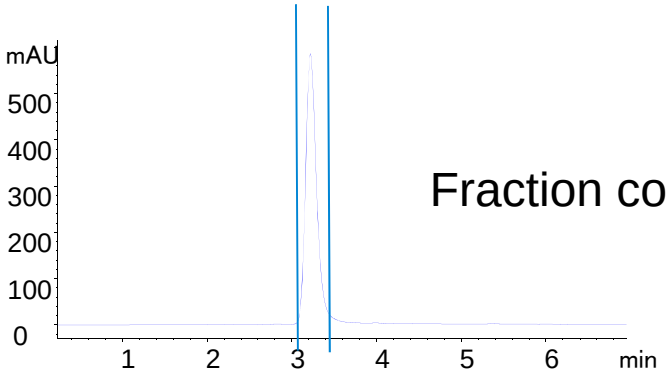
- Higher peak capacity with 2D-LC
- Improved separation using othogonal column combination: HILIC x RP
- Proof of concept with the clear differentiation of stressed and non-stressed antibody samples
- Ideal combination of Agilent 1290 Infinity 2D-LC Solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS

Case Study 2 Combining Two Techniques Protein A and WCX

- Aggregation Studies
- Charge Variant Analysis
- Peptide Mapping
- Glycan Profiling
- Titer Analysis
- And others…

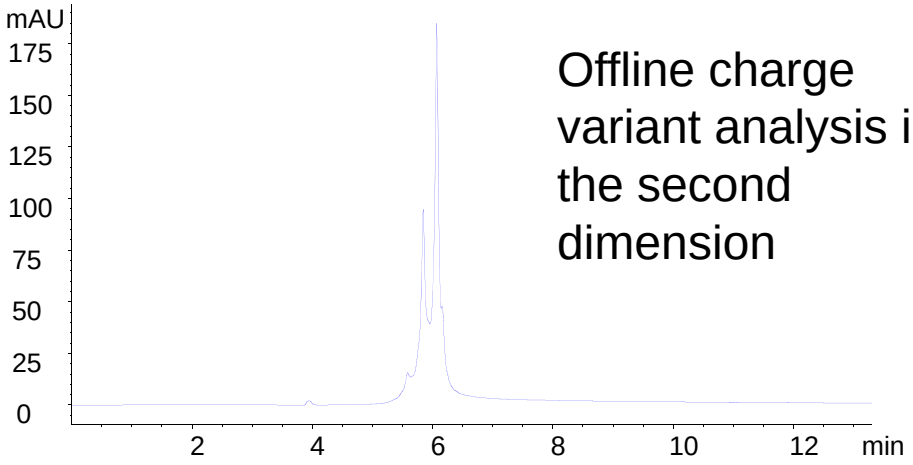
Combination of Two Analyses

Offline Approach



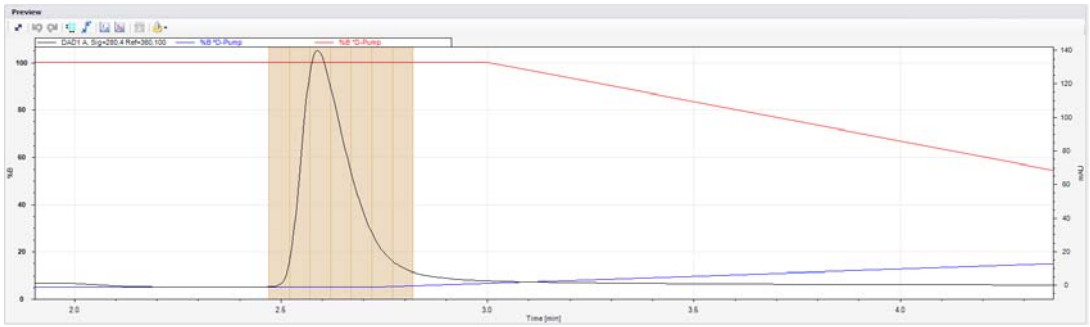
Fraction collection after first dimension

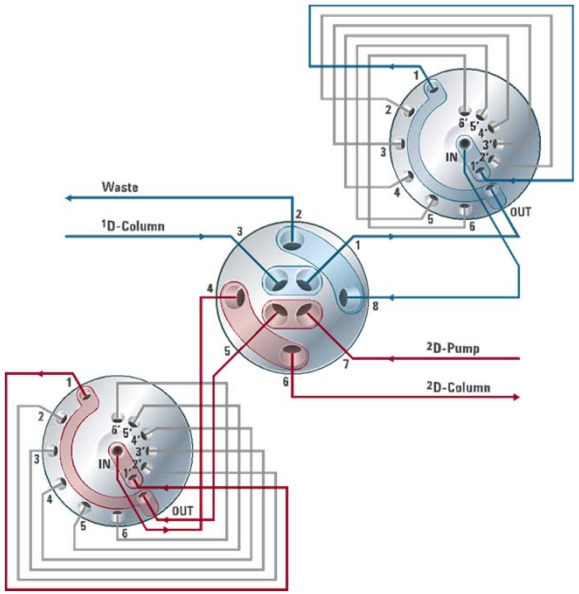
Requires hands-on time!



Offline charge variant analysis in the second dimension

Characterization of Monoclonal Antibodies
Protein A and Weak Cation Exchange Chromatography

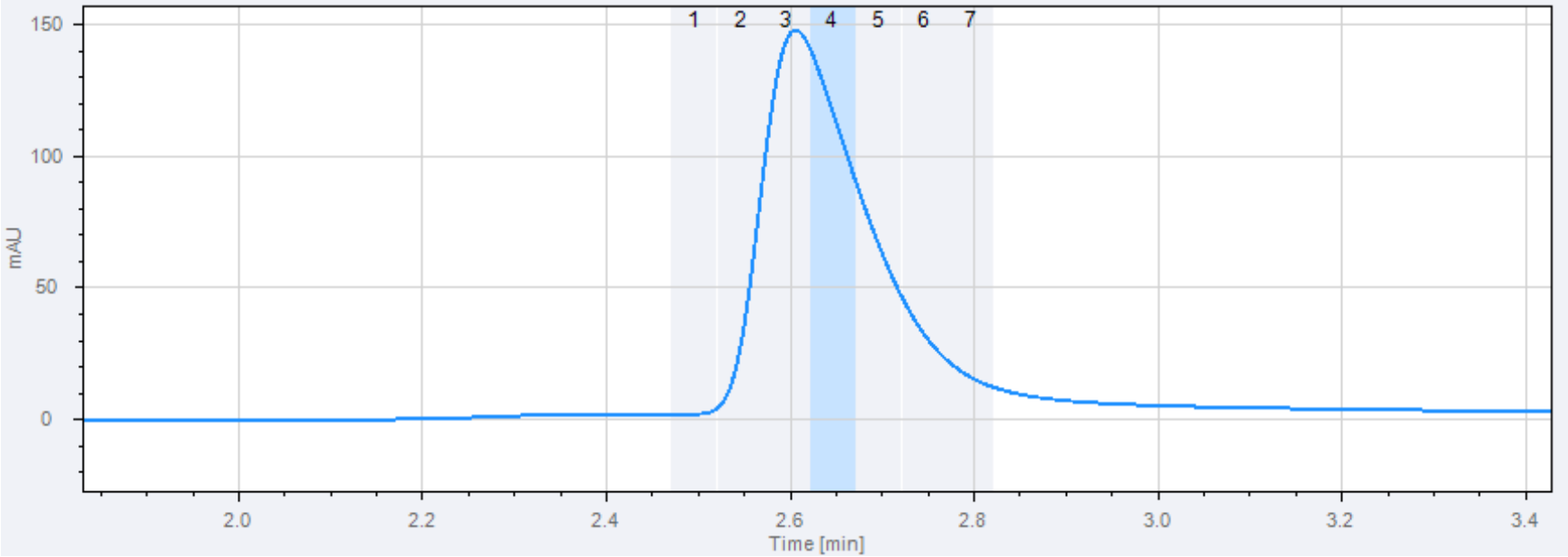




High Resolution Multiple Heart Cuts

Focus: Combination of two important Quality Attribute Analyses

2D-LC with High Resolution Sampling
Protein A Titer Analysis (First Dimension)



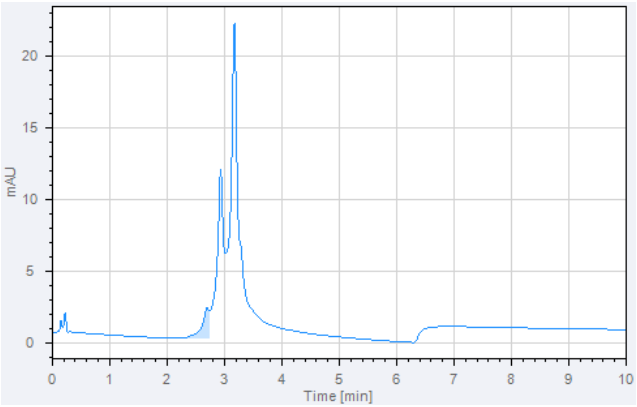
Consecutive Cuts Across the Peak

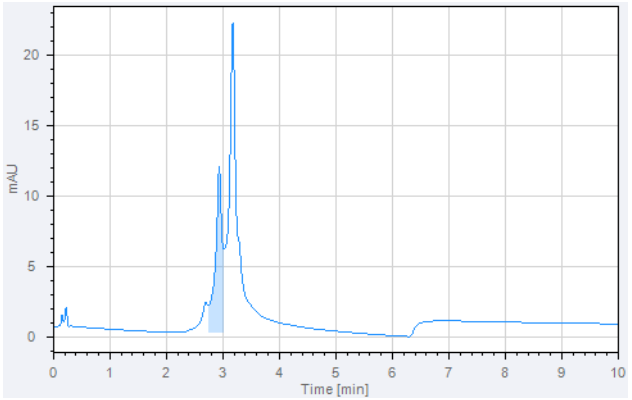
2D-LC with High Resolution Sampling

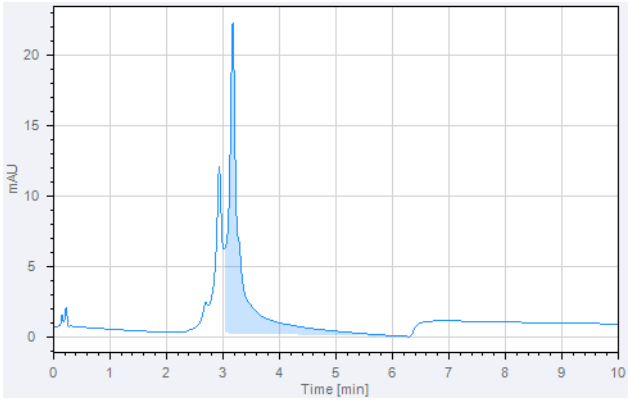
Weak Cation Exchange Chromatography (Second Dimension)

Peak table (²D)

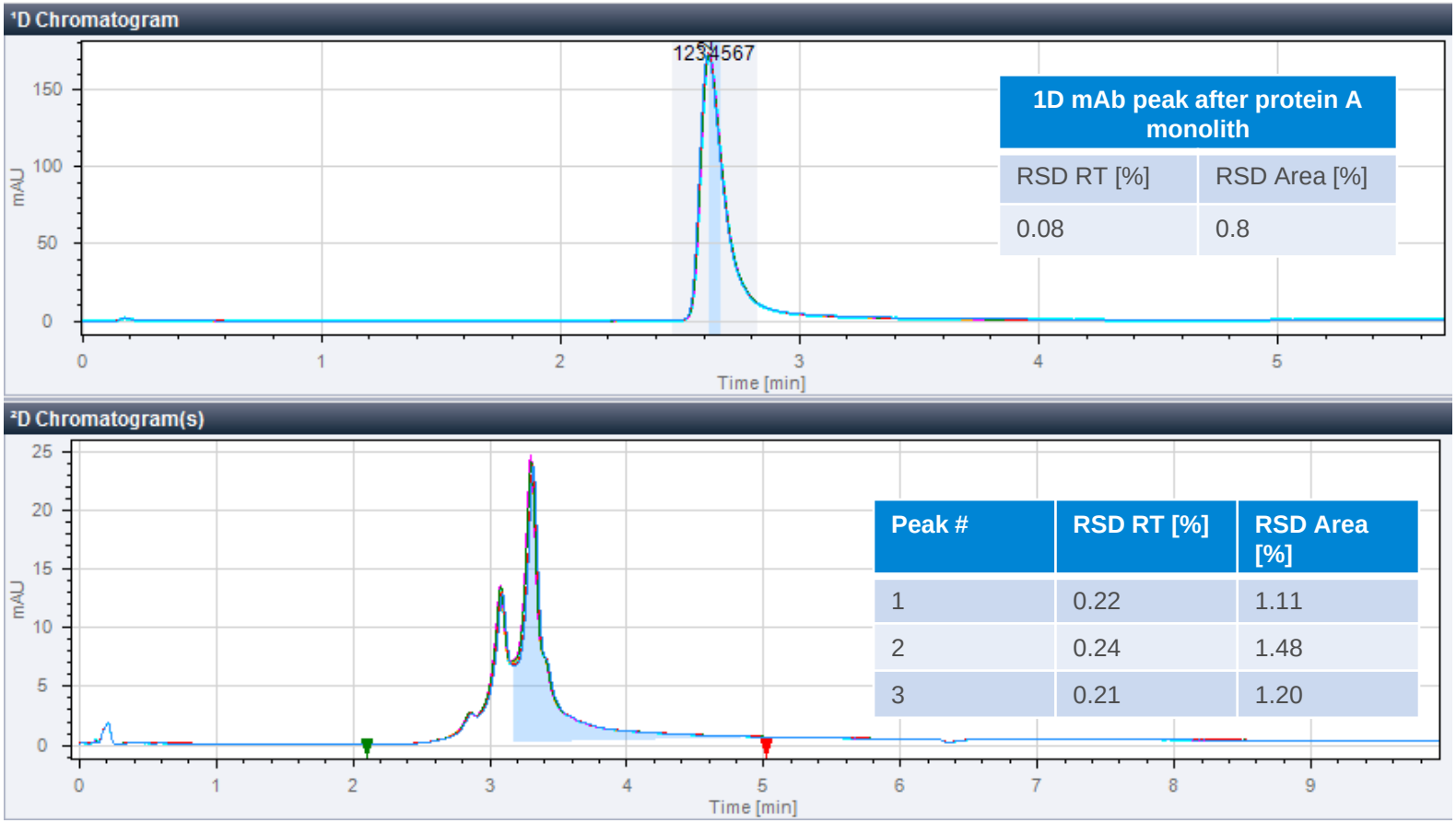
Cut #	²D Retention time [min]	Area	Height	Width	Symmetry
Compound: 3					
4	2.946	78.259	9.293	0.115	1.494
		78.259			
Compound: 4					
4	3.184	250.323	17.263	0.188	0.424
		250.323			
Compound: 5					
4	2.706	16.746	1.827	0.123	2.747
		16.746			







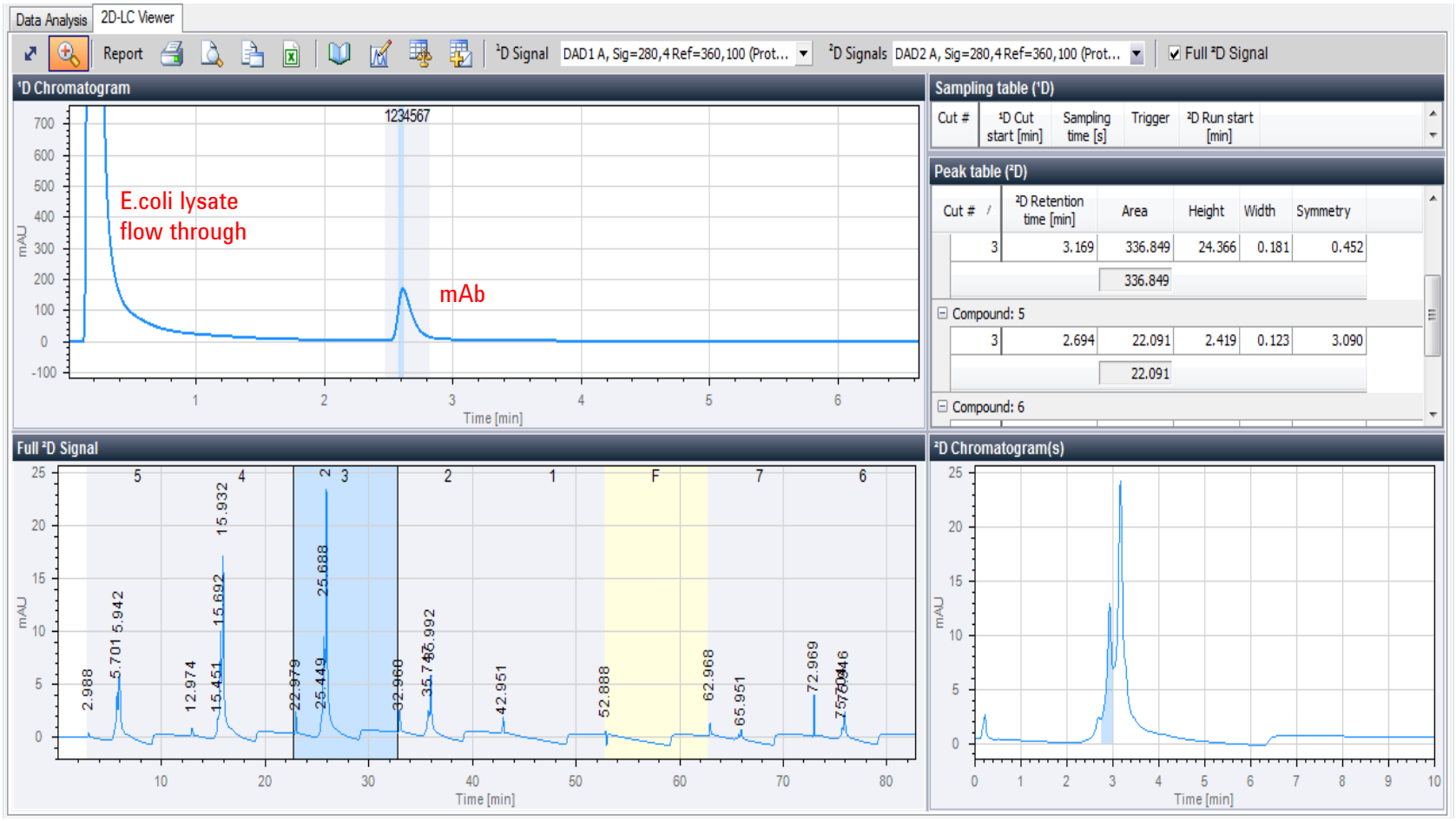
Precision of Retention Time and Area



1D mAb peak after protein A monolith	
RSD RT [%]	RSD Area [%]
0.08	0.8

Peak #	RSD RT [%]	RSD Area [%]
1	0.22	1.11
2	0.24	1.48
3	0.21	1.20

Mimicking a Cell Lysate mAb Process Control Sample



High Resolution Multiple Heart Cuts Protein A - WCX Summary

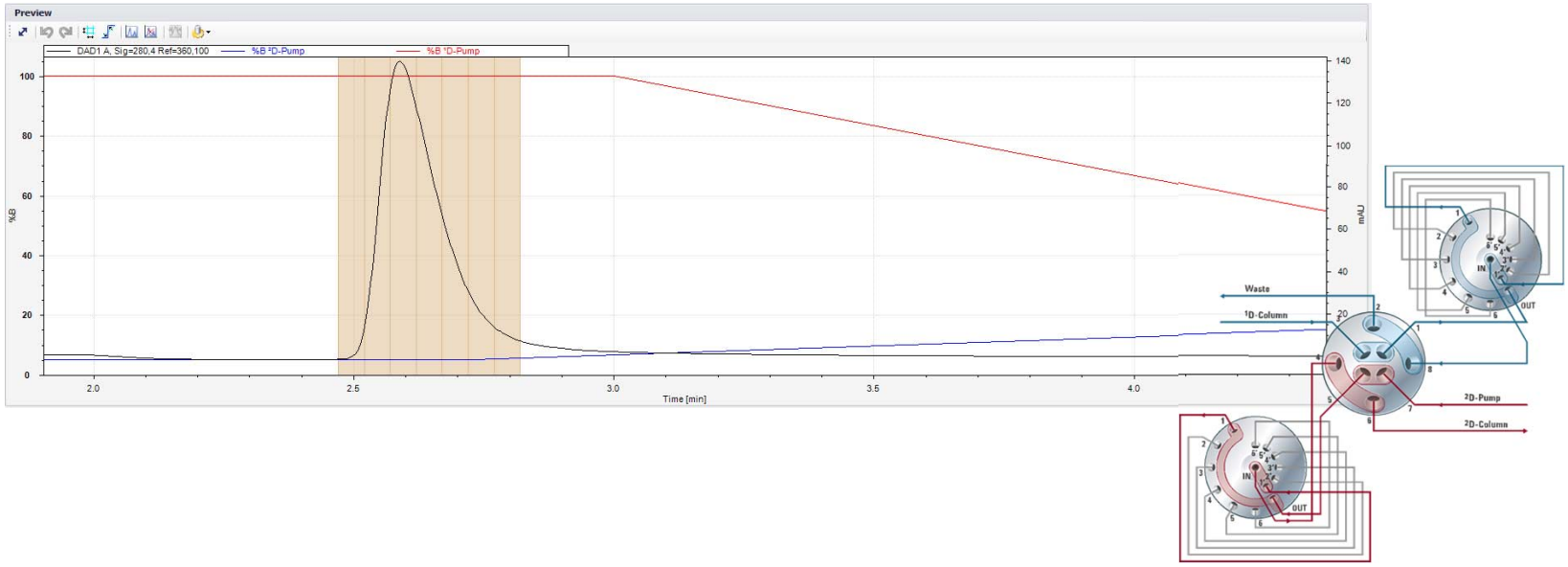
- Fully automated combination of two different quality attribute analyses

→ Protein A titer analysis and charge variants

- No hands-on time required between the two analyses
- High precision of retention time and area found in both dimensions
- Also suitable for process control samples

Characterization of Monoclonal Antibodies

Size Exclusion and Weak Cation Exchange Chromatography

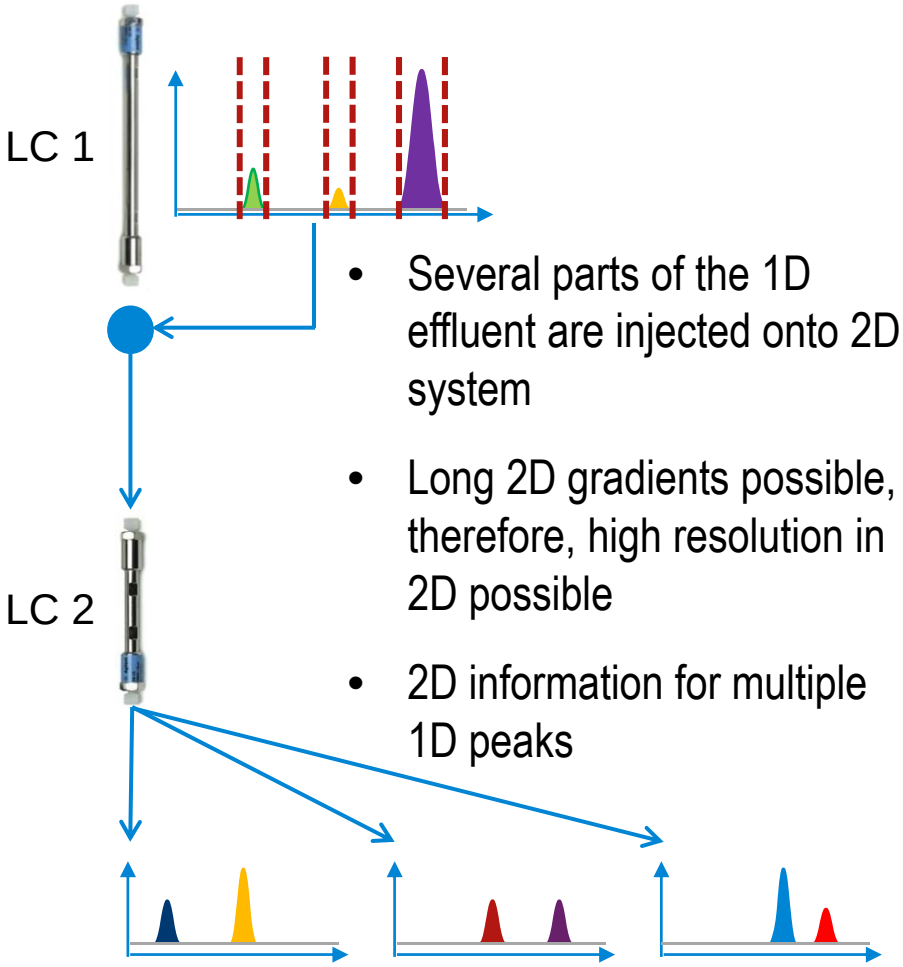


Focus: Combination of two important Quality Attribute Analyses

Case Study 3 Combining Two Techniques SEC and WCX

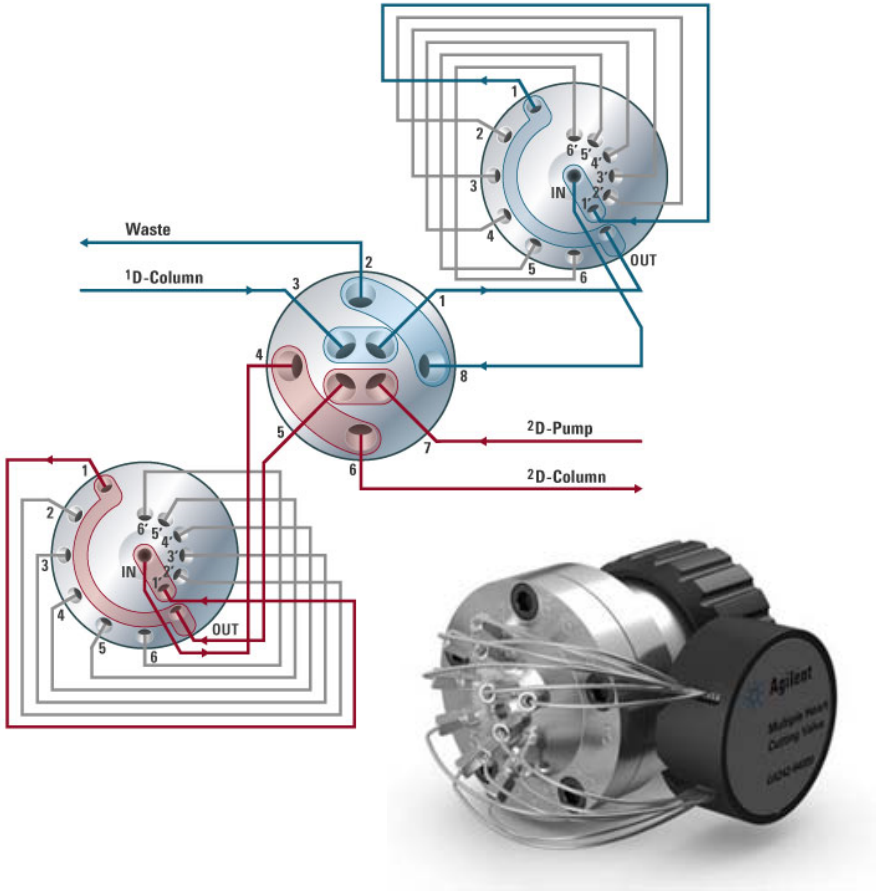
- Aggregation Studies
- Charge Variant Analysis
- Peptide Mapping
- Glycan Profiling
- Titer Analysis
- And others…

Multiple Heart-Cutting 2D-LC

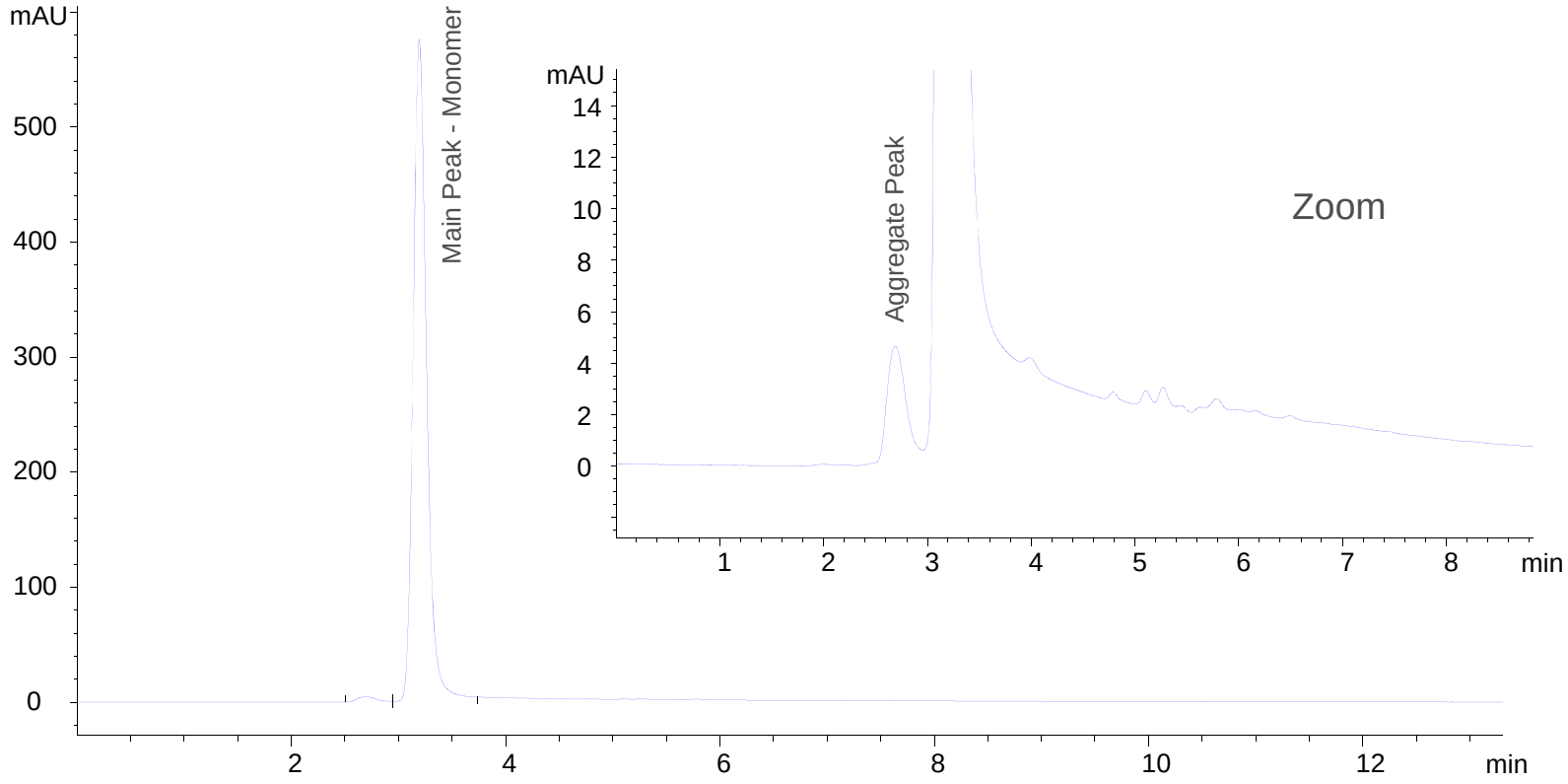


- Several parts of the 1D effluent are injected onto 2D system
- Long 2D gradients possible, therefore, high resolution in 2D possible
- 2D information for multiple 1D peaks

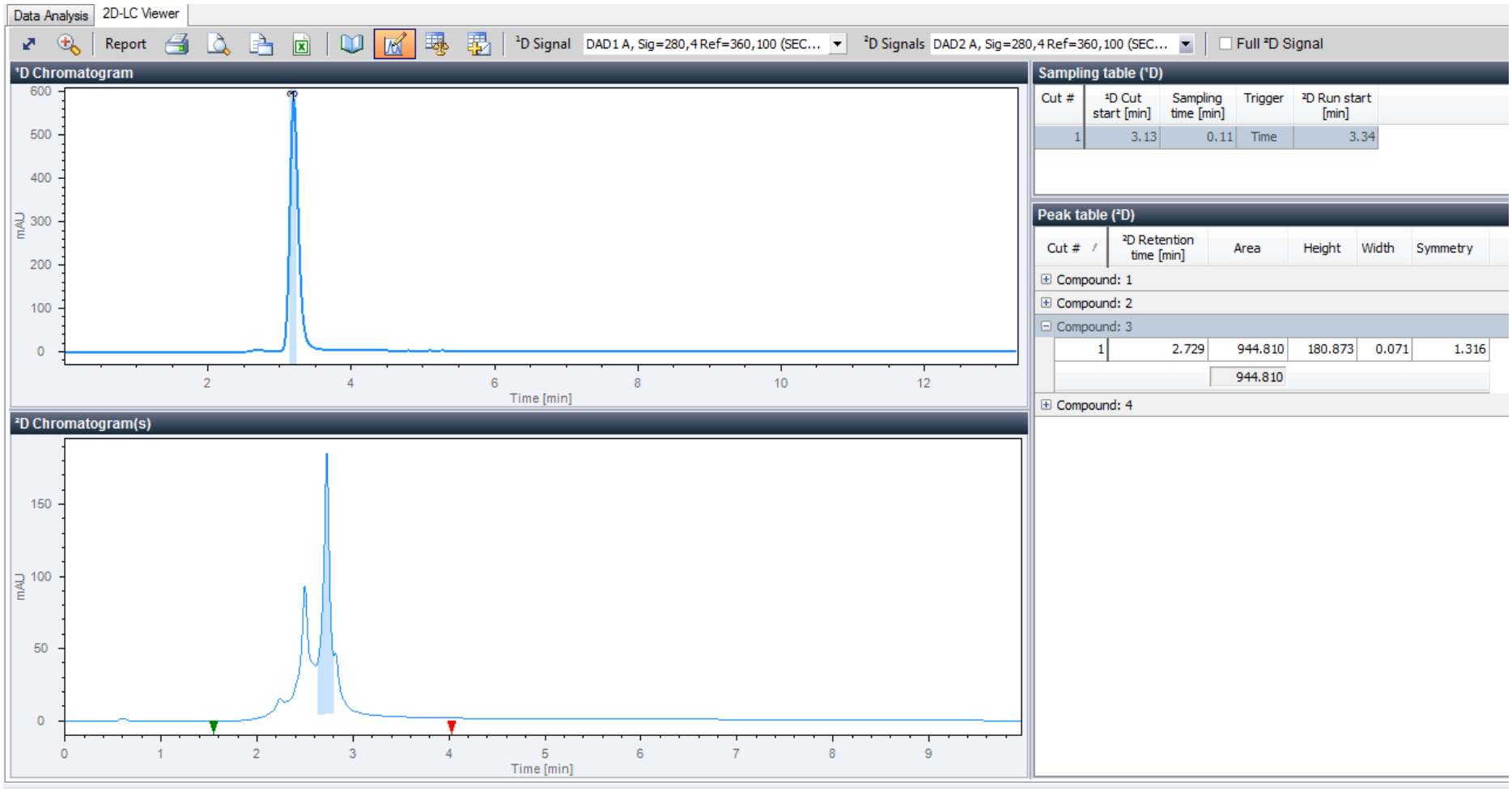
Valve and loop configuration



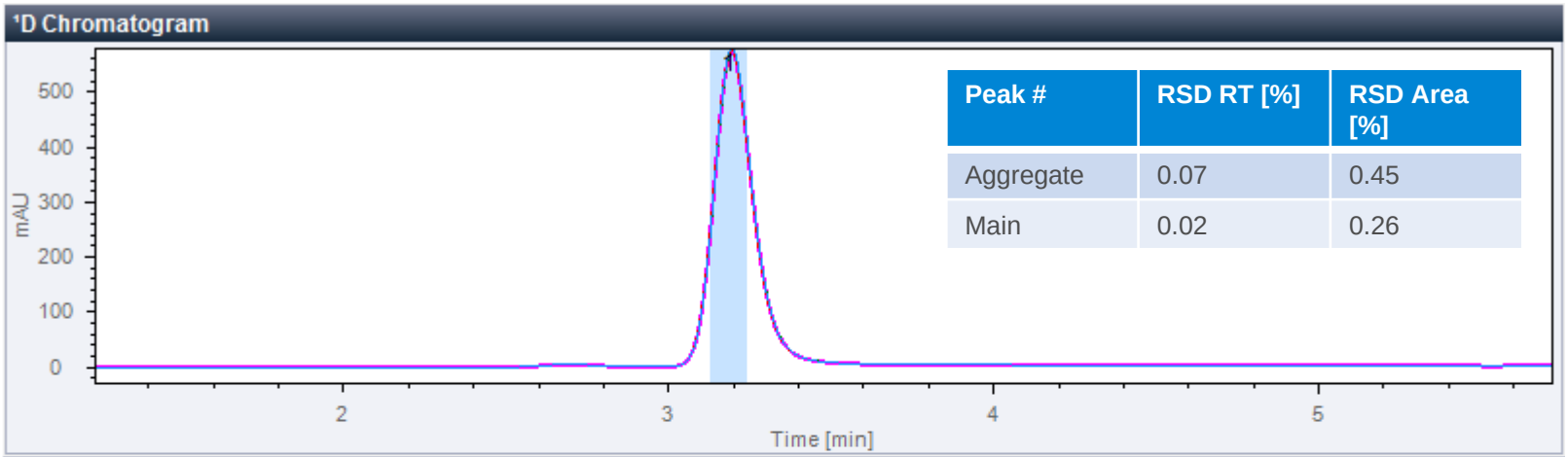
Aggregation Analysis of mAb
SEC in the First Dimension



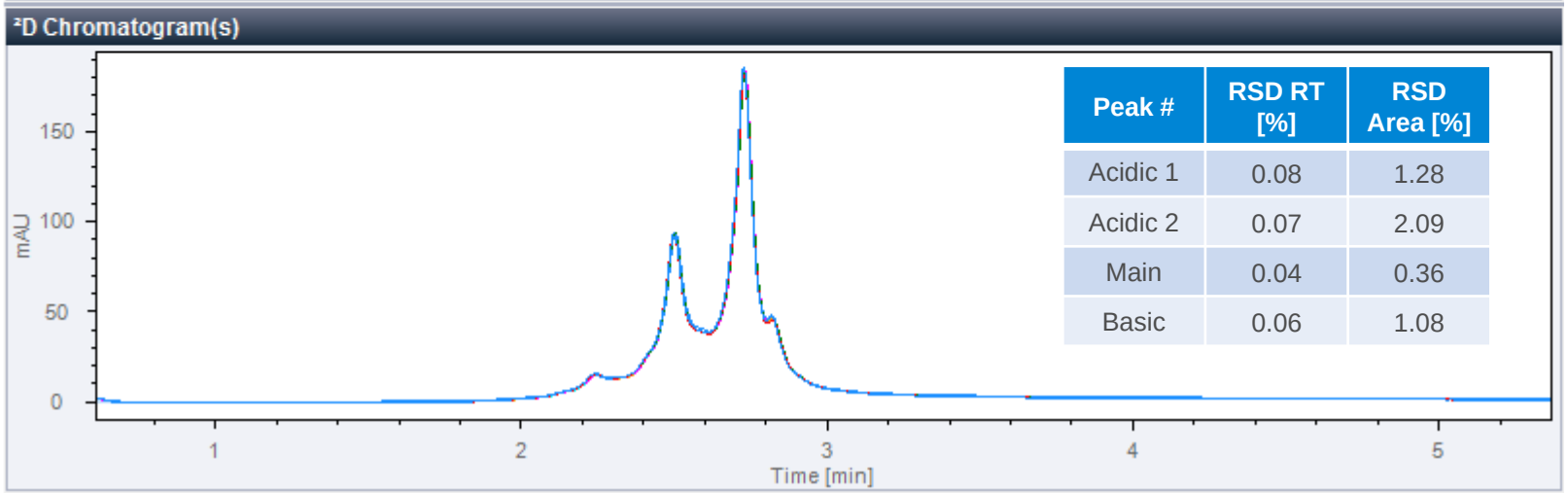
Heart Cuts from SEC Sent to WCX



2D LC Multiple Heart Cut SEC – WCX Precision of Retention Time and Area



Peak #	RSD RT [%]	RSD Area [%]
Aggregate	0.07	0.45
Main	0.02	0.26



Peak #	RSD RT [%]	RSD Area [%]
Acidic 1	0.08	1.28
Acidic 2	0.07	2.09
Main	0.04	0.36
Basic	0.06	1.08

Case Study 3 Combining SEC and WCX Summary

- Fully automated combination of two different quality attribute analyses

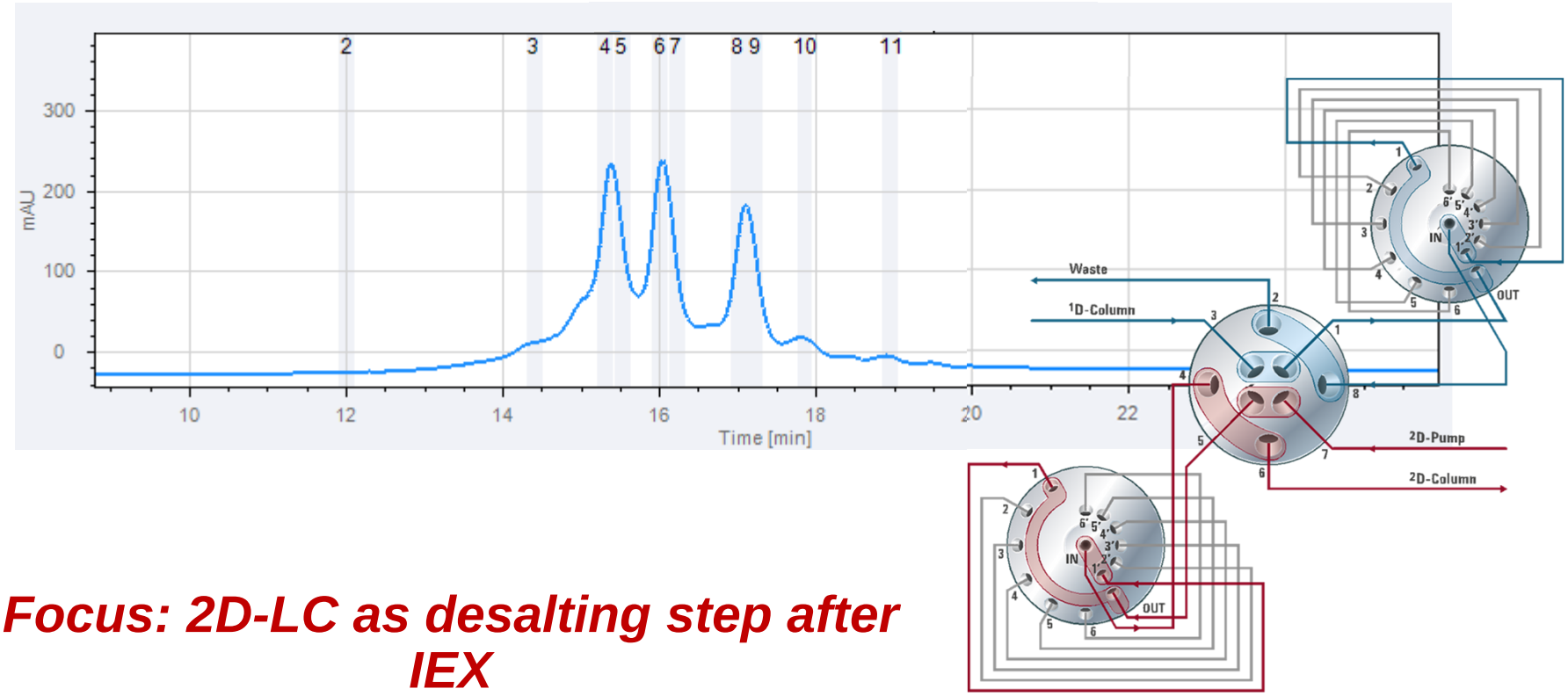
→ Size Exclusion and Charge Variants

- No hands-on time required between the two analyses

- High precision of retention time and area found in both dimensions

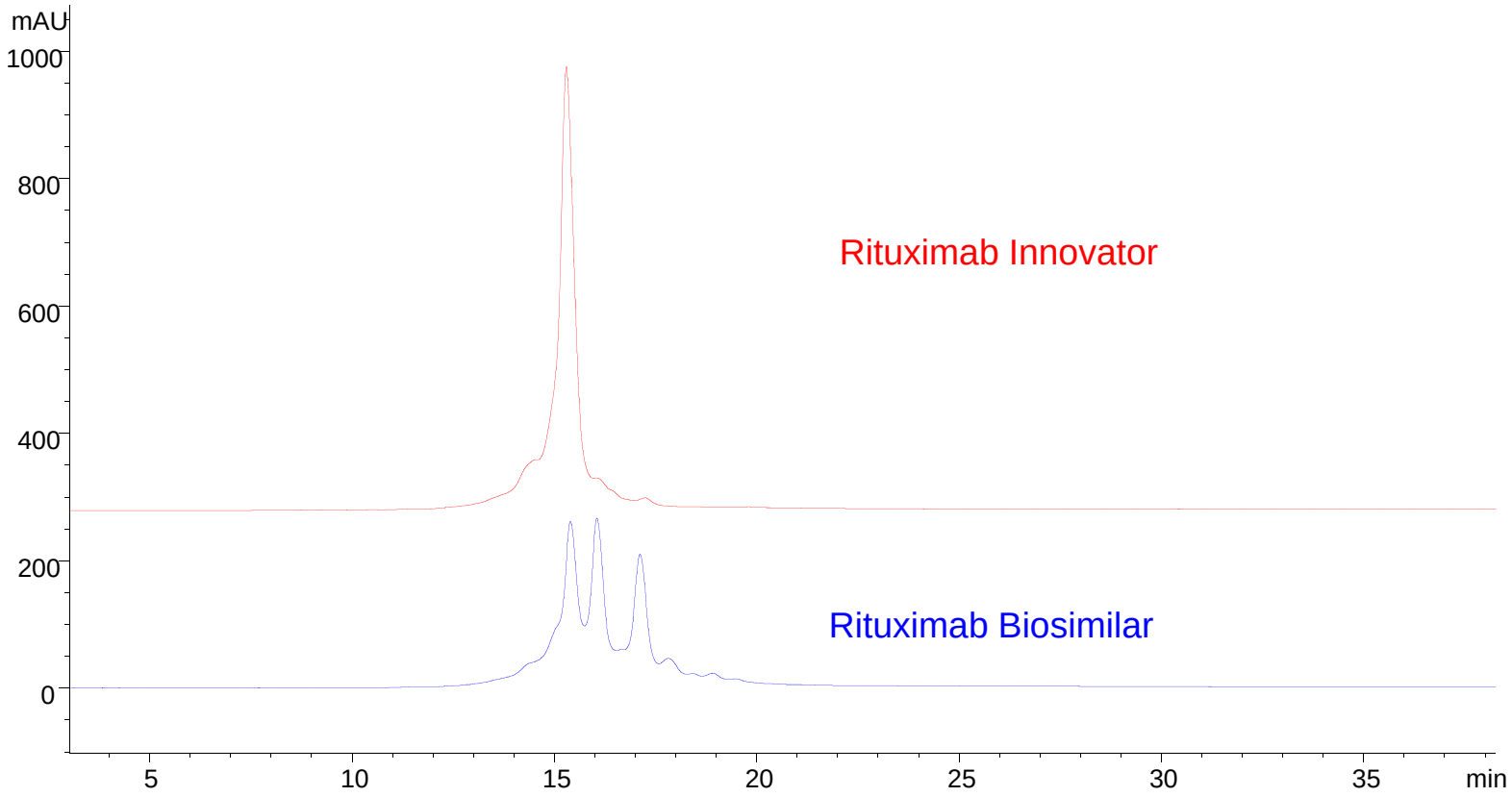
Case Study 4 Charge Variants Analysis LCMS

Ion Exchange and Reversed-Phase Chromatography

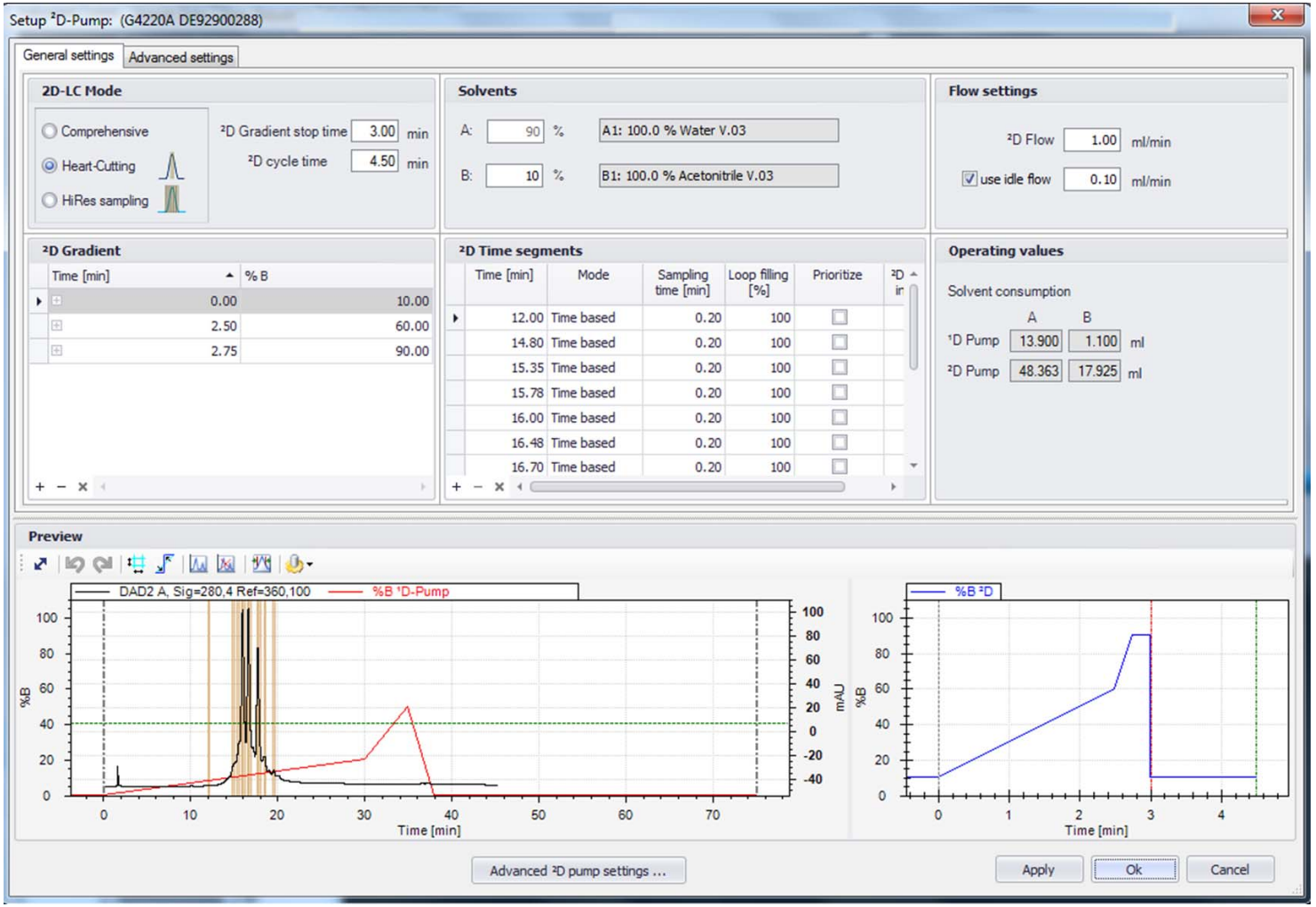


Focus: 2D-LC as desalting step after IEX

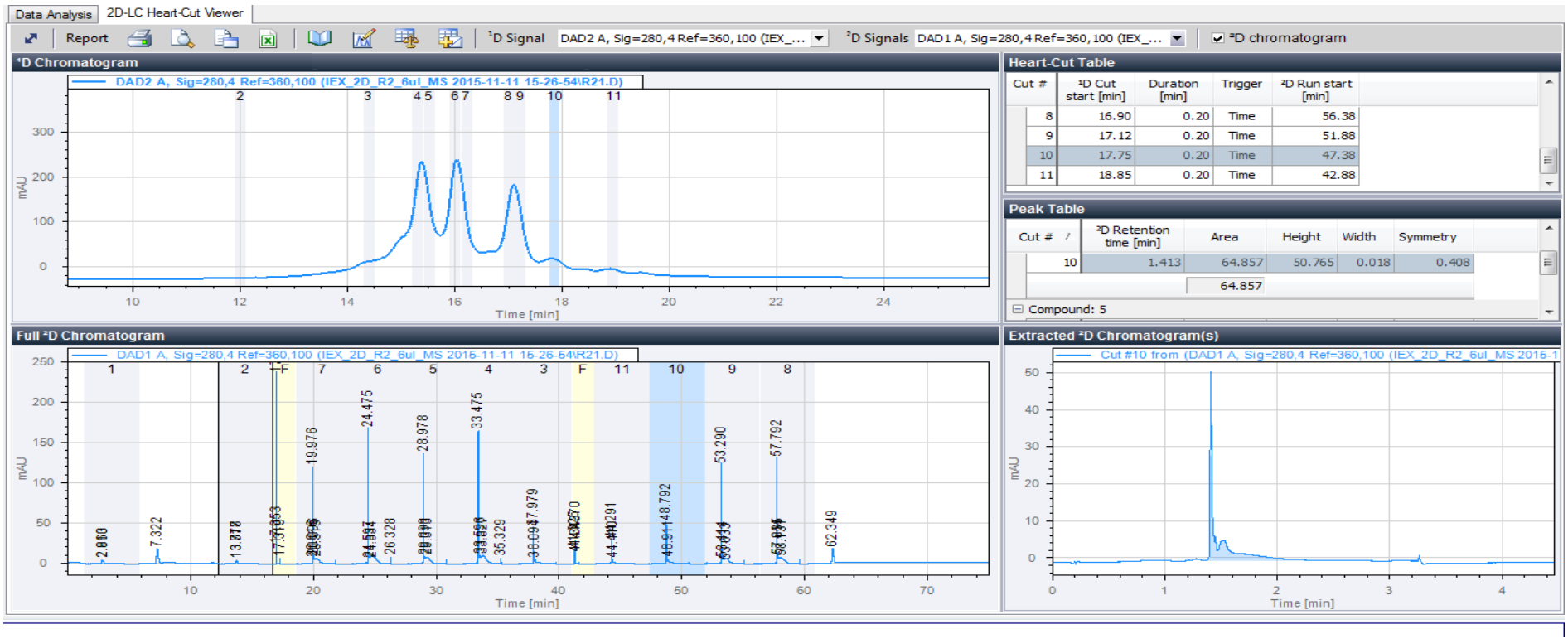
Charge profiles of Rituximab
Innovator and Biosimilar



WCX (first dimension) and Reverse Phase (second dimension)

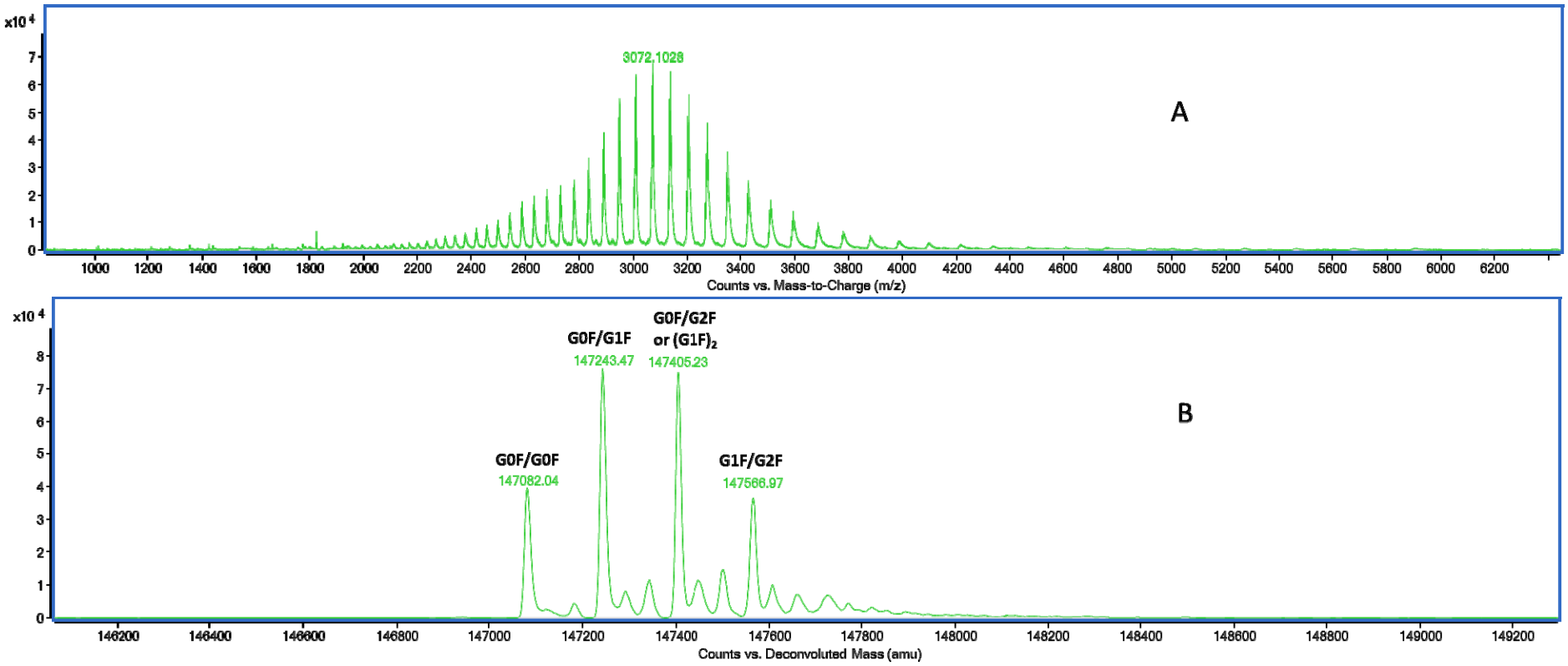


2D LC Data View of UV Results



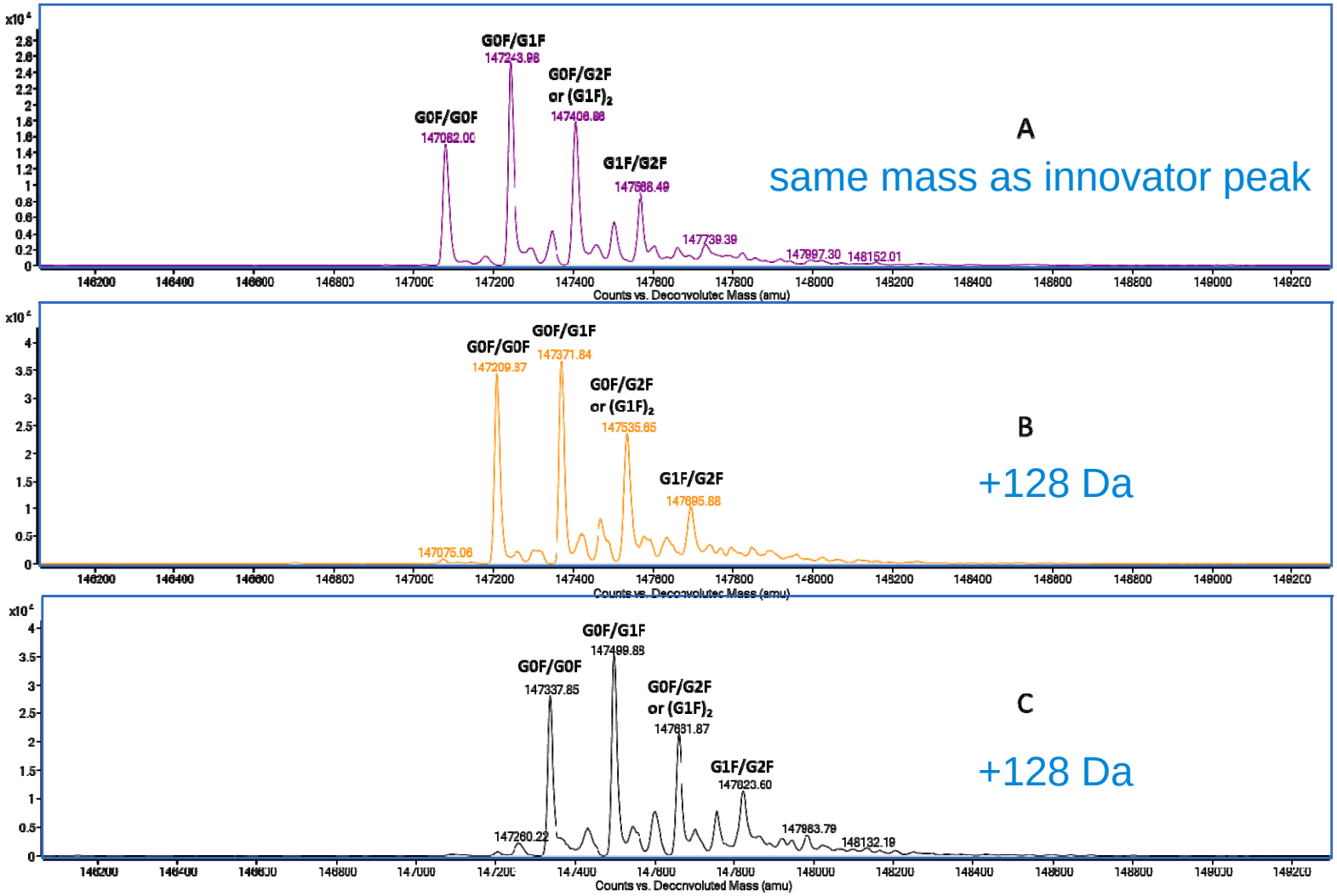
Online MS detection

Intact mass analysis of Innovator Rituximab – 1 peak



Online MS detection

Intact Mass Analysis of Biosimilar Rituximab – 3 peaks



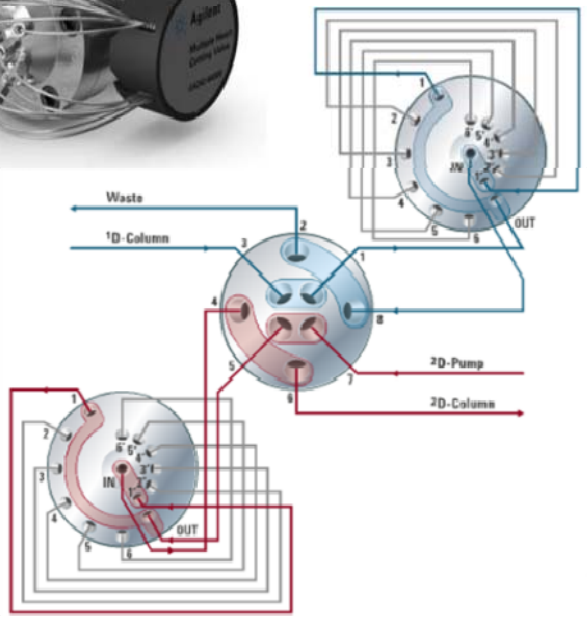
Case Study 4 Charge Variants Analysis LCMS Summary

- Innovator and Biosimilar Rituximab charge variants were analyzed using weak cation exchange chromatography
- Online 2DLC-MS qualification was enabled by automated desalting and denaturation
- The three peaks of the biosimilar vs one peak of the innovator were qualified as c-terminal lysine variants (mass shift of 128 Da)

Further information can be found…

- Schneider, S. 2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography, Agilent Technologies Application Note, publication number 5991-6673EN, **2016.**
- Schneider, S. Online 2D-LC Characterization of Monoclonal Antibodies Using Protein A and Weak Cation Exchange Chromatography, Agilent Technologies Application Note, publication number 5991-6848EN, **2016.**
- Schneider, S. Online 2D-LC Characterization of Monoclonal Antibodies with Size Exclusion and Weak Cation Exchange Chromatography, Agilent Technologies Application Note, publication number 5991-6906EN, **2016.**
- Vanhoenacker, G et al.; Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution Part 2: HILIC × RPLC-MS, Agilent Technologies Application Note, publication number 5991-4530EN, **2014**.
- Schneider, S; Naegele, E; Krieger, S. Online 2D-LC Analysis of Complex N-Glycans in Biopharmaceuticals Using the Agilent 1290 Infi nity 2D-LC Solution Agilent Technologies Application Note, publication number 5991-5349EN, **2015**.

Online 2D-LC Analysis of Complex N-Glycans in Biopharmaceuticals



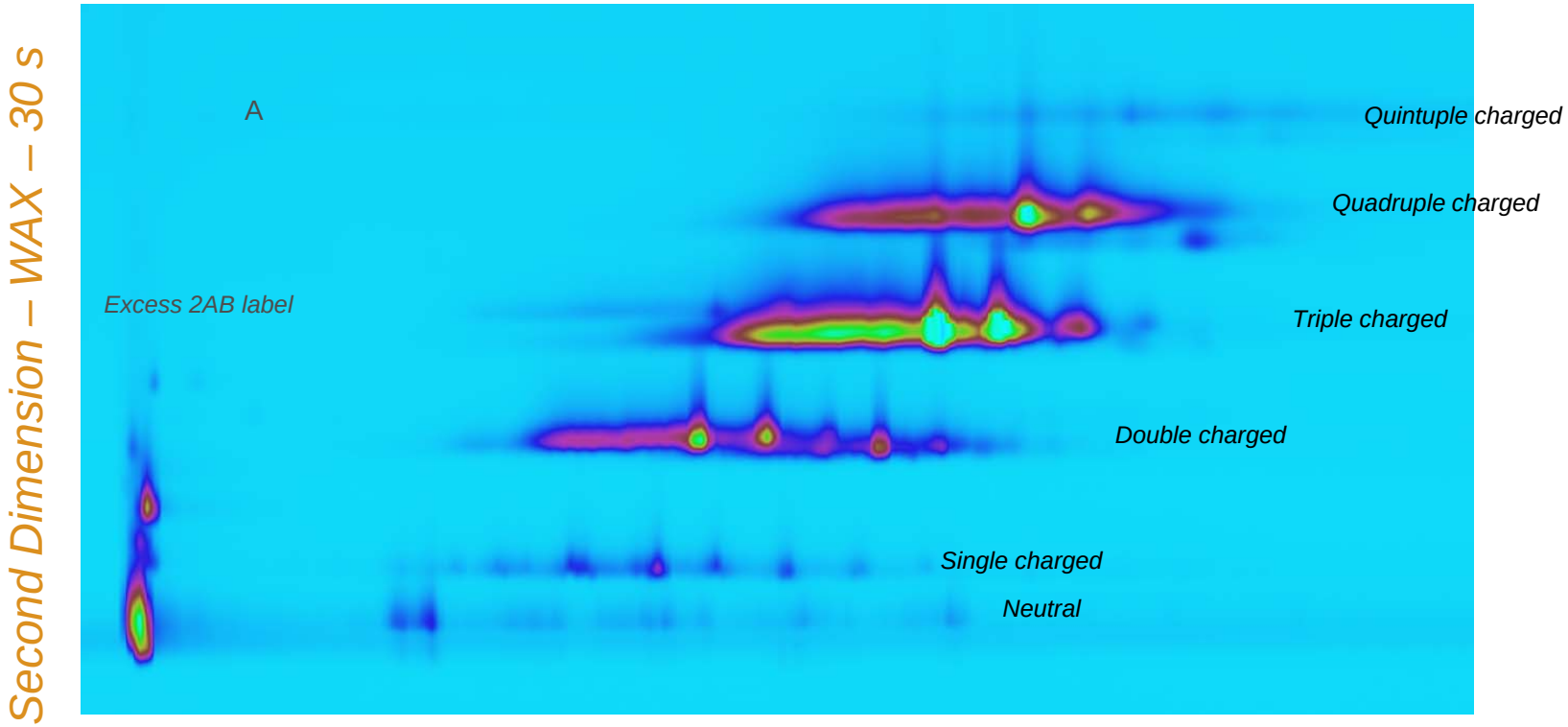
Comprehensive 2D-LC of Glycans

HILIC / WAX

- First Dimension:
  - Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm
    Total run time 165 min (Stoptime + Posttime)

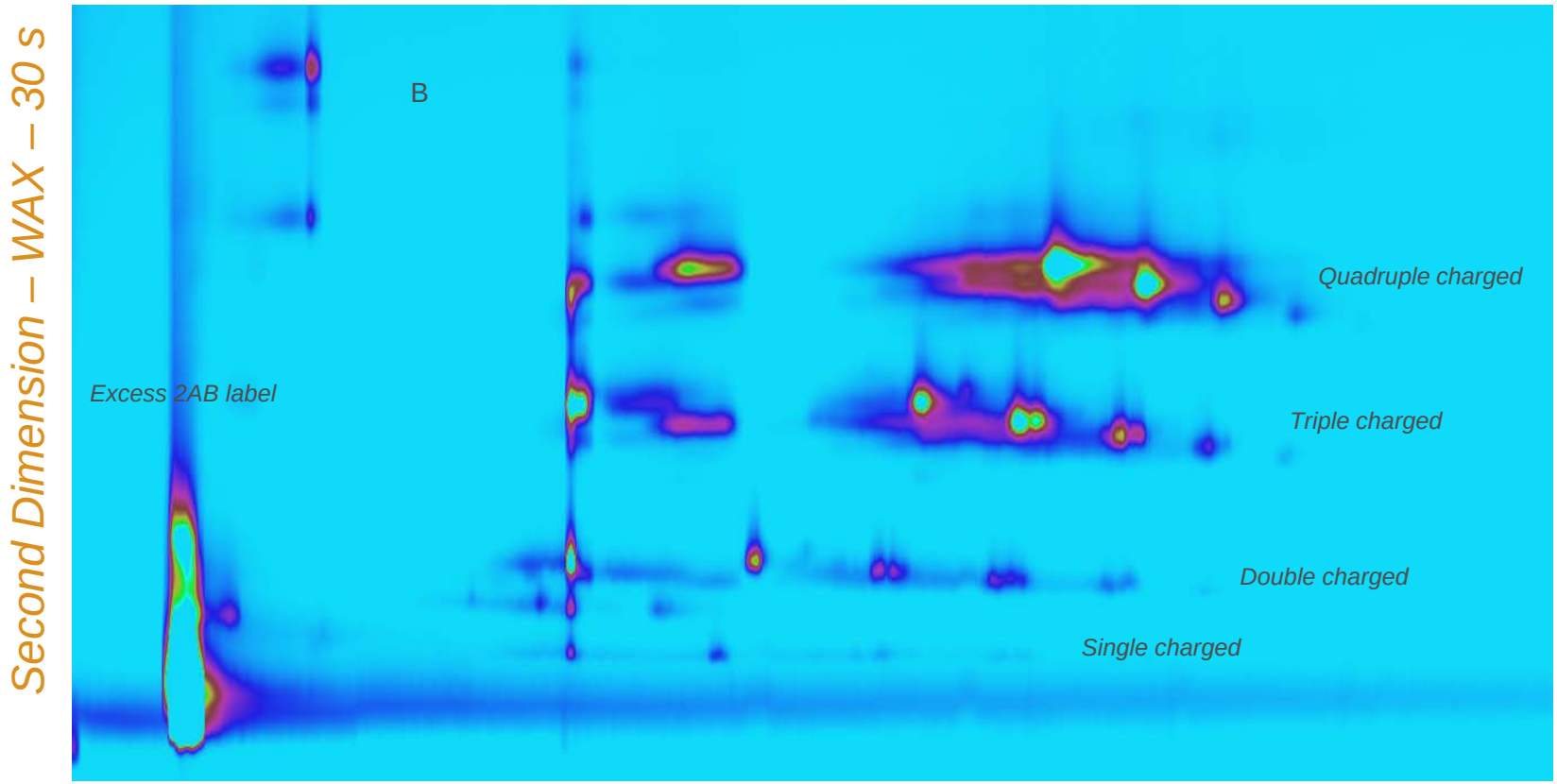
- Second Dimension:
  - Agilent Bio WAX column, 2.1 × 50 mm, 5 µm
    30 seconds 2D gradients

Comprehensive 2D-LC of Fetuin HILIC/WAX



http://web.expasy.org/glycomod/ for the determination of glycan structures

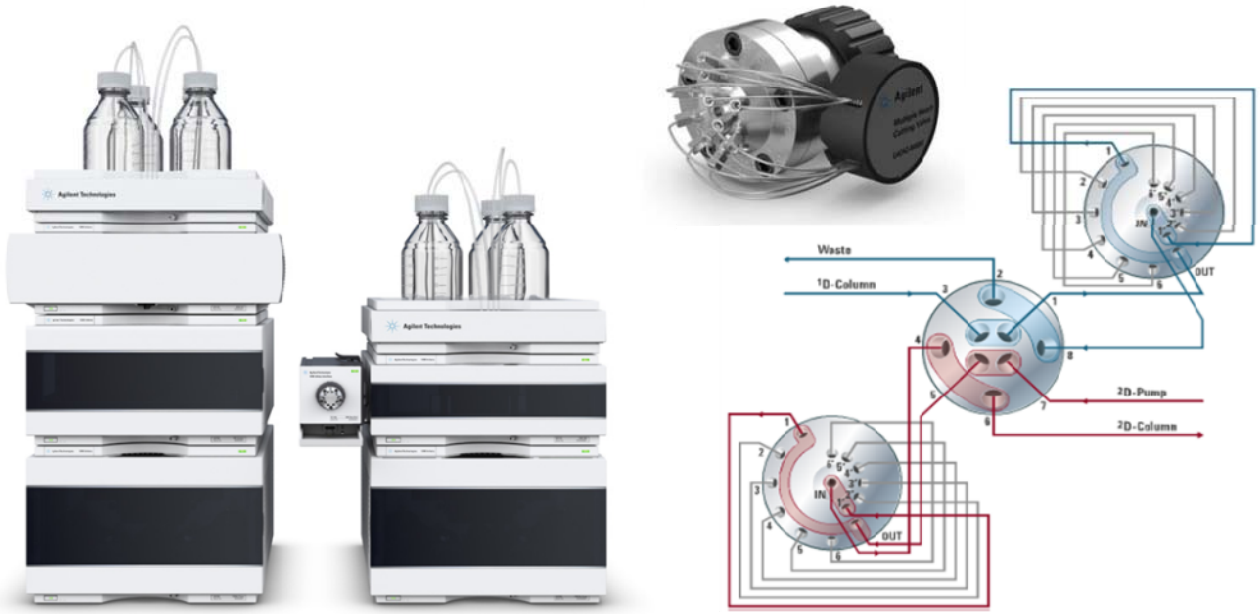
Comprehensive 2D-LC of EPO HILIC/WAX



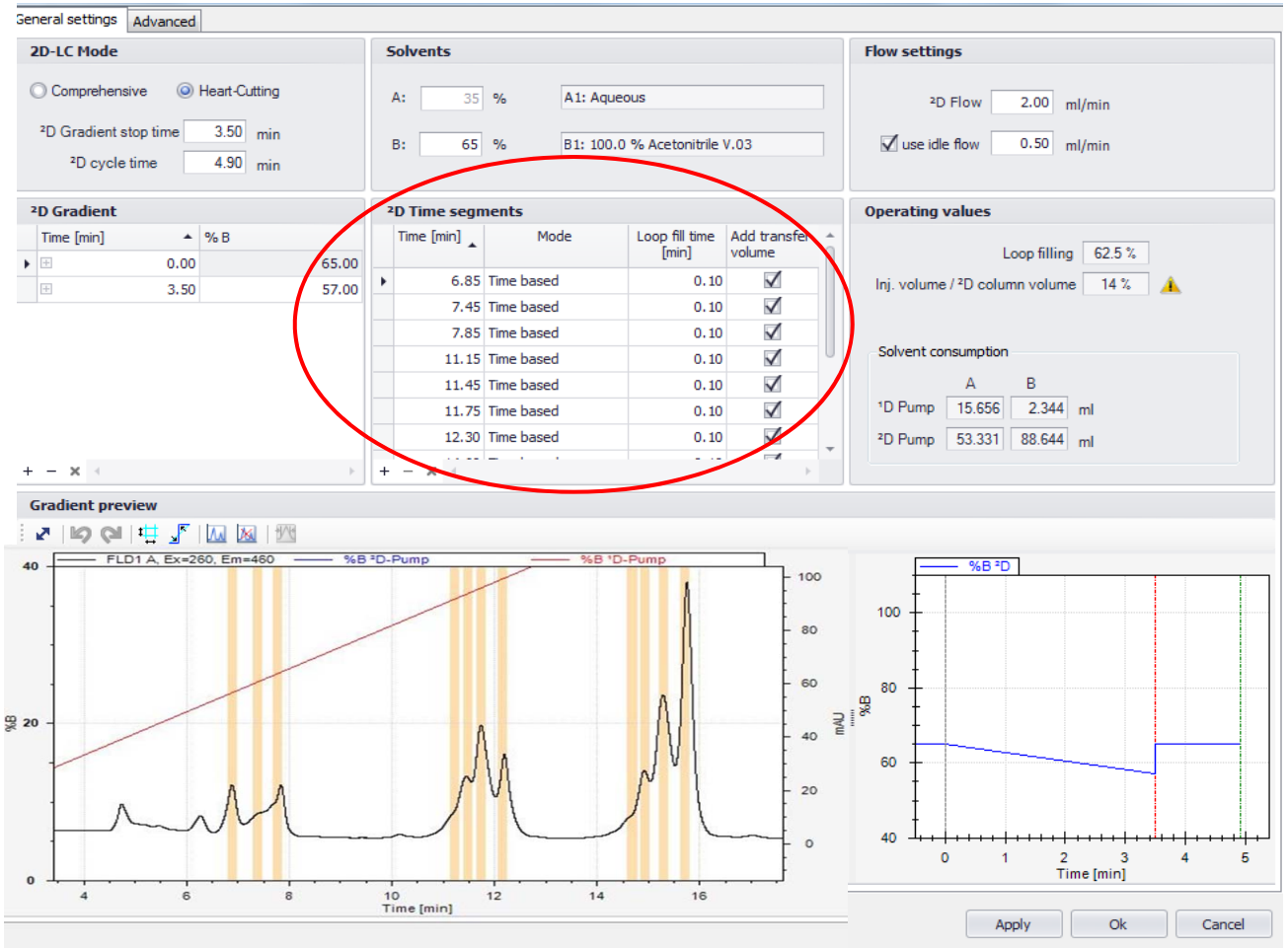
Comprehensive 2D-LC Analysis of Glycans Summary

- Comprehensive HILIC/WAX – high peak capacity
- Complete automation possible within a run time of about 110 minutes
- Easy data analysis and interpretation due to the grouping of the glycans according to their charge in the second dimension, enabling simultaneous charge profiling

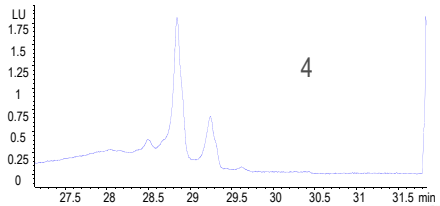
Multiple Heart-Cutting 2D-LC of Glycans

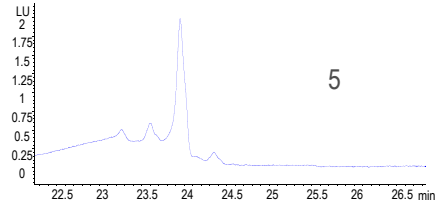


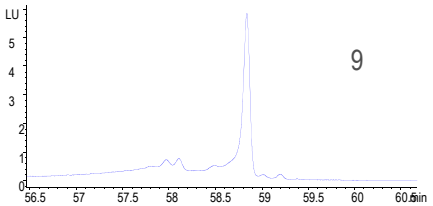
Time-Based Multiple Heart-Cutting of EPO 2D-LC Method Setup

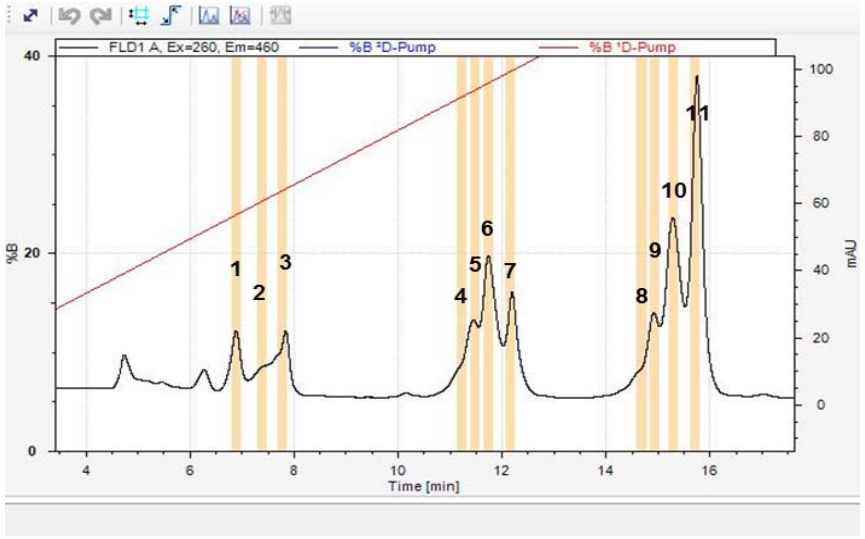


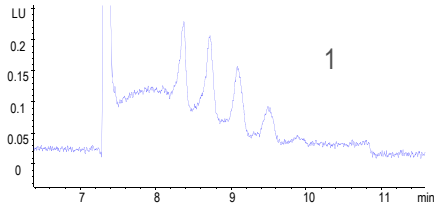
Time-Based Multiple Heart-Cutting of EPO

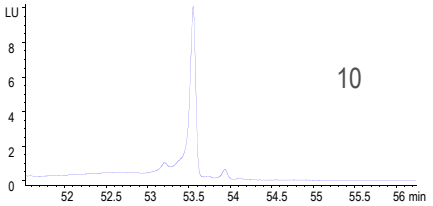


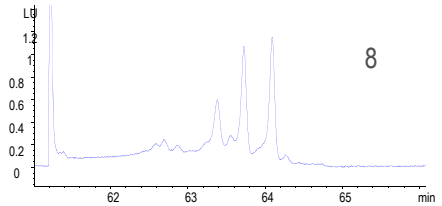












Time-Based Multiple Heart-Cutting of EPO Summary

- Multiple heart-cutting WAX/HILIC High Resolution
- Higher flexibility – no limitation to super short gradients and high flow rates in the second dimension
- Facilitates the combination of WAX/HILIC having WAX in the first and HILIC in the second dimension enabling partly fill of the sample loops
- Time saving of over 70 % compared to the offline WAX/HILIC analysis with 4 hours vs only 70 minutes

Thank you for your attention

Questions?