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COMING OF AGE OF MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY

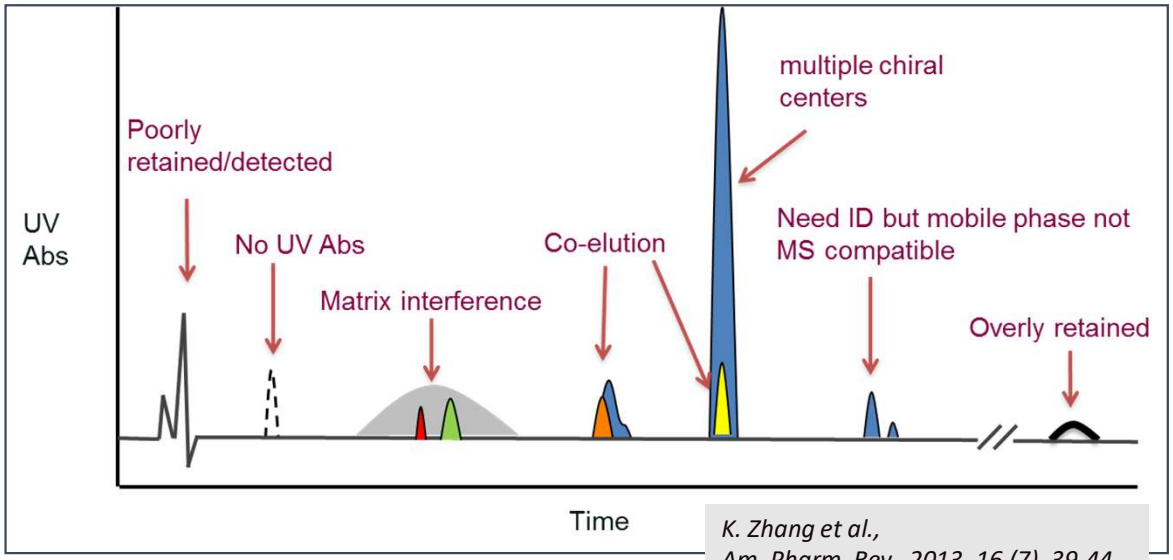
BRIDGING THE GAP BETWEEN RESEARCH AND MODERN ANALYTICAL WORKFLOW

Gerd Vanhoenacker

Why do we use 2D-LC (or mD-LC)?

- Peak capacity
- Non-MS compatible mobile phase
- Additional selectivity
  (matrix, co-elution, …)
- Multi-attribute methods
- Automate/eliminate/streamline workflows and sample preparation steps
- …

- **Green Analytical Chemistry?**

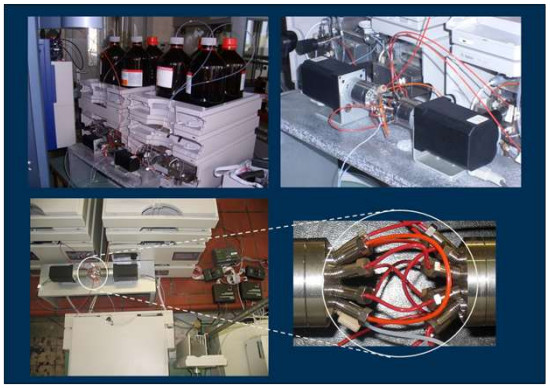


K. Zhang et al.,
Am. Pharm. Rev., 2013, 16 (7), 39-44.

The evolution of 2D-LC: from research to routine

- Analytical power
  - Multidimensional chromatography provides superior peak capacity, resolving complex analytes that standard methods miss.

- Bridging the gap
  - While 2D-GC is already industry-standard, 2D-LC has historically been limited by hardware and software complexity.



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2D-LC Hardware

^{1}D Pump
- Flow rate
- Delay volume

^{1}D Injection
- Autosampler
- Manual

^{1}D Column
- Internal diameter
- Performance

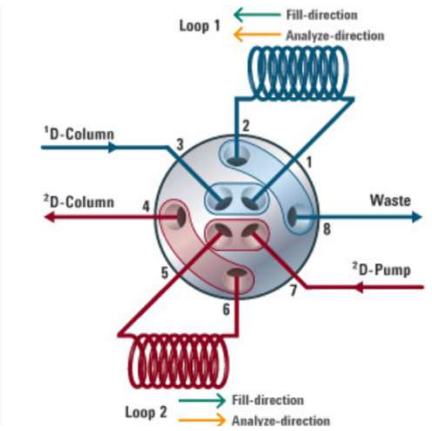
^{1}D Detection
- Optional
- Monitor first dimension

^{2}D Pump
- Speed
- Power
- Delay volume

Modulator
- Trap/collect LC 1 (Dimension 1) effluent
- Transfer/re-inject on LC 2 (Dimension 2)

^{2}D Column
- Column length
- Particle size
- Temperature

^{2}D Detection
- Speed
- Selectivity
- Hyphenation



Agilent 1290 Infinity III 2D-LC Solution
Hardware & Software

Heart-Cutting 2D-LC	**LC-LC**
Multiple Heart-Cutting 2D-LC	**mLC-LC**
Selective Comprehensive 2D-LC (High Resolution Sampling)	**sLC×LC (HRS)**
Comprehensive 2D-LC	**LC×LC**

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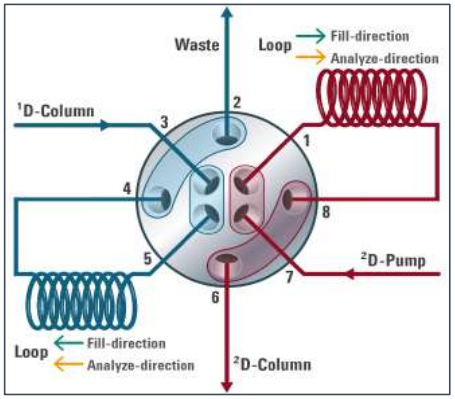
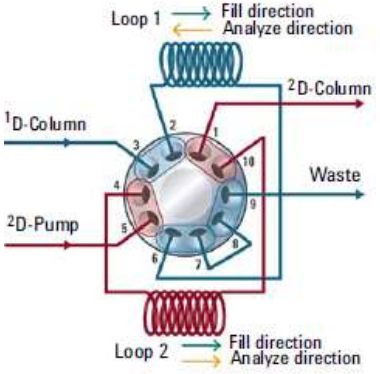
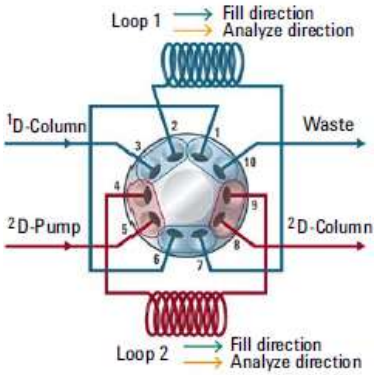
2D-LC Tools

Hardware

2ps/4pt duo valve
LC×LC
Fully identical flow paths





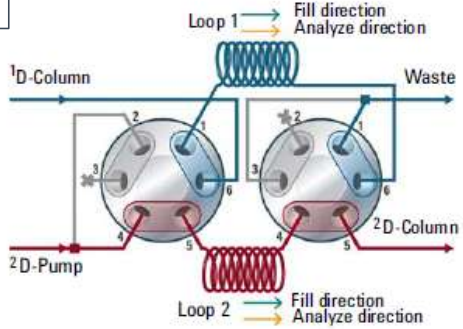


2ps/10pt (with bridging capillary)
LC×LC

Assymmetric Symmetric

Software

- System configuration
- Acquisition and control settings
- Data analysis
- Method development?



2ps/6pt valve x2
LC×LC

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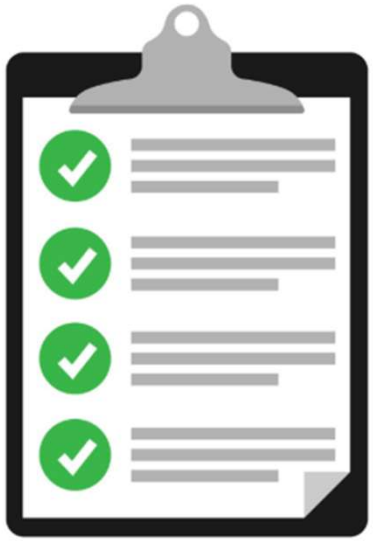
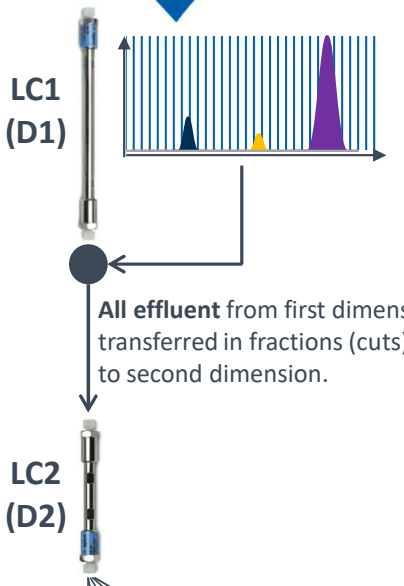
Use in routine analytical laboratories?

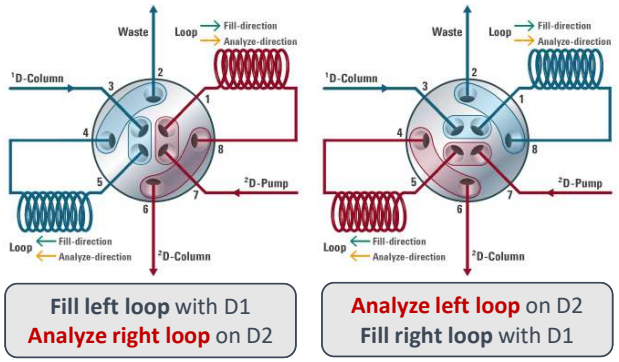


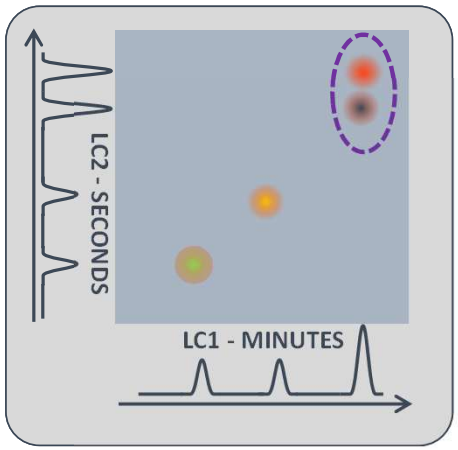
- Precision
- Reproducibility
- Accuracy
- Quantitative capabilities
- Ease-of-use, reduced complexity (hardware **AND** software)
- Hyphenation
- …

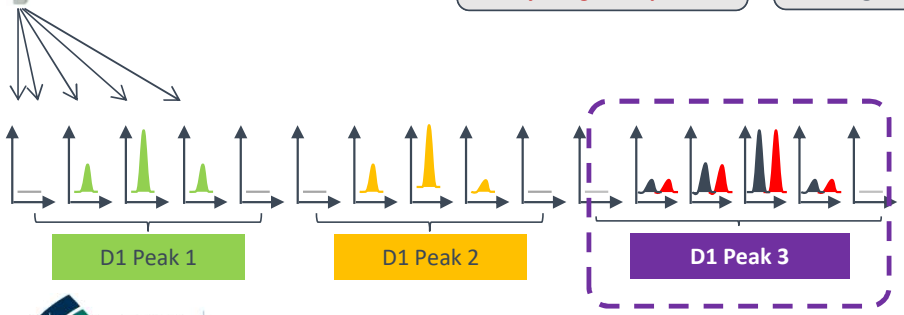
- **Greener technology?**

Comprehensive 2D-LC (LC×LC)









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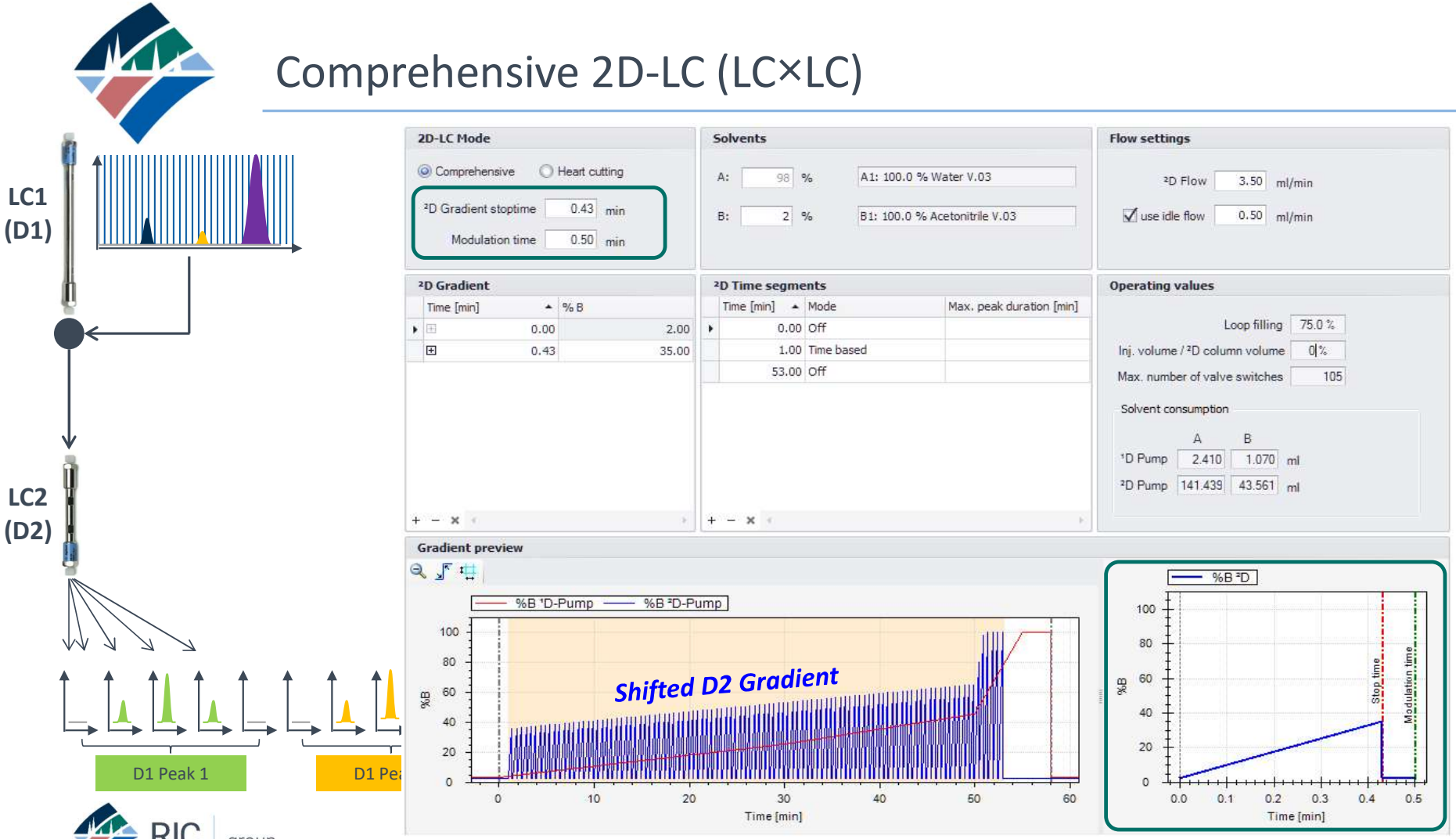
Comprehensive 2D-LC (LC×LC)



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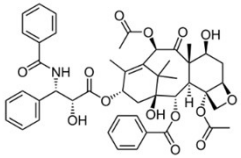
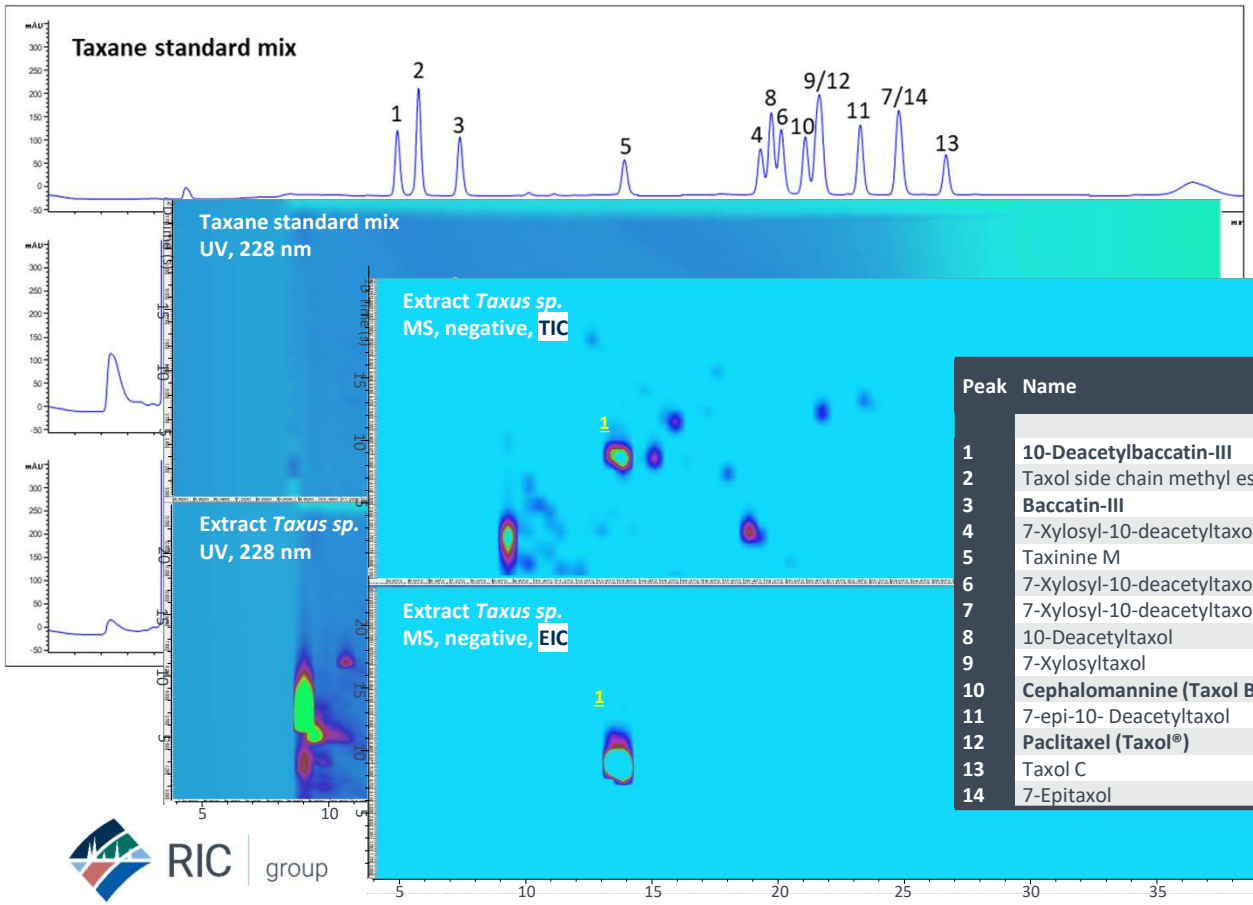
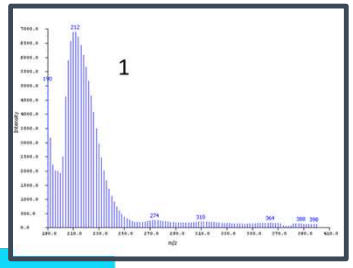
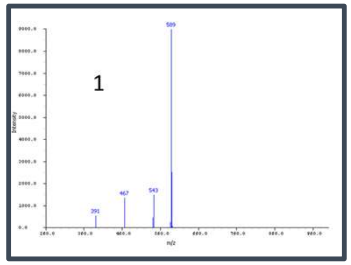
LC×LC, Natural Products – Taxanes



Agilent Technologies Publication Number 5991-3576EN







Peak	Name	R^2 (0.4-30 µg/mL)	Peak volume precision (RSD%, 10 µg/mL, n=6)	Recovery (%, spike 4 µg/mL)	
				Extract 1	Extract 2
1	**10-Deacetylbaccatin-III**	0.99999	0.35	102.3	118.8
2	Taxol side chain methyl ester	0.99997	0.34	76.2	72.9
3	**Baccatin-III**	0.99995	0.41	73.9	78.7
4	7-Xylosyl-10-deacetyltaxol B	0.99992	0.49	96.6	104.1
5	Taxinine M	0.99996	0.29	99.3	121.4
6	7-Xylosyl-10-deacetyltaxol	0.99997	0.20	95.4	102.4
7	7-Xylosyl-10-deacetyltaxol C	0.99999	0.33	96.4	102.4
8	10-Deacetyltaxol	1.00000	0.15	99.0	108.2
9	7-Xylosyltaxol	0.99999	0.17	96.8	102.9
10	**Cephalomannine (Taxol B)**	0.99994	0.80	99.6	102.9
11	7-epi-10- Deacetyltaxol	0.99997	0.34	92.4	98.9
12	**Paclitaxel (Taxol®)**	0.99995	0.54	95.5	86.5
13	Taxol C	0.99999	0.47	95.3	101.3
14	7-Epitaxol	0.99998	0.32	96.5	102.0

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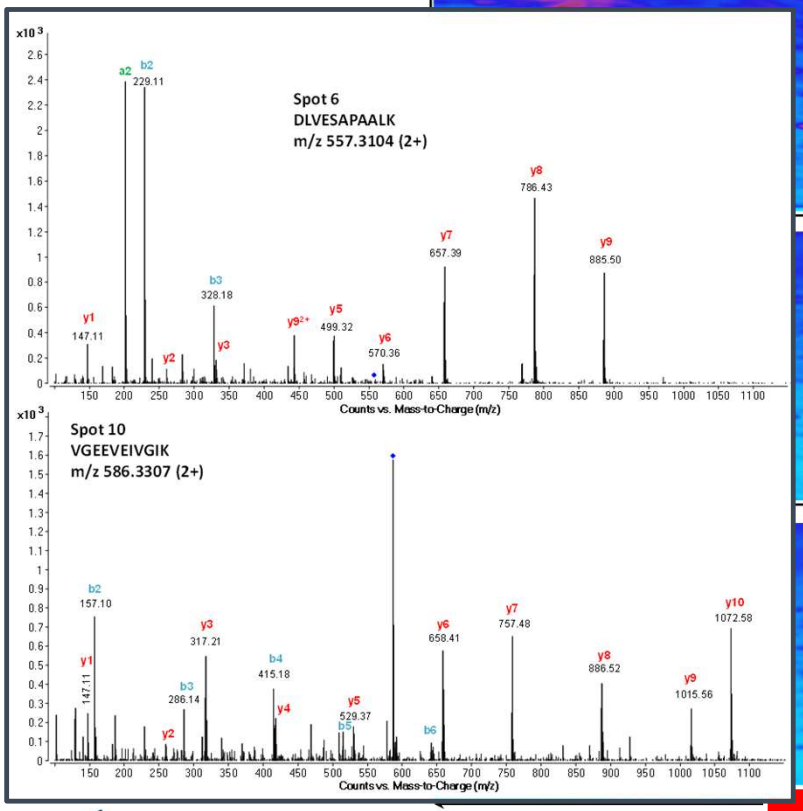
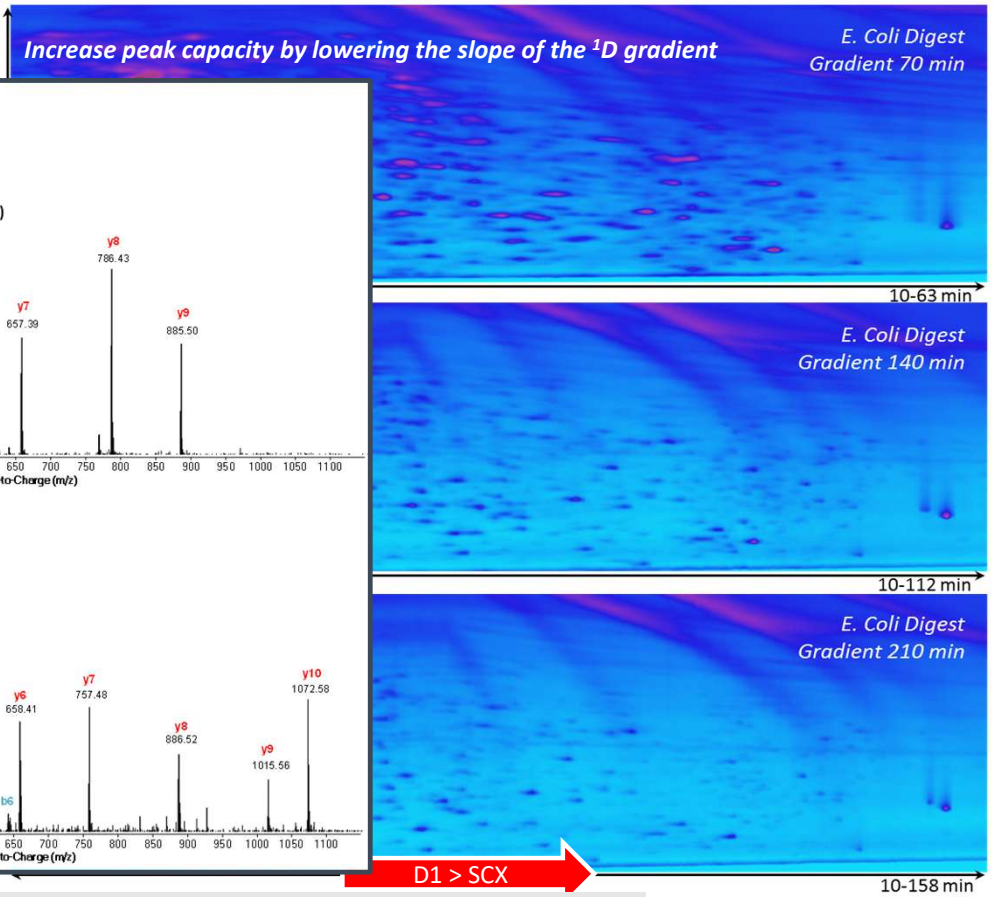
Peak capacity - Screening complex samples





1D-LC Peak Capacity
- ***HPLC: 1050 in 500 min***
- ***UHPLC: 800 in 180 min***

$n_{c,2D}$	1930 (theory)
$\mathbf{n'_{c,2D}}$	**1150 (effective)** ***in 80 min***

$n_{c,2D}$	3140
$\mathbf{n'_{c,2D}}$	**1880** ***in 2.5 hours***

$n_{c,2D}$	*3750*
$\mathbf{n'_{c,2D}}$	***2250 in <4 hours***

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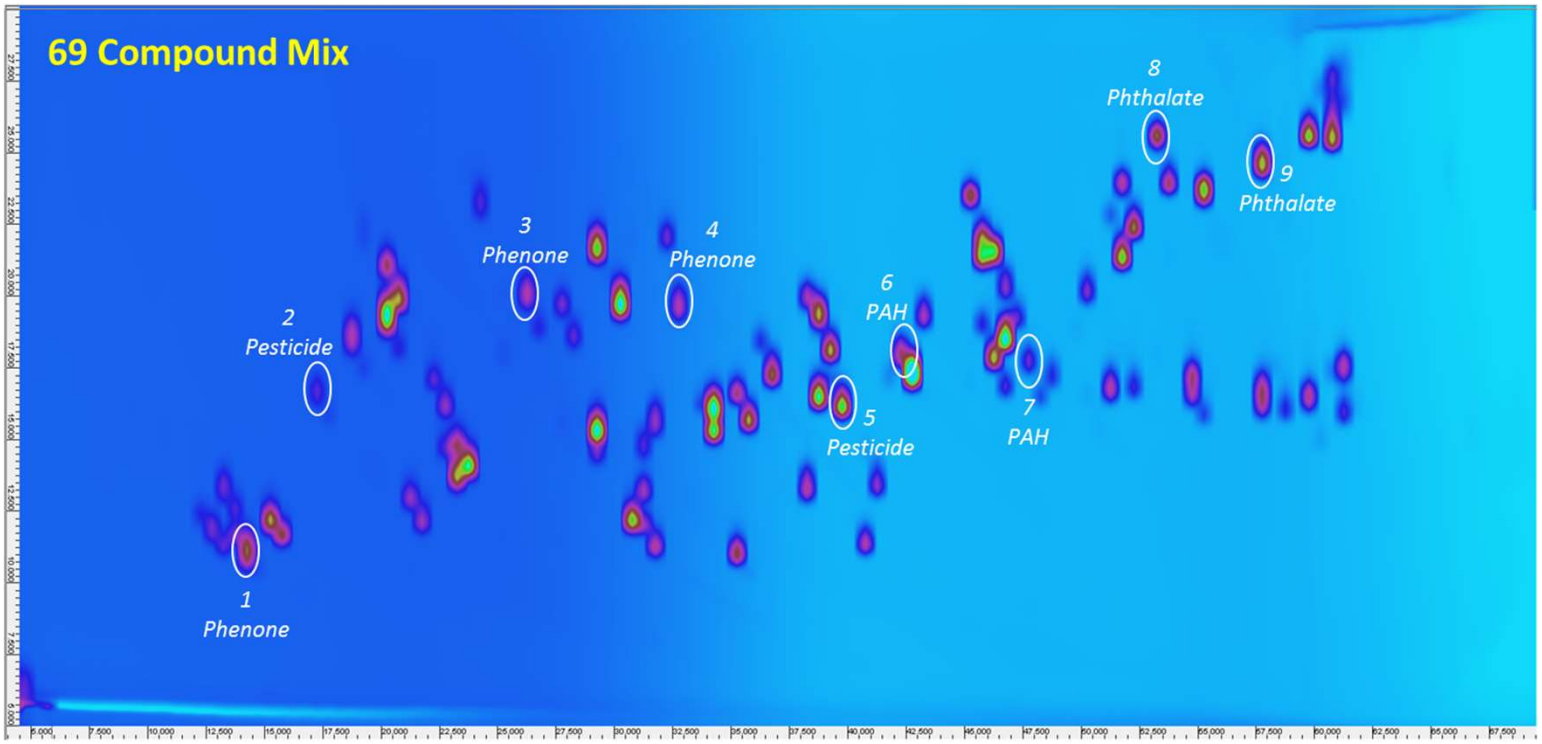
Agilent Technologies Publication Number 5991-5179EN

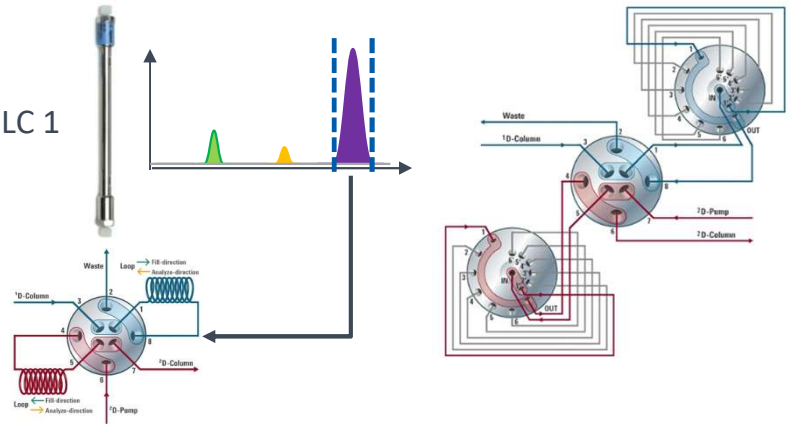
Precision LC×LC



Agilent Technologies Publication Number 5991-6123EN

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

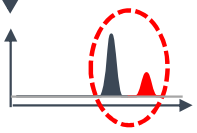
LC 1



LC 2

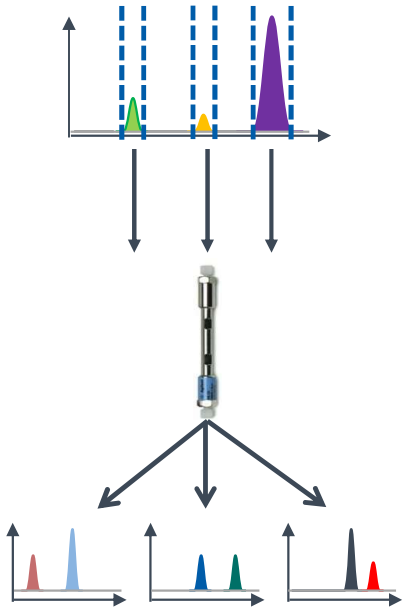
Limitations of standard dual-loop set-up:

- How to sample more than one peak/region?
- How can we sample larger regions, broader peaks, higher volumes (flow-rate D1)?



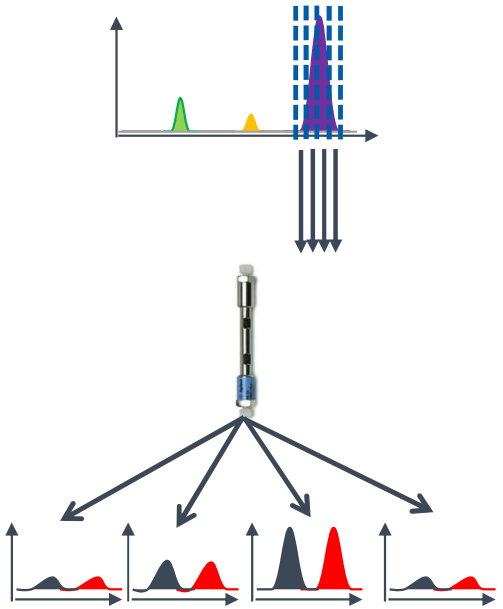
Multiple Heart-Cutting (mLC-LC, MHC)

Multiple parts or peaks from first dimension column are collected in loops and transferred to second dimension column.



Selective Comprehensive (sLC×LC), High Resolution Sampling (HRS)

A complete peak or zone from first dimension column is collected in loops and transferred to second dimension column.



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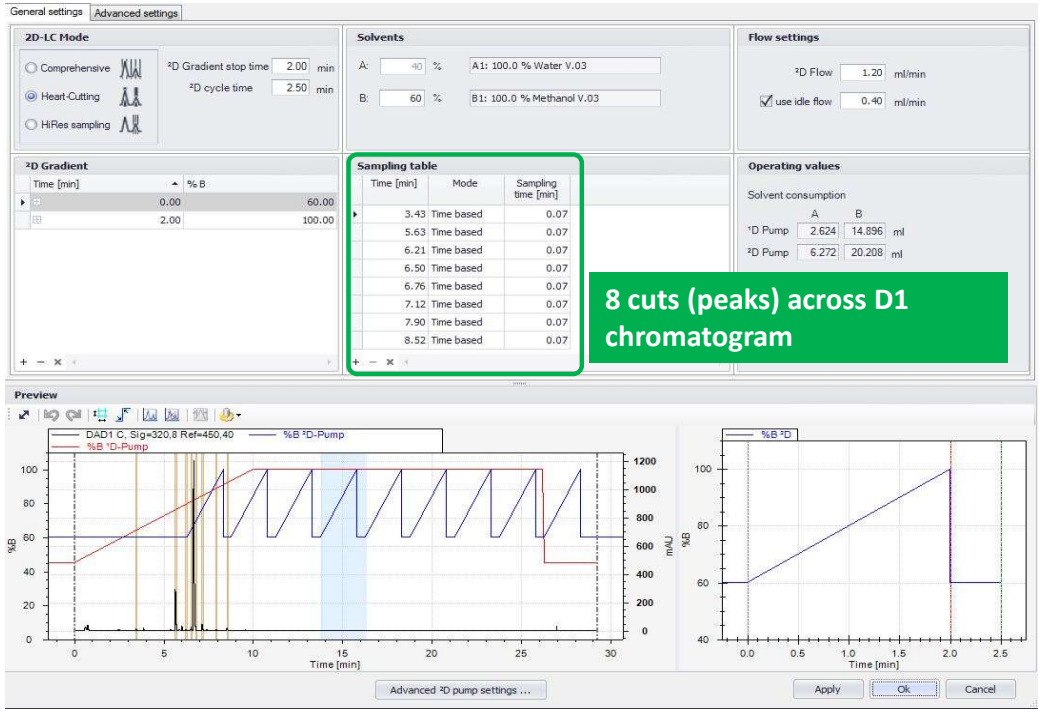
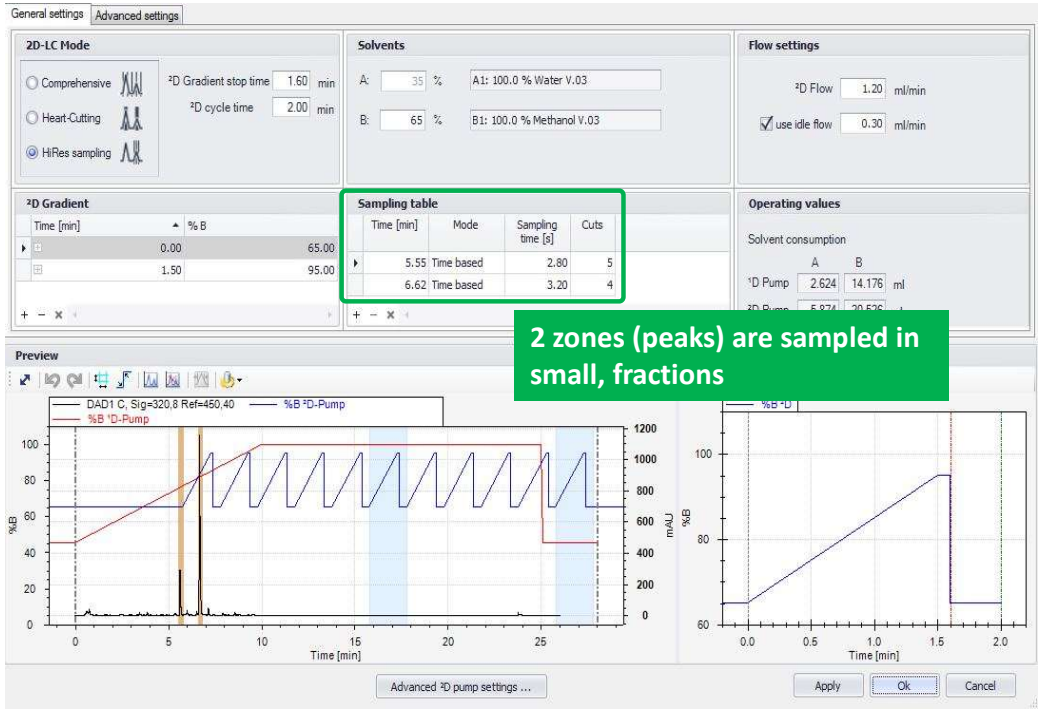
Heart-cutting 2D-LC (LC-LC)

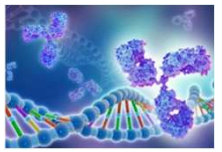
Multiple Heart-Cutting (MHC)



High Resolution Sampling (HRS)

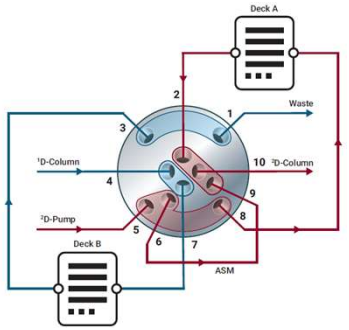


Heart-cutting 2D-LC (LC-LC)



Peptones:

- **Water-soluble sources of mainly amino acids and peptides**
- **Supplemented to growth media to support cell growth and recombinant protein production**



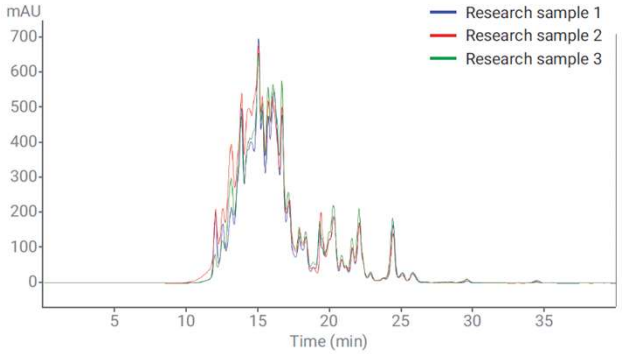
Aim:

- Demonstrate retention time and peak area stability in both dimensions
- Determine and characterize differences between peptone samples (batch, origin, …)

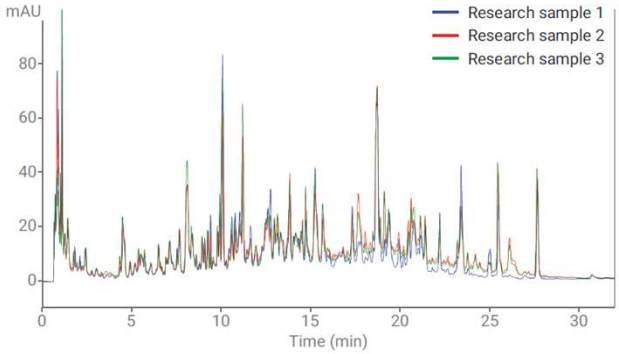
Sample:
Casein hydrolysates

First dimension:
Size exclusion chromatography (SEC)
Second dimension:
Reversed-phase liquid chromatography (RPLC)
Detection:
DAD and MS

1D SEC



1D RPLC



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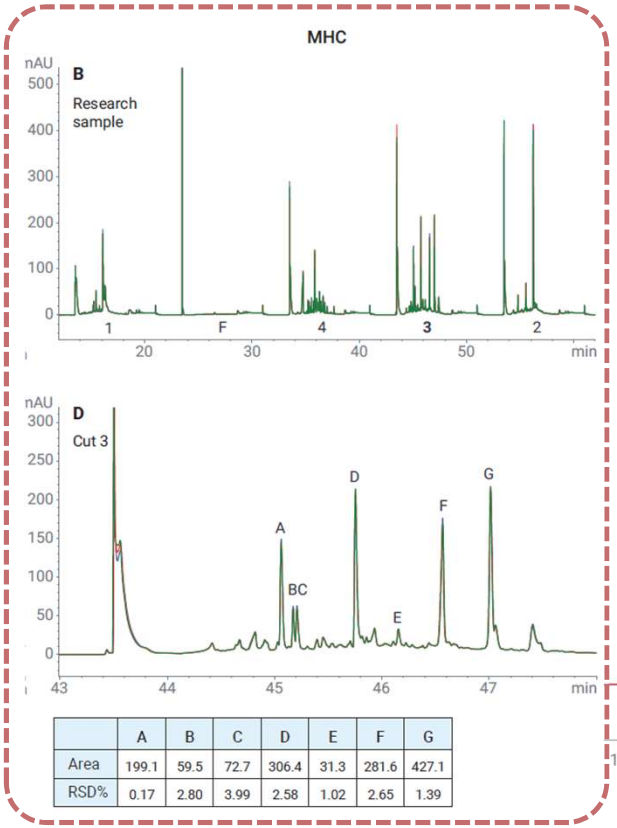
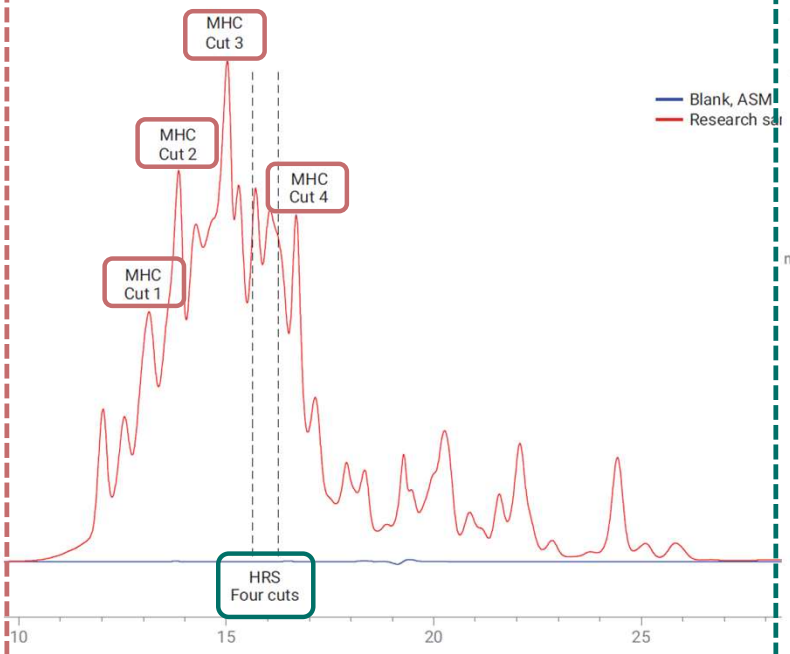
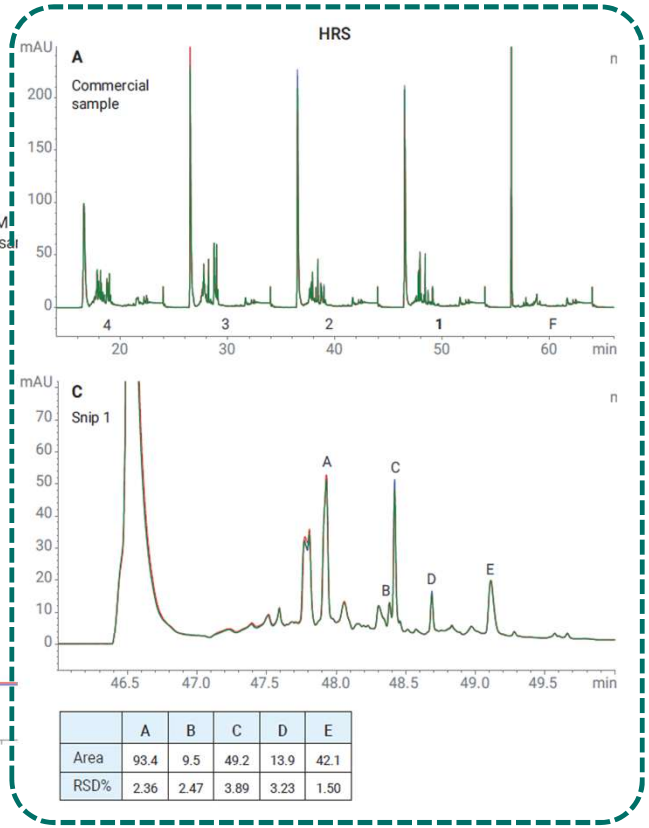
Agilent Technologies Publication Number 5994-0926EN

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



	A	B	C	D	E	F	G
Area	199.1	59.5	72.7	306.4	31.3	281.6	427.1
RSD%	0.17	2.80	3.99	2.58	1.02	2.65	1.39





	A	B	C	D	E
Area	93.4	9.5	49.2	13.9	42.1
RSD%	2.36	2.47	3.89	3.23	1.50

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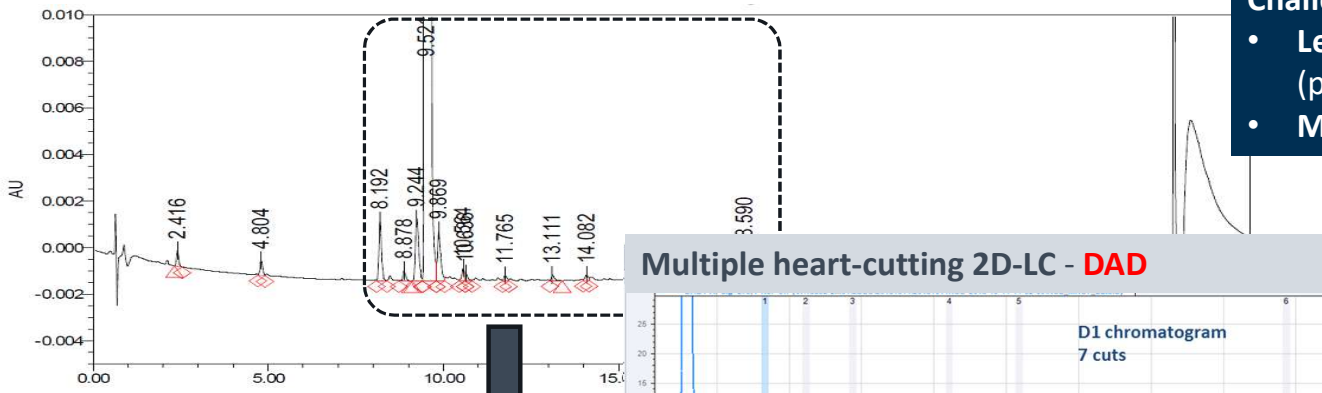
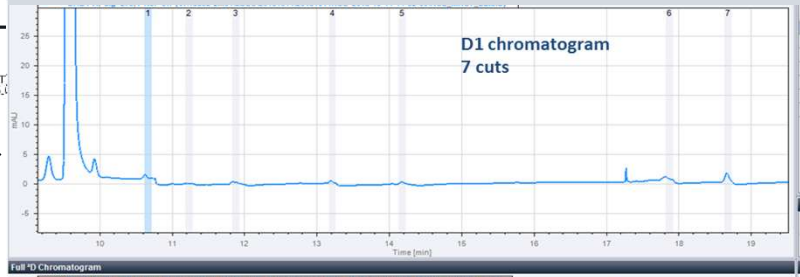
Impurity ID in pharmaceutical products

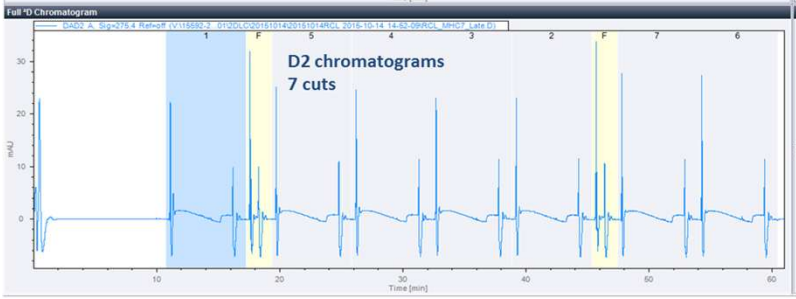
Challenge:

- **Legacy method, not compatible with MS** (phosphate buffered mobile phase)
- **Multiple compounds/impurities to be identified**



Multiple heart-cutting 2D-LC - DAD





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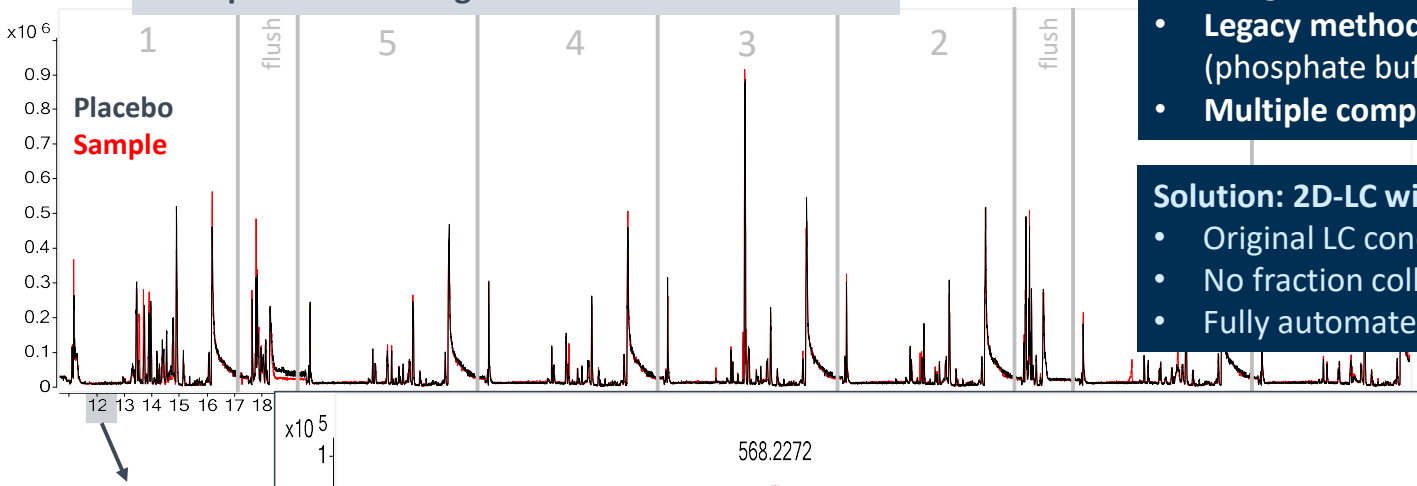
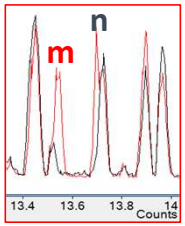
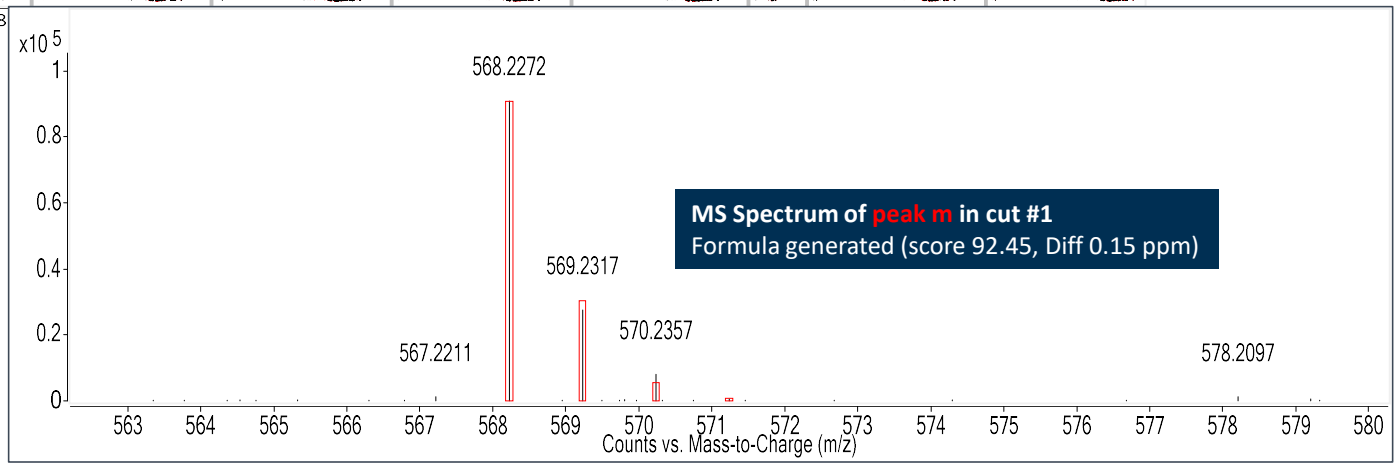
Impurity ID in pharmaceutical products

Multiple heart-cutting 2D-LC - HRMS







MS Spectrum of peak m in cut #1
Formula generated (score 92.45, Diff 0.15 ppm)

Challenge:

- **Legacy method, not compatible with MS** (phosphate buffered mobile phase)
- **Multiple compounds/impurities to be identified**

Solution: 2D-LC with HRMS

- Original LC conditions were not modified
- No fraction collection, intermediate steps
- Fully automated and traceable

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Quantitative PAH analysis in complex HPI products

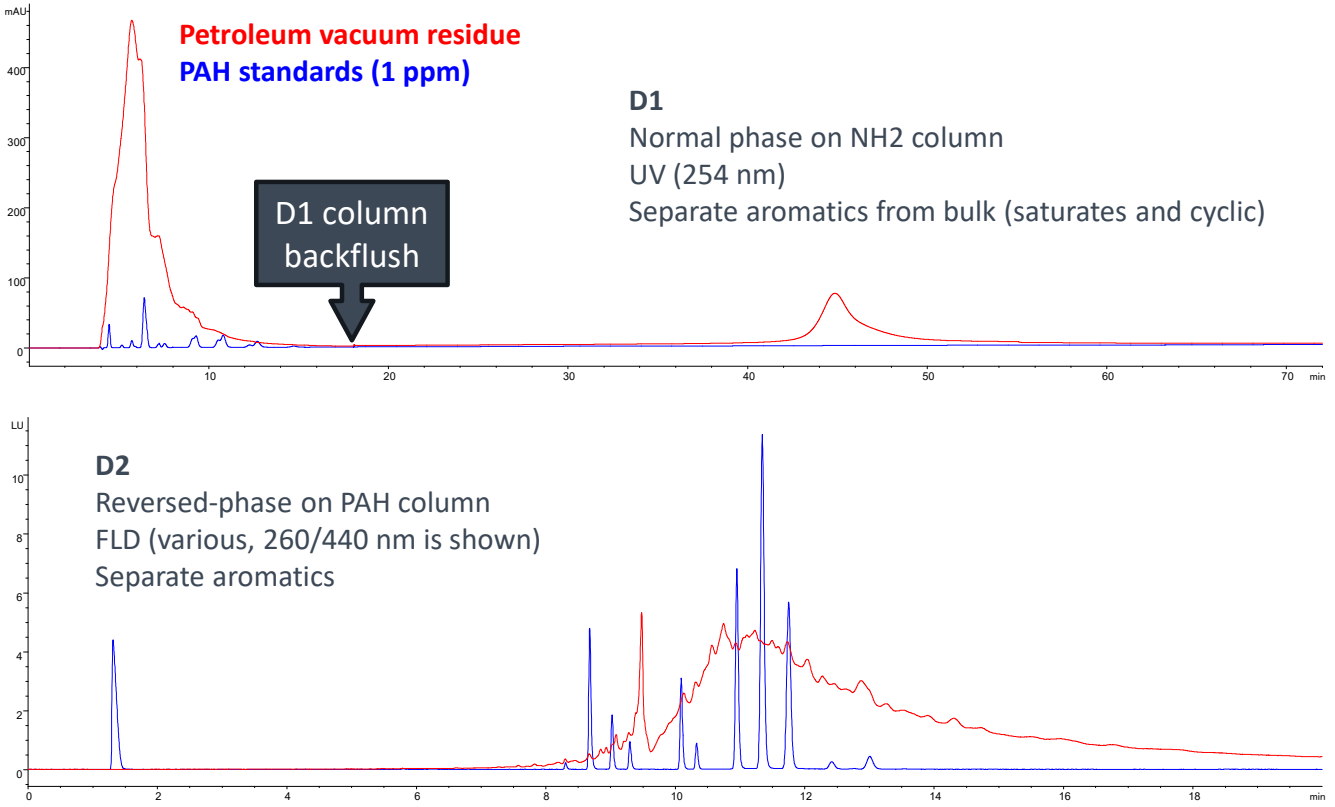
Polycyclic aromatic hydrocarbons (PAH) in petroleum products

Aim:
Determine individual PAHs in complex petrochemical samples using HPLC

Sample:
Petroleum vacuum residue (complex and severely contaminated part of crude oil) dissolved in iso-octane/cyclohexane

First dimension:
Normal phase, with additional valve for backflush to remove polar compounds

Second dimension:
Reversed-phase on PAH column, optimized for each heart-cut, individual PAH



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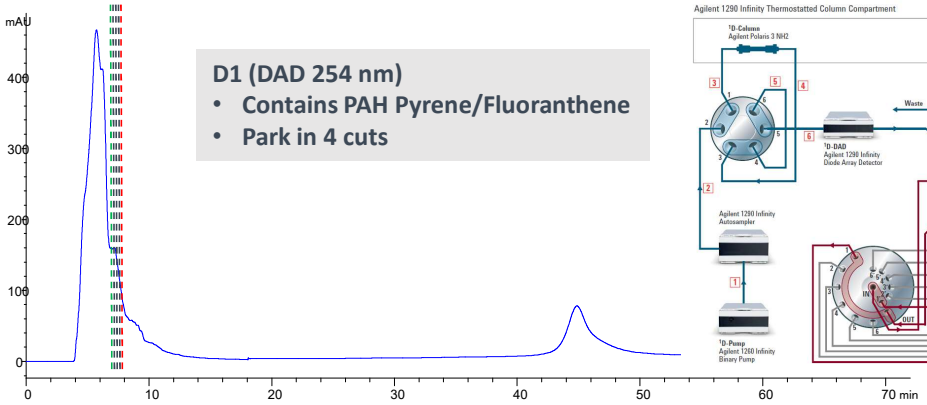
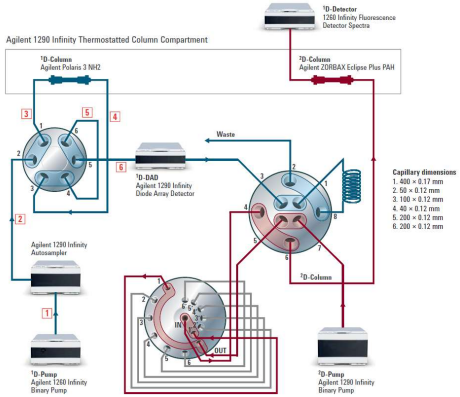
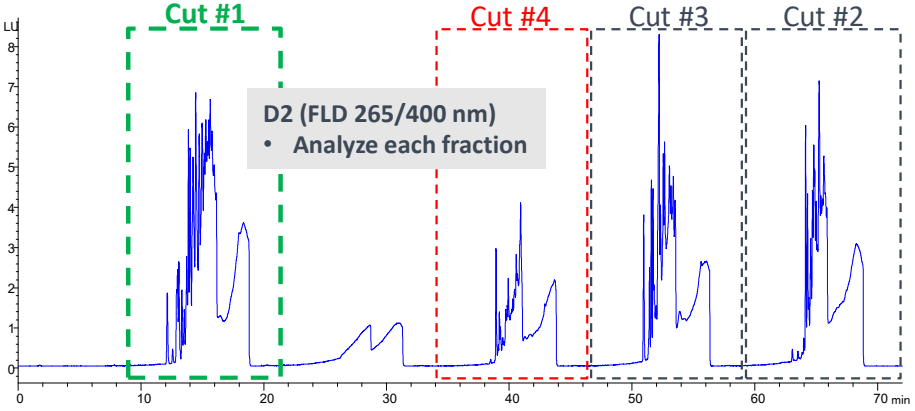
Agilent Technologies Publication Number 5991-6549EN

Quantitative PAH analysis in complex HPI products

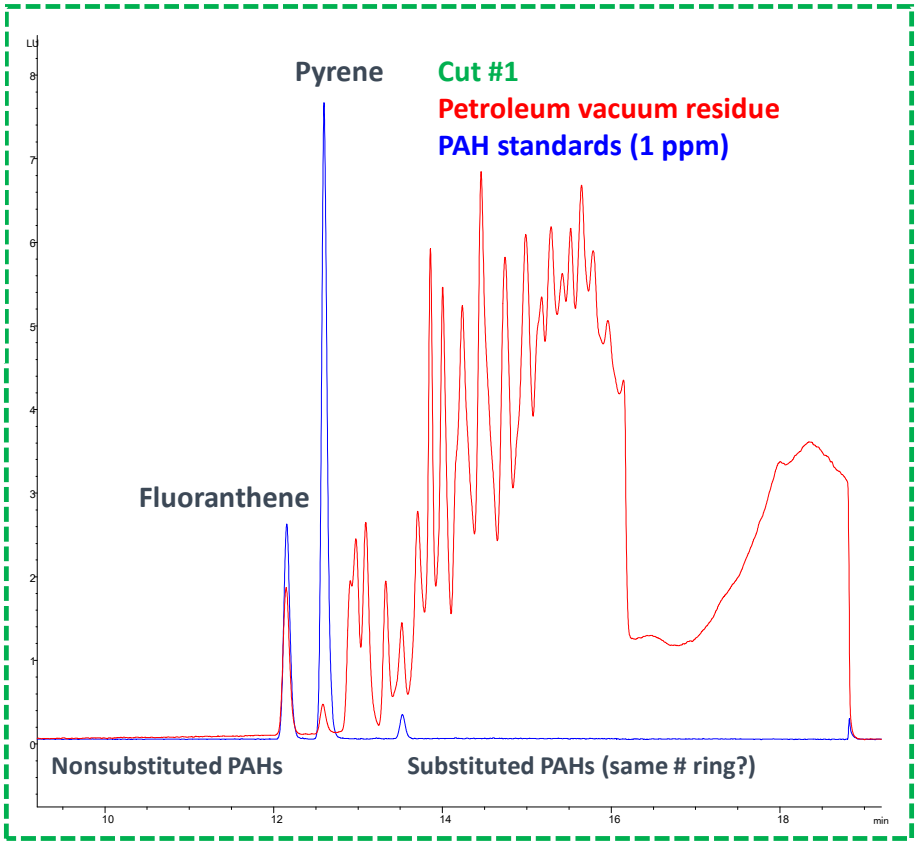


D1 (DAD 254 nm)
- Contains PAH Pyrene/Fluoranthene
- Park in 4 cuts





D2 (FLD 265/400 nm)
- Analyze each fraction



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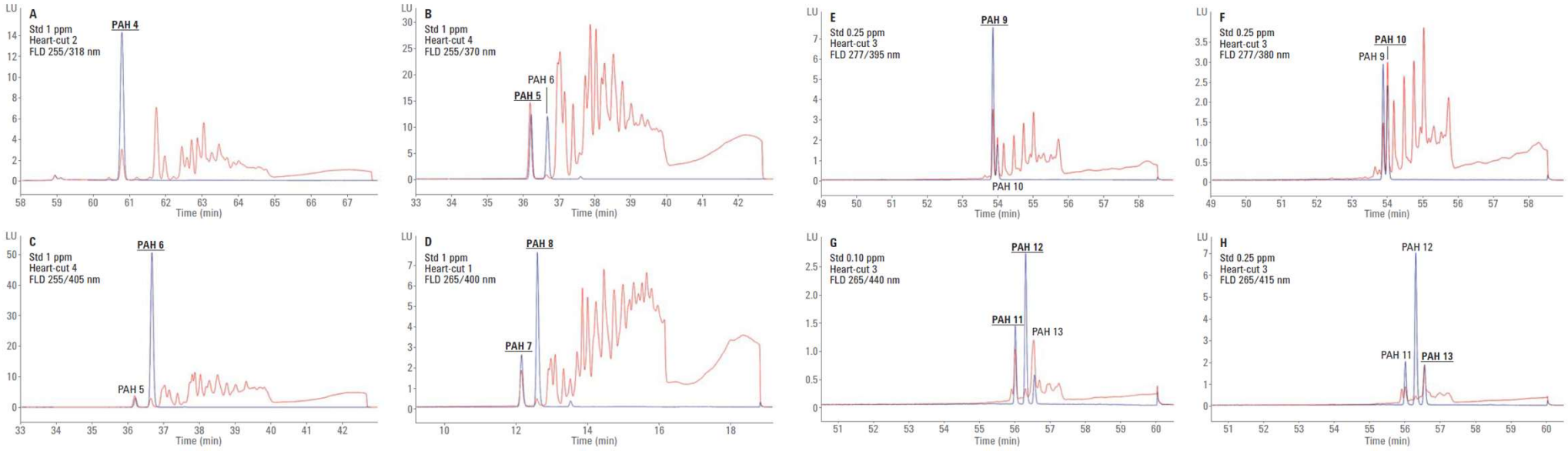
Quantitative PAH analysis in complex HPI products



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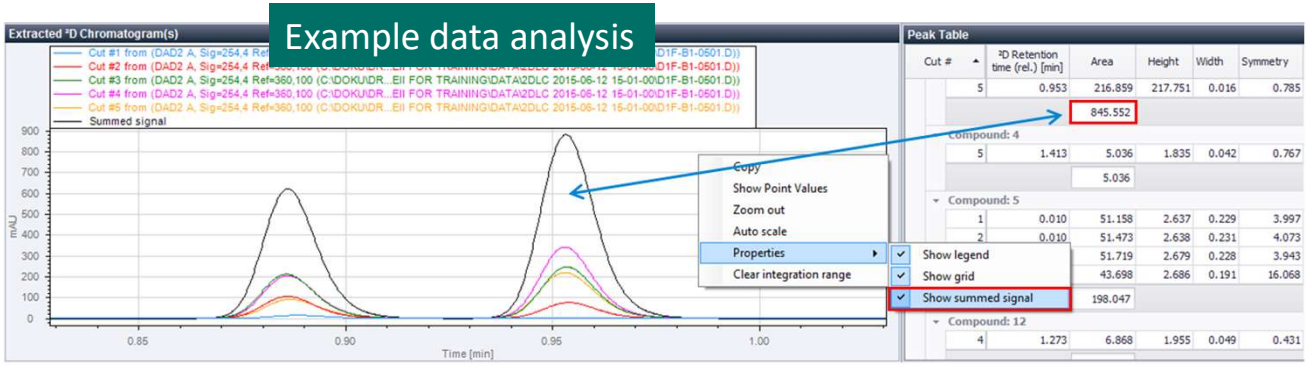
Quantitative PAH analysis in complex HPI products



PAH	FLD WL	Calibration (R^2)	Precision (RSD%)	Concentration extract (ppm)
Benzo(a)anthracene	EX = 277/EM = 395	1.00000	1.27	0.093
Chrysene	EX = 277/EM = 380	0.99998	2.13	0.272
Benzo(b)fluoranthene	EX = 265/EM = 440	0.99999	0.83	0.085
Benzo(k)fluoranthene	EX = 265/EM = 440	0.99998	0.90	0.012
Benzo(a)pyrene	EX = 265/EM = 415	0.99997	1.37	0.077

Data based on area sum of 4 cuts (summed signal)

Calibration:
0.05, 0.1, 0.25, 0.5, 1 ppm (n=1)
Precision:
1 ppm (n=5, consecutive injections)

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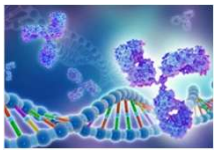
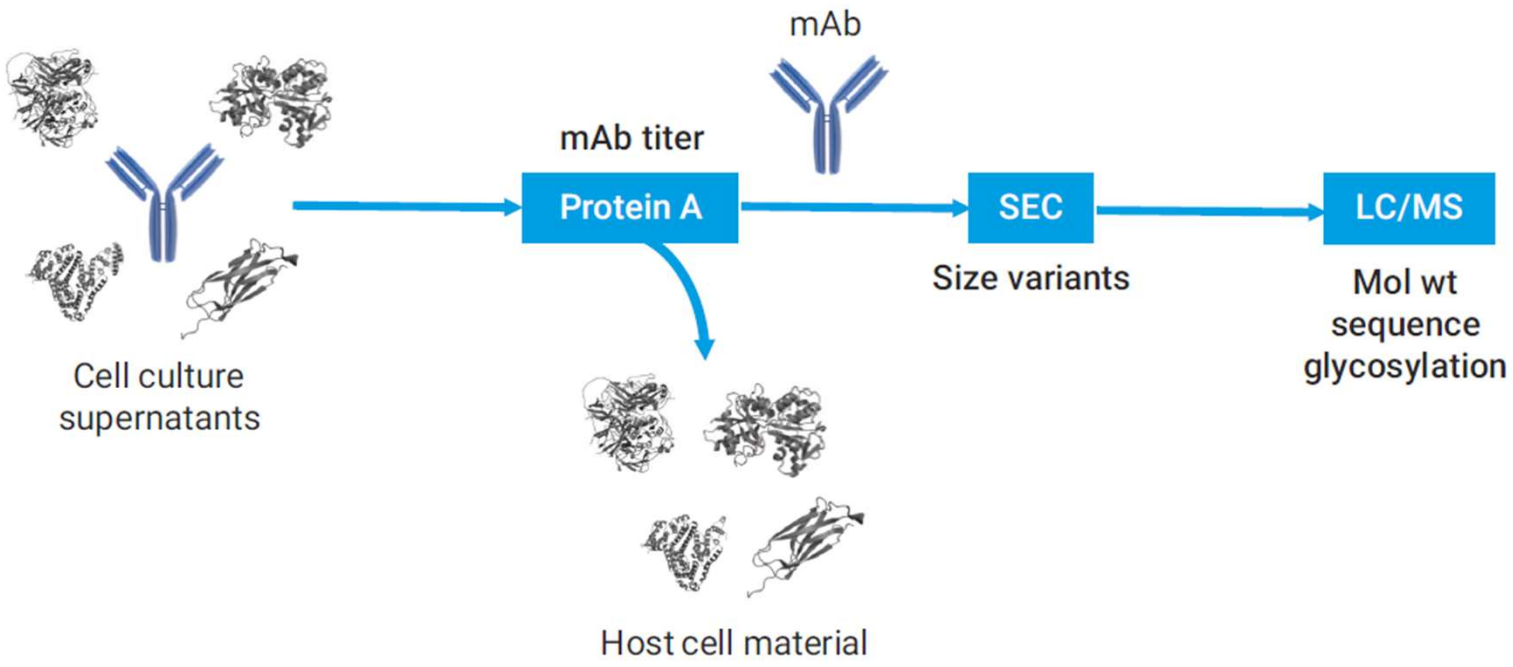
Multi-attribute analysis using **3D-LC**/MS



Analysis of monoclonal antibodies (mAbs)

Simultaneous determination of

- mAb titer
- size variants
- MW
- AA sequence
- PTMs



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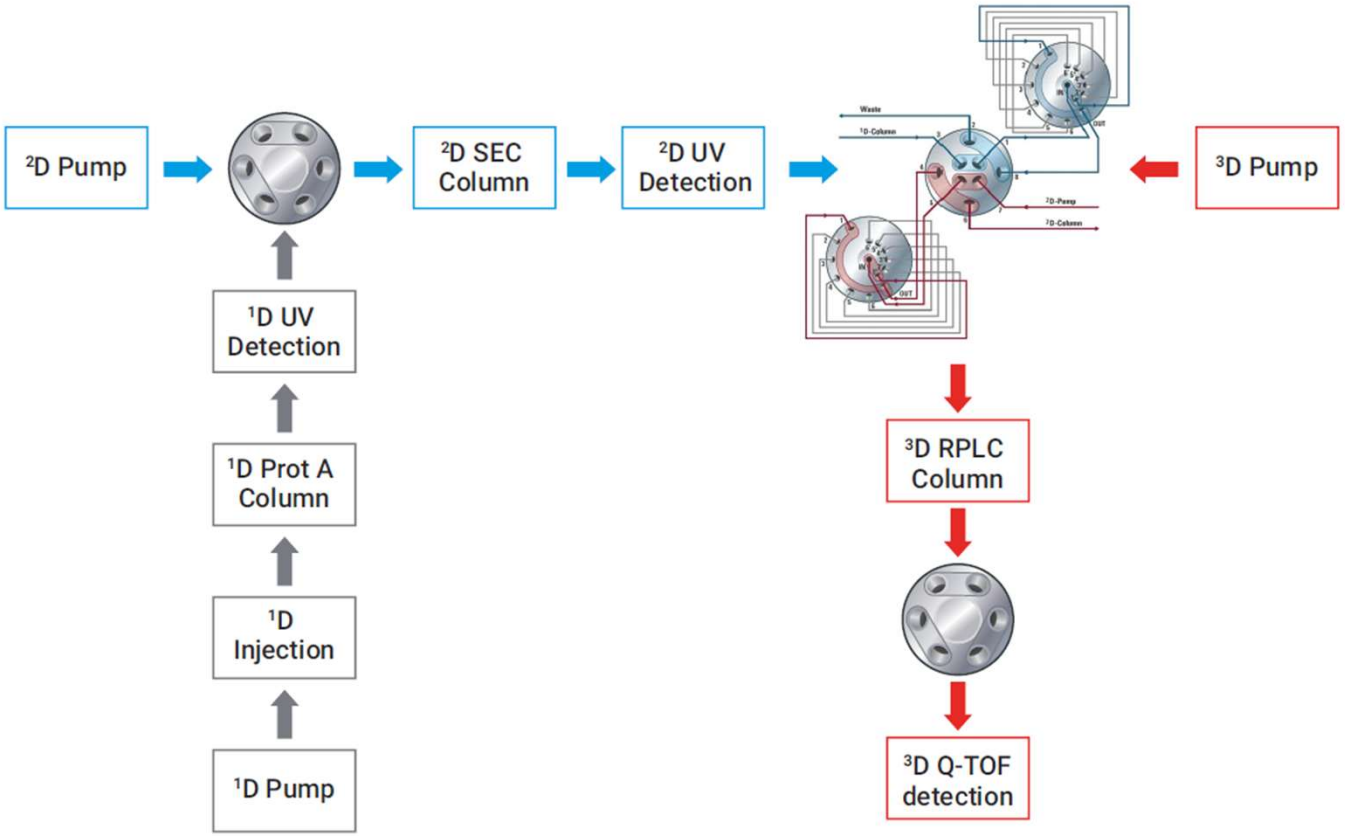
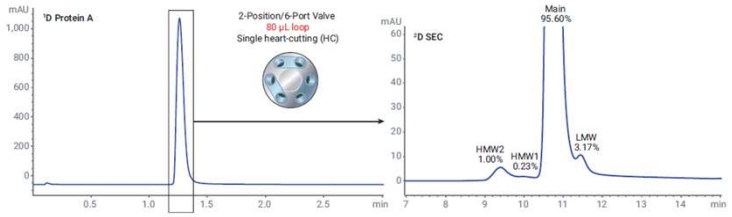
Agilent Technologies Publication Number 5994-0947EN

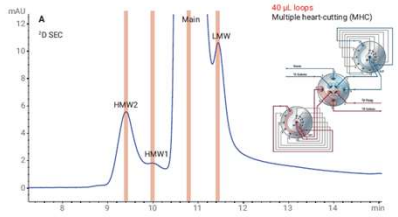
Multi-attribute analysis using **3D-LC**/MS



Multi-attribute analysis using **3D-LC**/MS

DAD 280 nm
Area RSD: 0.71%



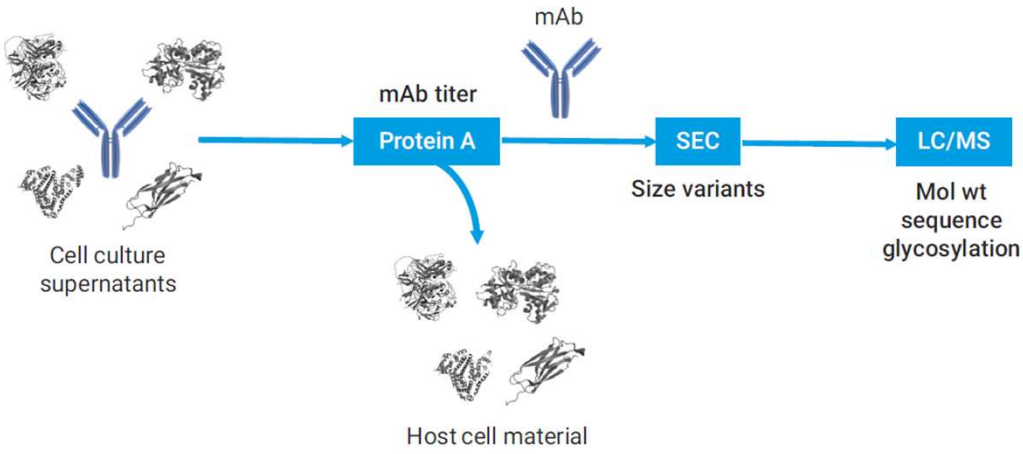


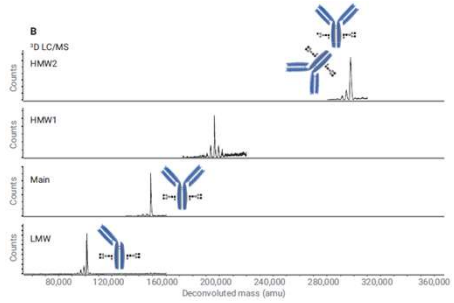
DAD 220 nm
Area RSD: 0.66% (Main)
Area RSD: 1.81-2.84% (Size variants)

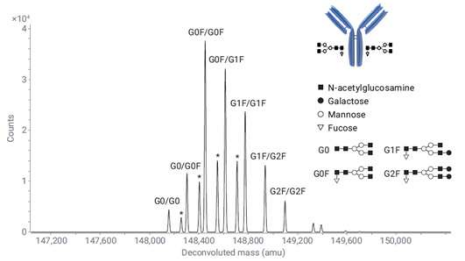
Analysis of monoclonal antibodies (mAbs)

Simultaneous determination of

- mAb titer
- size variants
- MW
- AA sequence
- PTMs







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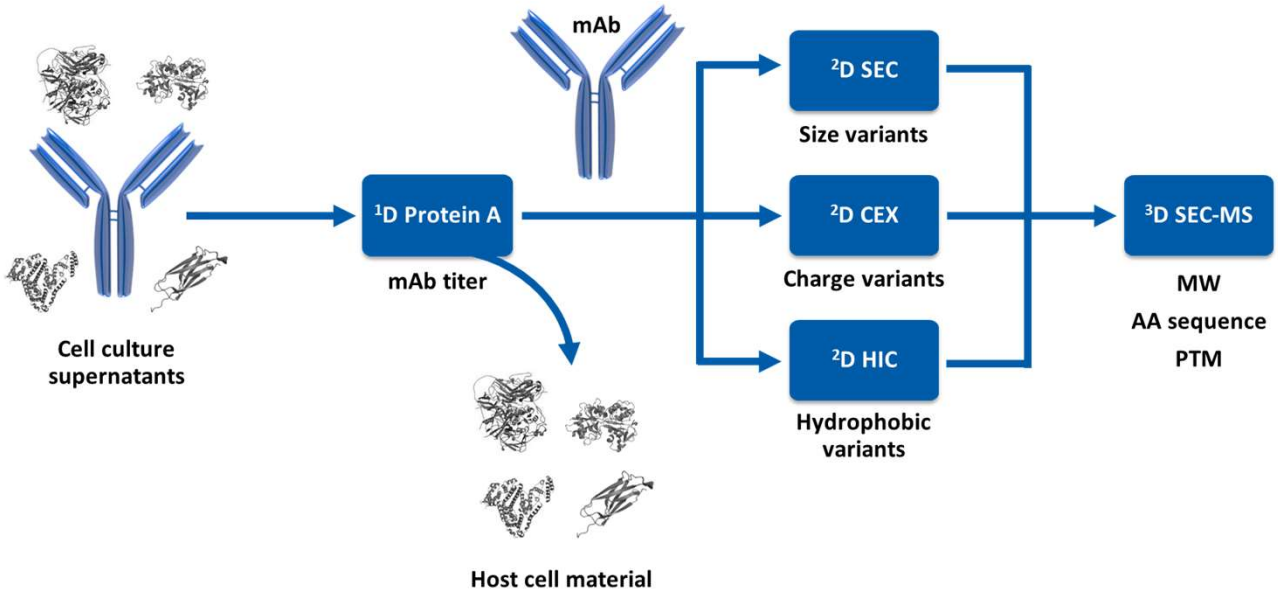
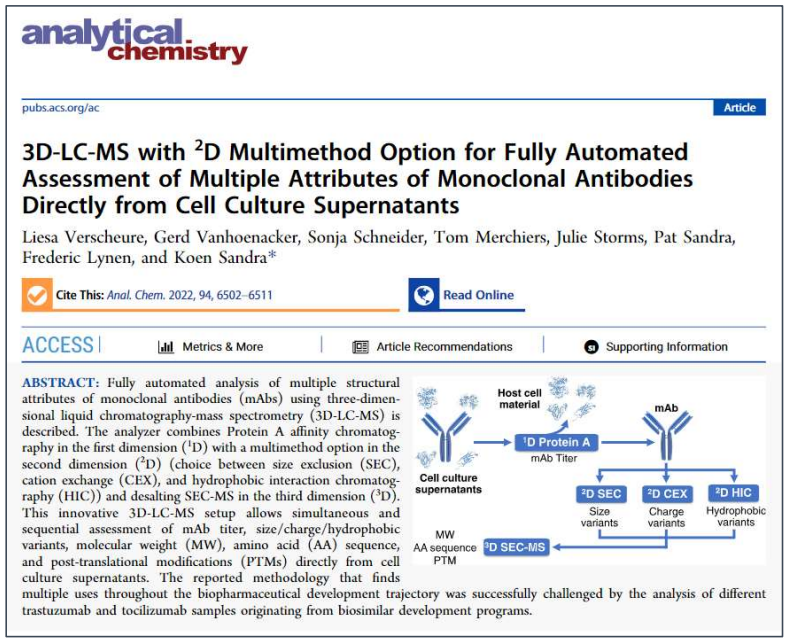
Multi-attribute analysis using 3D-LC **with ^{2}D Multimethod option**

Verscheure L. et al.
Anal. Chem. 94 (2022) 6502-6511





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3D-LC-MS with ^{2}D Multimethod Option for Fully Automated Assessment of Multiple Attributes of Monoclonal Antibodies Directly from Cell Culture Supernatants

Liesa Verscheure, Gerd Vanhoenacker, Sonja Schneider, Tom Merchiers, Julie Storms, Pat Sandra, Frederic Lynen, and Koen Sandra*

Cite This: *Anal. Chem.* 2022, 94, 6502−6511 Read Online

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ABSTRACT: Fully automated analysis of multiple structural attributes of monoclonal antibodies (mAbs) using three-dimensional liquid chromatography-mass spectrometry (3D-LC-MS) is described. The analyzer combines Protein A affinity chromatography in the first dimension (^{1}D) with a multimethod option in the second dimension (^{2}D) (choice between size exclusion (SEC), cation exchange (CEX), and hydrophobic interaction chromatography (HIC)) and desalting SEC-MS in the third dimension (^{3}D). This innovative 3D-LC-MS setup allows simultaneous and sequential assessment of mAb titer, size/charge/hydrophobic variants, molecular weight (MW), amino acid (AA) sequence, and post-translational modifications (PTMs) directly from cell culture supernatants. The reported methodology that finds multiple uses throughout the biopharmaceutical development trajectory was successfully challenged by the analysis of different trastuzumab and tocilizumab samples originating from biosimilar development programs.

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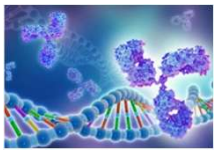
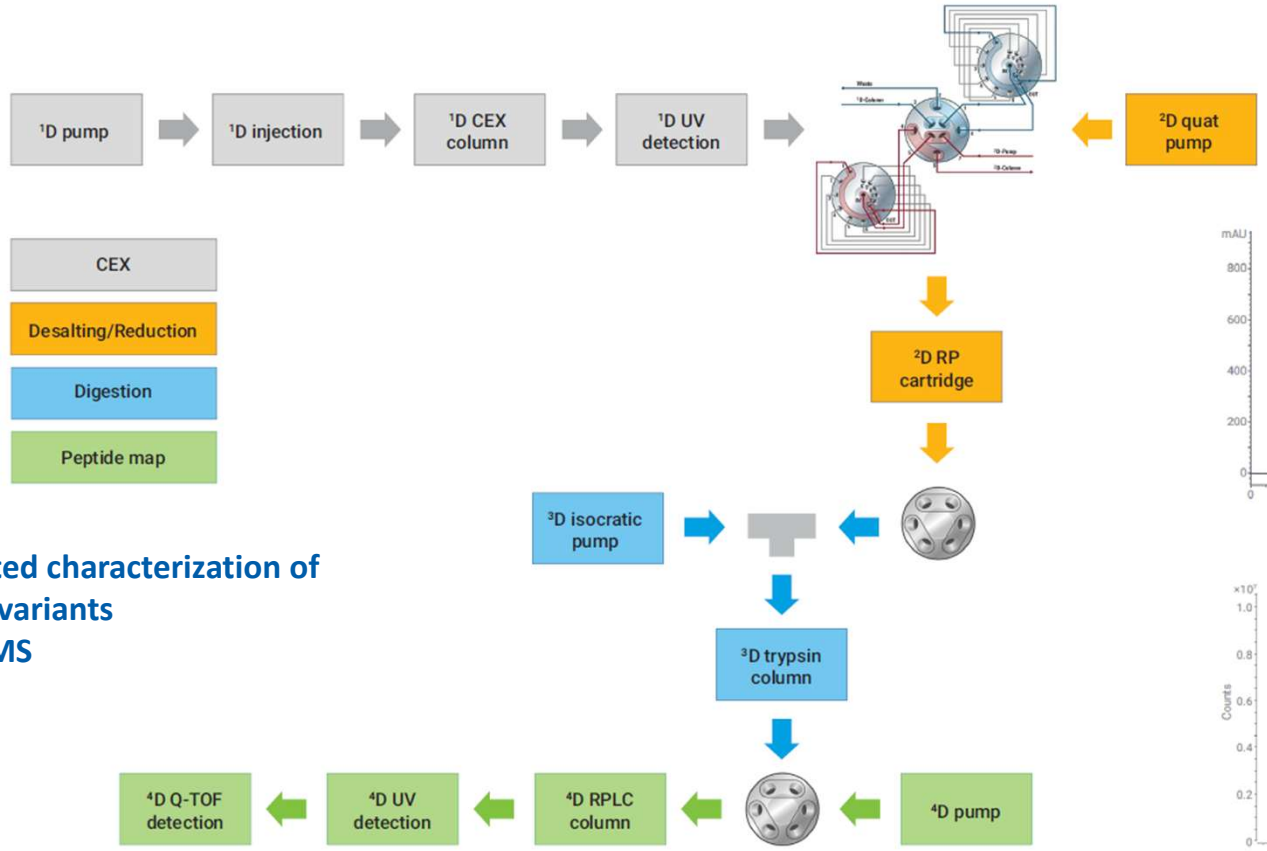
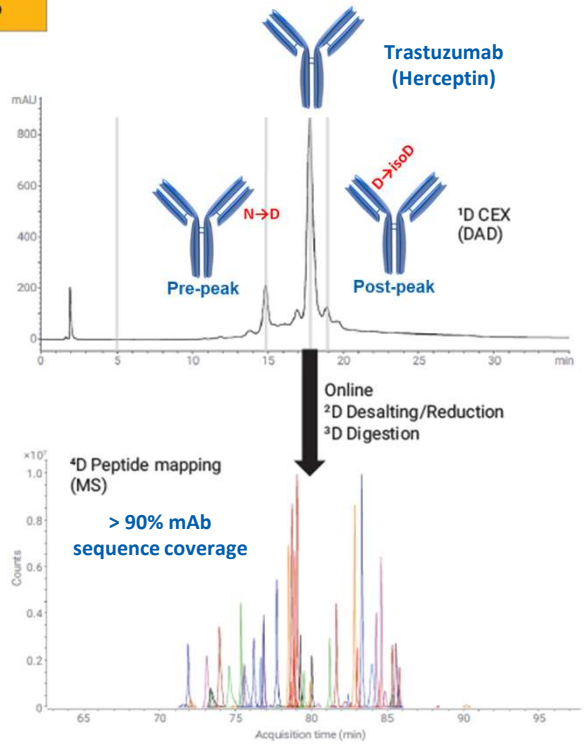
Automate workflows using **4D-LC**/MS





Verscheure L. et al.
J. Chrom. A 1653 (2021) 462409



Fully automated characterization of mAbs charge variants using 4D-LC/MS

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Conclusion

- 2D-LC (mD-LC) advantages:
  - Automation, versatility, and increased throughput
  - Reduced material requirements
  - Limited sample loss and artifacts
  - Maximized information gathering
  - Negligible user intervention
- 2D-LC can be considered as a green alternative for more traditional workflows
- 2D-LC is ready for day-to-day workflows, transitioning from a niche research technique to a standard analytical tool
- 2D-LC is not always easy and may (not always) require more experienced operators

Acknowledgements

- Liesa Verscheure, Shauni Detremmerie, Isabel Vandenheede, Eline De Rore, Jelle De Vos, Mabelle Meersseman, Ruben t'Kindt, Pat Sandra, Koen Sandra (**RIC group, Belgium**)

- Sonja Schneider (**Agilent Technologies, Germany**)

- Colleagues from the various industries we serve

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