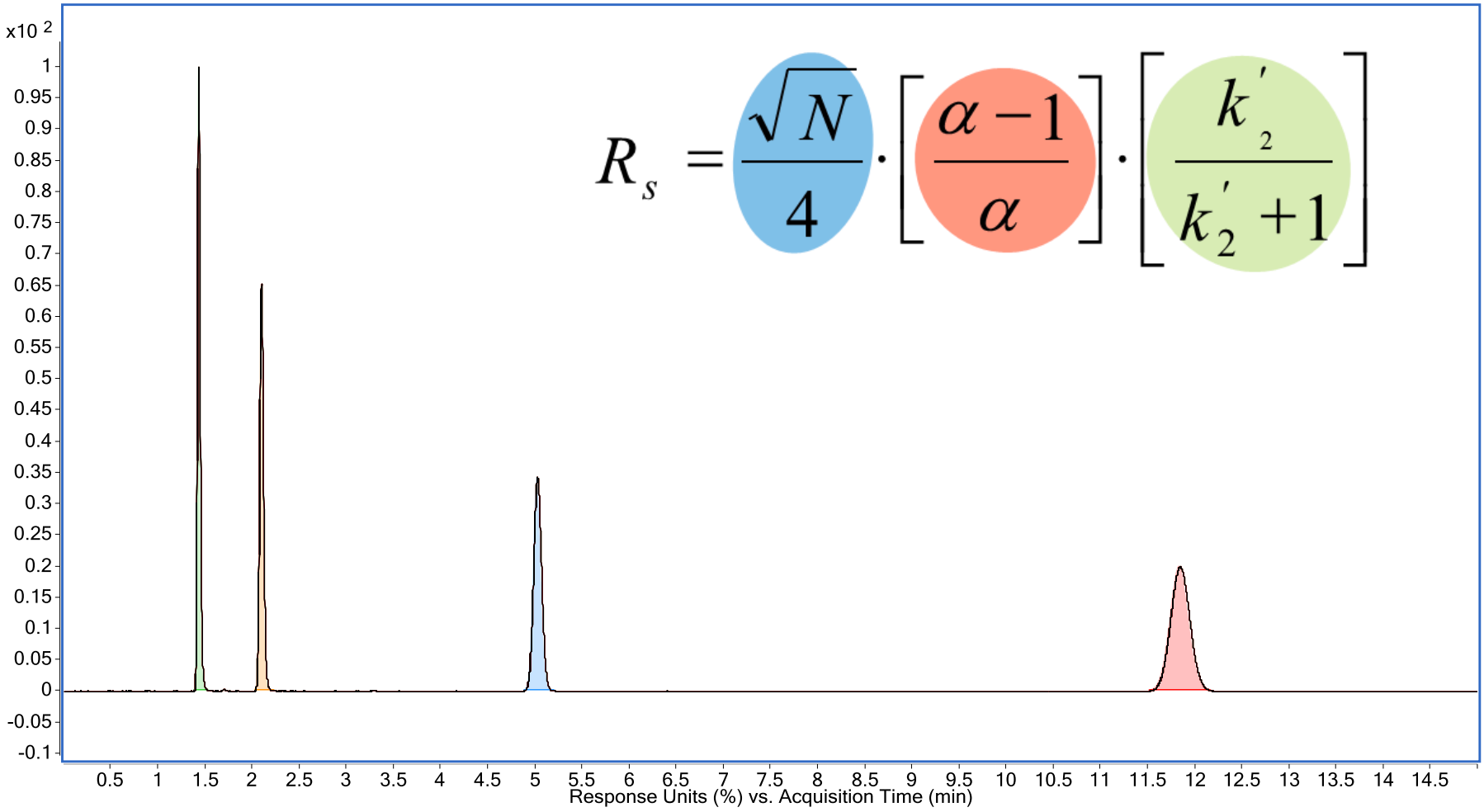
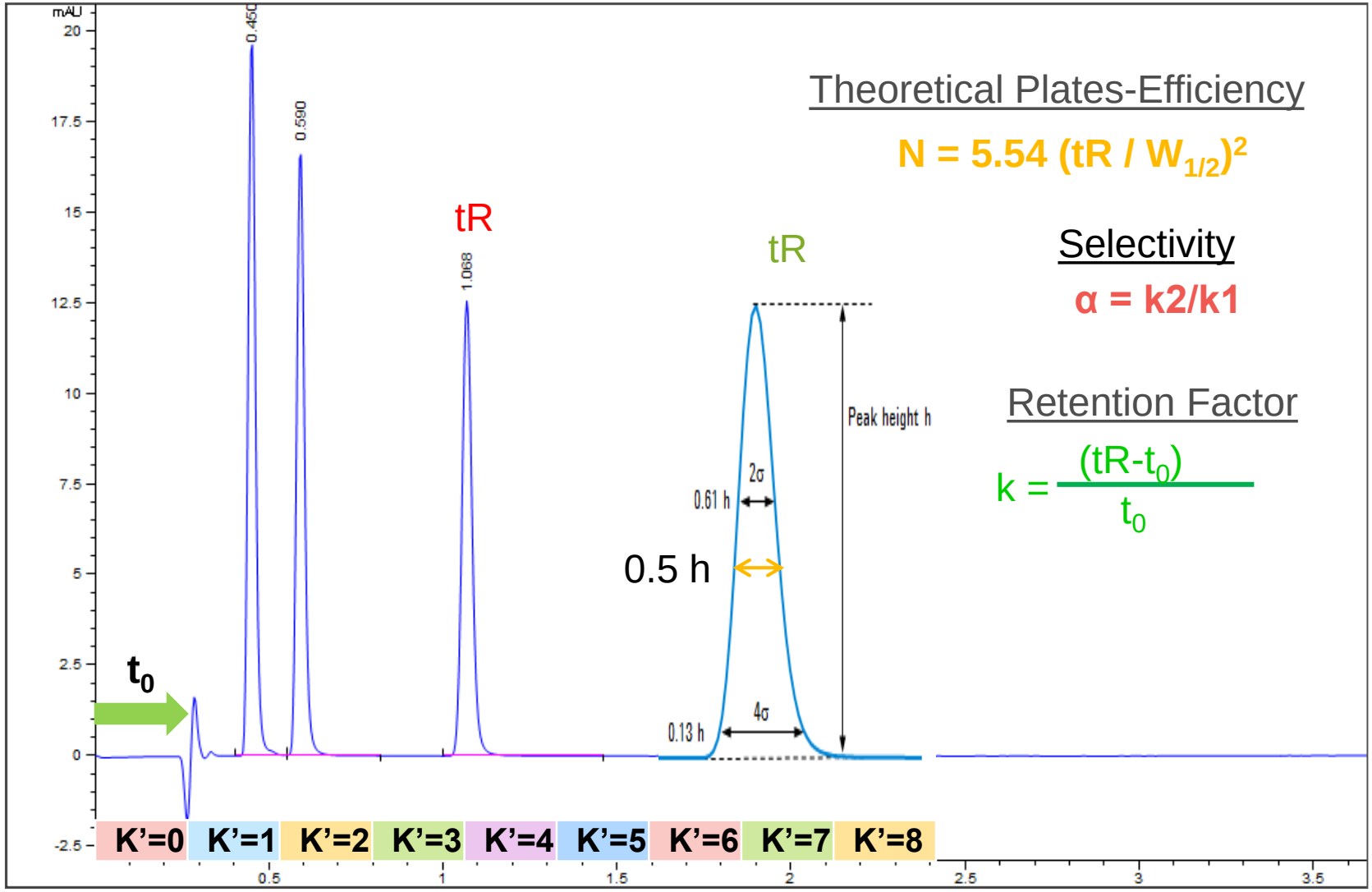
Two Dimensional Liquid Chromatography: Theory and Applications

N = Number of theoretical. plates	Column length, particle size
α = Selectivity	Stationary and mobile phase, temperature
k = Retention	Stationary and mobile phase

$$R_s = \frac{\sqrt{N}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k'_2}{k'_2 + 1}\right]$$





Theoretical Plates-Efficiency

$$N = 5.54 \, (tR / W_{1/2})^2$$

Selectivity

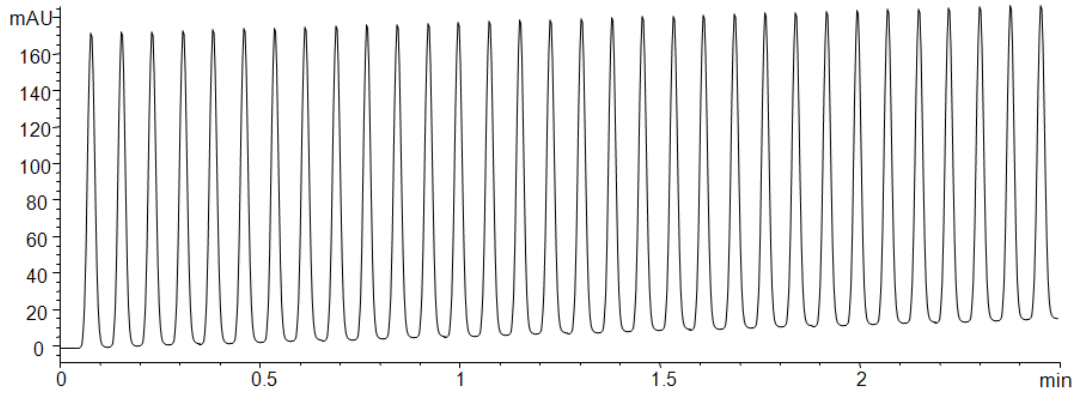
$$\alpha = k2/k1$$

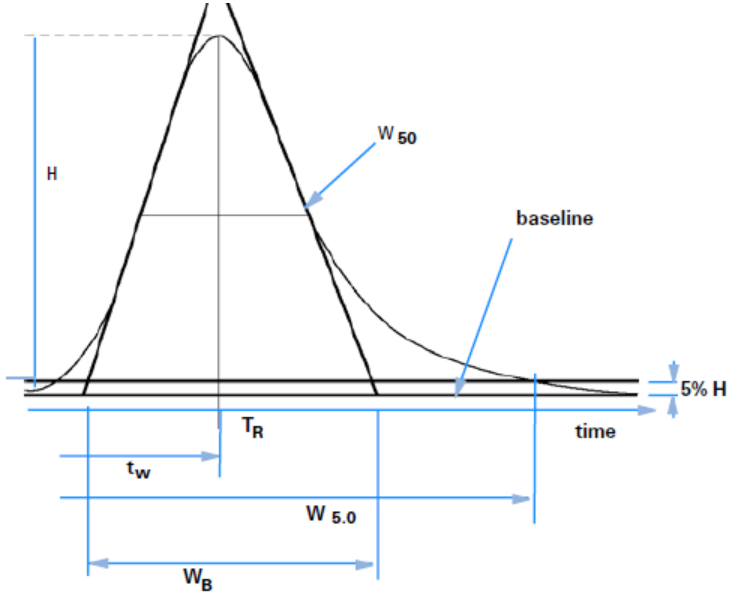
Retention Factor

$$k = \frac{(tR - t_0)}{t_0}$$

Peak capacity as a measure of resolving power

Peak capacity is the number of peaks, which can be separated in a given time with a given resolution (Rs = 1)





The peak capacity can be calculated from the gradient time t_g and the average peak width $\overline{w}$:

$$n = 1 + \frac{t_g}{\overline{w}}$$

The importance of peak capacity
Statistical theory of component overlap

"… using the statistical theory of peak overlap …"

"… peak resolution is severely compromised when the number of components present in a sample overrates 1/3 of the peak capacity."

J.M. Davis, J.C. Giddings, Anal. Chem. 55 (1983) 418

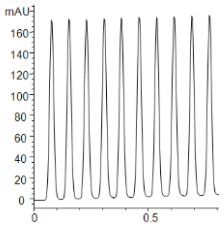
"…in order to resolve 98% of the components, the peak capacity must exceed the number of components by a factor of 100."

J.C. Giddings, J. Chromatogr. A 703 (1995) 3

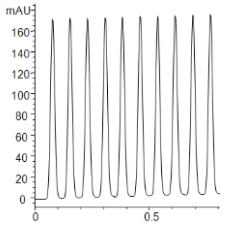
Peak capacity in comprehensive 2D-LC

LC + **LC**

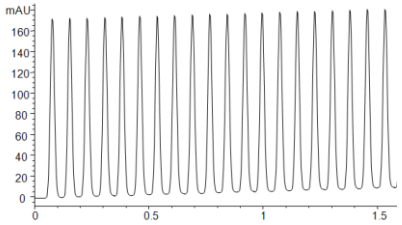
$$P = f(\sqrt{N})$$



Peak capacity = 10

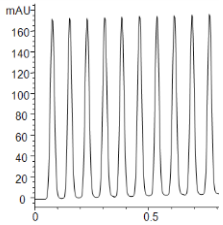


Peak capacity = 10

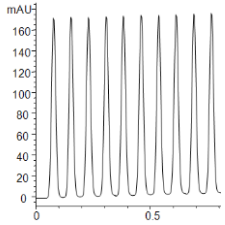


Peak capacity = 14

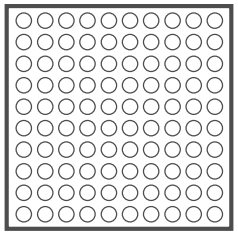
LC × **LC**



Peak capacity = 10



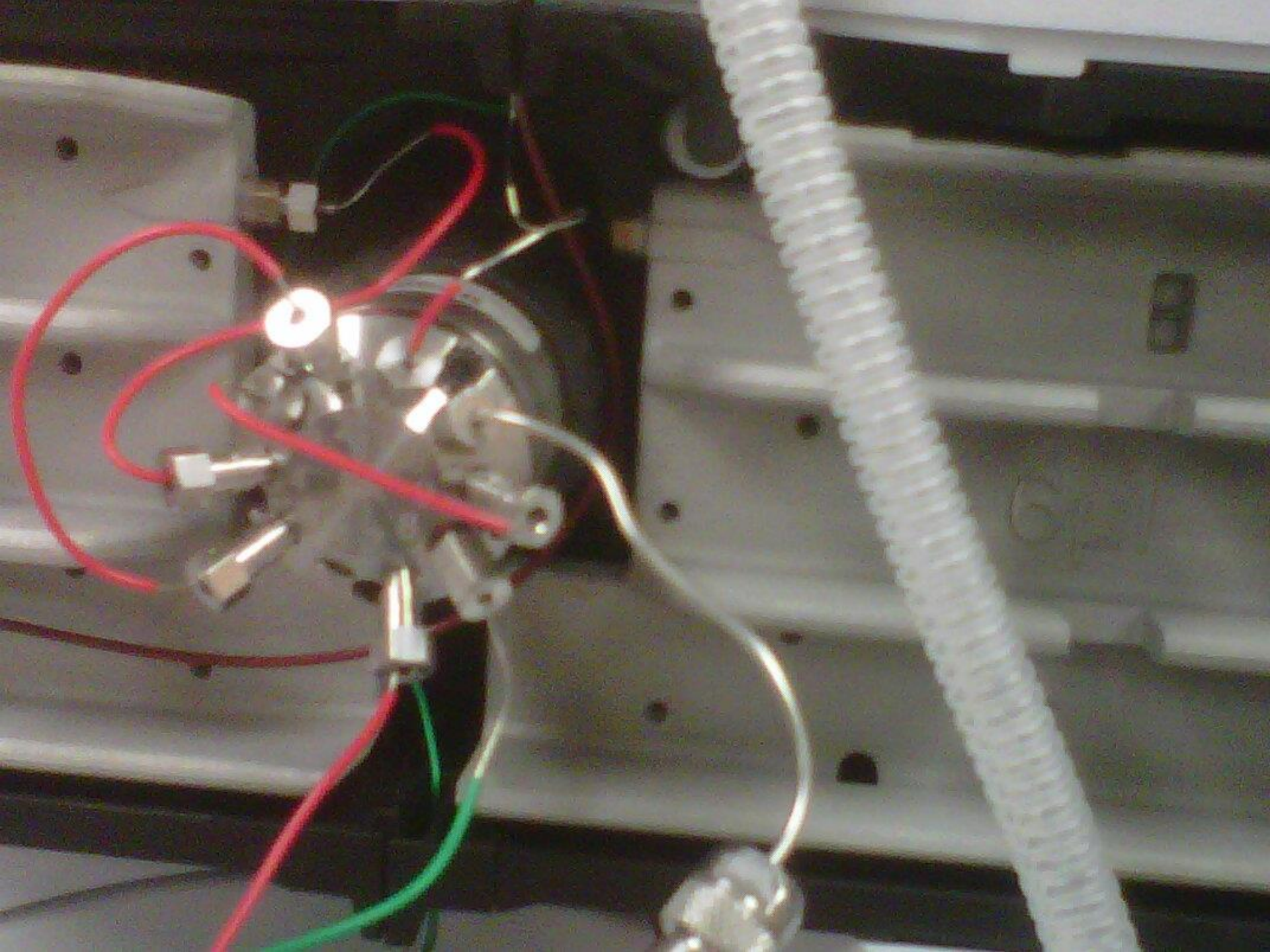
Peak capacity = 10



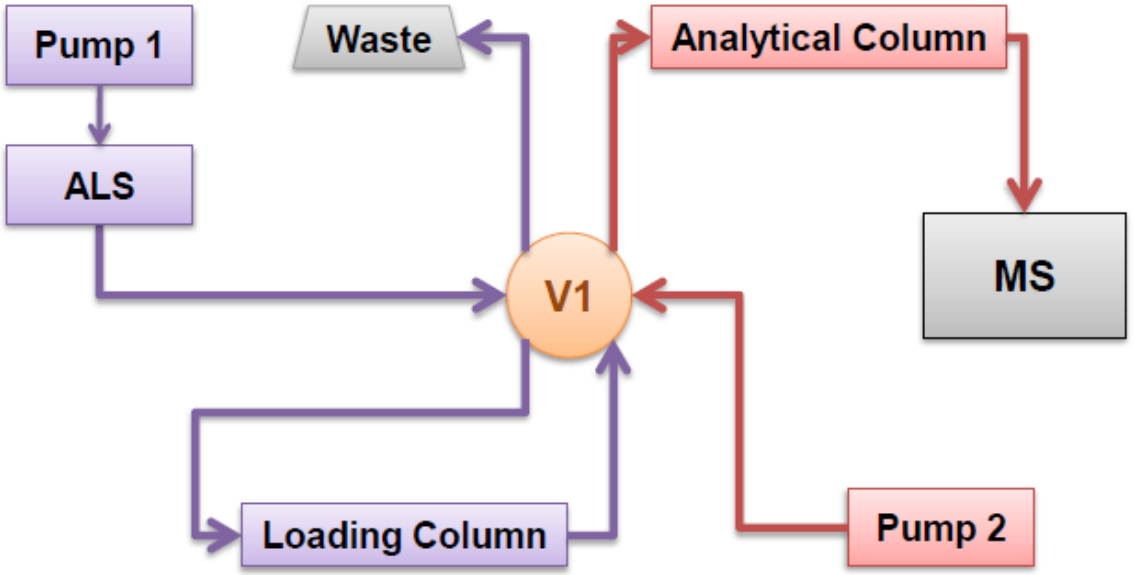
Peak capacity = 100 !

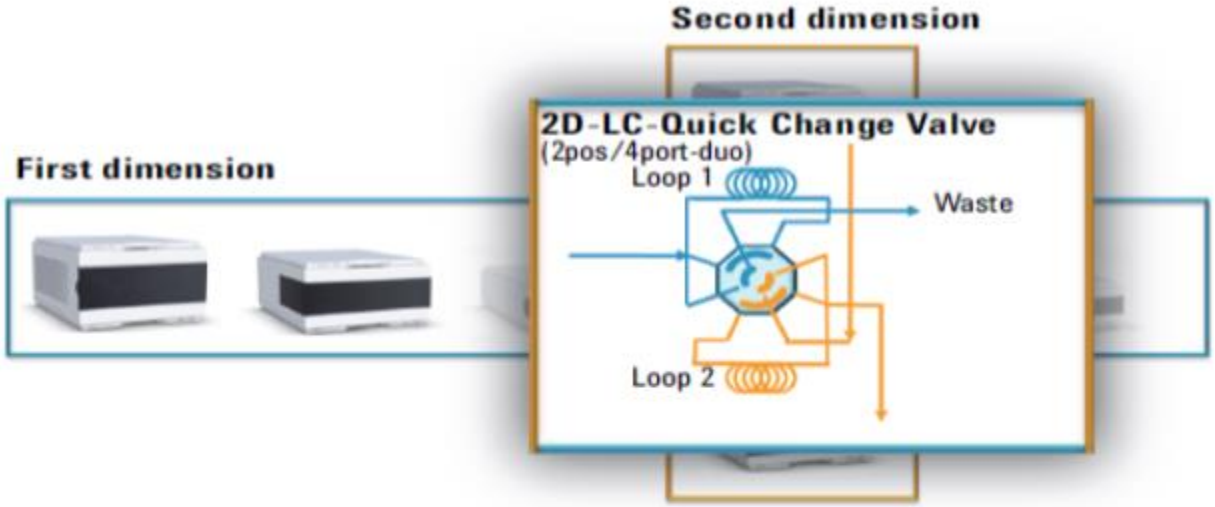
2D or not 2D, when is the question?

- Sample cleanup by removing matrix or interfering compounds
- Increase sample throughput (two separations going on at once)
- Trace enrichment of major compounds of interest (column focusing)
- ***Increased peak capacity***
- ***Second dimension mobile phase amenable to mass spectrometry***
- ***Further resolution of a complex mixture that cannot be separated on a single mode/column***

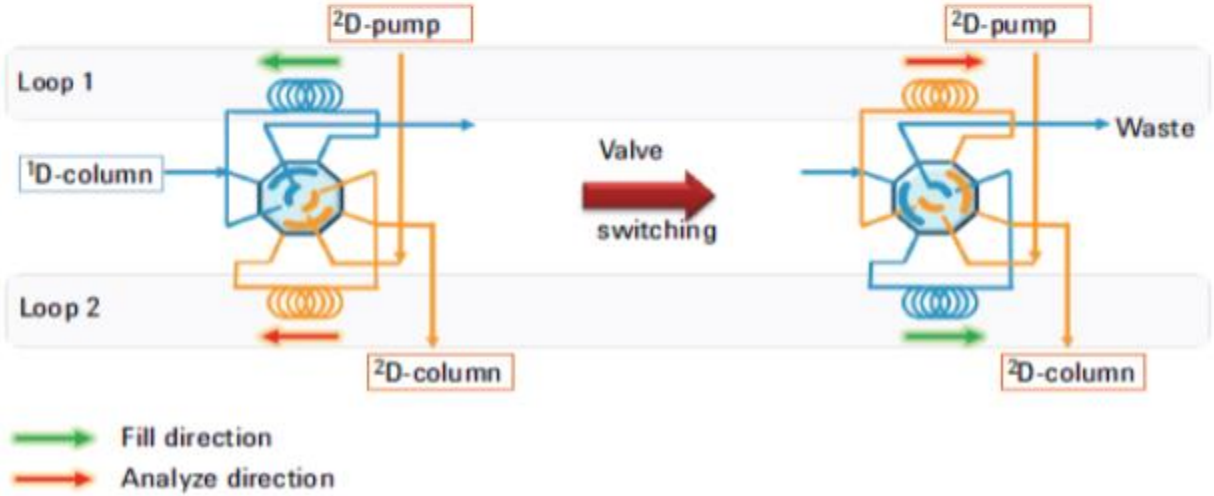


2-D On-Line Sample Cleanup and Enrichment/Heart-Cut LC-MS System

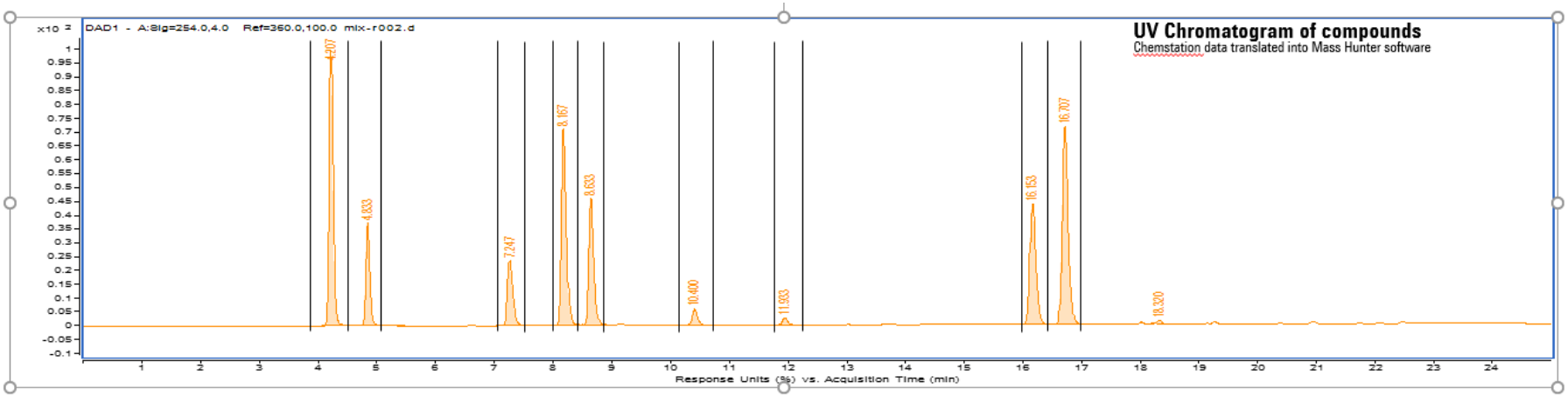


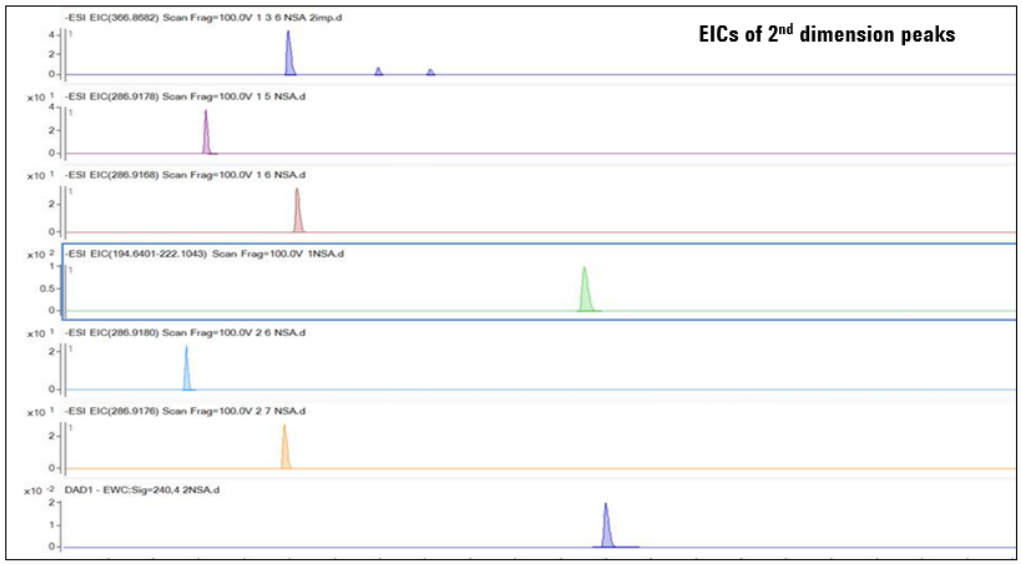


2D-LC-Quick-Change Valve configuration (overview)



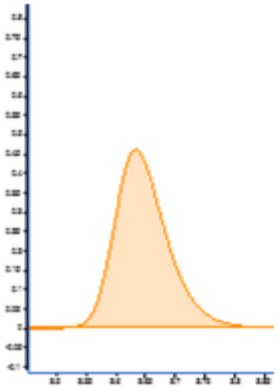
Changing Mobile Phases



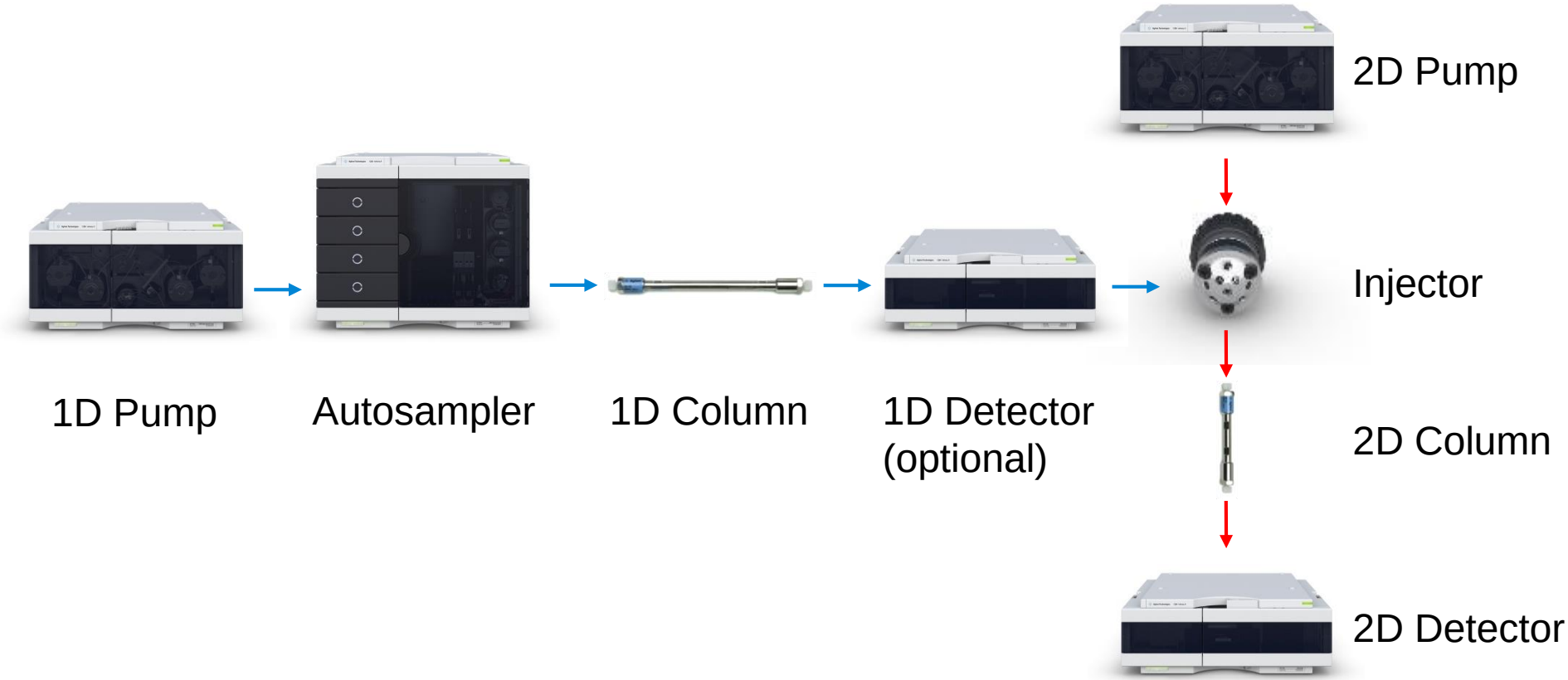


Peak volume

0.4 ml/min x 0.3 minutes = 1.2 ml volume



Two-dimensional LC
System configuration



Principles of Two Dimensional HPLC

- Long efficient first column retains and separate sample components in one chromatography mode (first dimension)
- The eluent flows through valve with injection loops (Comprehensive or Heart Cutting modes)
- The loop content is automatically introduced into a 2nd, fast column (UHPLC) for an orthogonal separation mode
- Recent work with heart-cutting has shown second column may not necessarily be UHPLC column

Two Approaches to 2D HPLC

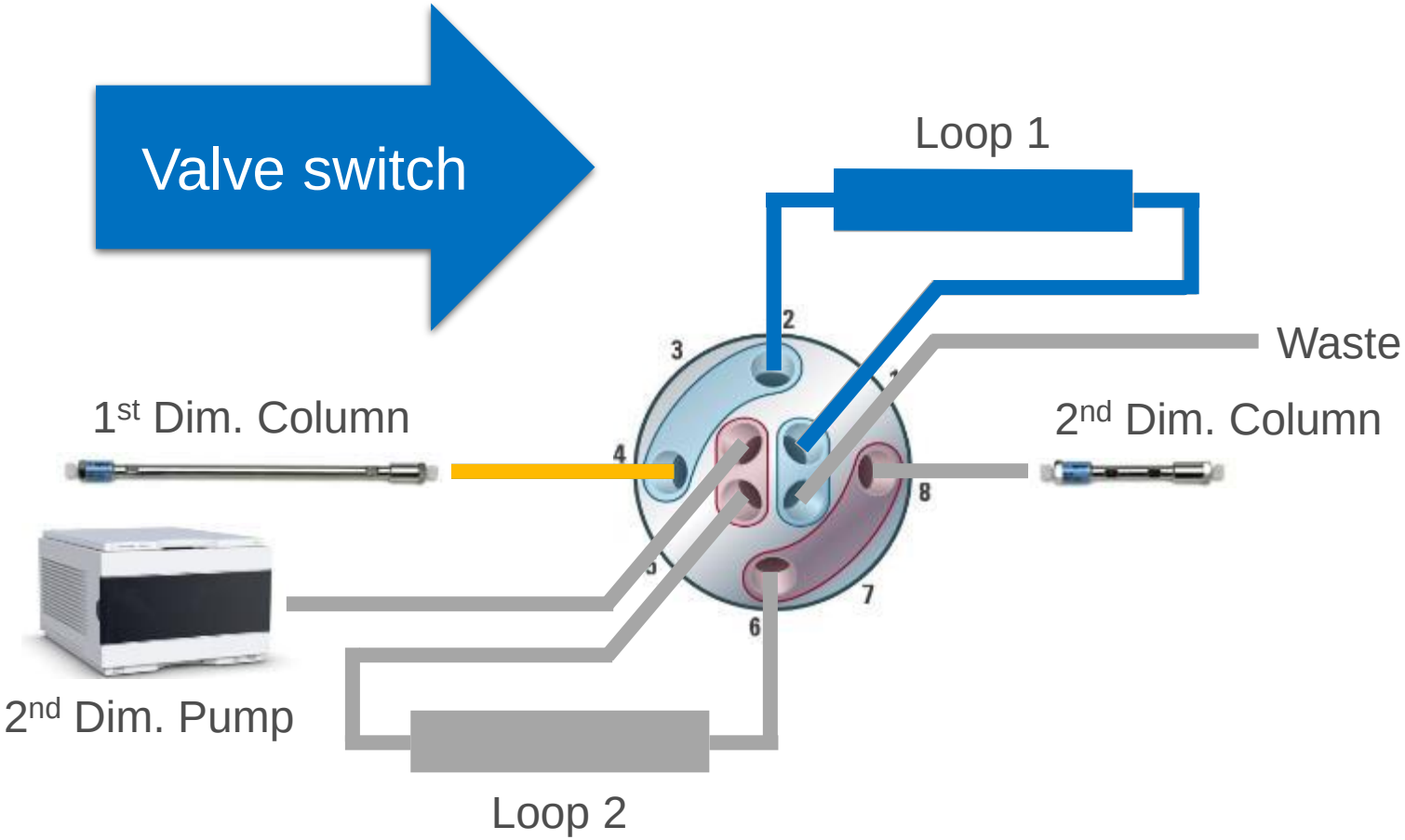
- **Comprehensive**: All of the sample from the first column is ‘trapped & released’ on to the second column in sequential fractions throughout the first dimension run

- **Heart-cutting:** Detector in the first dimension detects peaks to be trapped and released on to the second dimension column

COMPREHENSIVE 2D-LC

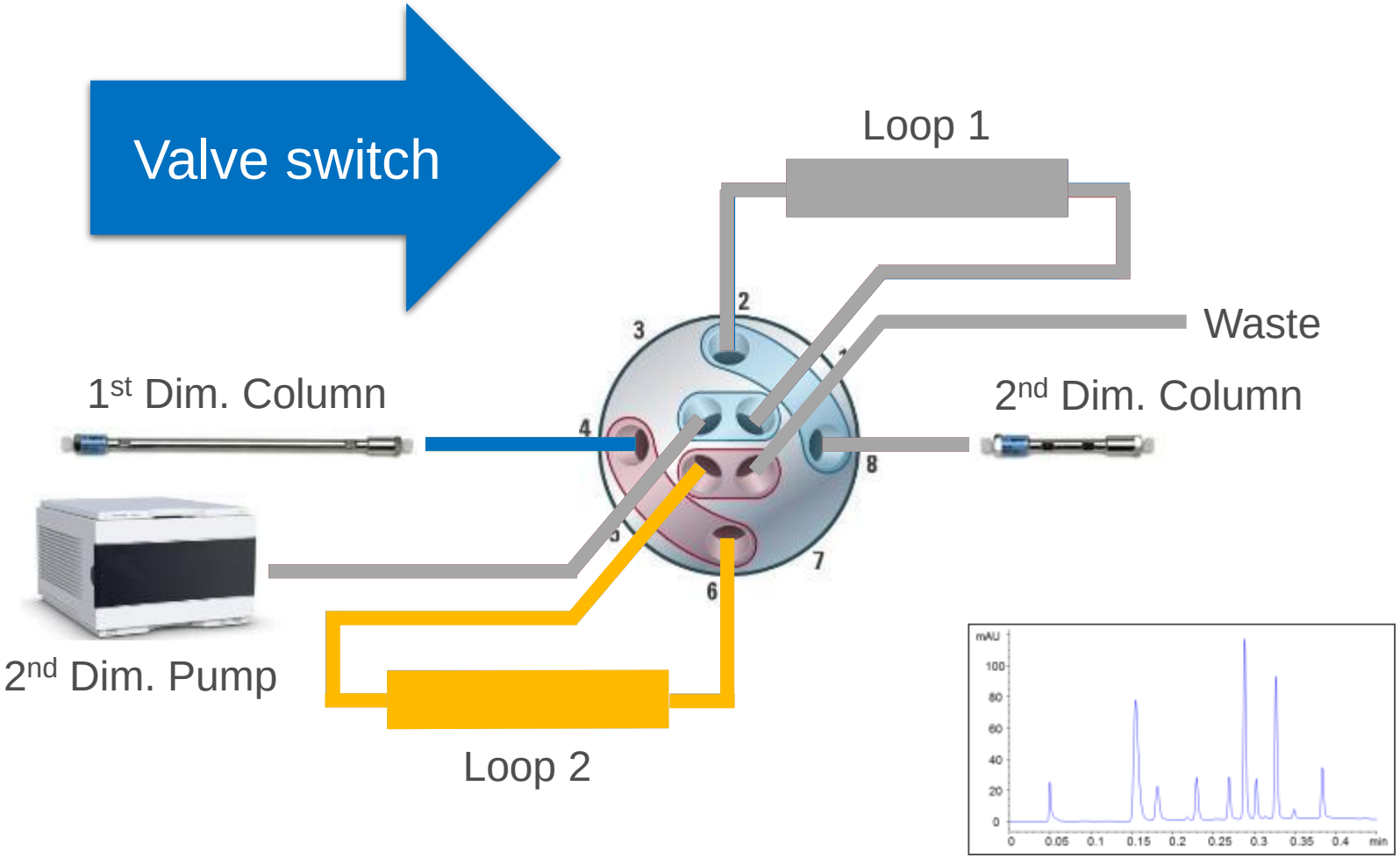
Comprehensive 2D-LC

Operating Principle



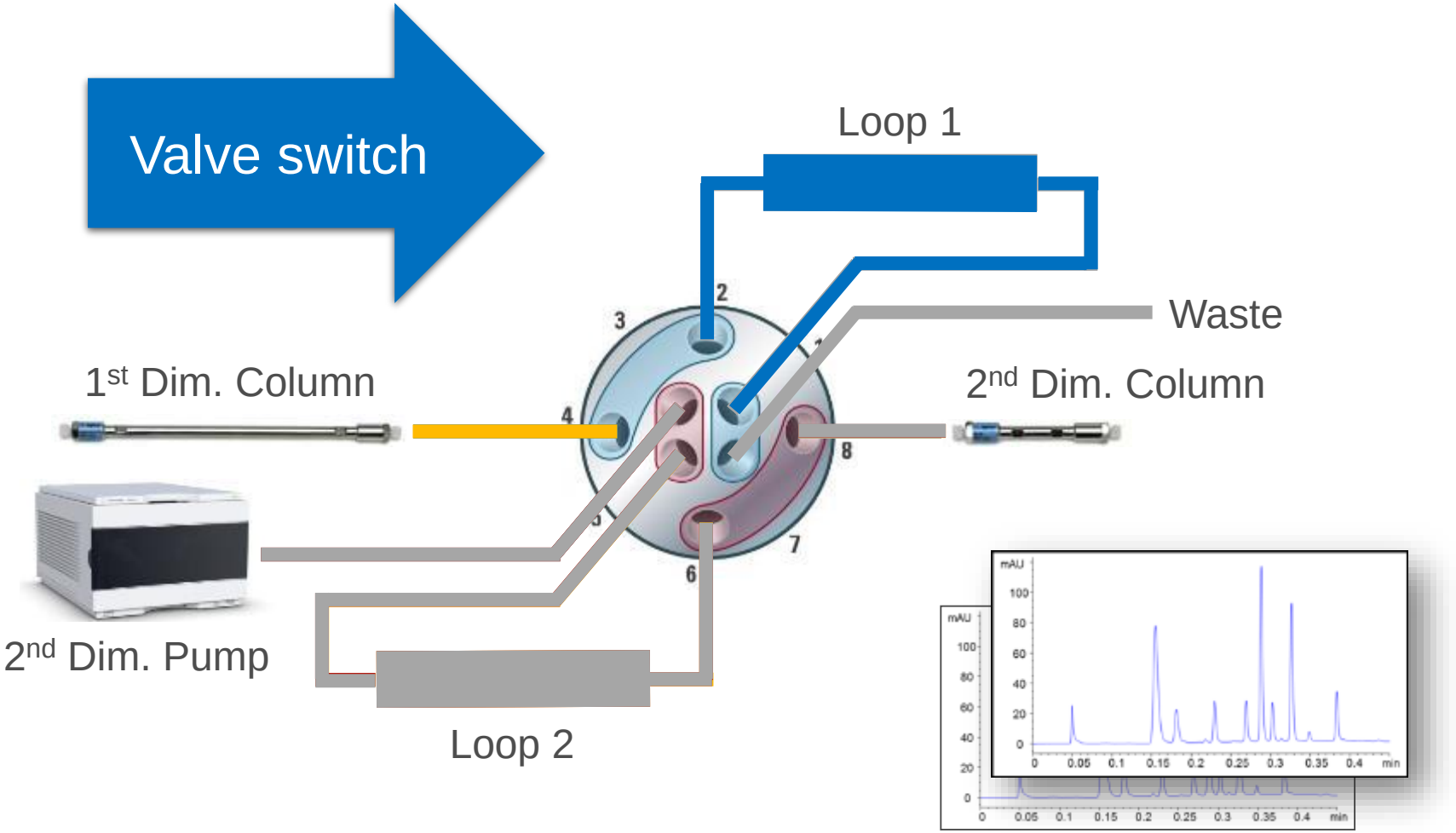
Comprehensive 2D-LC

Operating Principle



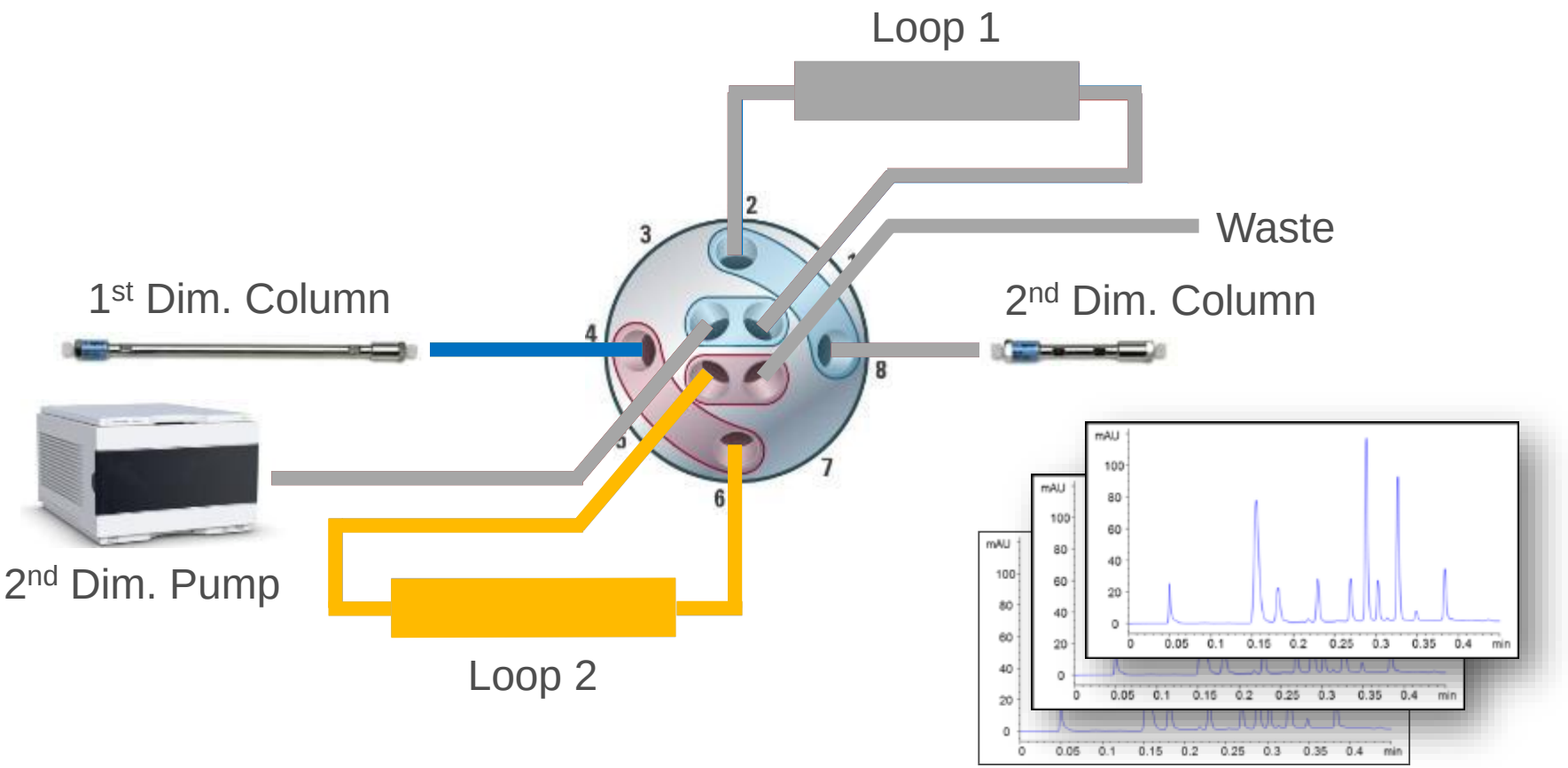
Comprehensive 2D-LC

Operating Principle

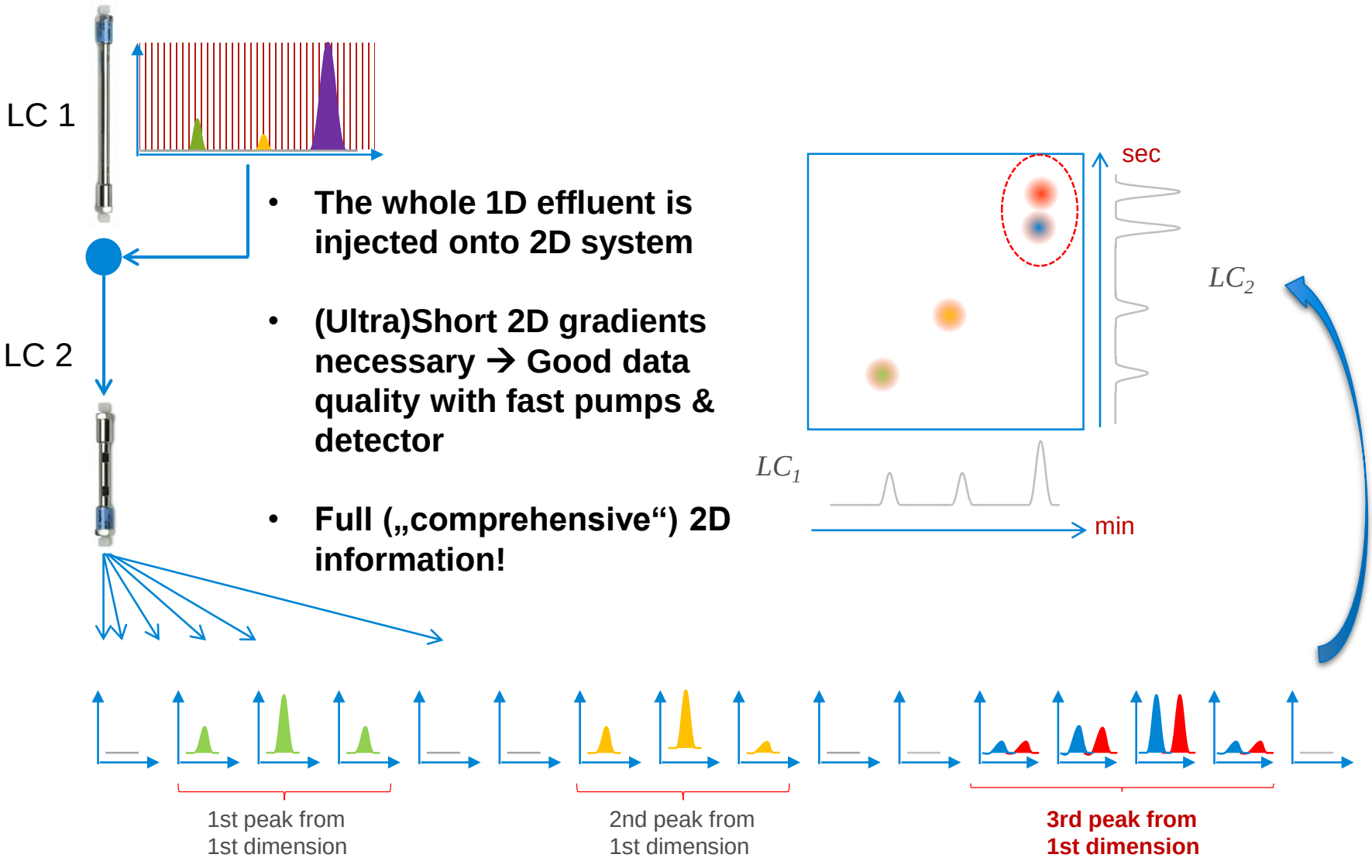


Comprehensive 2D-LC

Operating Principle

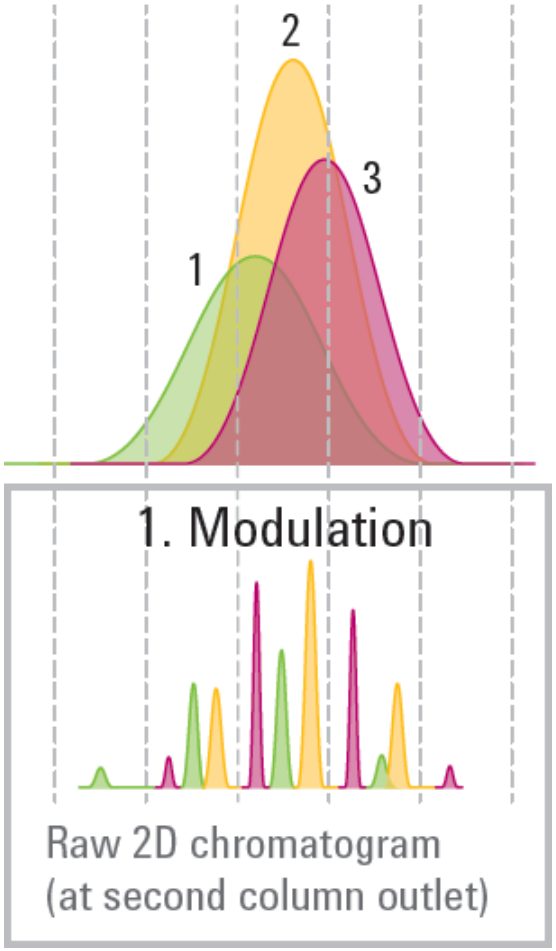


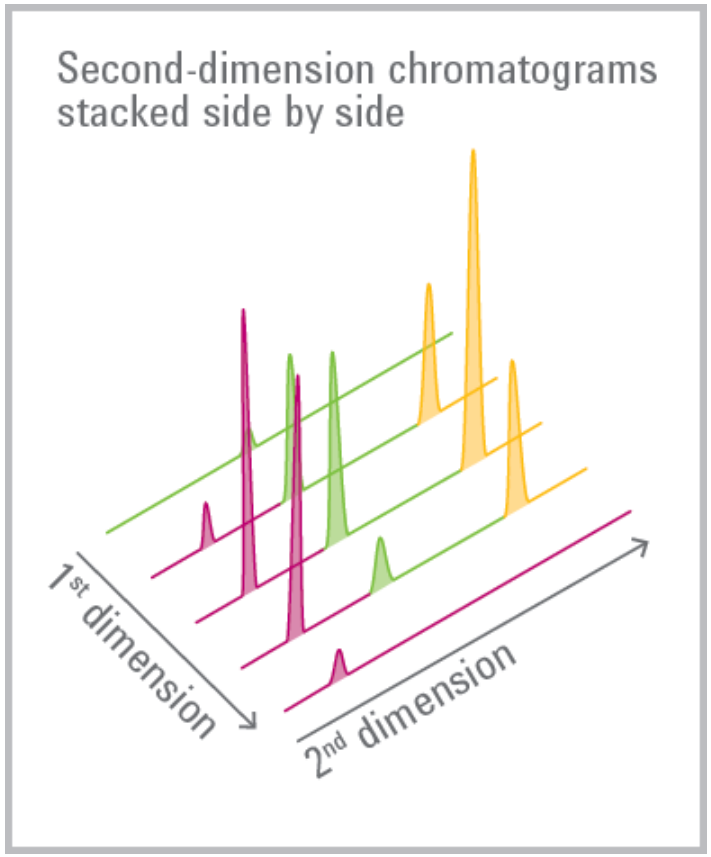
Comprehensive 2D-LC



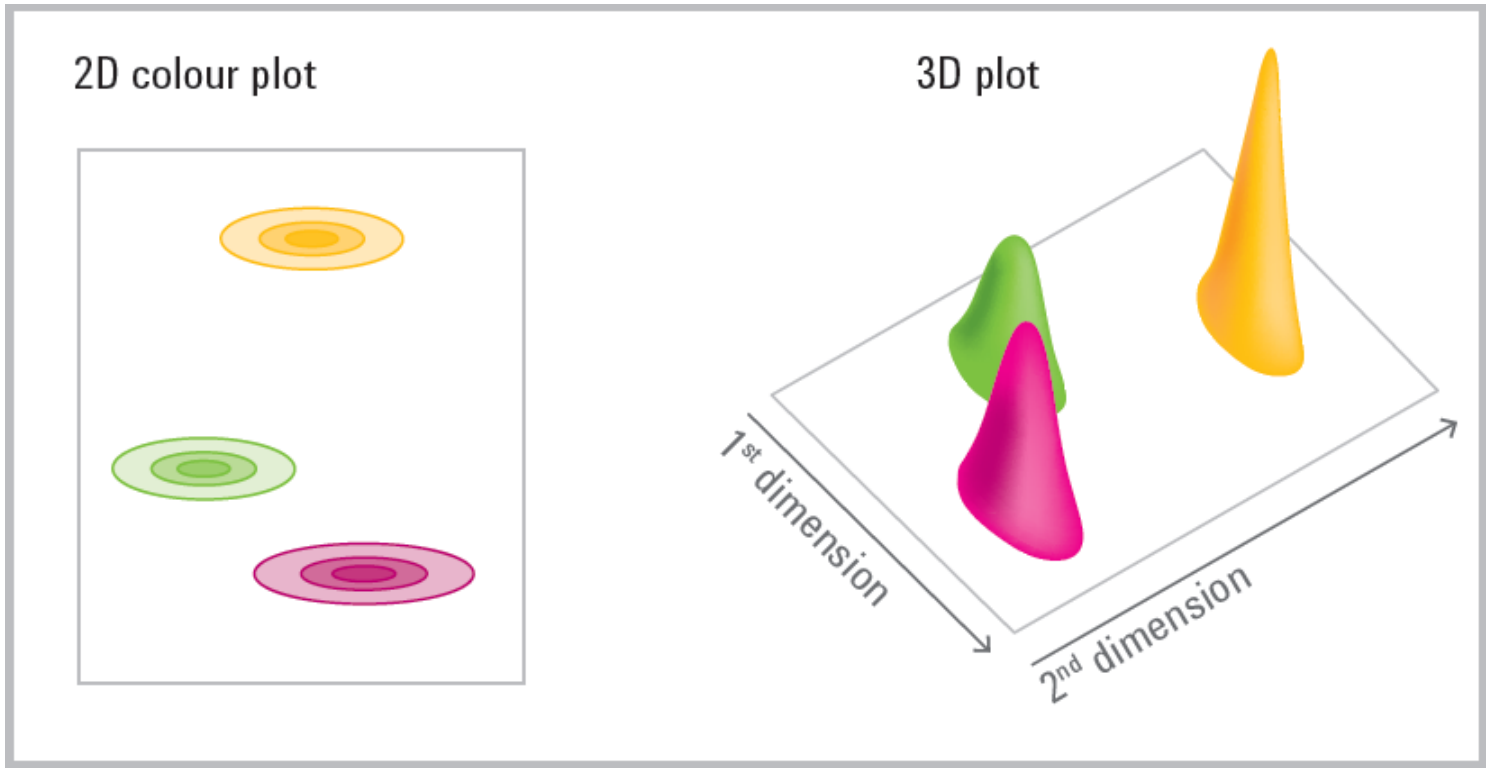
- **The whole 1D effluent is injected onto 2D system**
- **(Ultra)Short 2D gradients necessary → Good data quality with fast pumps & detector**
- **Full („comprehensive“) 2D information!**

Comprehensive 2D-LC Generation of 2D and 3D plots using Imaging Software





Comprehensive 2D-LC
Generation of 2D and 3D plots



Comprehensive 2D-LC
Fingerprinting Analysis of Different Types of Beer

Fingerprinting analysis of different types of beer by comprehensive 2D-LC enables classification of beer samples.

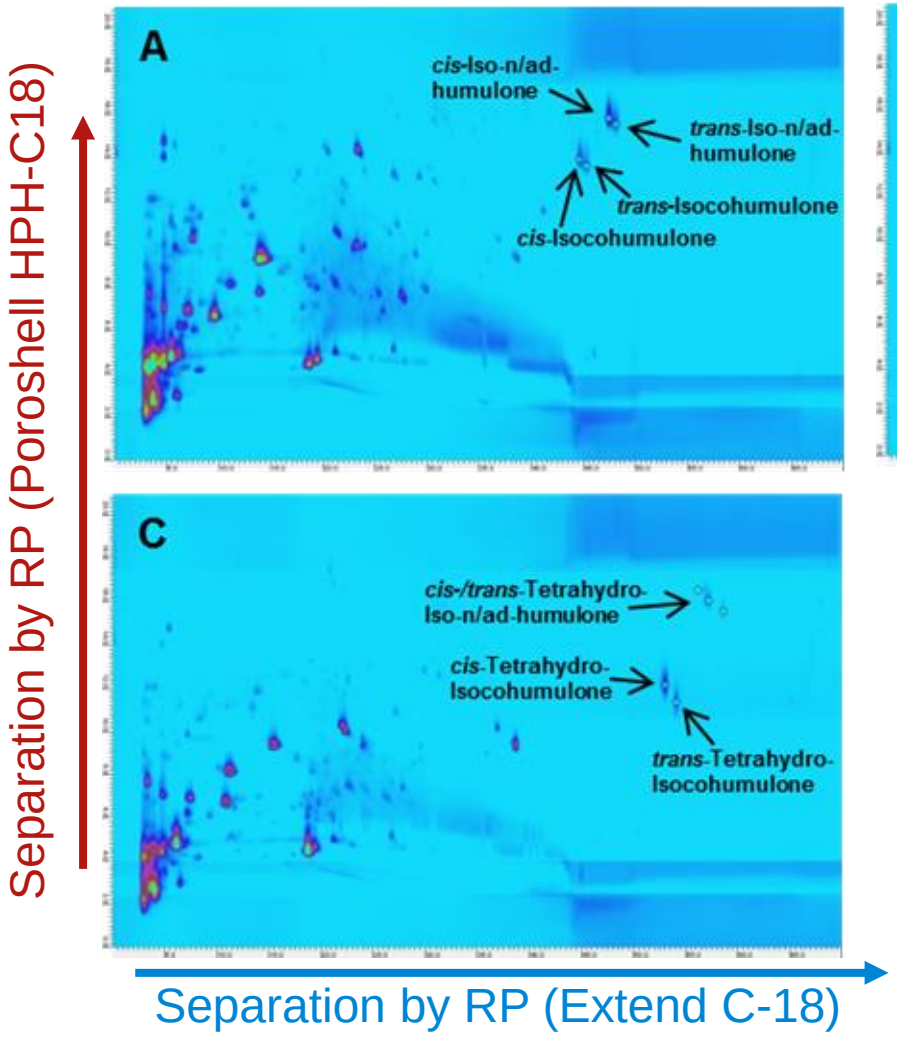
- Beer bitterness achieved by adding hops (*Humulus lupulus* L.) during wort boiling; iso-α-acids (isohumulones) and polyphenolic compounds responsible for beer bitterness.
- Iso-α-acids are light sensitive; light exposure leads to the formation of off-flavors (light struck flavor).
- Reduced iso-α-acids used in the brewing industry to enhance light stability; in Germany only natural hop compounds may be used (*Reinheitsgebot*).

Data from Agilent Application Note 5991-5521EN

Fingerprinting Analysis of Different Types of Beer

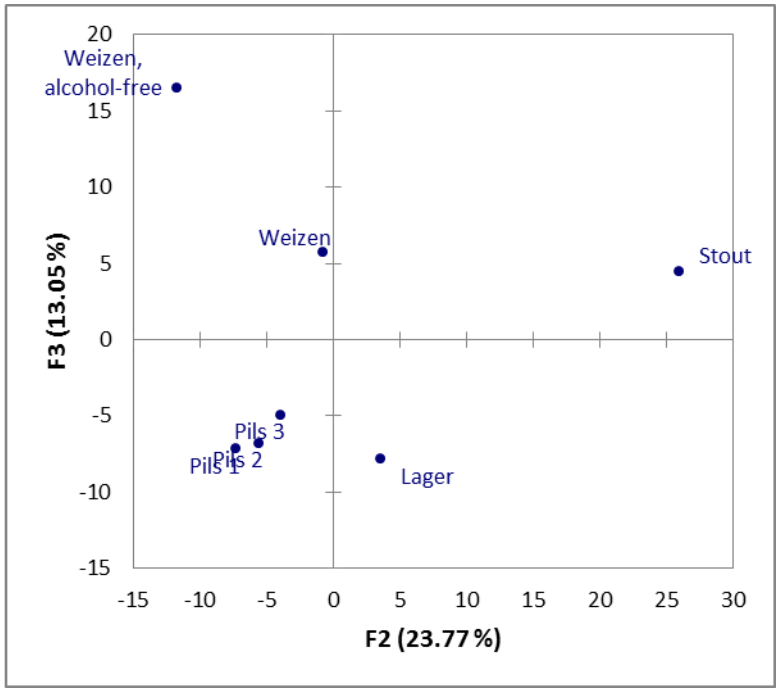
Analyses of Different Beer Samples

Detection: DAD at 270 nm and MS (Q-TOF)



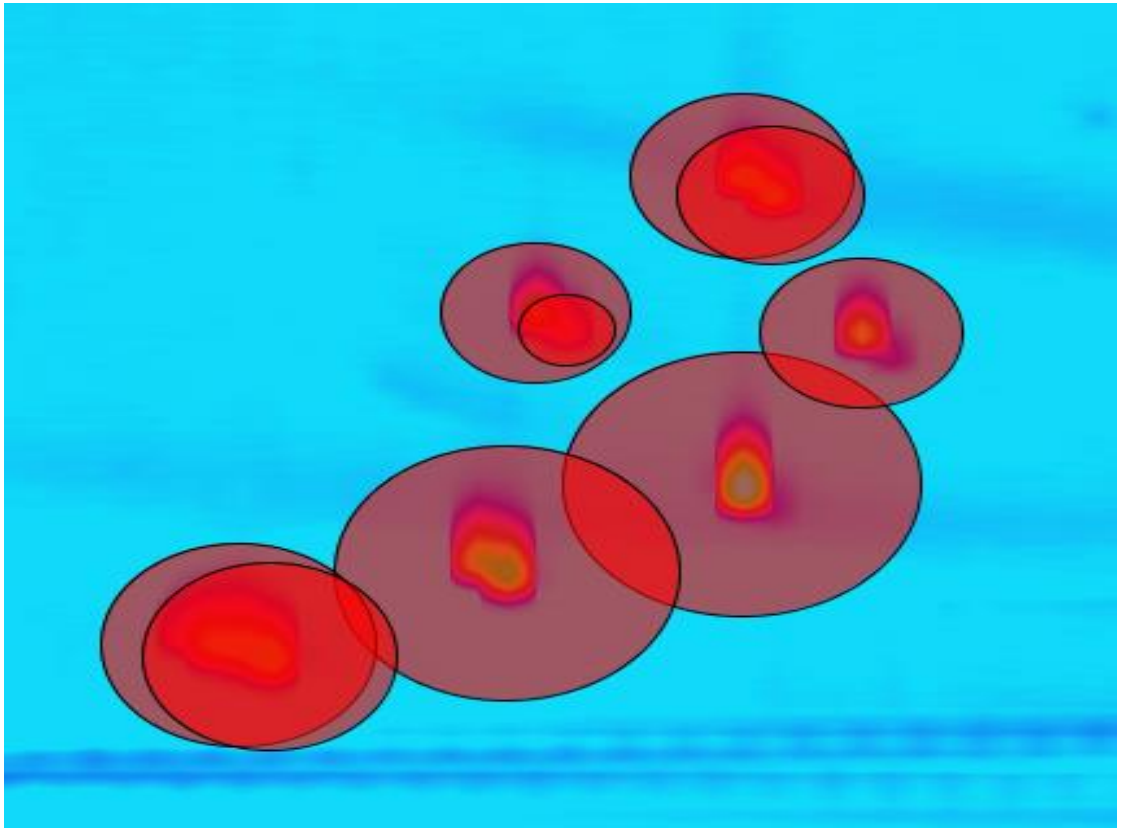
(A) German Weizen beer
(B) German Pils beer
(C) American Lager beer

Fingerprinting Analysis of Different Types of Beer
Classification by Principal Component Analysis



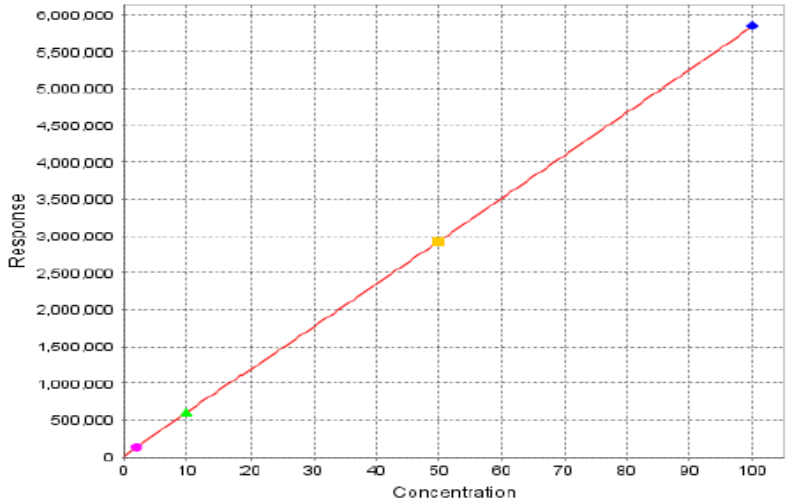
PCA: Classification of beer samples according to their type

Calibrations May Be Done with Imaging Software Using ‘Blobs’ (Cones)



Calibration May Be Generated from Multiple Blobs

Calibration Table



Response Vs. Concentration for Caffeine

Calibration Table for Caffeine

Concentration	Response
100.00000	5849561.82981
10.00000	608875.13394
2.00000	140641.53676
50.00000	2918411.05514

Road Blocks to 2D Chromatography

- Typically first dimension gradient is a long, slow gradient followed by rapid, repeated gradients on the second dimension so a very low delay volume pump is needed capable of ballistic gradients
- Difficult to coordinate timing between first and second dimension gradient
- Difficult to coordinate valve timing between first and second dimension
- Difficult to coordinate heart-cutting first dimension detector with trapping valve
- Any changes to one time table necessitates changes to all the other tables

Complex Gradient and Valve Switch Tables

Capillary pump 1:
Gradient across SCX column

Time	%B
0	0
6	0
135	10
200	20
230	30
280	50
295	100
320	100
320.1	0
350	0

Capillary pump 2:
Gradient across analytical column

Time	%B	Time	%B
0	3	175	3
5	3	201.1	65
26.1	65	201.2	3
26.2	3	210	3
35	3	236.1	65
61.1	65	236.2	3
61.2	3	245	3
70	3	271.1	65
96.1	65	271.2	3
96.2	3	280	3
105	3	306.1	65
131.1	65	306.2	3
131.2	3	315	3
140	3	340	65
166.1	65	340.1	90
166.2	3	345	90

6-port valve:
timetable

Time	Position
0	column 2
26.1	column 1
35	column 2
61.1	column 1
70	column 2
96.1	column 1
105	column 2
131.1	column 1
140	column 2
166.1	column 1
175	column 2
201.1	column 1
210	column 2
236.1	column 1
245	column 2
271.1	column 1
280	column 2
306.1	column 1
315	column 2
345	column 1

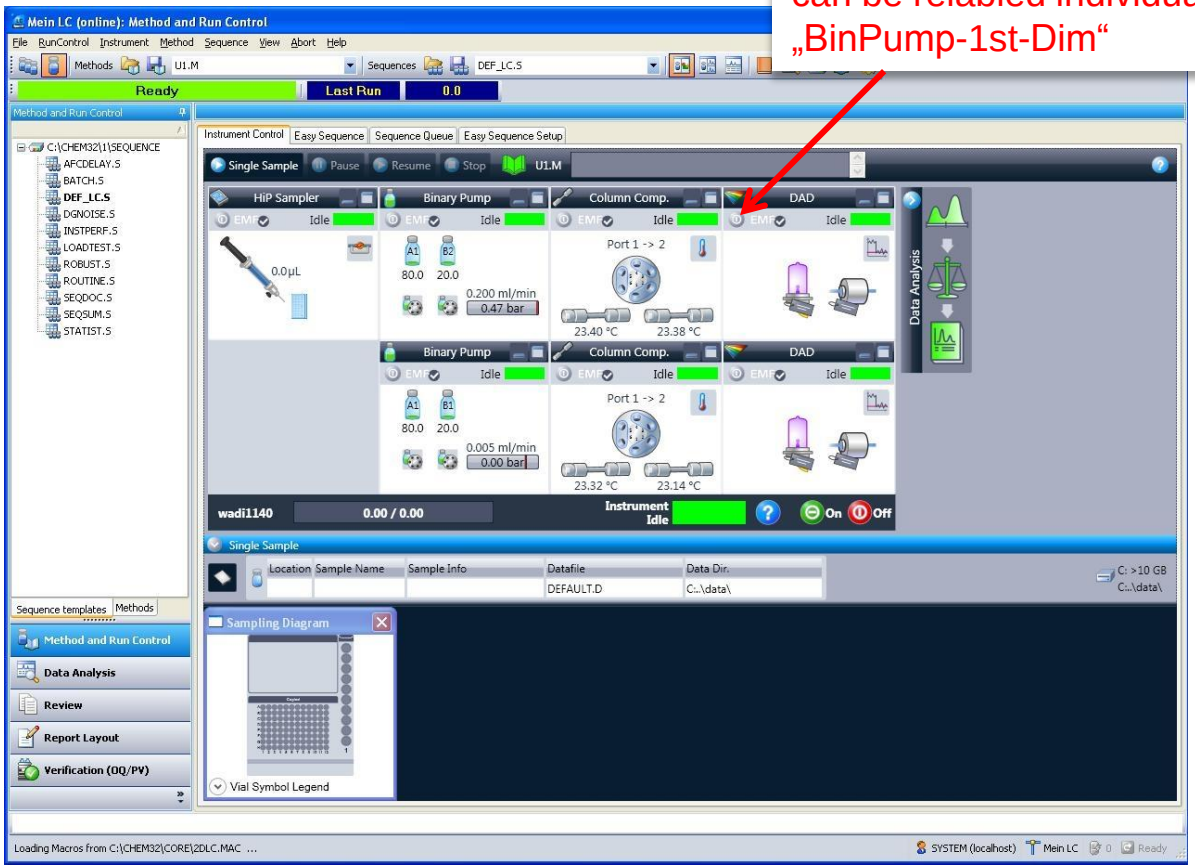
10-port valve:
timetable

Time	Position
0	Pos 1
5	Pos 2
30	Pos 1
65	Pos 2
100	Pos 1
135	Pos 2
170	Pos 1
205	Pos 2
240	Pos 1
275	Pos 2
310	Pos 1

Agilent 2D-LC Add-On Software

ChemStation Dashboard:

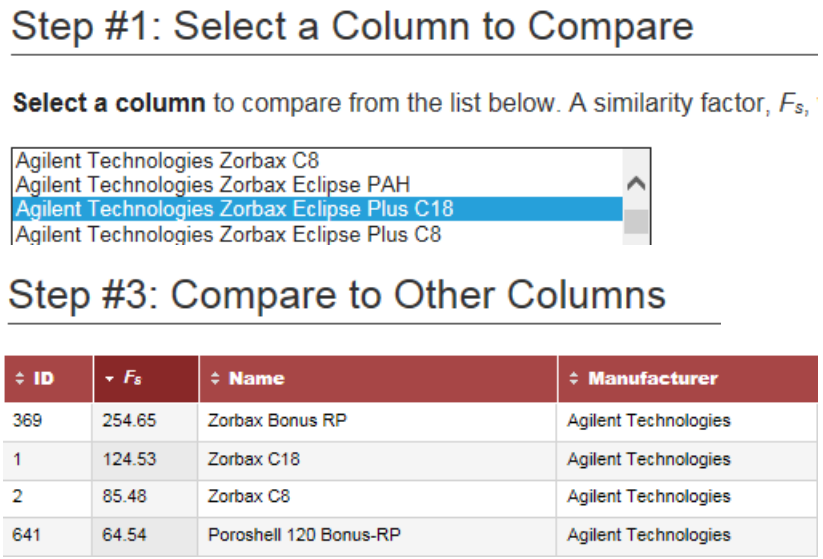
All modules in one dashboard
can be relabled individually, e.g.
„BinPump-1st-Dim“



Combinations of Separation Mechanisms
RPLC: Hydrophobic Subtraction Model

Hydrophobic Subtraction Model:

- Describes the interactions between RP columns and different analytes according to hydrophobicity, steric hindrance, acidity, basicity and ion-exchange capacity
- Calculation of a column selectivity factor (F_s) as a metric for the selectivity differences between two RP columns
- Characteristics of more than 600 RP columns are available as a web-based database (www.hplccolumns.org)
- Keep in mind that retention and selectivity not only depend on the column, but also on the mobile phase (organic solvent, pH) and the temperature



2D-LC System Configuration

“One screen for the entire system”

Define ^{1}D / ^{2}D pump

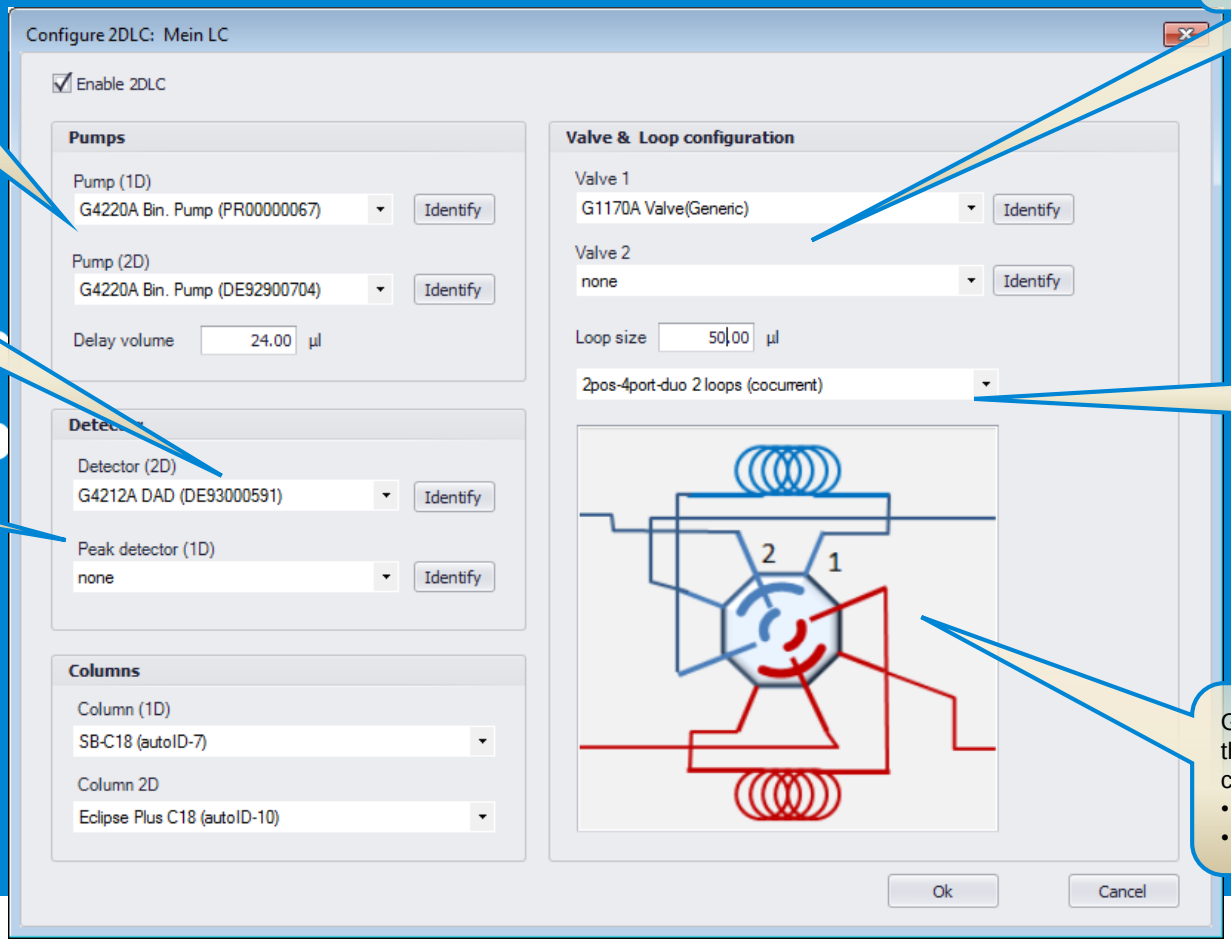
Define detector in the second dimension

Define peak detector (optional)

Select the valve(s) to be used for 2D-LC injection

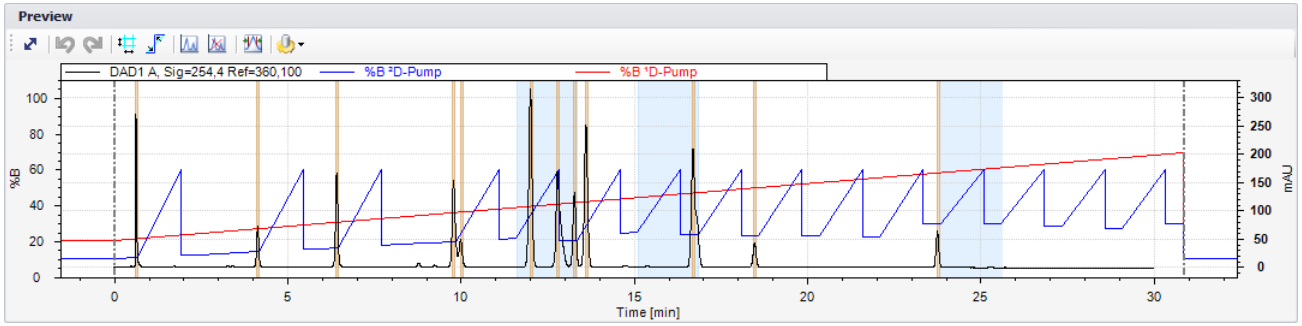
Select a possible valve / loop configuration

Graphical representation of the selected valve / loop configuration:
- Flow path ^{1}D & ^{2}D
- Animated valve switching



Considerations for method development
Shifted gradient

Using a shifted second dimension gradient means that over the course of the 2D-LC run, the second dimension gradient is changed in order to adapt to the hydrophobicity of the compounds eluting from the first dimension.



²D Gradient

Time [min]	% B
0.00	10.00
Shift	
20.00	30.00
1.25	60.00

In comparison, using a full second dimension gradient means that every fraction injected to 2D experiences the same 2D gradient.

Using RPLC in both dimensions, compounds with weak retention on the first dimension RP column will tend to show weak retention also on the second dimension RP column. In this case, the 2D gradient can be shifted such that each fraction injected to the 2D experiences a shallower gradient, thereby increasing 2D resolution.

Method User Interface

2D-LC specific parameters of the 2D-pump

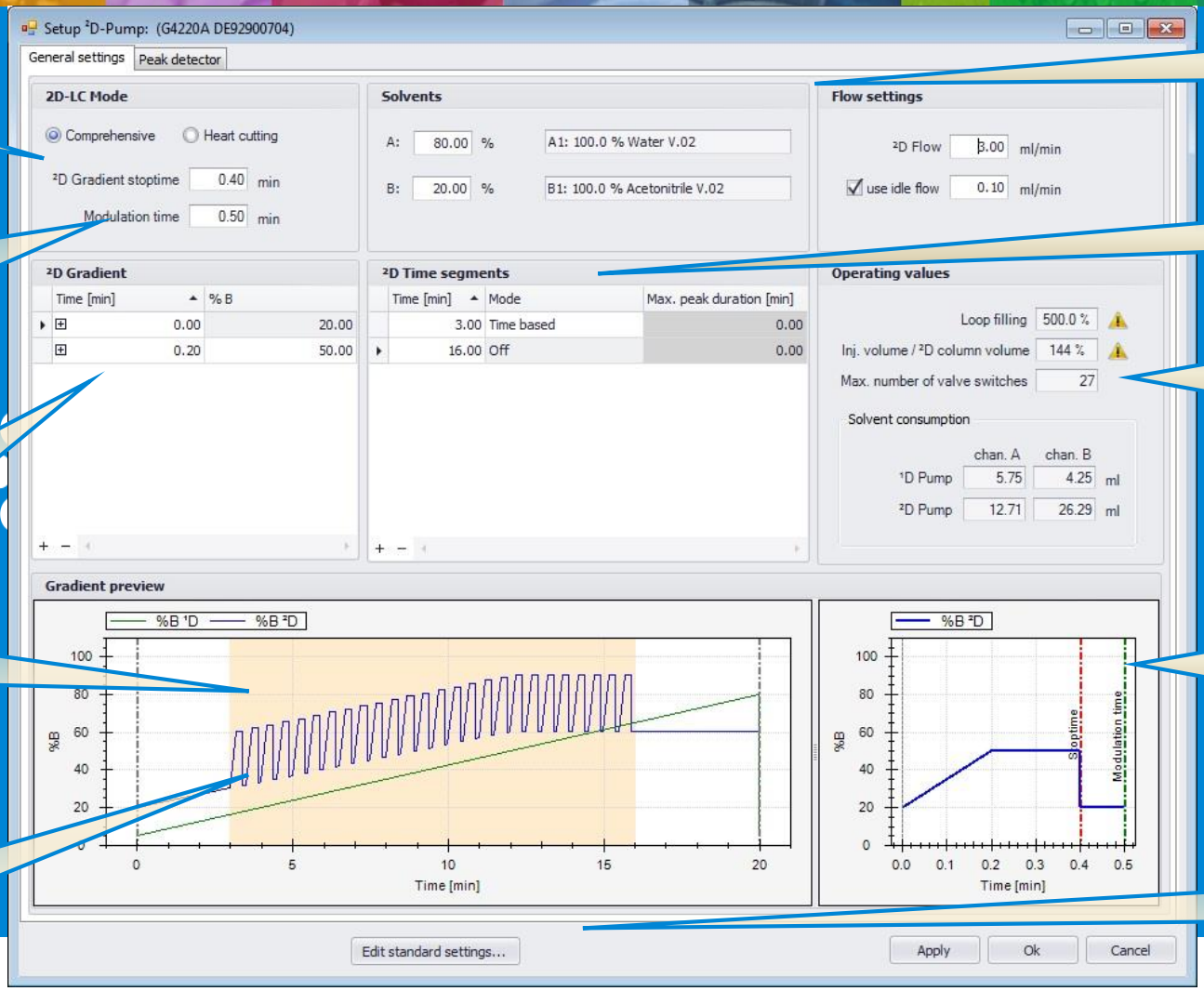
Select the 2D-LC mode: comprehensive / heart-cutting

Define repetition of 2nd dimension gradient (Modulation time)

Define the gradient of the 2nd dimension

Show rollout of gradient in the 2nd dim over the runtime of the 1st dimension

Graphical editing of gradient shift



Solvent & Flow-Settings

Define time window(s) where the selected 2DLC mode is active

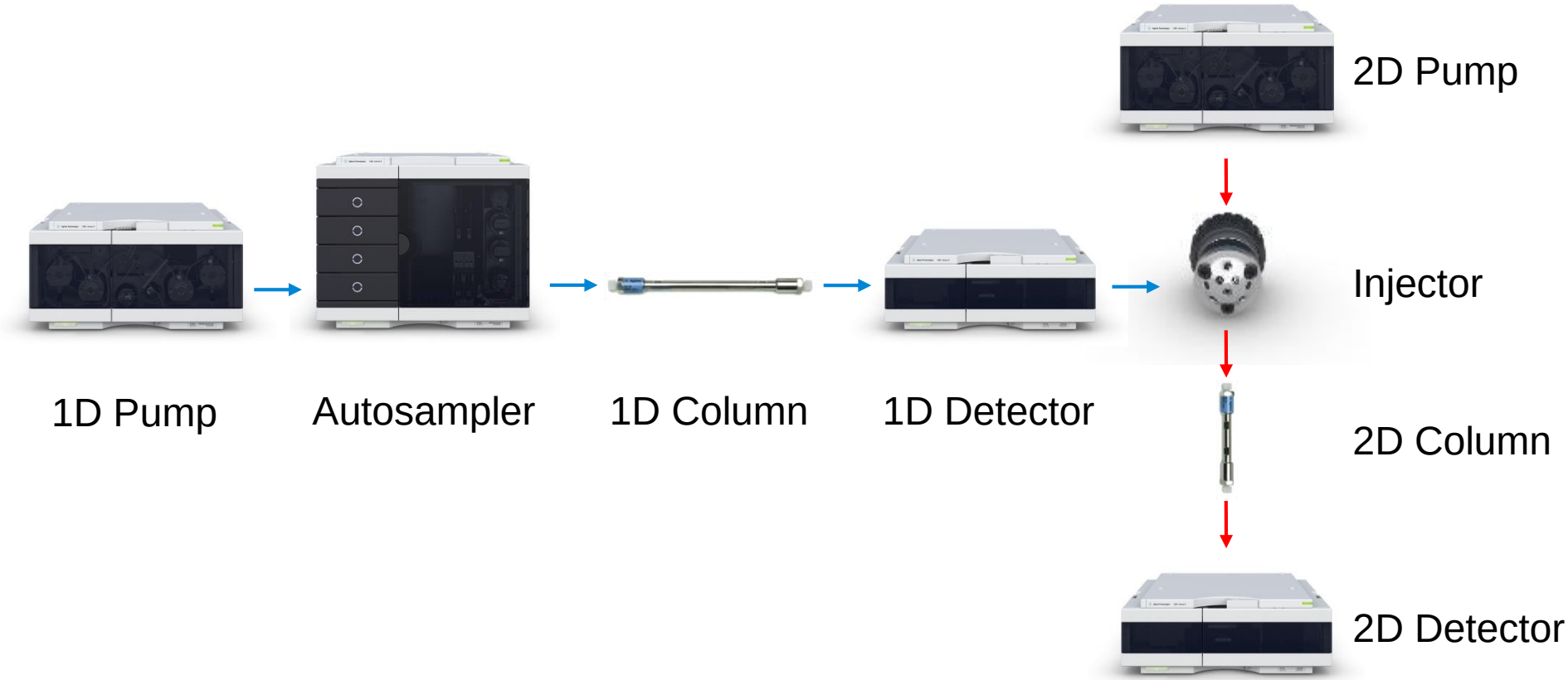
Operation values, warnings

Close-up of ²D-gradient

Access to standard method UI of the pump

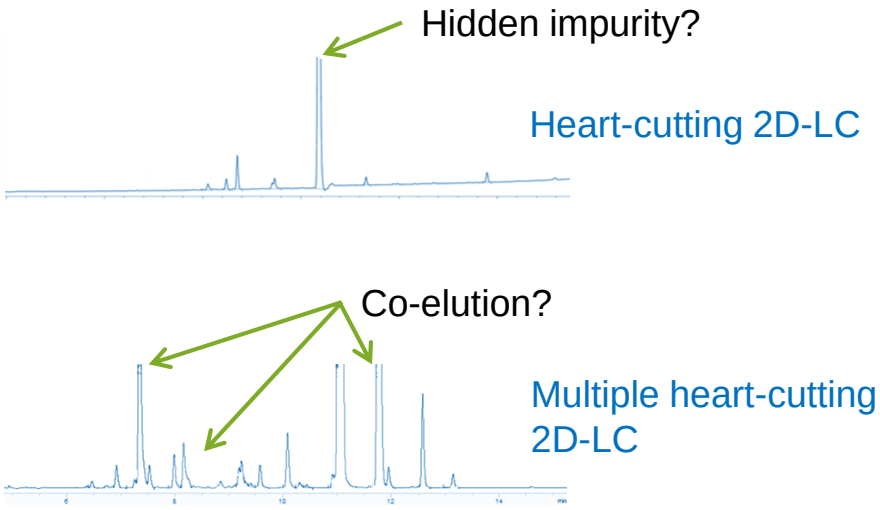
HEART-CUTTING TWO-DIMENSIONAL LC

Two-dimensional LC
System configuration

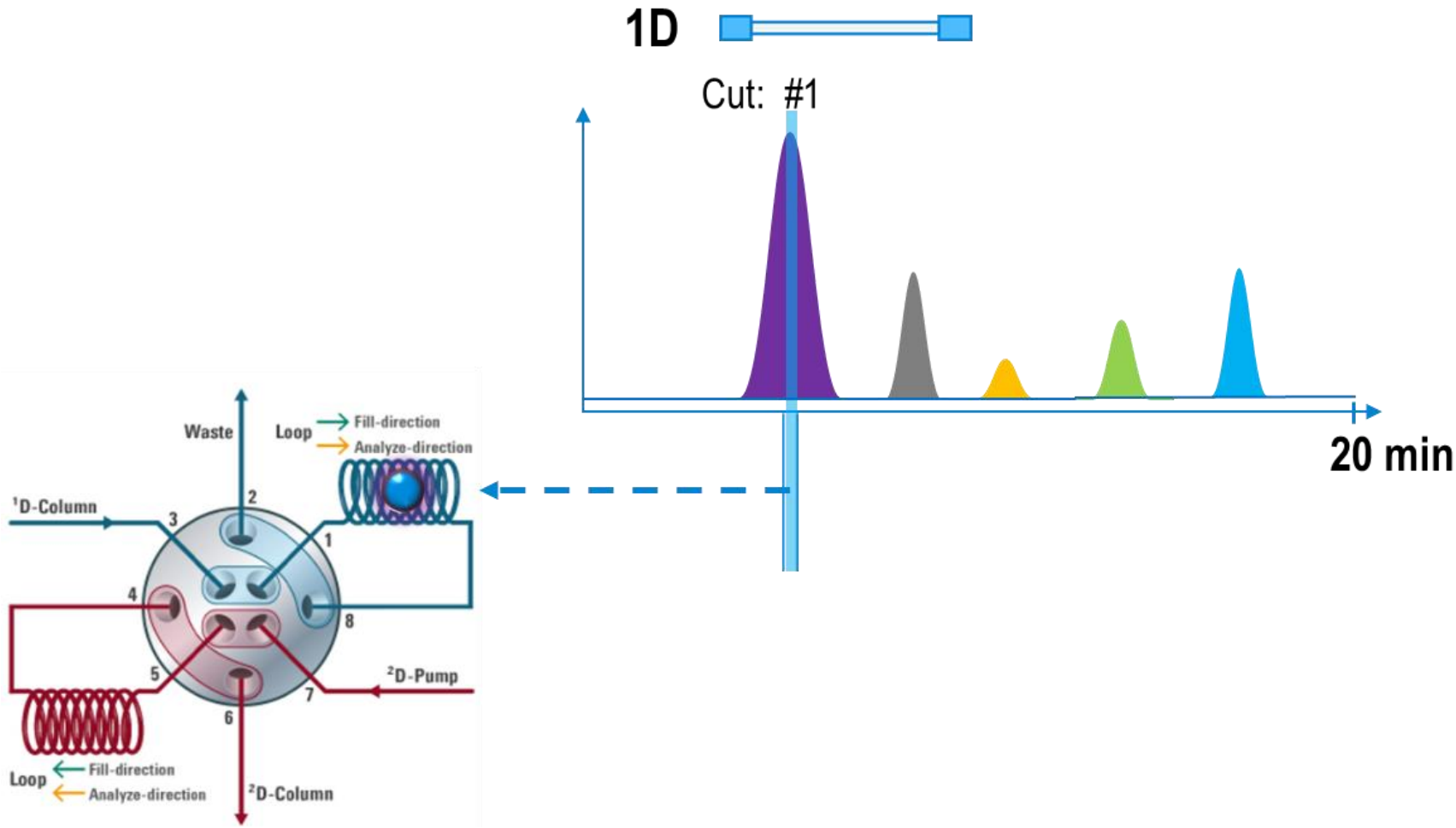


Why heart cutting two-dimensional LC?

- 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 Workflow with Dual Loop
First Cut Sent to ^{2}D



Heart-cutting 2D-LC
Achiral-Chiral 2D-LC Analysis of Pharmaceutical Substances

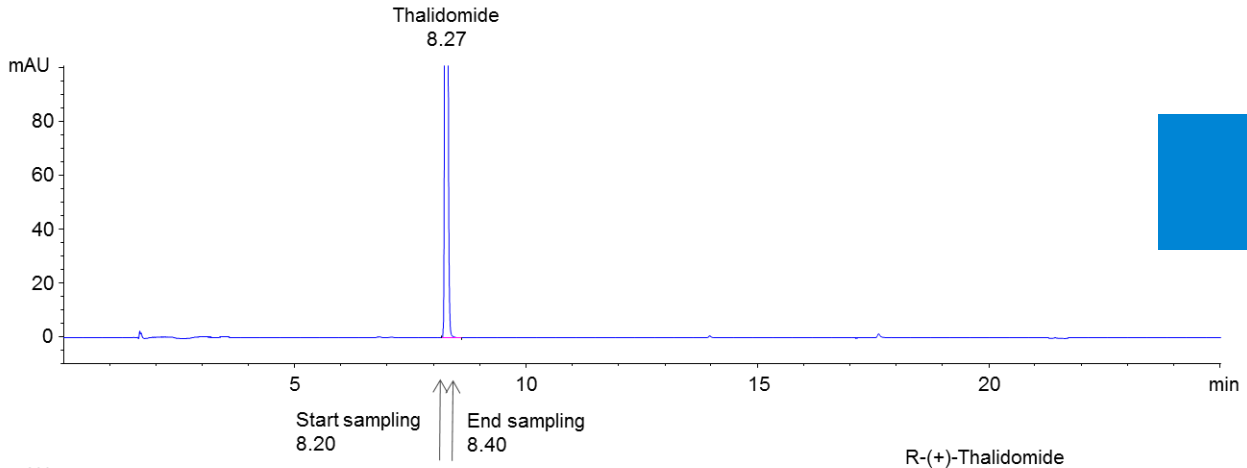
Determination of achiral impurities and enantiomeric excess in a single run. Chiral column in the second dimension.

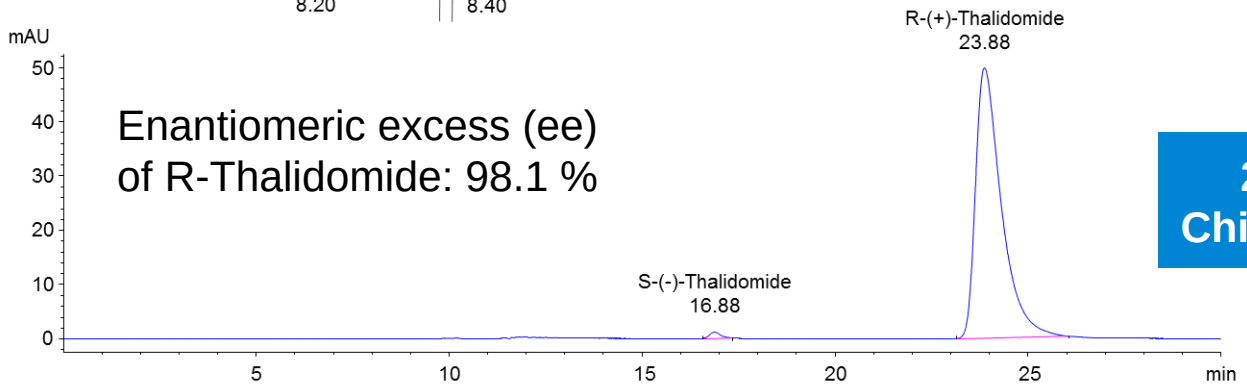
- ICH guideline Q3A (R2): Impurities at or above 0.05 % in new drug substances need to be reported
- Enantiomers of chiral drugs: Often differences in pharmacokinetic behavior and pharmacological activity
- Heart-cutting 2D-LC: Simultaneous impurity analysis and separation of enantiomers in one analysis

Data from Agilent Application Note 5991-4664EN

Achiral-chiral 2D-LC of Pharmaceutical Substances

Main compound R-Thalidomide with trace S-Thalidomide





Enantiomeric excess (ee)
of R-Thalidomide: 98.1 %

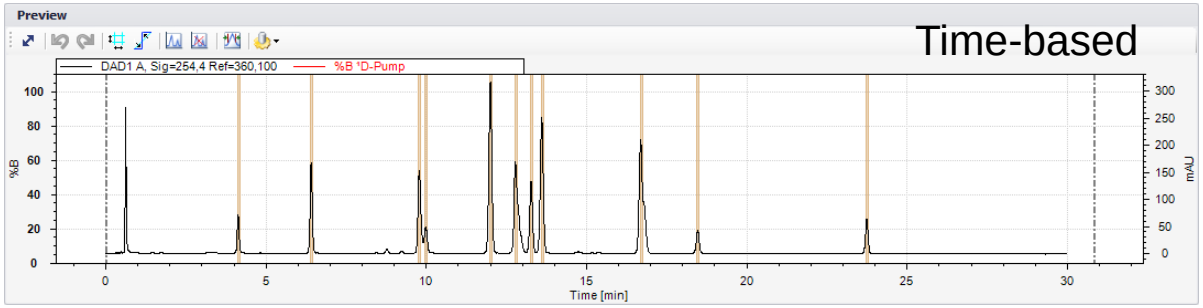
1st Dim: C18

2nd Dim: Chiral column

Time-based and peak-based operation

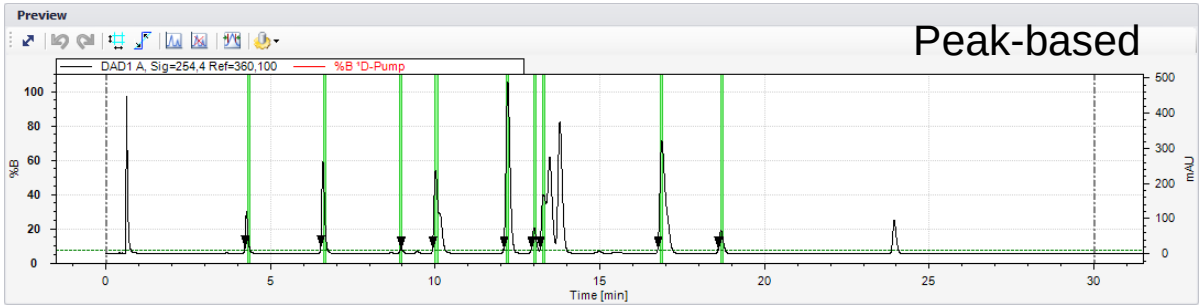
(Multiple) Heart-cutting can be performed either time-based or peak-based.

- Time-based means that heart-cut times are defined in a timetable. This timetable can be constructed according to the first dimension retention time of peaks in a reference chromatogram.
- Peak-based means that heart-cutting is triggered by the first dimension detector.



Timetable

Time [min]	Mode	Sampling time [min]	Add transfer volume
4.11	Time based	0.07	☑
6.39	Time based	0.07	☑
9.76	Time based	0.07	☑
9.97	Time based	0.07	☑
11.98	Time based	0.07	☑
12.76	Time based	0.07	☑
13.25	Time based	0.07	☑



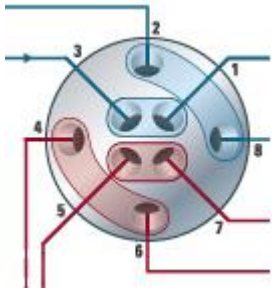
Timetable

Time [min]	Mode	Sampling time [min]	Add transfer volume
3.00	Peak based	0.14	
20.00	Off		

What happens after the 1D detector?
Transfer volume

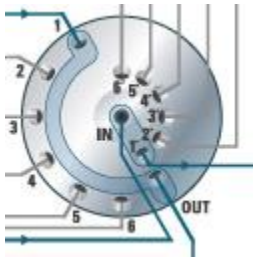
1D detector

transfer volume

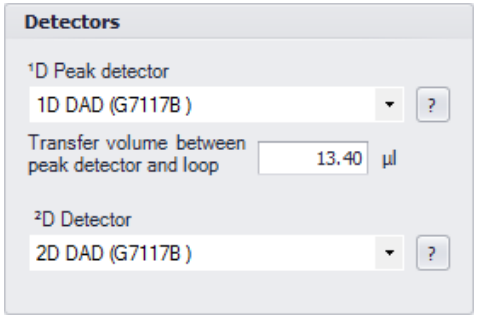


2D-LC valve

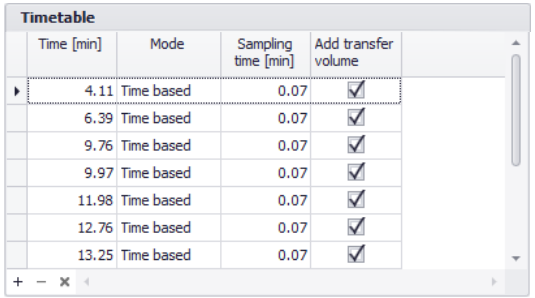
more transfer volume

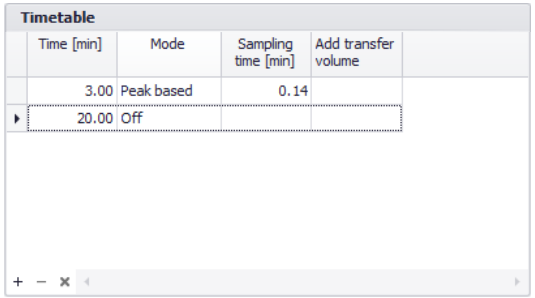


MHC valves



2D-LC configuration





2D-LC setup

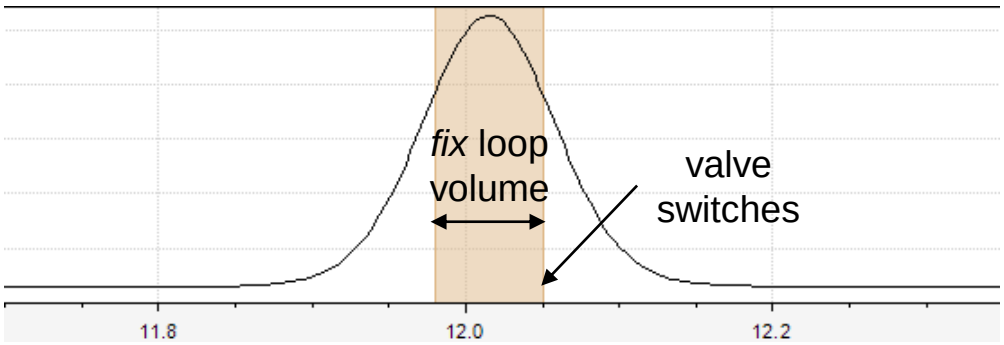
- For peak-based operation „add transfer volume“ is compulsory as the transfer volume always needs to be considered due to the time difference between peak detection and arrival in loop.
- For time-based operation, using the transfer volume is optional but checked by default. Unchecking this option switches the valve at times indicated in the timetable. If a reference chromatogram acquired by the 1D detector is used for timetable setup, the transfer volume must be considered!

Time-based operation
Sampling time and loop filling

In multiple heart-cutting 2D-LC, sampling of the 1D effluent is achieved by switching the valve at the end of the heart-cut. Typically, loop overfill is used.

Before a heart-cut is taken, the 1D effluent is already flowing through the loop that the next heart-cut is going to be sampled in.

For time-based operation, a fix sampling time (according to the fix loop volume of 40 µL and the 1D flow rate) starts with the heart-cut time defined in the 2D time segments table. The loop filling results from the 1D effluent flowing through the loop before the defined heart-cut time.



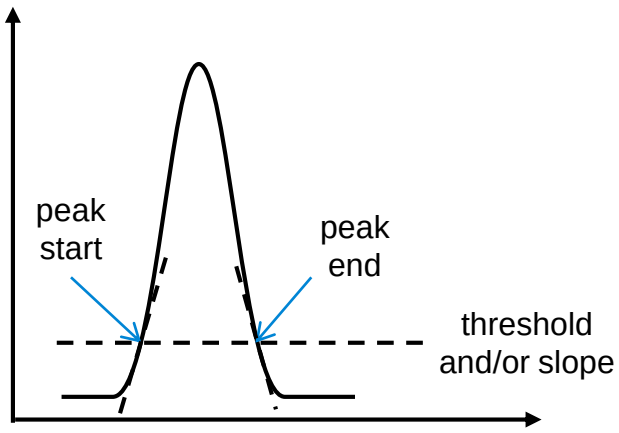
Fix sampling time according to 40 µL loop volume and 1D flow of 600 µL/min

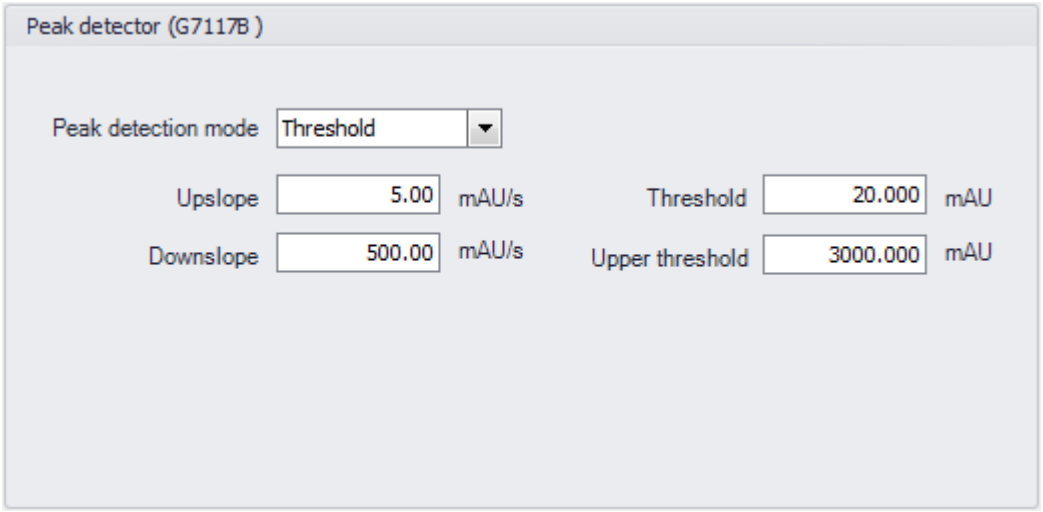
Timetable

Time [min]	Mode	Sampling time [min]	Loop filling [%]
11.98	Time based	0.07	> 200

Loop filling due to 1D effluent flowing through loop before sampling

Peak-based operation





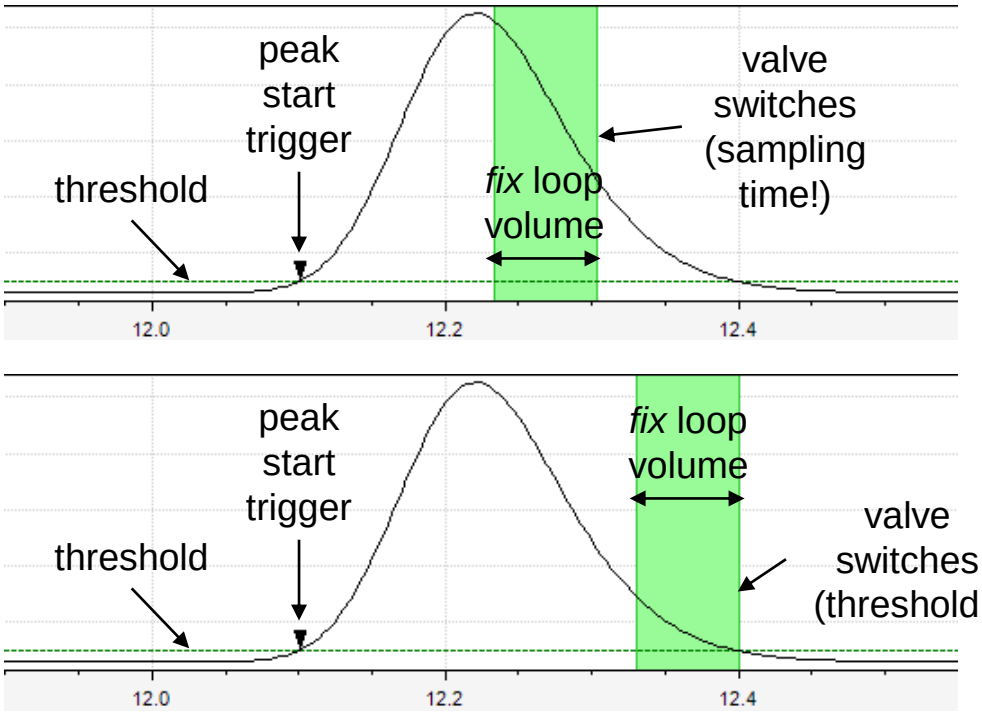
2D-LC setup

In peak-based operation, heart-cutting is triggered upon peak detection at the first dimension detector. Parameters for peak detection are set similar to integrator settings by threshold and/or slope.

Peak-based operation
Triggering and sampling time

In peak-based operation the valve switch occurs:

- if the sampling time has elapsed (sampling time controls cut position!)
- or if the signal falls below threshold/slope, whichever comes first!



Timetable

	Time [min]	Mode	Sampling time [min]
▸	3.00	Peak based	0.20

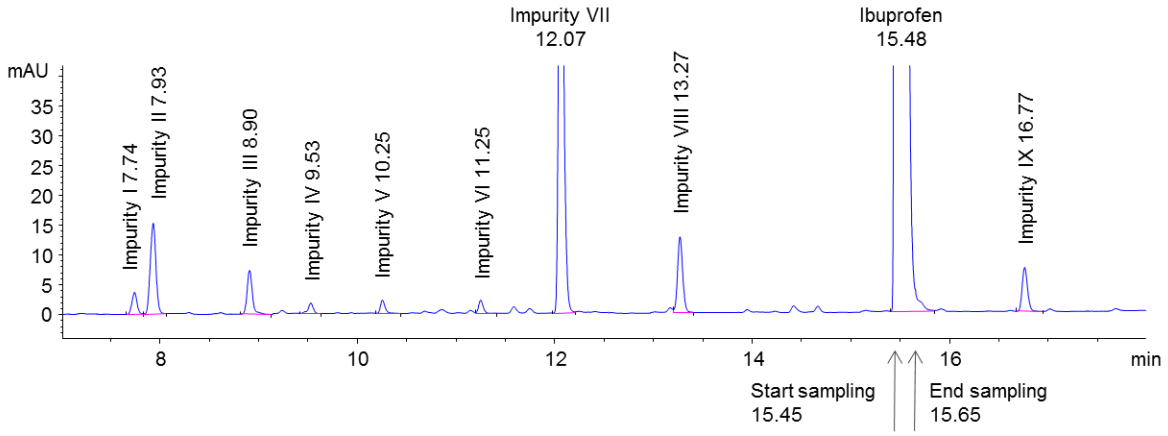
variable sampling time

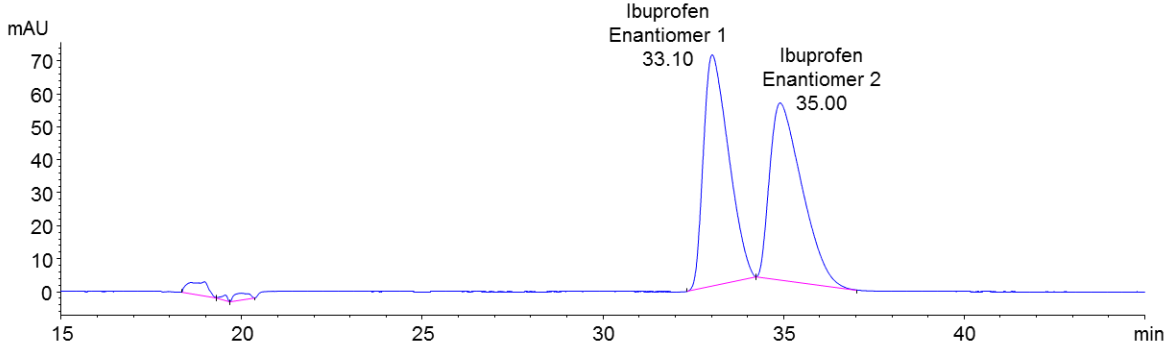
Timetable

	Time [min]	Mode	Sampling time [min]
	3.00	Peak based	0.50

Achiral-chiral 2D-LC of Pharmaceutical Substances

Ibuprofen (racemic)

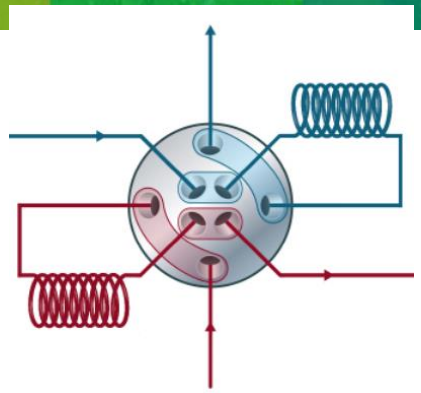


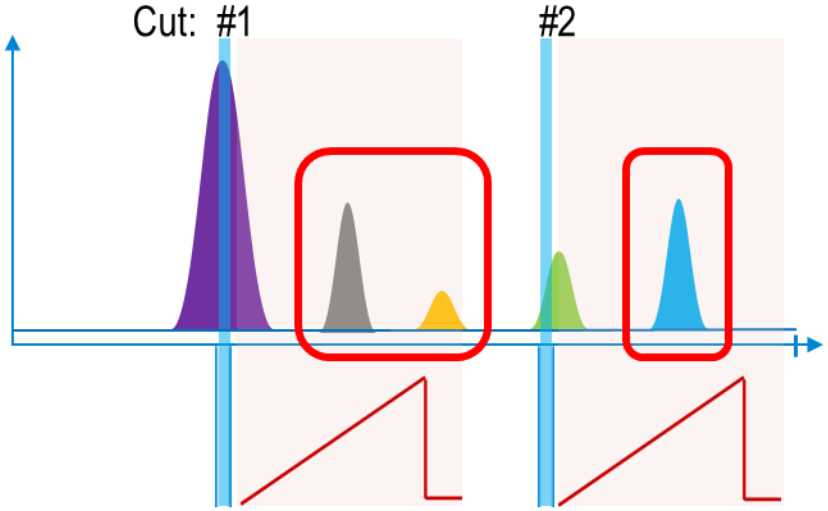


1st Dim: C18

2nd Dim: Chiral column

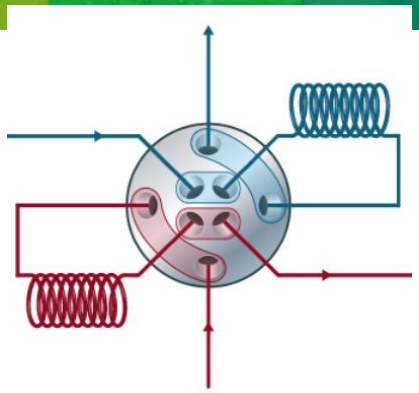
Limit of (Single) Heart-Cutting

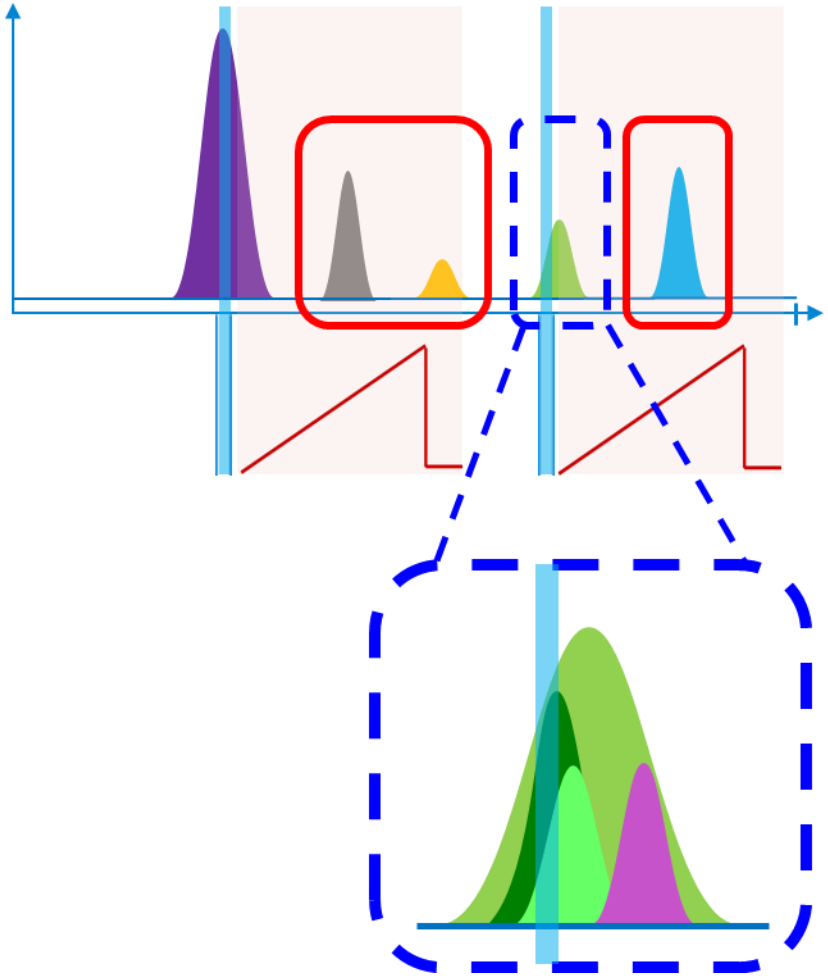




- Skipped interesting peaks during 2D cycle.

Limit of (Single) Heart-Cutting

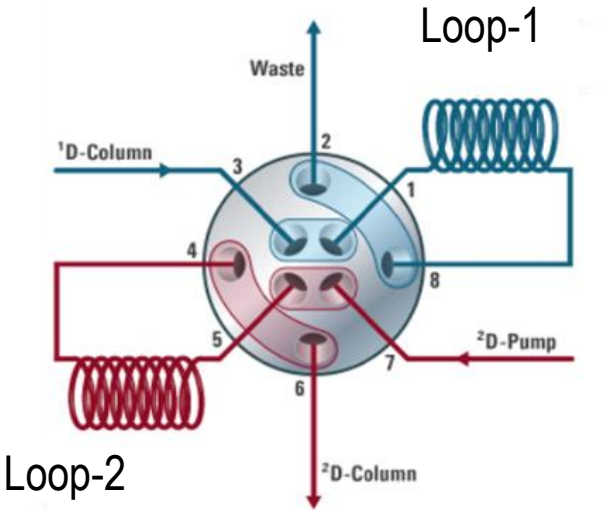


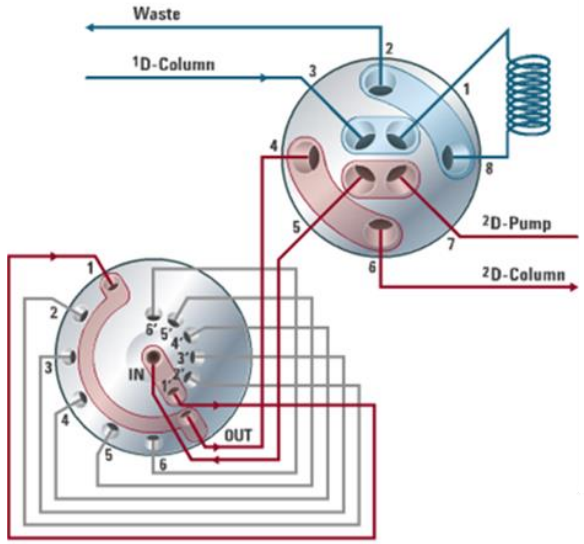


- Skipped interesting peaks during 2D cycle.
- There may be an additional co-eluting peak located at a different position than that where the heart cut was made.

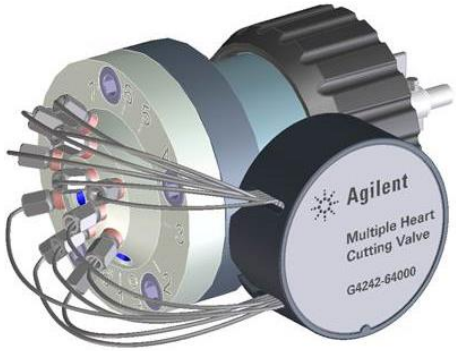
New Multiple Heart-Cutting (MHC) Solution

From Dual Loop to Multiple Heart Cut





Deck with 6 loops



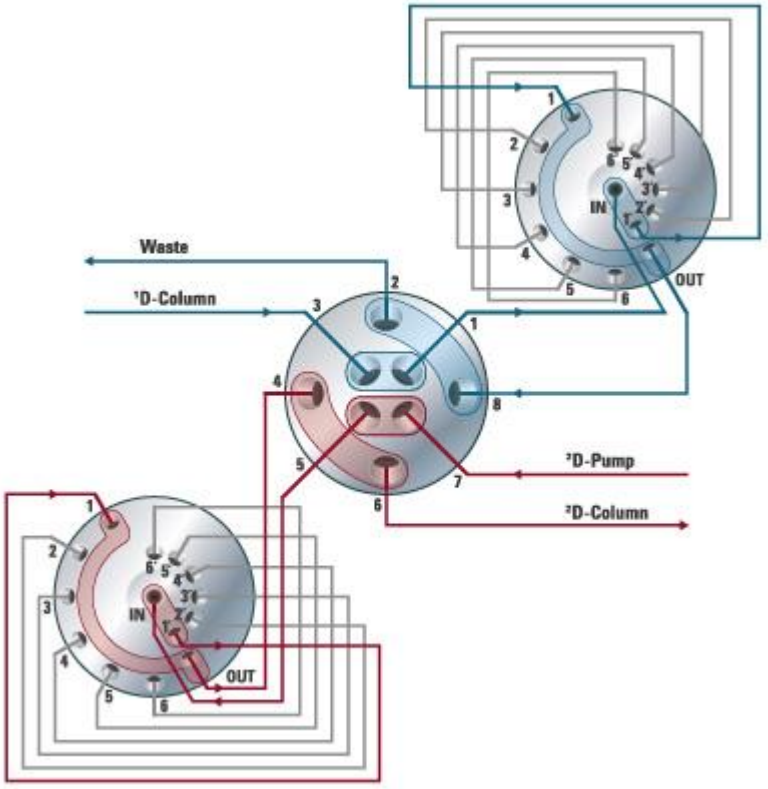
One of the loops replaced by 6-column selector valve fitted with 6 loops:

Parking deck cluster offering 7 sampling positions.

Dual-Deck MultipleHeart-Cut

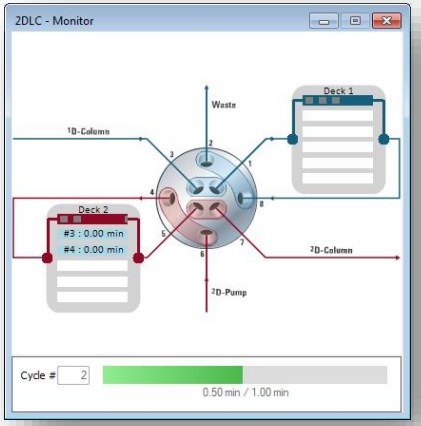
Unmatched Multiple Heart-Cutting 2D-LC Usability

Smart Valve-Loop Setup with 6 or 12 loops
→ 2D-LC valve + one or two 6/14 valves



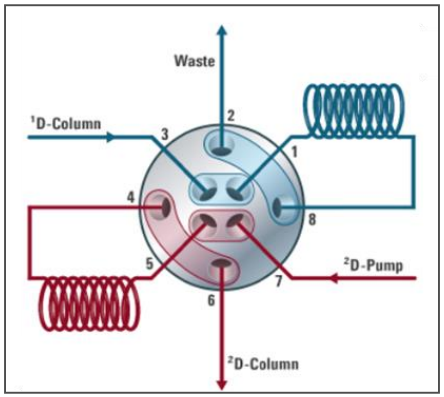
Pre-aligned loop-valve kits, just add to the existing 2D-LC system

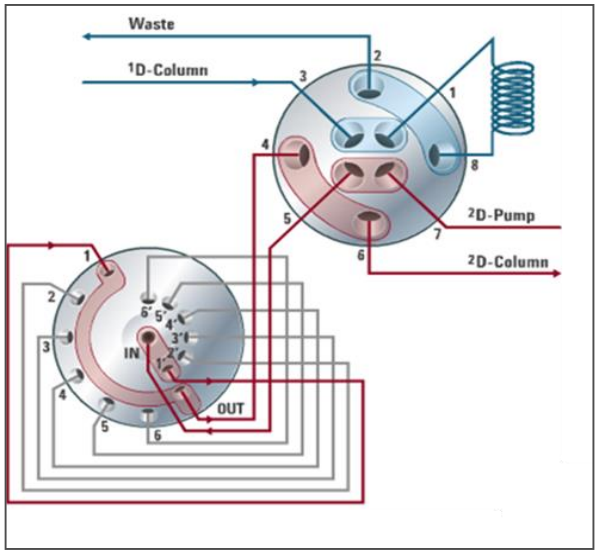
Online status monitoring



New Multiple Heart-Cutting (MHC) Solution

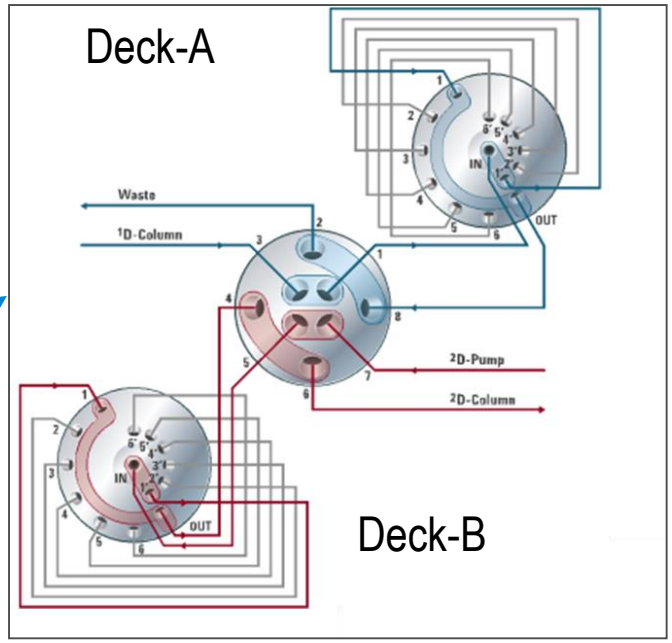
From dual loop to MHC





Deck with 6 loops

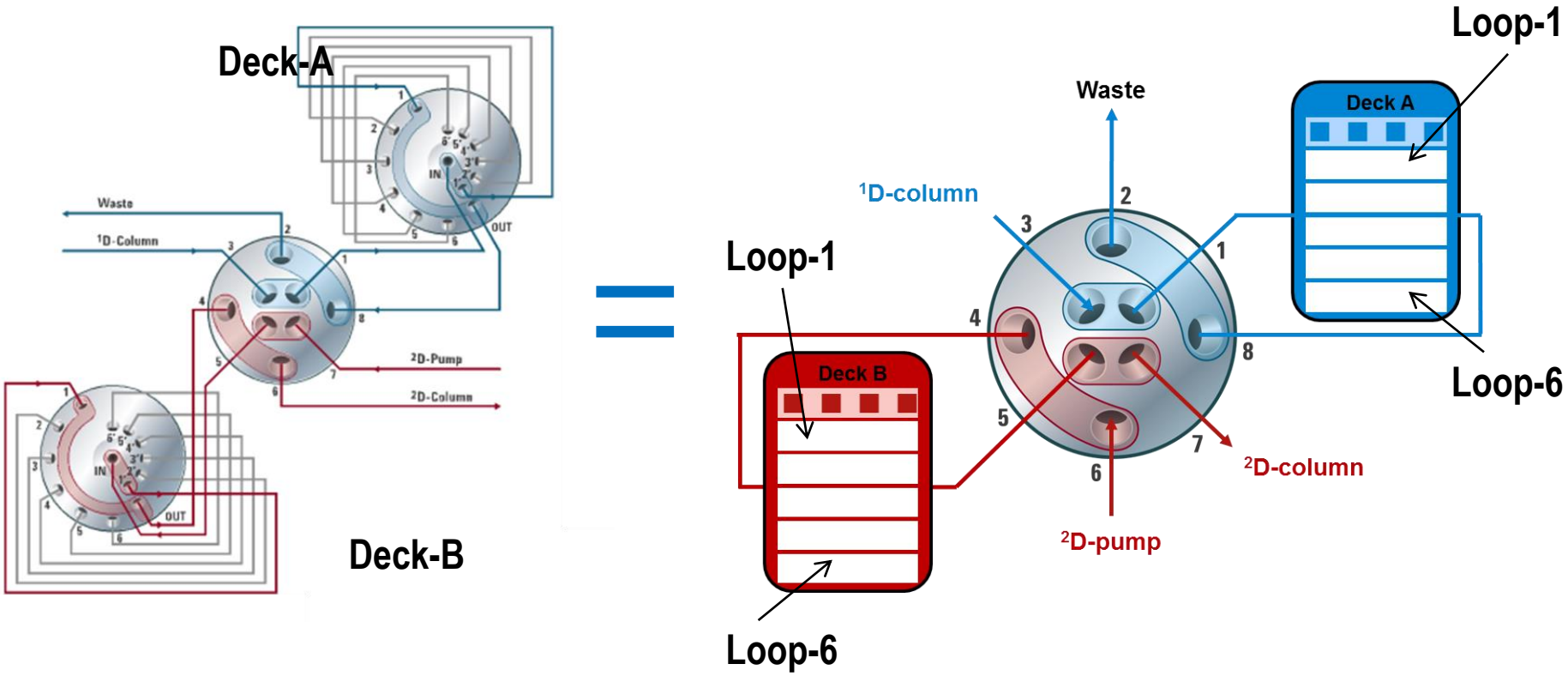
One of the loops replaced with selector valve fitted with 6 loops **Parking deck cluster** offering 7 sampling positions.



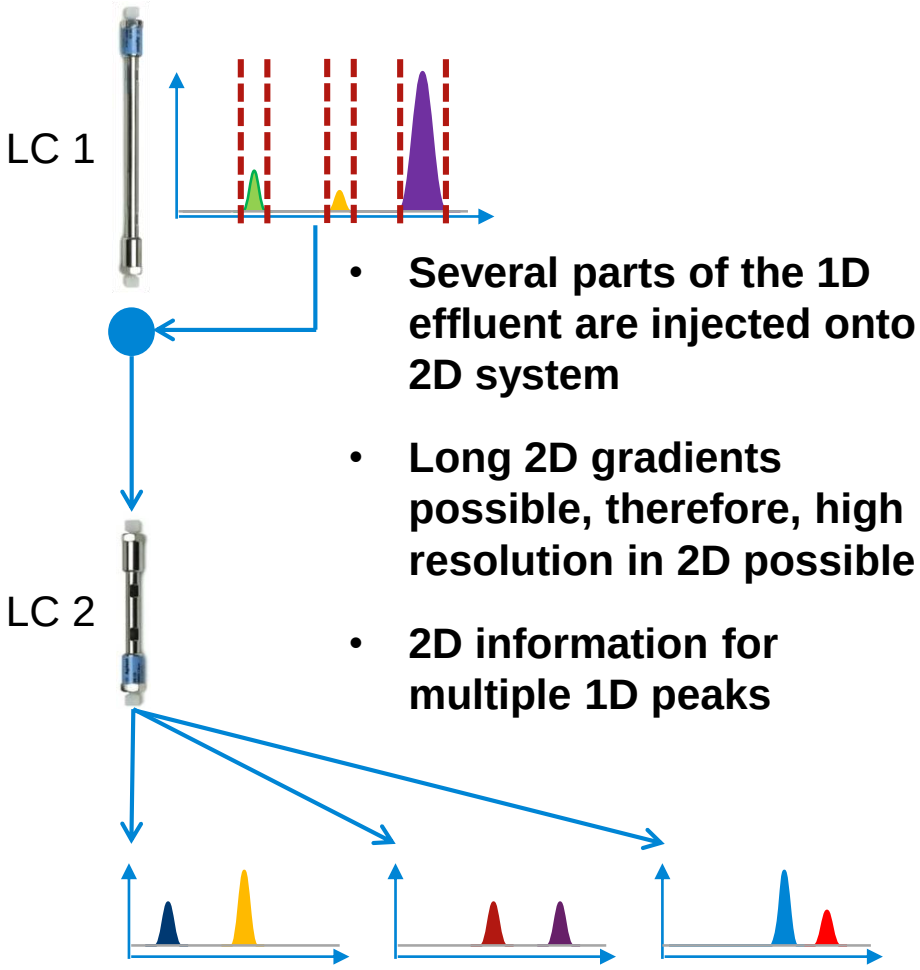
Both loops replaced with two selector valves fitted with 6 loops each **Parking deck cluster** offering 12 sampling positions.

New Multiple Heart-Cutting (MHC) Solution

Different view for better illustration

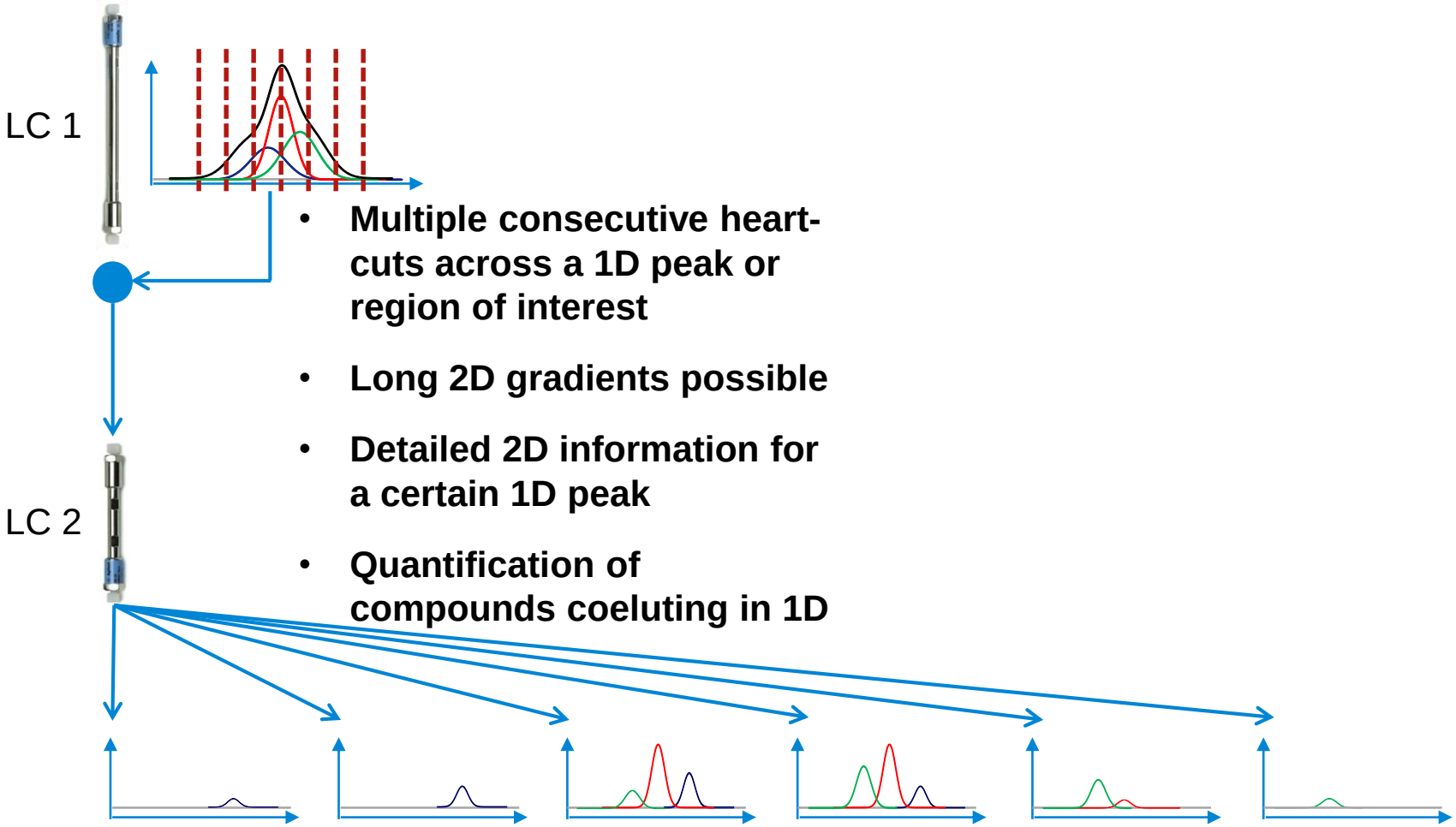


Multiple heart-cutting 2D-LC



- **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**

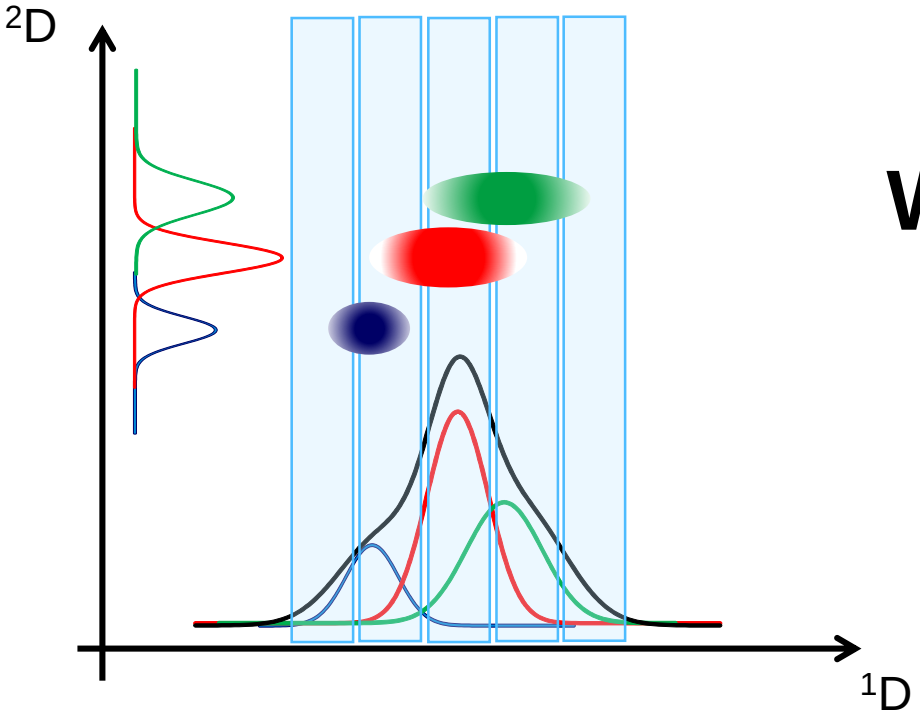
High resolution sampling 2D-LC



- **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**

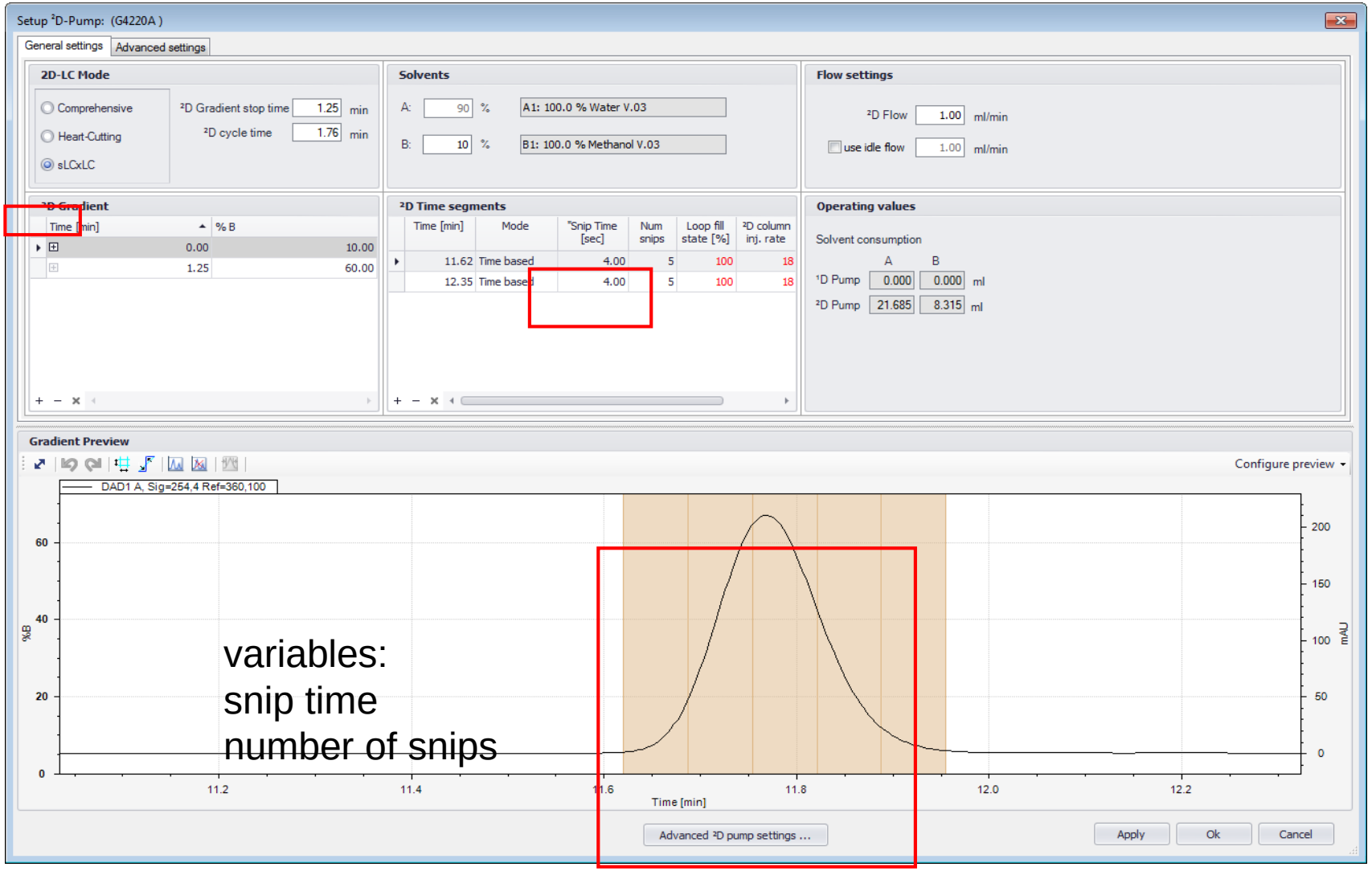
1290 Infinity II 2D-LC Solution with Multiple Heart-Cutting

High Resolution Sampling



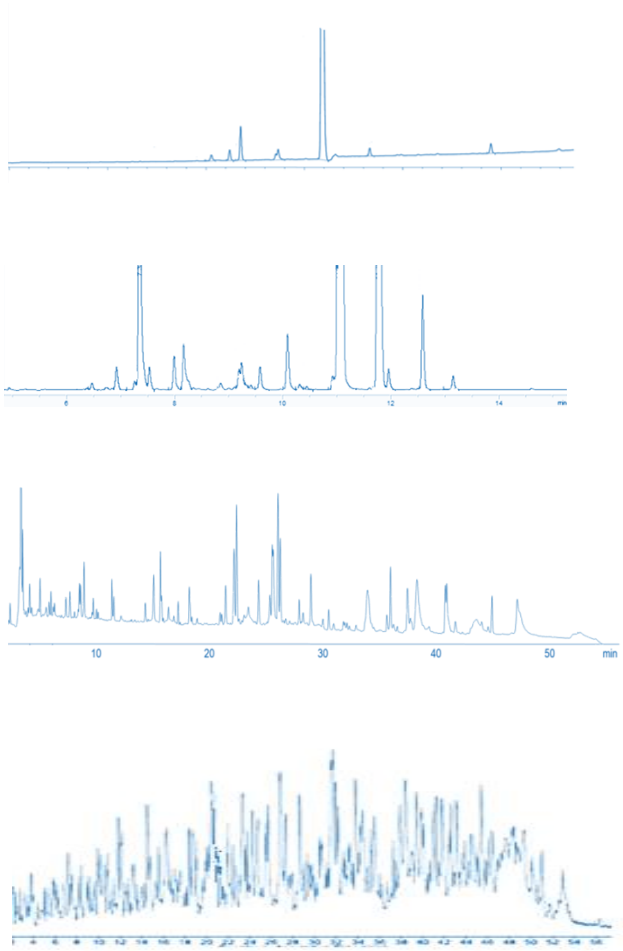
Where to take the cut?

High Resolution Heart-Cutting



1290 Infinity II 2D-LC Solution

Sample complexity



API impurity profiling

Complex formulations

Bio-pharmaceuticals

Natural products, biological samples....

Heart-cutting 2D-LC

Multiple heart-cutting 2D-LC

Comprehensive 2D-LC

Comprehensive 2D-LC/(IMS)-MS/MS

Multiple Heart-cutting 2D-LC
Method Development for Impurity Analysis

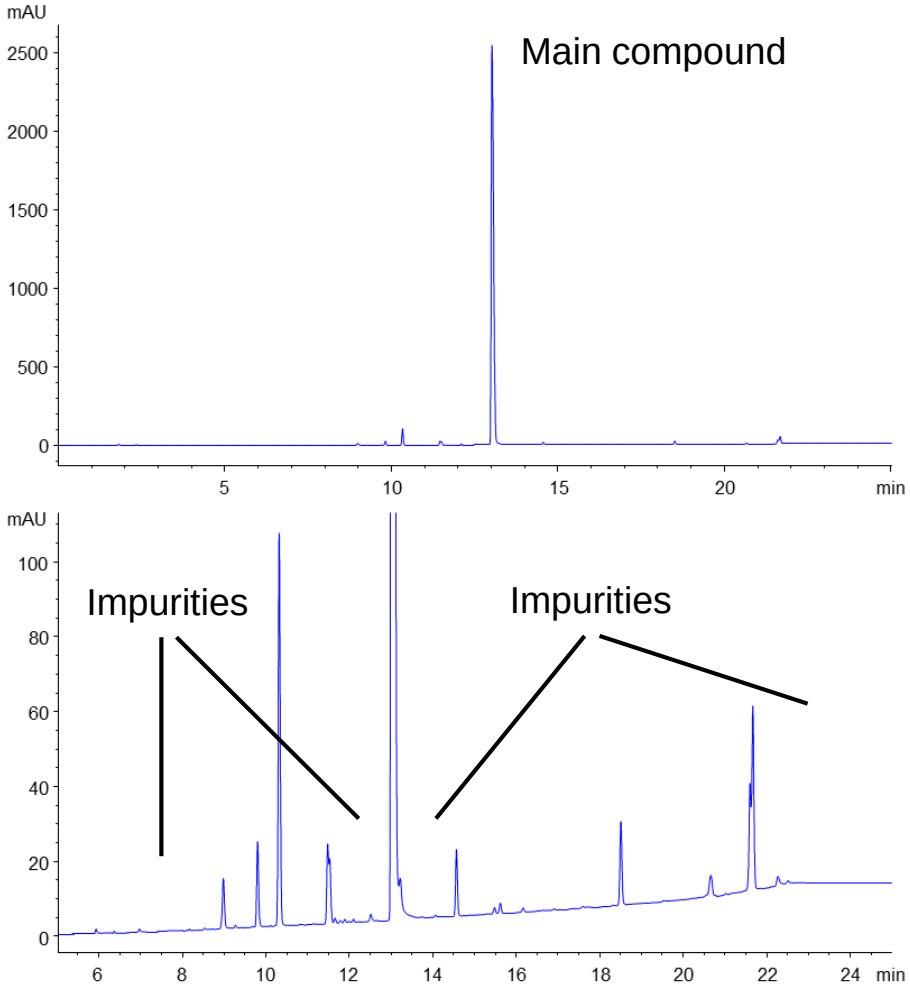
Impurity profiling for method development. Increase probability to detect all impurities, even under the main peak.

- Analysis of a standard mixture representing the analysis of impurities in pharmaceutical compounds or fine chemicals.
- Heart-cutting of the main compound and all impurities to enable detection of co-elutions.
- Multiple cuts across main compound enables „walking through the peak“.

Data from Agilent Application Note, not published yet

Method Development for Impurity Analysis

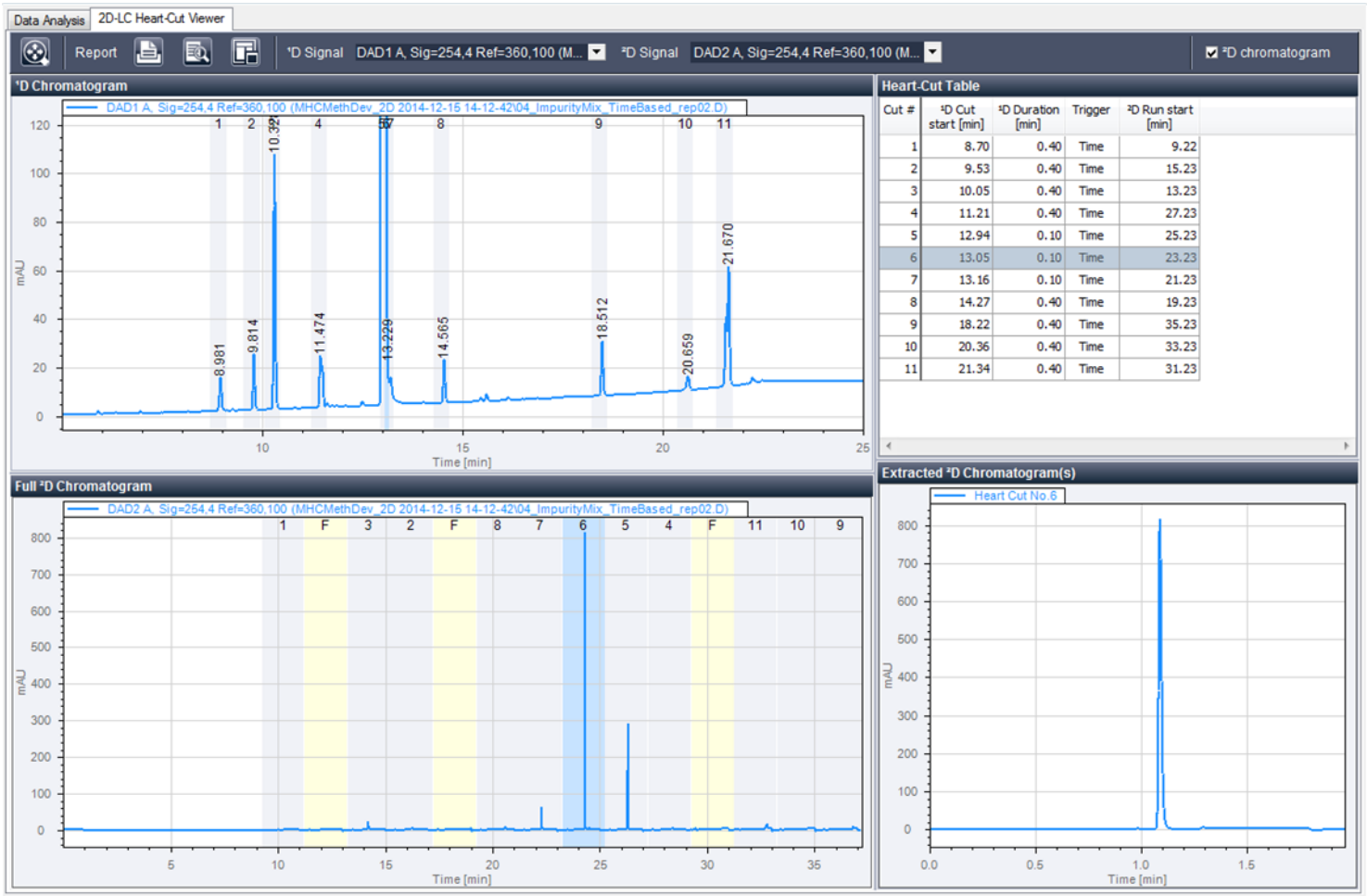
Potential Co-elutions in Onedimensional Analysis



- Impurities hidden underneath the main compound peak?
- Coelutions of impurities?

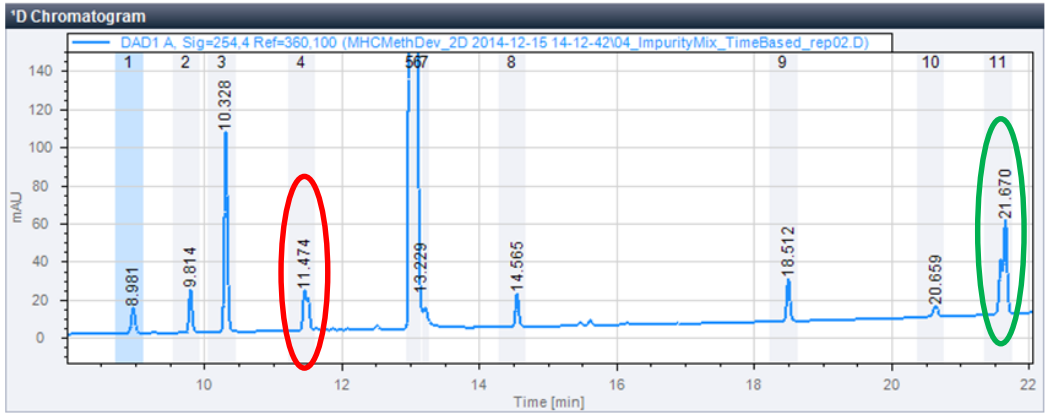
Method Development for Impurity Analysis

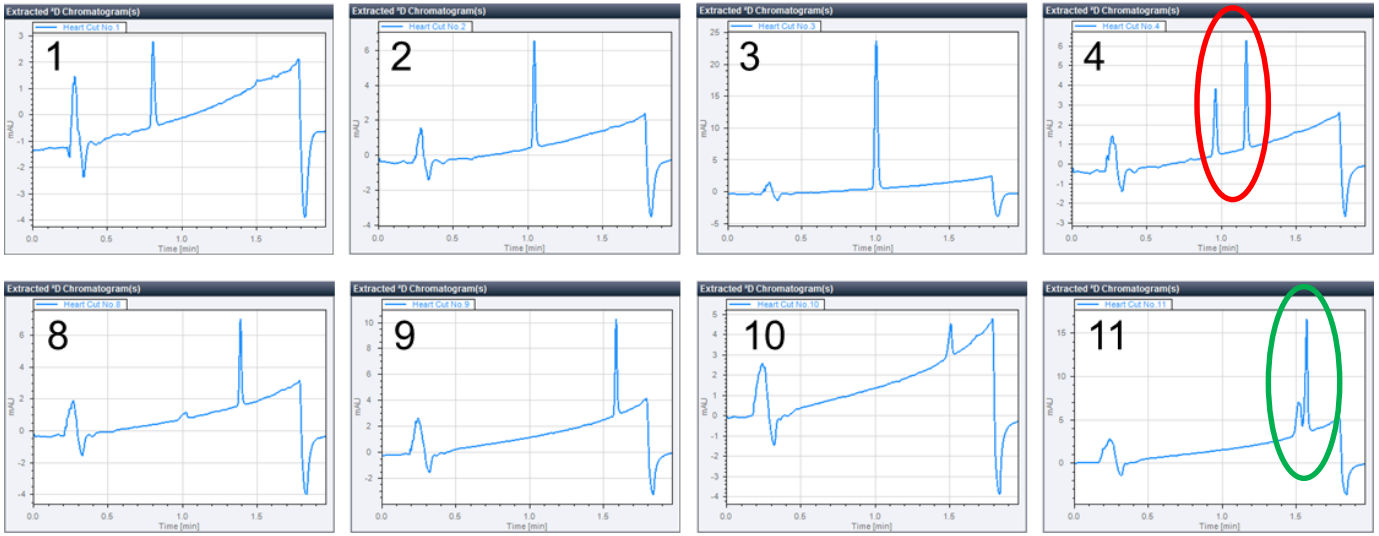
Time-based Heart-cutting of Main Compound and Impurities



Method Development for Impurity Analysis

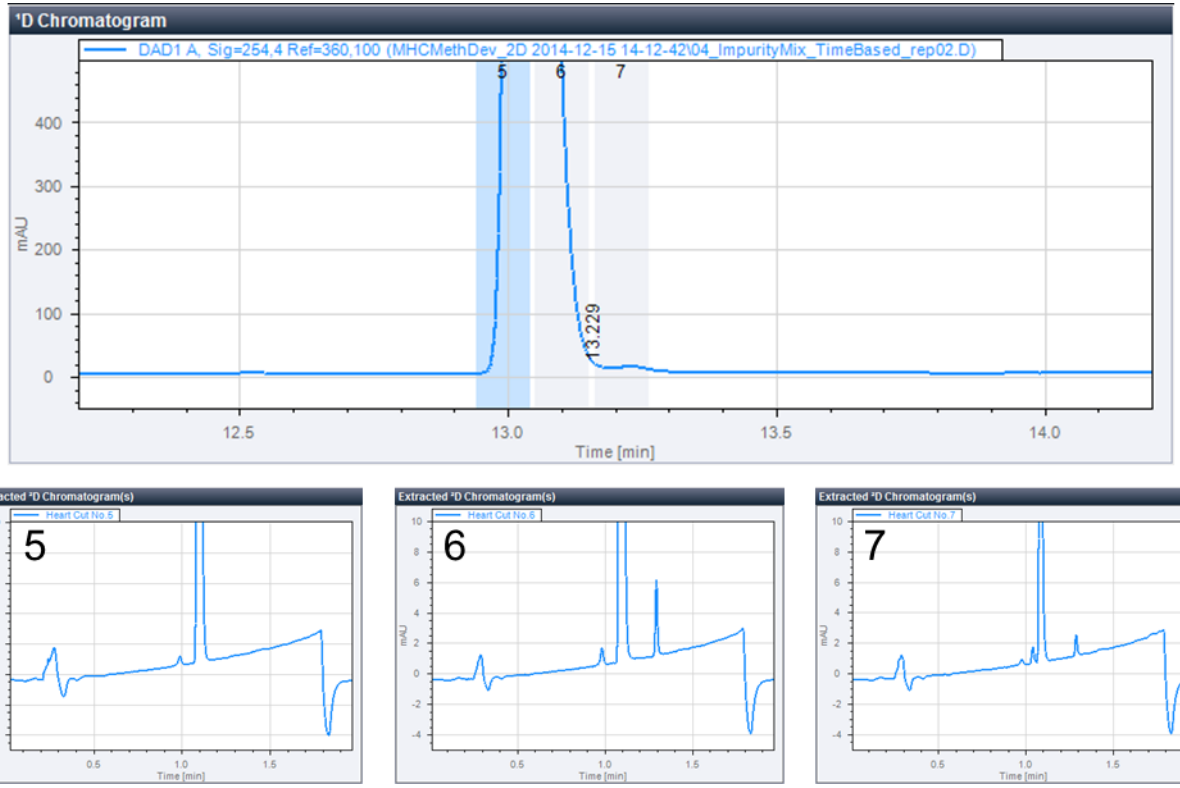
Heart-cutting 2D-LC Reveals Co-elutions of Impurities





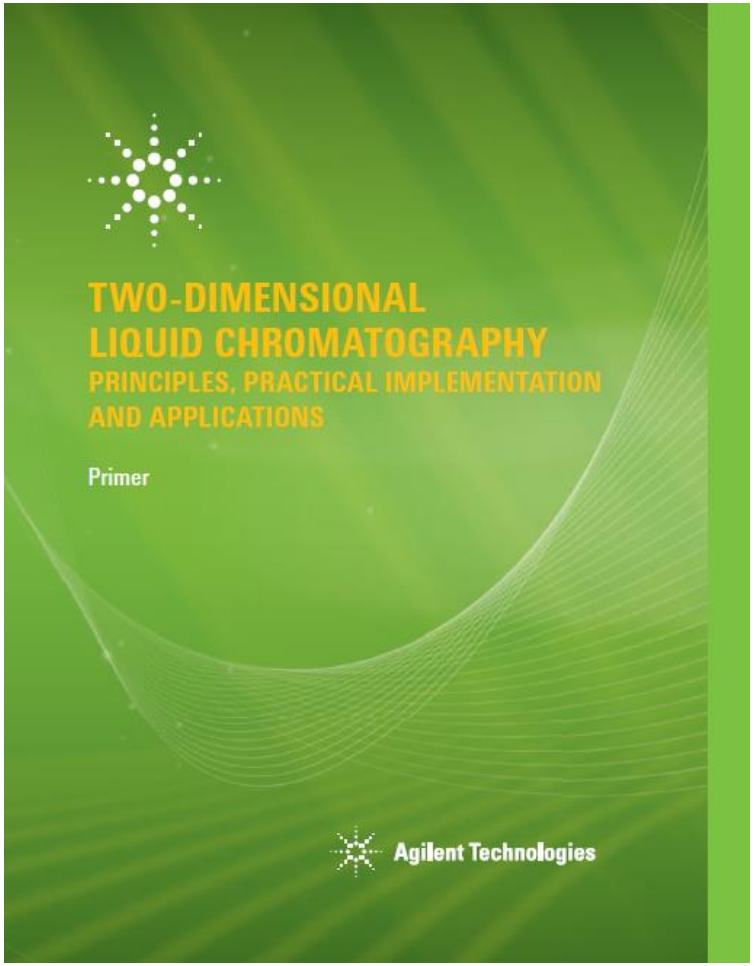
Method Development for Impurity Analysis

Multiple Cuts Across the Main Compound Peak



- Two impurities completely hidden under main compound peak
- Partial coelution of a third impurity with the main compound

Further reading



2D-LC Primer

Available online

http://www.agilent.com/cs/library/primers/public/5991-2359EN.pdf

What do you think?