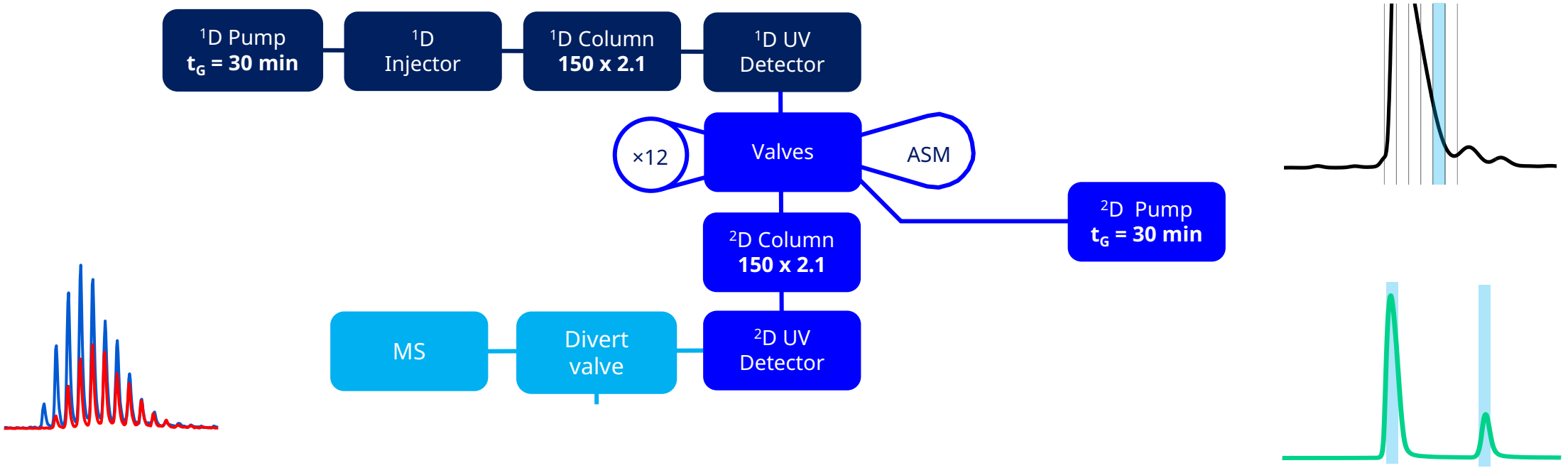
Application of multiple heart-cutting 2D-LC-MS for analysis of pharmaceutical peptides

16th Multidimensional Chromatography Workshop, Liege, Belgium, Feb. 3-5 2025

P. Petersson, Ferring Pharmaceuticals, Kastrup, Denmark

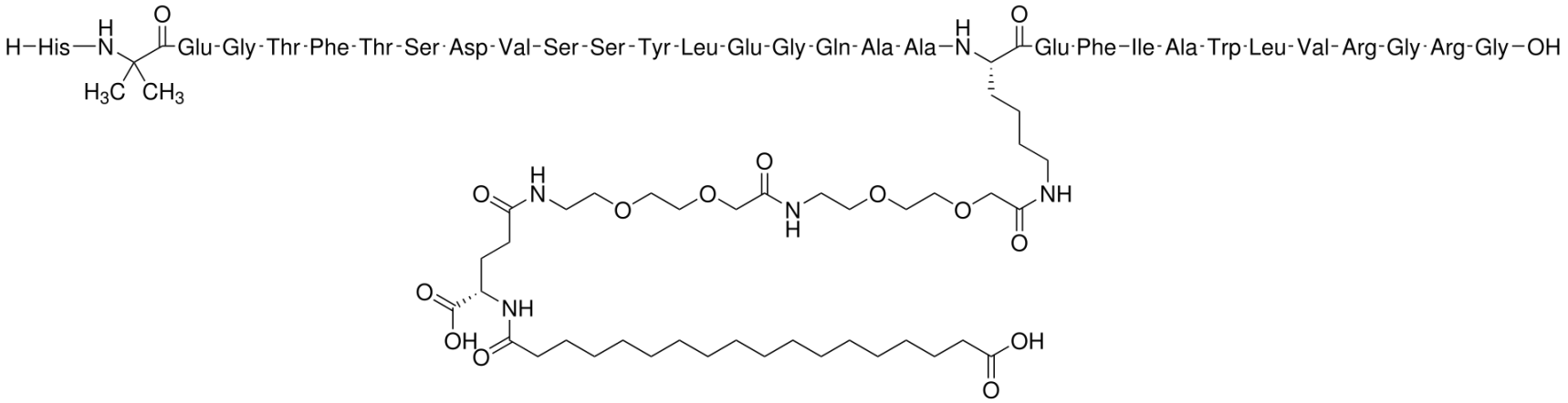
Outline

- Identification of peptide related impurities in salt-based methods by 2D-LC-MS
- Strategy for 2D-LC-MS-based peak purity analysis of pharmaceutical peptides



Background

- A very hot topic right now within the biopharma industry is GLP-1 receptor agonists for treatment of obesity, type 2 diabetes and cardiovascular problems
- Dec. 2024 Novo Nordisk, Eli Lilly, Pfizer, Amgen, *et al.* were running approx. 60 clinical trials involving oral formulations of these peptides [1]



- Despite this hype and recent years trend towards biopharmaceuticals there are only 4 publications on 2D-LC for determination of related impurities in therapeutic peptides

[1] Företagens megafajt om nästa generations bantningspiller, E. Fagerström, Sydsvenskan 28 Dec 2024, A8

Background

- Multiple Heart-Cutting 2D-LC-MS: Towards Real Time Determination of Related Impurities of Bio-Pharmaceuticals in Salt Based Separation Methods, P. Petersson, K. Haselmann, Stephan Buckenmaier, J. Chromatogr. A, 1468 (2016) 95-101
- A selective comprehensive reversed-phase x reversed-phase 2D-liquid chromatography approach with multiple complementary detectors as advanced generic method for the quality control of synthetic and therapeutic peptides, R. Karongo, T. Ikegami, D.R. Stoll, M.J. Laemmerhofer, Chromatogr. A, 1627 (2020) 461430
- A Strategy for Assessing Peak Purity of Pharmaceutical Peptides in Reversed-Phase Chromatography Methods using 2D-LC-MS. Part I: Selection of Columns and Mobile Phases, P. Petersson, S. Buckenmaier, M.R. Euerby, D.R. Stoll, J. Chromatogr. A, 1693 (2023) 463874
- A Strategy for Assessing Peak Purity of Pharmaceutical Peptides in Reversed-Phase Chromatography Methods using 2D-LC-MS. Part II: Development of Second-Dimension Gradient Conditions, D.R. Stoll, M. Sylvester, M.R. Euerby, S. Buckenmaier, P. Petersson, J. Chromatogr. A, 1693 (2023) 463873

Identification of peptide related impurities in salt-based methods by multiple heart-cutting 2D-LC-MS

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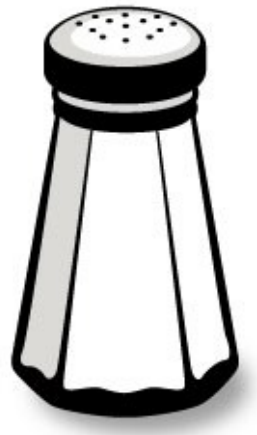
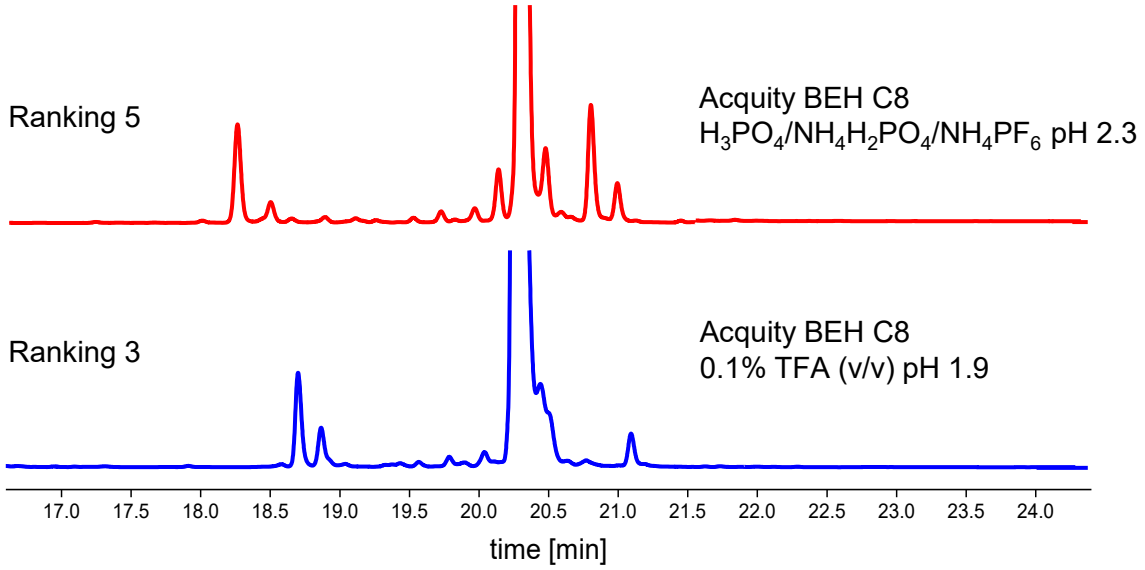
Determination of peptide related impurities in salt-based methods by 2D-LC-MS



- Common misperception that TFA always is the best additive for peptide analysis
- 15 years of method development at Novo Nordisk and Ferring Pharmaceuticals suggest that salt based eluents often are better [2,3]
- Salt based eluents such as:
  - $NH_4H_xPO_4$
  - $NH_4H_xPO_4$ /NaCl
  - $NH_4H_xPO_4$ /Na_2SO_4 (kosmotropic)
  - $NH_4H_xPO_4$/NH_4PF_6 (chaotropic)
- Often provide better peak shape and selectivity than TFA
- ~50% of investigated peptides

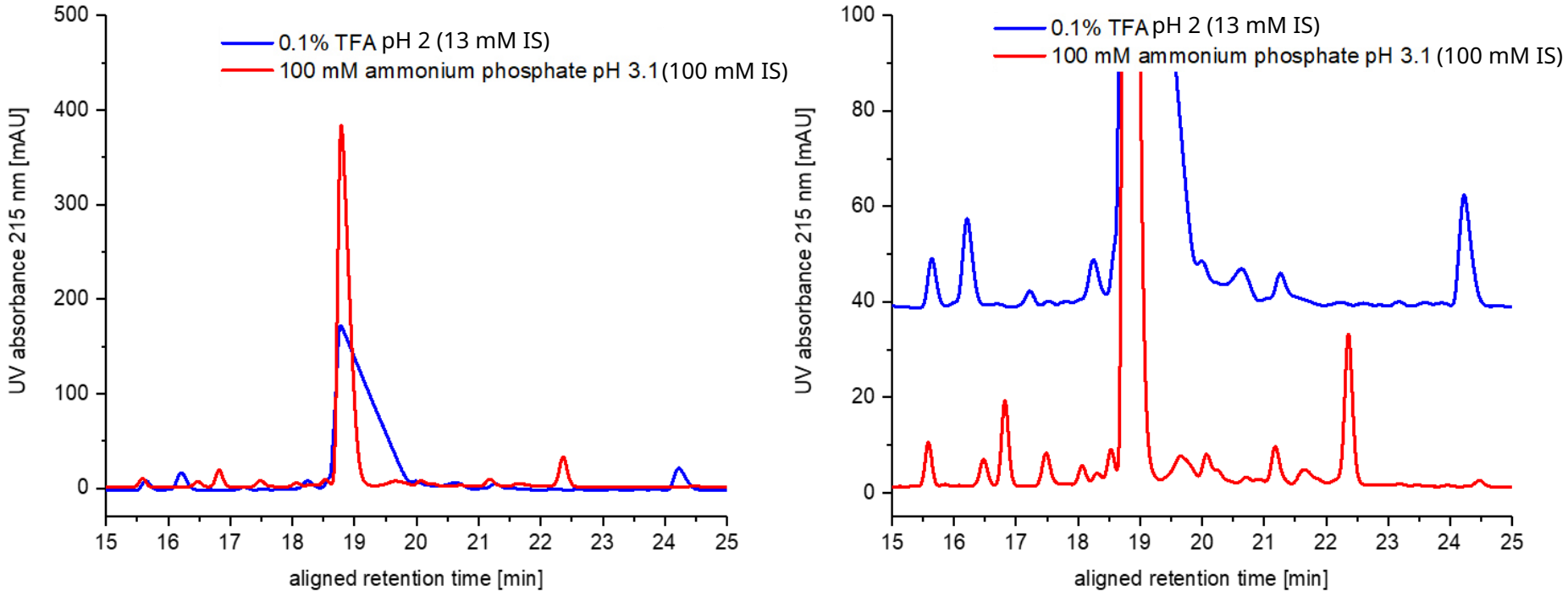


[2] Investigation into reversed-phase chromatography peptide separation systems part V: Establishment of a screening strategy for development of methods for assessment of pharmaceutical peptide's purity, M.Y. Cheung, J. Bruce, M.R. Euerby, J.K. Field, P. Petersson, J. Chromatogr. A, 2022, 1668, 462888.

[3] Method development for reversed-phase separations of peptides: A rational screening strategy for column and mobile phase combinations with complementary selectivity, J.K. Field, J. Brucec, S. Buckenmaier, M.Y. Cheung, M.R. Euerby, K.F. Haselmann, J.F. Lau, D. Stoll, M. Sylvester, H. Thøgersen, Patrik Petersson, , LCGC Europe, November/December 2022, Volume 35, Issue 10, pages 440-449.

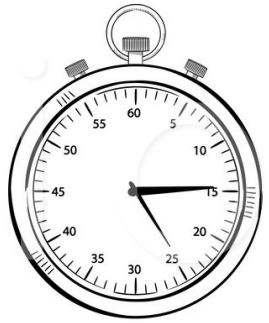
Determination of peptide related impurities in salt-based methods by 2D-LC-MS

- Another example

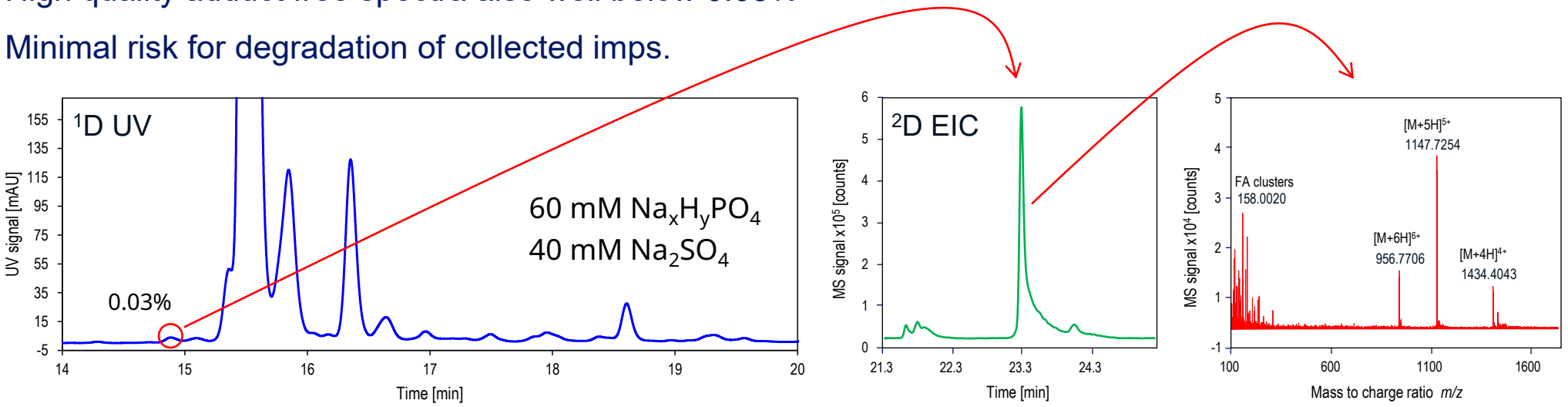


- Consequently, many RPC methods are salt-based and therefore not MS compatible

Identification of peptide related impurities in salt-based methods by 2D-LC-MS



- Multiple Heart-Cutting 2D-LC-MS employing a rapid desalting gradient as ^{2}D provides almost real time MS data without adducts and clusters for impurities at relevant levels (<0.05%)
- An example: Determination of impurities in degraded insulin [4]
  - 13 cuts from a 38 min gradient with sodium, phosphate and sulphate in the eluent
  - Each cut analyzed with a 4 min desalting gradient in the ^{2}D – total time for data collection 63 min
  - High quality adduct free spectra also well below 0.03%
  - Minimal risk for degradation of collected imps.



[4] Multiple Heart-Cutting 2D-LC-MS: Towards Real Time Determination of Related Impurities of Bio-Pharmaceuticals in Salt Based Separation Methods, P. Petersson, K. Haselmann, Stephan Buckenmaier, J. Chromatogr. A, 1468 (2016) 95-101.

Strategy for 2D-LC-MS-based peak purity analysis of pharmaceutical peptides in RPC methods

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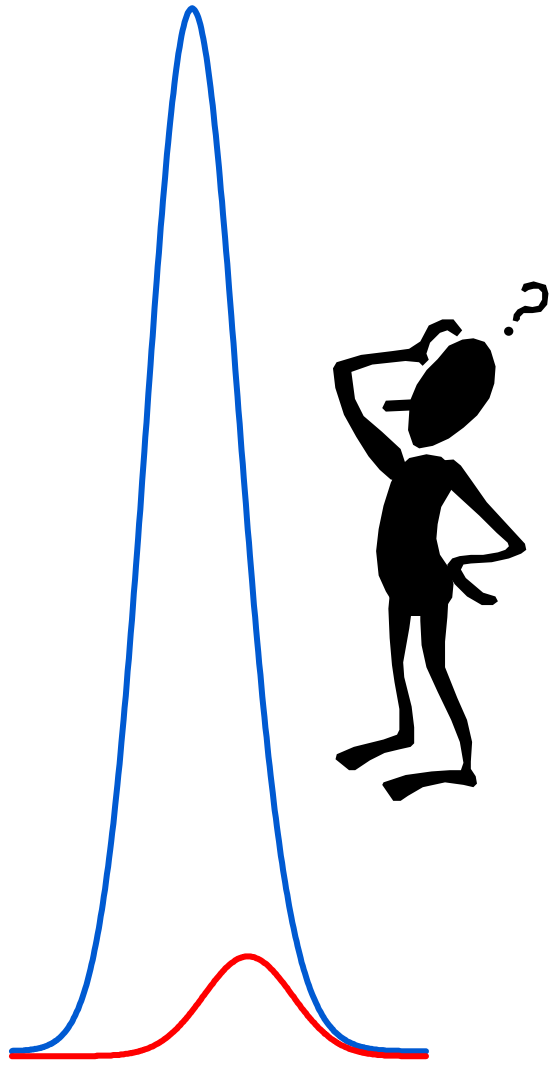
Peak purity analysis

- Once a purity method has been developed for a pharmaceutical drug product it is required to conduct a peak purity analysis
- Is something hiding in the main peak?



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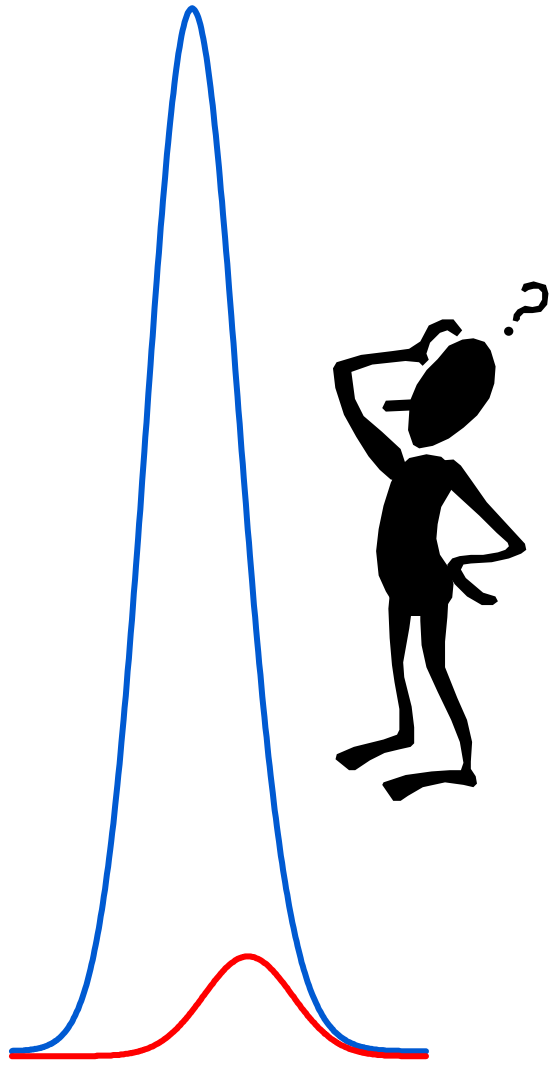
- Large number of publications on peak purity analysis by DAD (but is difficult)
  - Interpretation of DAD data is not conclusive
  - UV spectra of closely eluting impurities tend to be similar
  - The quality of UV spectra is poor for impurities at 0.05% level



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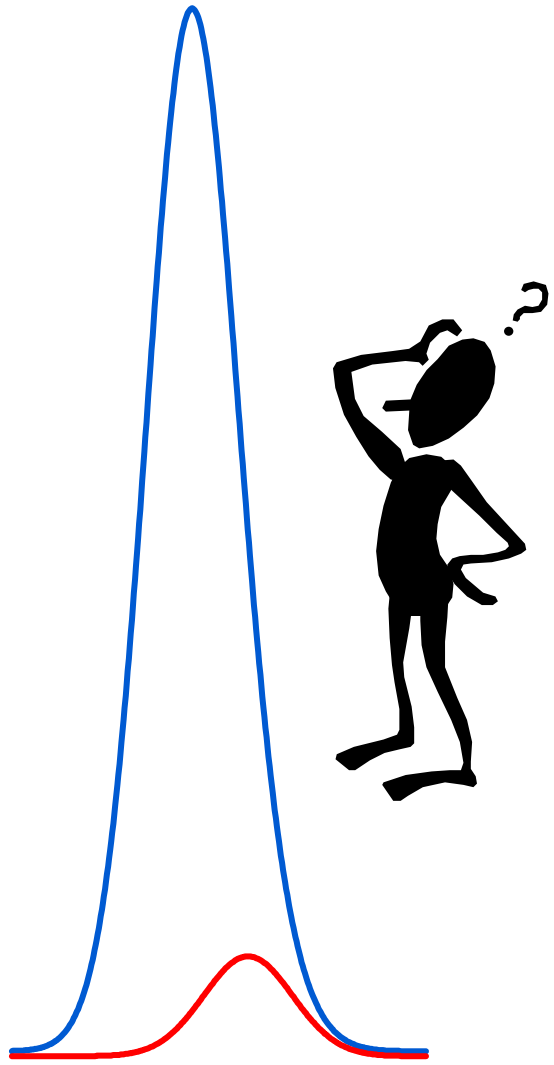
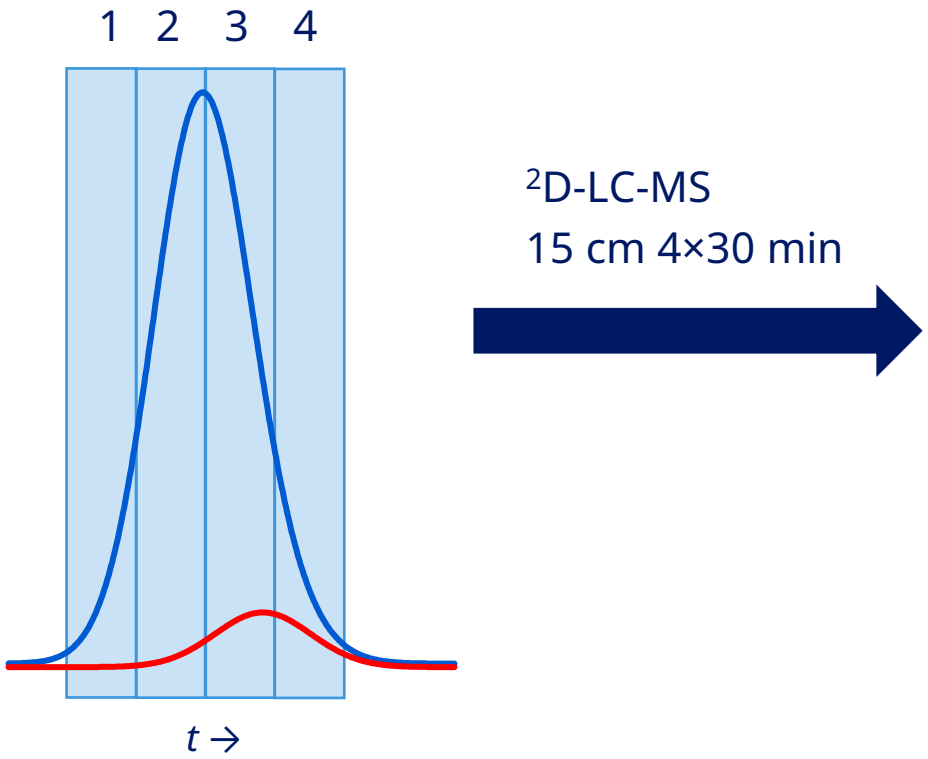
  - UV spectra of closely eluting impurities tend to be similar
  - The quality of UV spectra is poor for impurities at 0.05% level
- Peak purity analysis by MS is also difficult
  - The signal for impurities eluting under the main peak might be suppressed
  - (For small molecules an additional problem is that not all impurities ionize)
  - **Peptide impurities are often diastereomers from racemization and have the same *m/z* as the drug substance which prevents direct MS determination**



Peak purity analysis

- 2D-LC-MS is probably the best alternative for peak purity analysis also for methods with volatile eluents

^{1}D-LC-UV = the method to be evaluated
e.g. 15 cm 30 min



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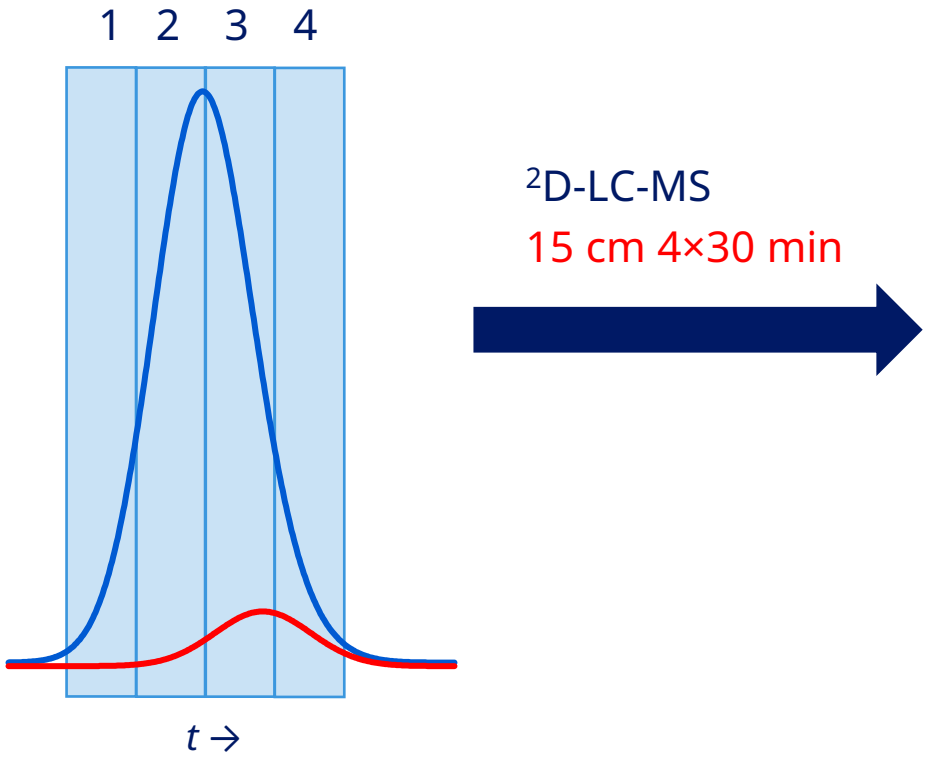
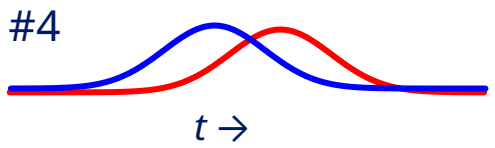


[2]D-LC-MS
15 cm 4×30 min

I) A more favorable imp./API ratio → better resolution



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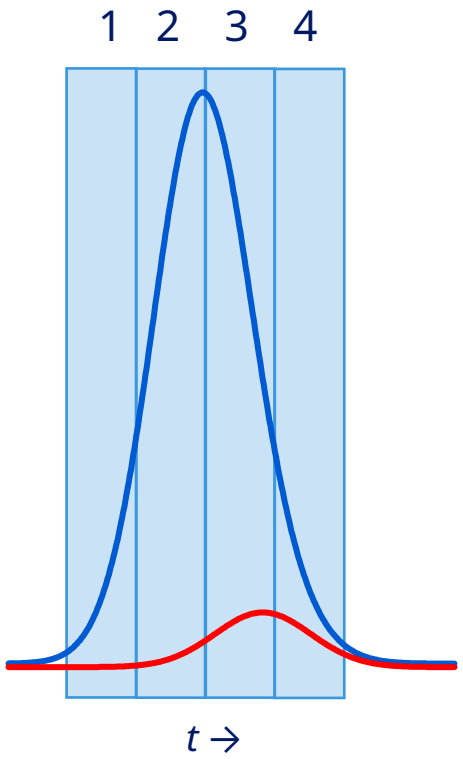

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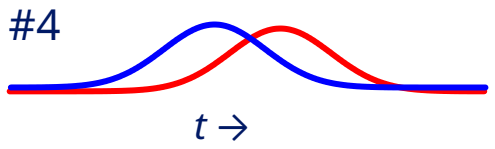
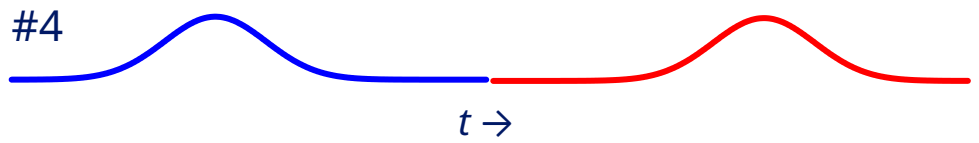


II) Other chromatographic selectivity → separation in time and better resolution



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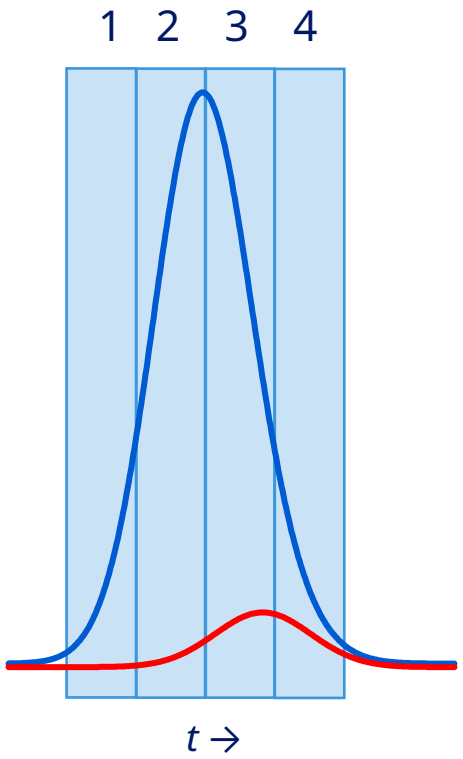

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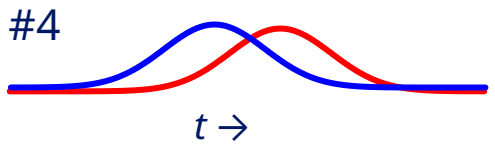

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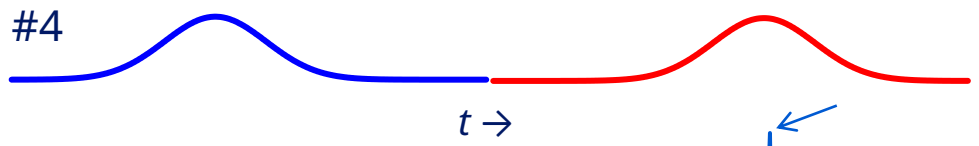
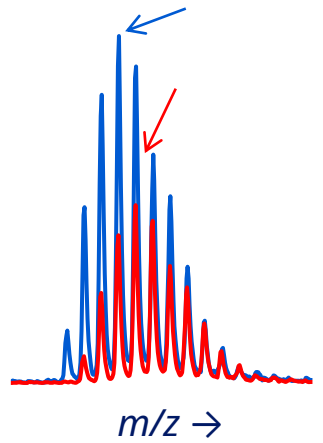


III) MS → separation in *m/z*
#4



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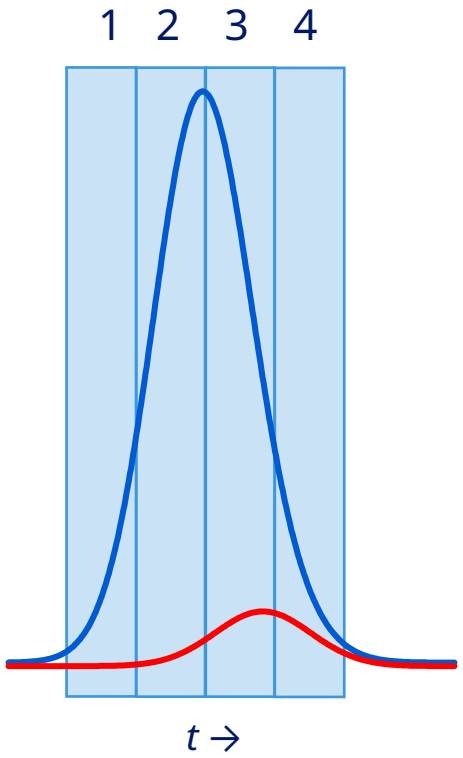

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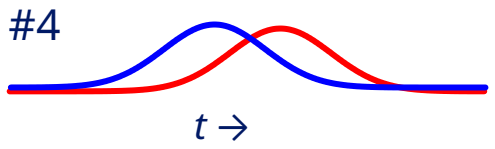

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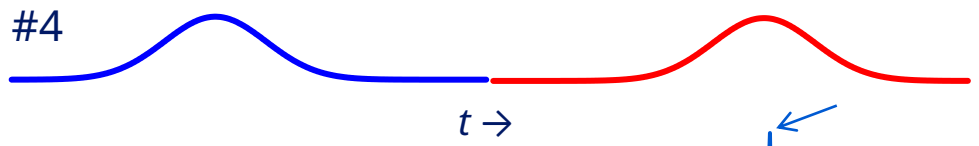

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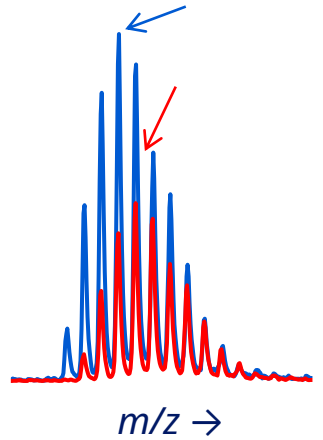

I+II+III combined → high probability to spot impurities

Strategy for 2D-LC-MS-based peak purity analysis of pharmaceutical peptides in RPC methods. Part I: Selection of ^{2}D columns and eluents with good isomer selectivity

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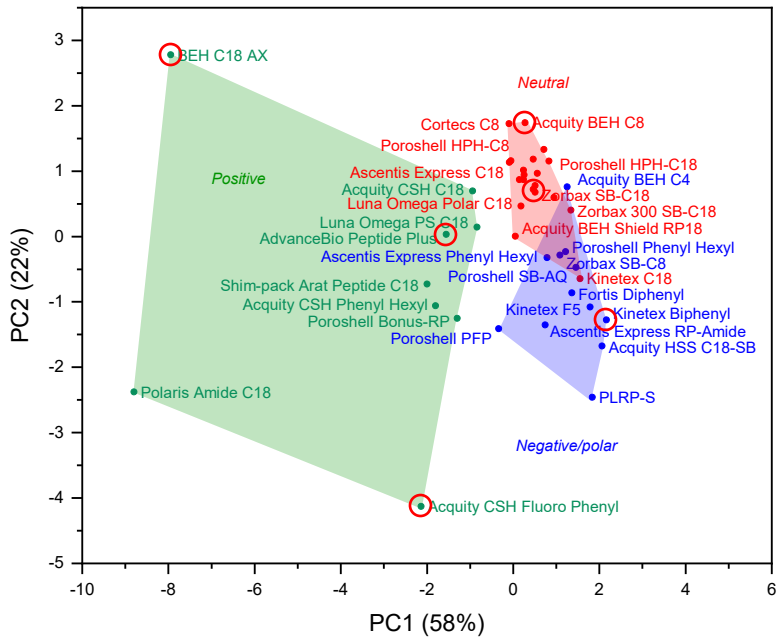
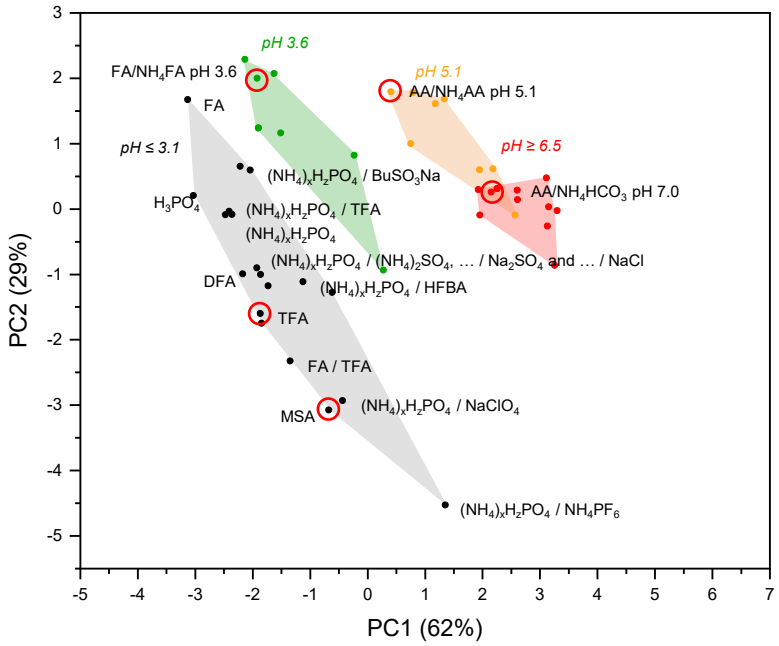
Selection of ^{2}D columns and eluents with good isomer selectivity

- 6 Columns and 5 MS compatible eluents were selected based on “The Peptide RPC Column Characterization Protocol” [5]
- The protocol is based on the retention of 9 peptides designed to
  - Reflect typical degradation pathways (oxidation, racemization, deamidation)
  - Probe hydrophobicity, H-bonding, π-π-interactions, polar interactions, steric interactions as well as pos. and neg. charge





[5] Investigation into reversed phase chromatography peptide separation systems part III: Establishing a Column Characterisation Database, J.K. Field, M.R. Euerby, P. Petersson, J. Chromatogr A., 2020, 1622, 461093

Selection of ^{2}D columns and eluents with good isomer selectivity

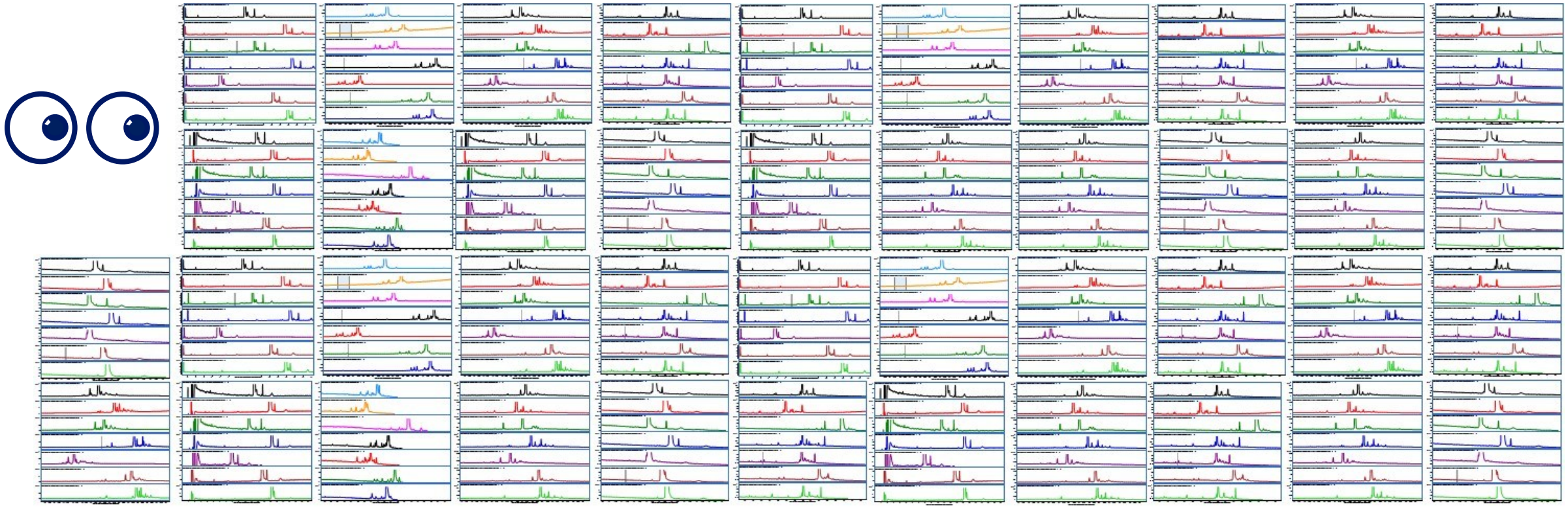
- In the current study characterization of columns and **MS compatible eluents** with focus on **isomer selectivity**
  - 6 diverse peptides
    - 1 < MW < 16 kDa
    - 4 < pI < 13
    - Forced degradation → *n* degradation products
    - In total 29 peptides which *m/z* we easily could track across all column/eluent combinations)
  - 12 D/L-diastereomers of GLP-2 [1-15]

- UV and MS detection to reflect
  - General selectivity at 215 nm
  - Isomer selectivity by EIC

Peptide	Amino acid sequence	MW [kDa]	pI [-]
Bradykinin	RPPGFSPFR	1.1	12.5
Rat GLP-2 (1-33)	HADGSFSDEMNTILDNLATRDFINWLIQTKITD	3.8	3.7
Bovine insulin	GIVEQCCASVCSLYQLENYCN-FVNQHLCGSHLVEALYLVCGERGFFYTPKA	5.7	5.3
Bovine ubiquitin	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEPIPPDQQRLI FAGKQLEDGRTLSDYNIQKESTLHLVLRLR	8.5	7.6
Chicken lysozyme	MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRGYS LGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINSRWWC NDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVSDGNGM NAWVAWRNRCKGTDVQAWIRGCRL	16.2	11.0
Bovine GLP-2 (1-15)	HADGSFSDEMNTVLD	1.6	3.4
[D-His1]-Bovine GLP-2 (1-15)	hADGSFSDEMNTVLD	1.6	3.4
[D-Asp3]-Bovine GLP-2 (1-15)	HAdGSFSDEMNTVLD	1.6	3.4
[D-Ser5]-Bovine GLP-2 (1-15)	HADGsFSDEMNTVLD	1.6	3.4
[D-Ser7]-Bovine GLP-2 (1-15)	HADGSFsDEMNTVLD	1.6	3.4
[isoAsp3]-Bovine GLP-2 (1-15)	HAiDGSFSDEMNTVLD	1.6	3.4
[D-isoAsp3]-Bovine GLP-2 (1-15)	HAidGSFSDEMNTVLD	1.6	3.4
[Asp11]-Bovine GLP-2 (1-15)	HADGSFSDEMDTVLD	1.6	3.3
[D-Asp11]-Bovine GLP-2 (1-15)	HADGSFSDEMdTVLD	1.6	3.3
[Asp21, Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRDGINWLIQTKITD	2.0	6.6
[D-Asp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRdGINWLIQTKITD	2.0	6.6
[isoAsp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRiDGINWLIQTKITD	2.0	6.6
[D-isoAsp21,Gly22,Ile27]-Bovine GLP-2 (16-33)	SLATRidGINWLIQTKITD	2.0	6.6

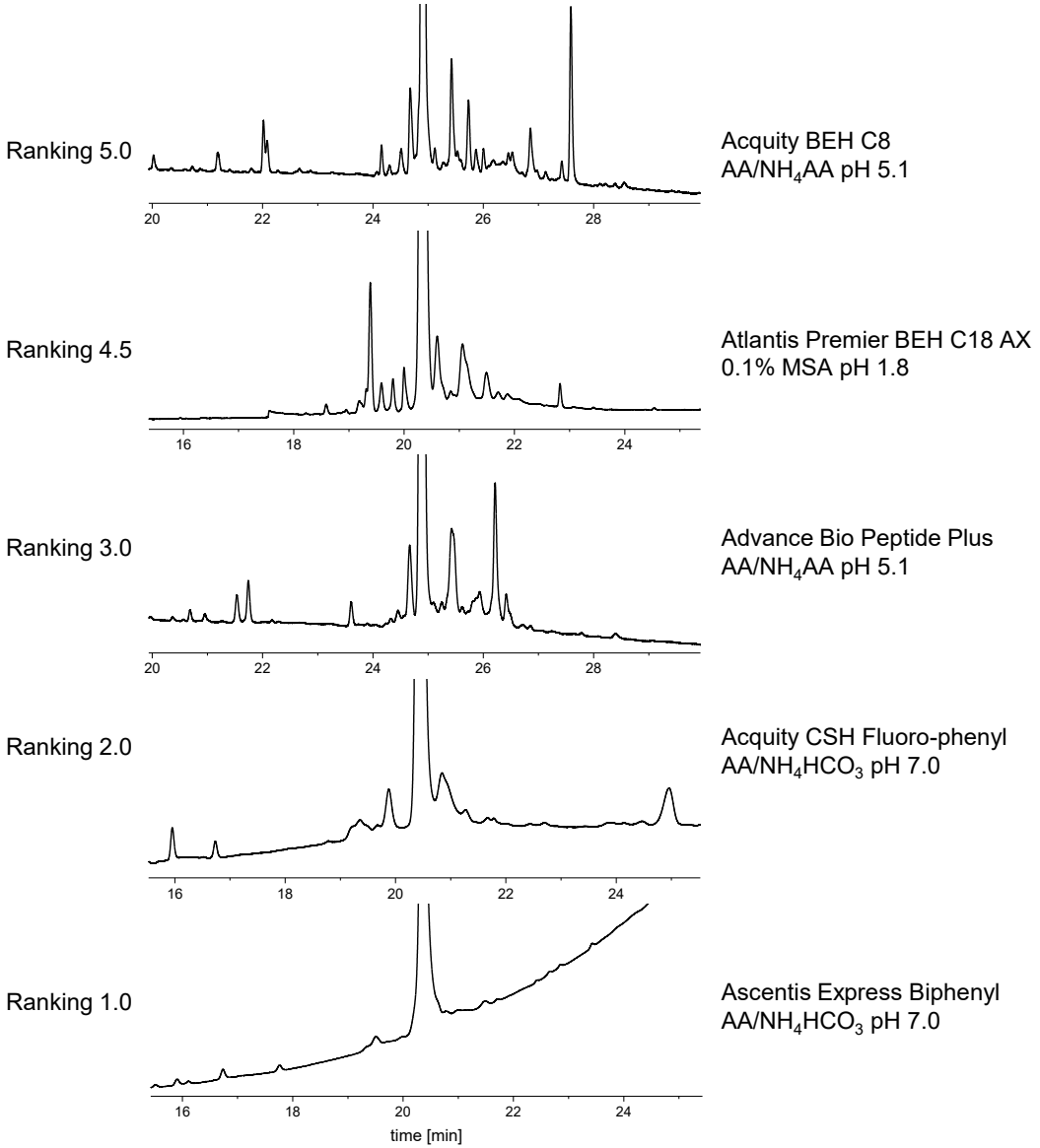
Selection of ^{2}D columns and eluents with good isomer selectivity

- 300 chromatograms collected
- Chromatograms for each sample visually ranked from 1 to 5 (5 = best performance)
- Ranking criteria = number of peaks, peak shape and resolution around the main peak



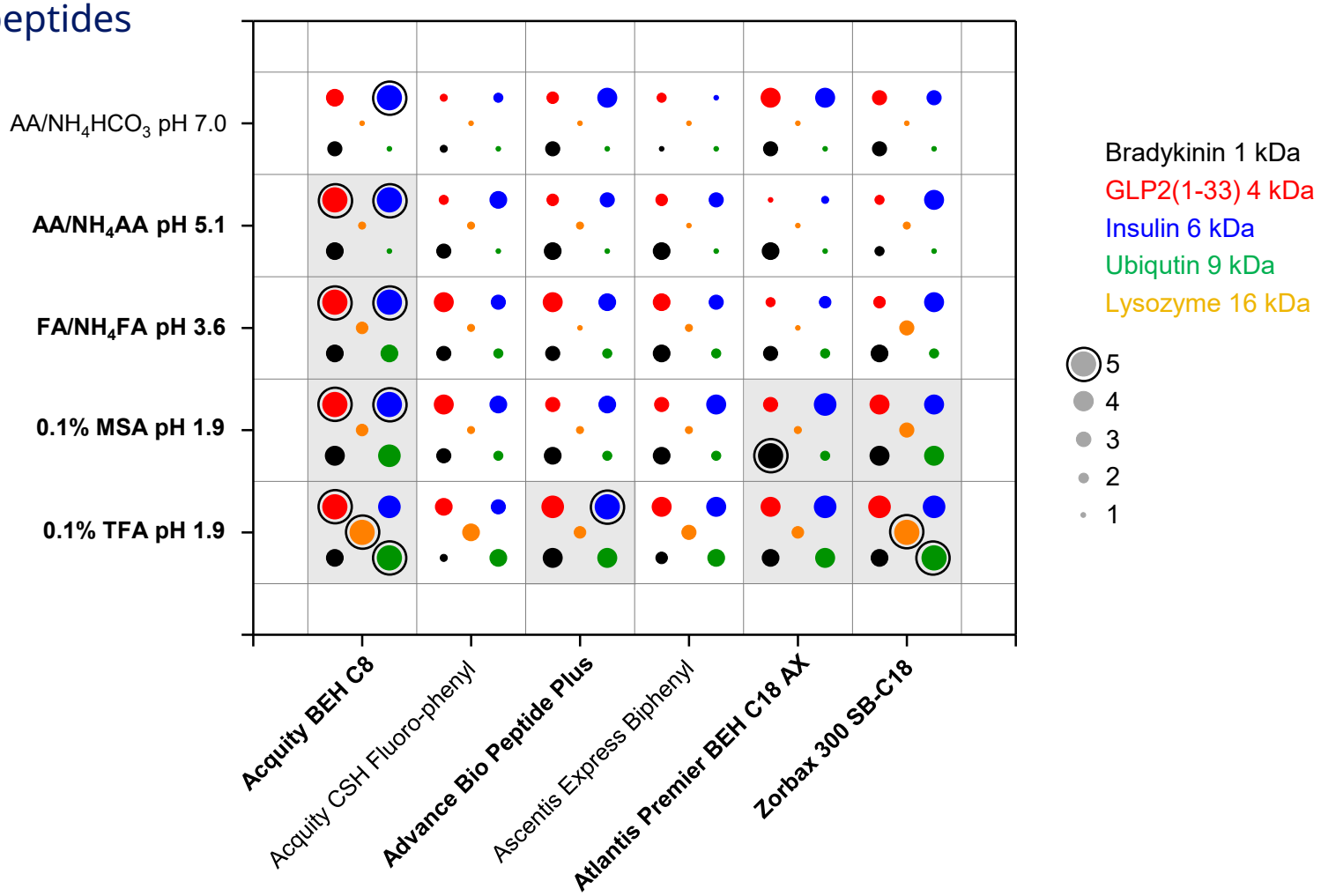
Selection of ^{2}D columns and eluents with good isomer selectivity

- Ranking examples for **general selectivity**
- Degraded insulin at 215 nm



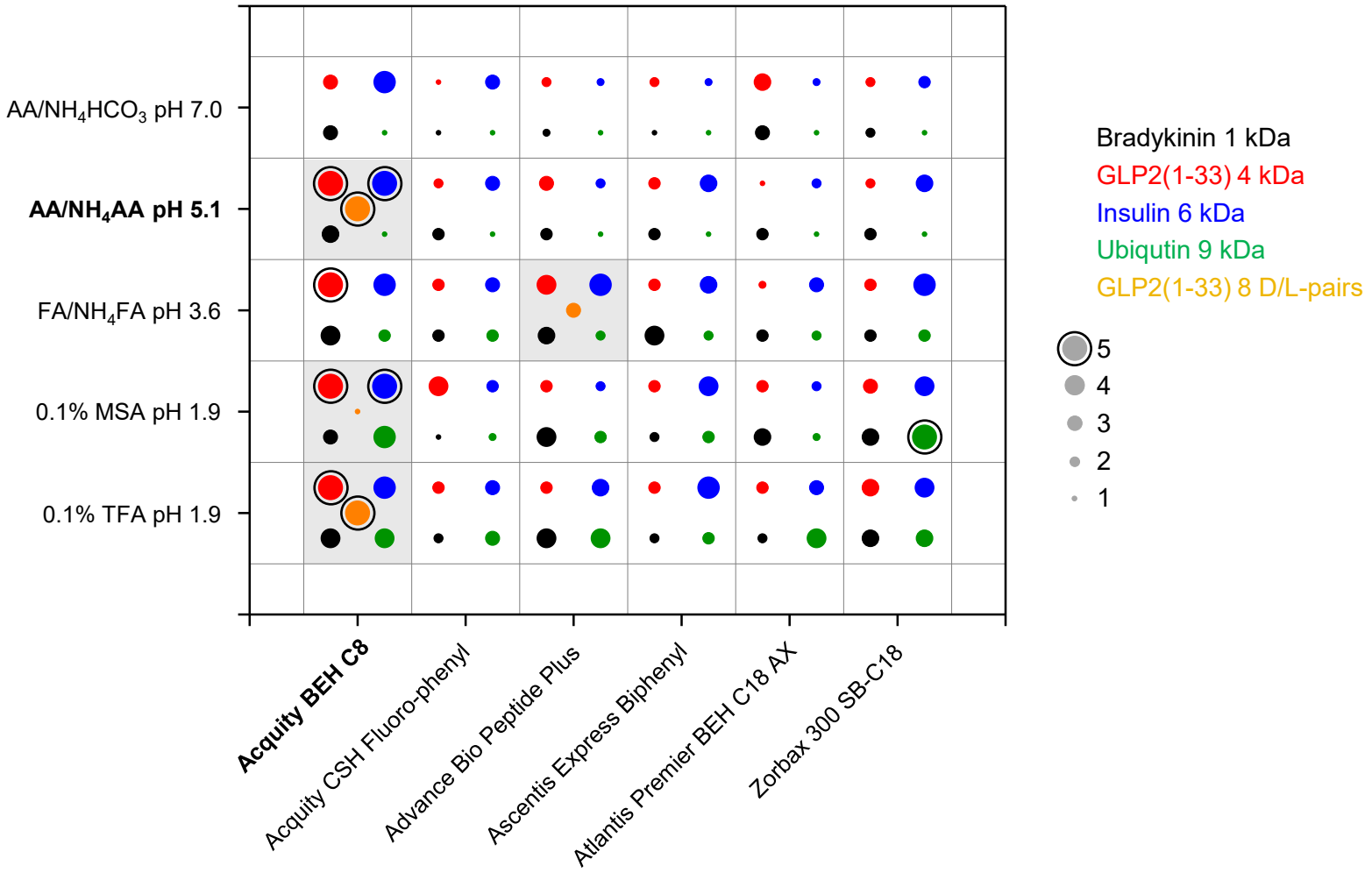
Selection of ^{2}D columns and eluents with good isomer selectivity

- Overview **general selectivity** – ranking of 215 nm data
- BEH C8 combined with 0.1% TFA got the highest ranking for general selectivity
- Other combinations better for certain peptides
- 9 more promising combinations



Selection of ^{2}D columns and eluents with good isomer selectivity

- Overview **isomeric selectivity** – ranking of EIC data
- BEH C8 combined with AA/NH_4AA pH 5 got the highest ranking followed by 0.1% TFA

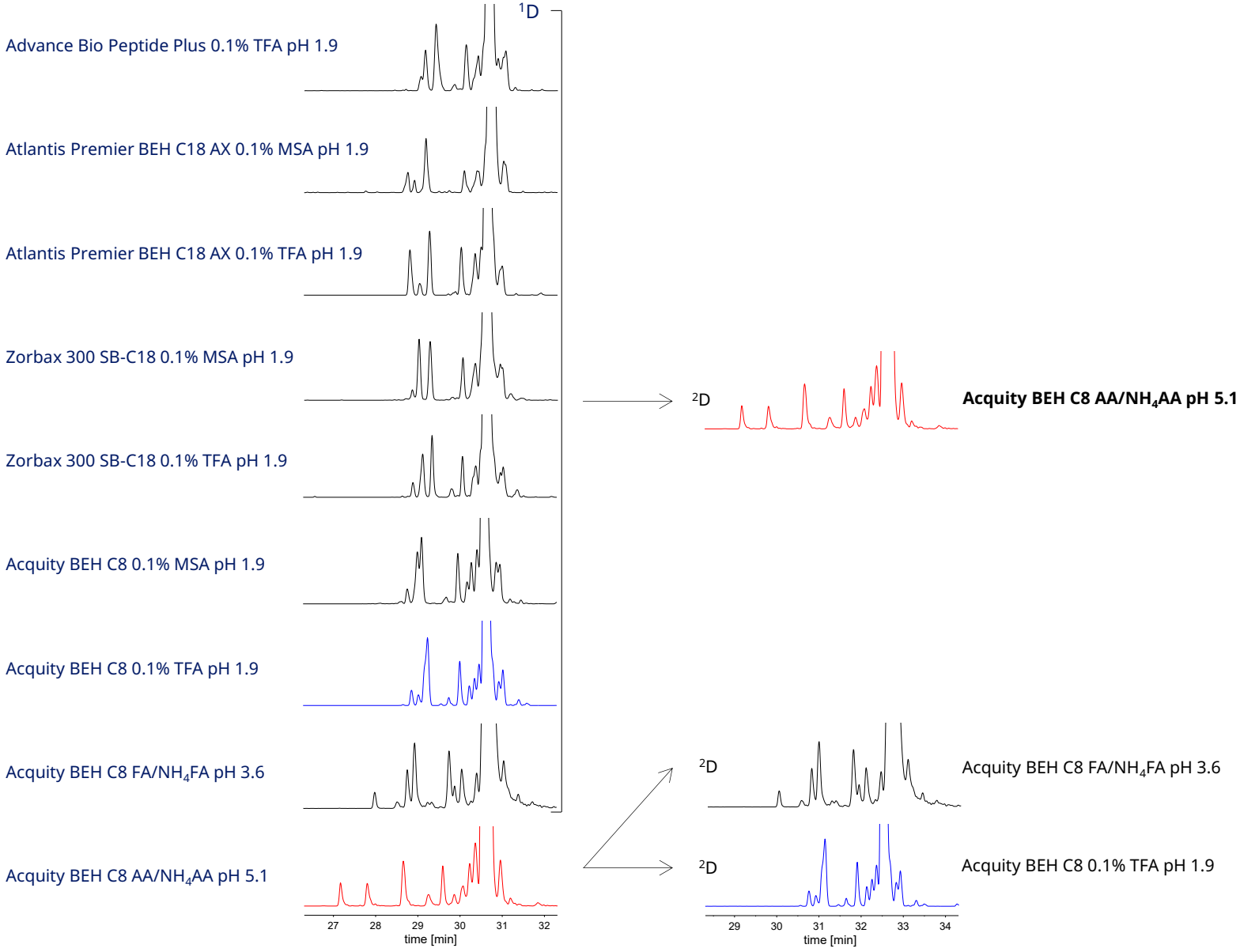


Selection of ^{2}D columns and eluents with good isomer selectivity

- An alternative comparison is a comparison of IECs for the 9 combinations that got the highest rankings for general selectivity at 215 nm
e.g. for GLP2(1-33) →

- **BEH C8 / AA/NH_4AA pH 5 again appears to be the most general combination**
- If this gives a poor peak shape try 0.1% TFA (>10 kDa)

- BEH C8 *vs.* C18 and other C8 with neutral character show minor differences



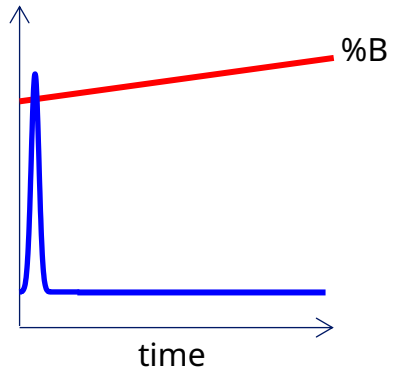
Strategy for 2D-LC-MS-based peak purity analysis of pharmaceutical peptides in RPC methods. Part II: Definition of a shallow ^{2}D gradient to maximize isomer selectivity

A Strategy for Assessing Peak Purity of Pharmaceutical Peptides in Reversed-Phase Chromatography Methods using 2D-LC-MS. Part II: Development of Second-Dimension Gradient Conditions, D.R. Stoll, M. Sylvester, M.R. Euerby, S. Buckenmaier, P. Petersson, J. Chromatogr. A, 2023, 463873.

Definition of a shallow ^{2}D gradient to maximize isomer selectivity

- A very shallow gradient is needed in the ^{2}D to maximize the resolution of isomers
- Selected / typical conditions: 30 min gradient and 0.15%/min at 0.3 mL/min on a 150 x 2.1 mm column
- **Initial %B is peptide dependent and needs to be optimized**
- This is challenging since peptides and other large molecules respond very strongly to small changes in %B [6]

$$\log k \propto 0.25\sqrt{M}\Phi$$



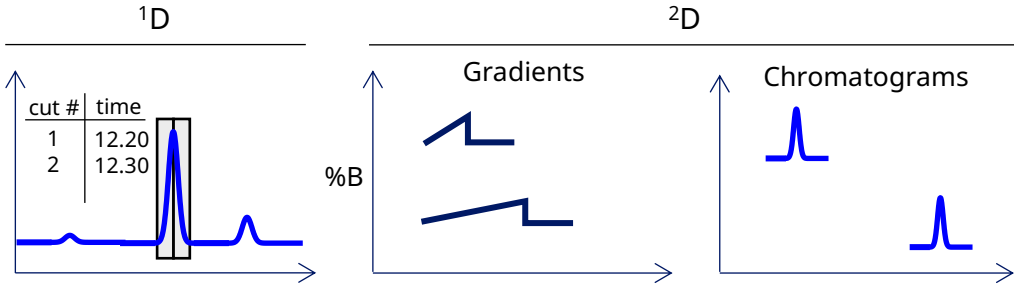
$%B_i$ too high

Too early elution!

[6] L.R. Snyder and J.W. Dolan, High Performance Gradient Elution, John Wiley & Sons, Hoboken, New Jersey, 2007, p. 230.

Definition of a shallow ^{2}D gradient to maximize isomer selectivity

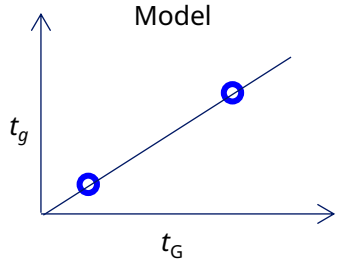
- Our approach to define the initial %B is based on linear solvent strength retention modelling [7-9]



1) Carry out one 2D-LC training experiment. Two ^{2}D gradients with different slope.

2) Build retention model using ^2t_g. Predict $^2\Phi_i$ for elution in 2nd the half of ^{2}D gradient.

$$\frac{t_g - t_m}{t_m} = k_{eff} = \frac{t_d}{t_m} + \frac{1}{b}\ln\left(\frac{b \cdot k_i\left(t_m - \frac{t_d}{k_i}\right)}{t_m} + 1\right)$$



[7] P. Schoenmakers, et al., J. Chromatogr. A, 1978, 519-537.
[8] P. Jandera / J. Chromatogr. A 1126 (2006) 195–218.
[9] L. R. Snyder, and J.W. Dolan, High performance gradient elution: The practical application of the liner-solvent strength model, John Wiley & Sons, Hoboken, New Jersey, 2007.

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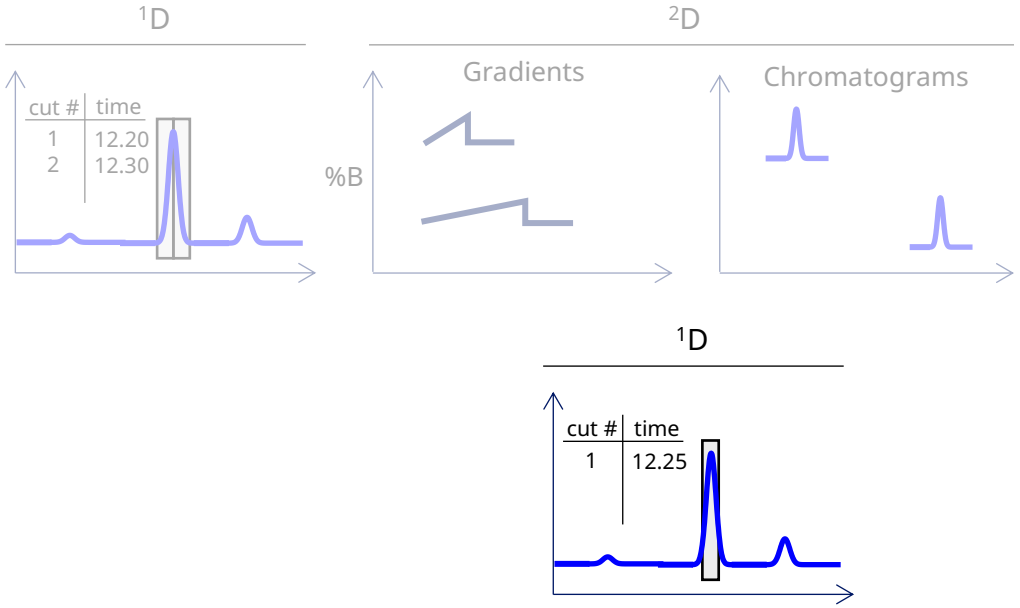

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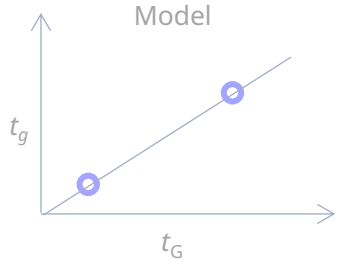

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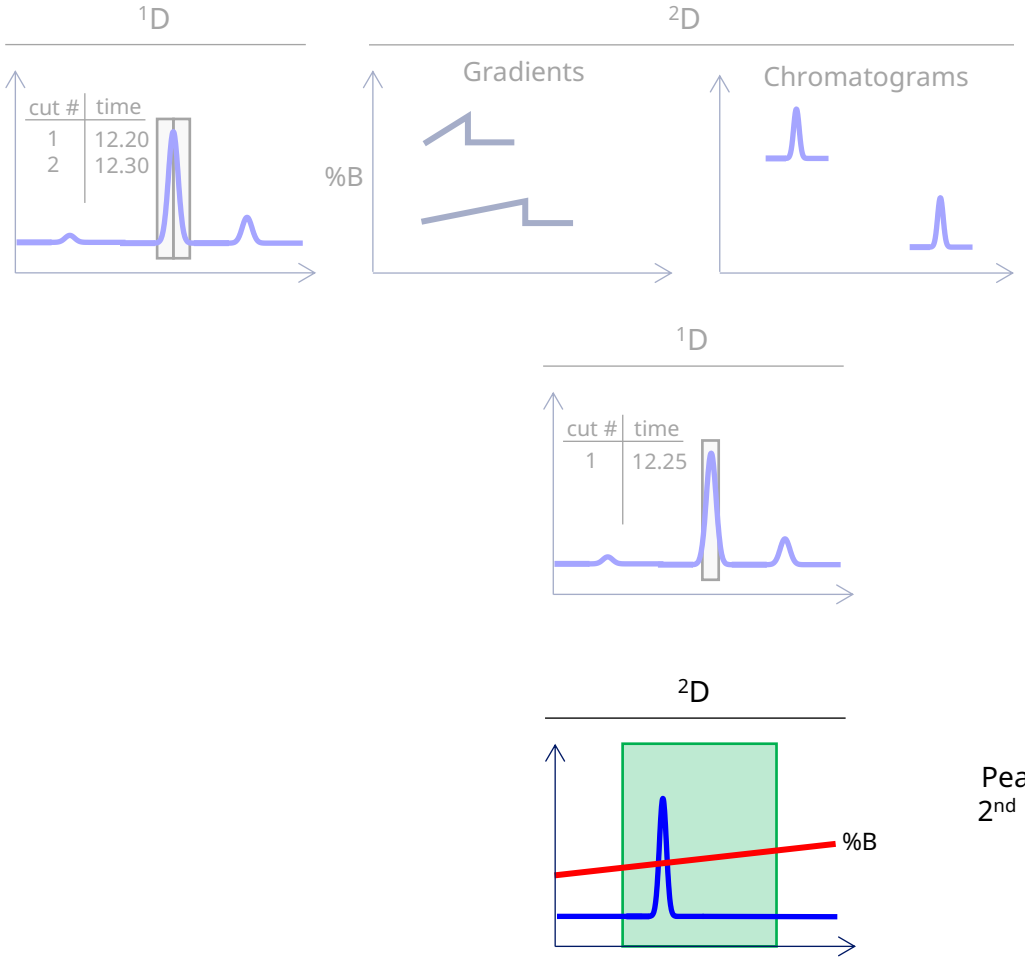

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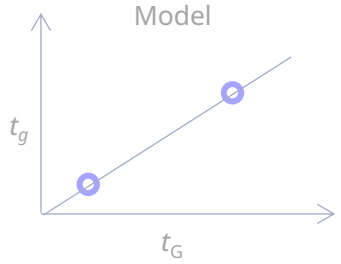
4) Evaluate retention time of main peak against the ^{2}D retention target window.

Peak in target window 2nd half of ^{2}D gradient?

Yes

5a) Method complete and we can perform the peak purity analysis with *n* cuts over the ^{1}D main peak.

$$\frac{t_g - t_m}{t_m} = k_{eff} = \frac{t_d}{t_m} + \frac{1}{b}\ln\left(\frac{b \cdot k_i\left(t_m - \frac{t_d}{k_i}\right)}{t_m} + 1\right)$$



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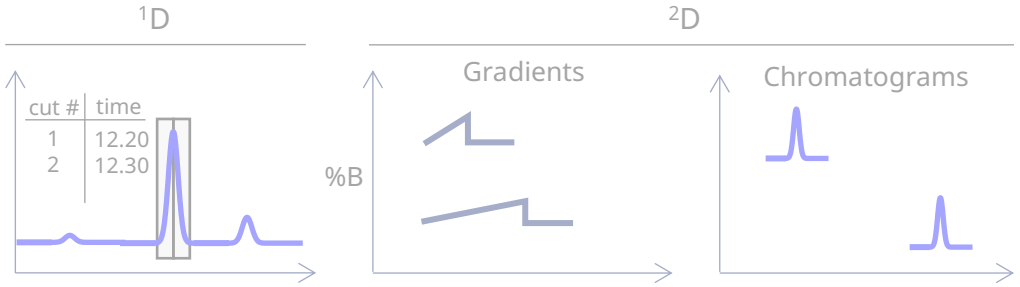

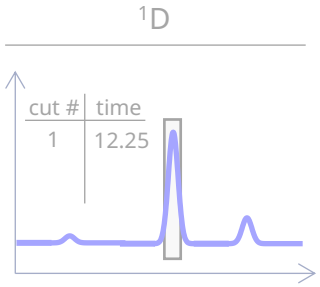

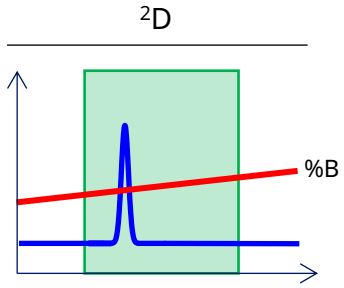

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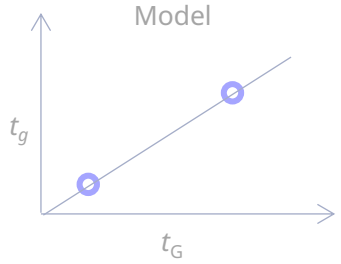
4) Evaluate retention time of main peak against the ^{2}D retention target window.

Peak in target window 2nd half of ^{2}D gradient?

No

5b) Adjust $^2\Phi_i$ after updating ^{2}D retention model using ^2t_g from Step #3.

$$\frac{t_g - t_m}{t_m} = k_{eff} = \frac{t_d}{t_m} + \frac{1}{b}\ln\left(\frac{b \cdot k_i\left(t_m - \frac{t_d}{k_i}\right)}{t_m} + 1\right)$$



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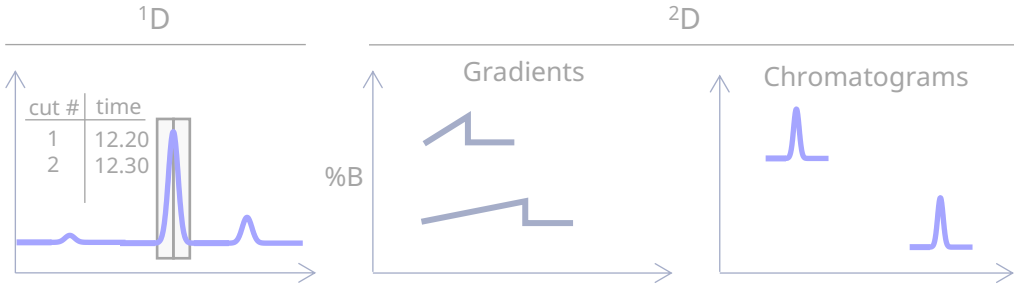

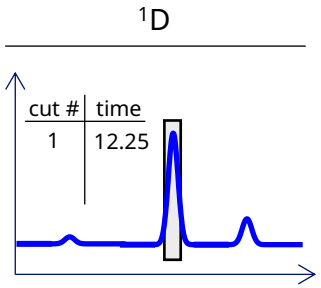

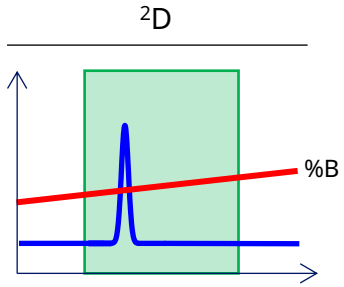

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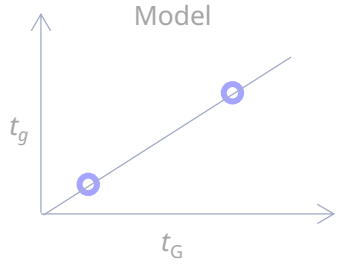

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No

5b) Adjust $^2\Phi_i$ after updating ^{2}D retention model using ^2t_g from Step #3.

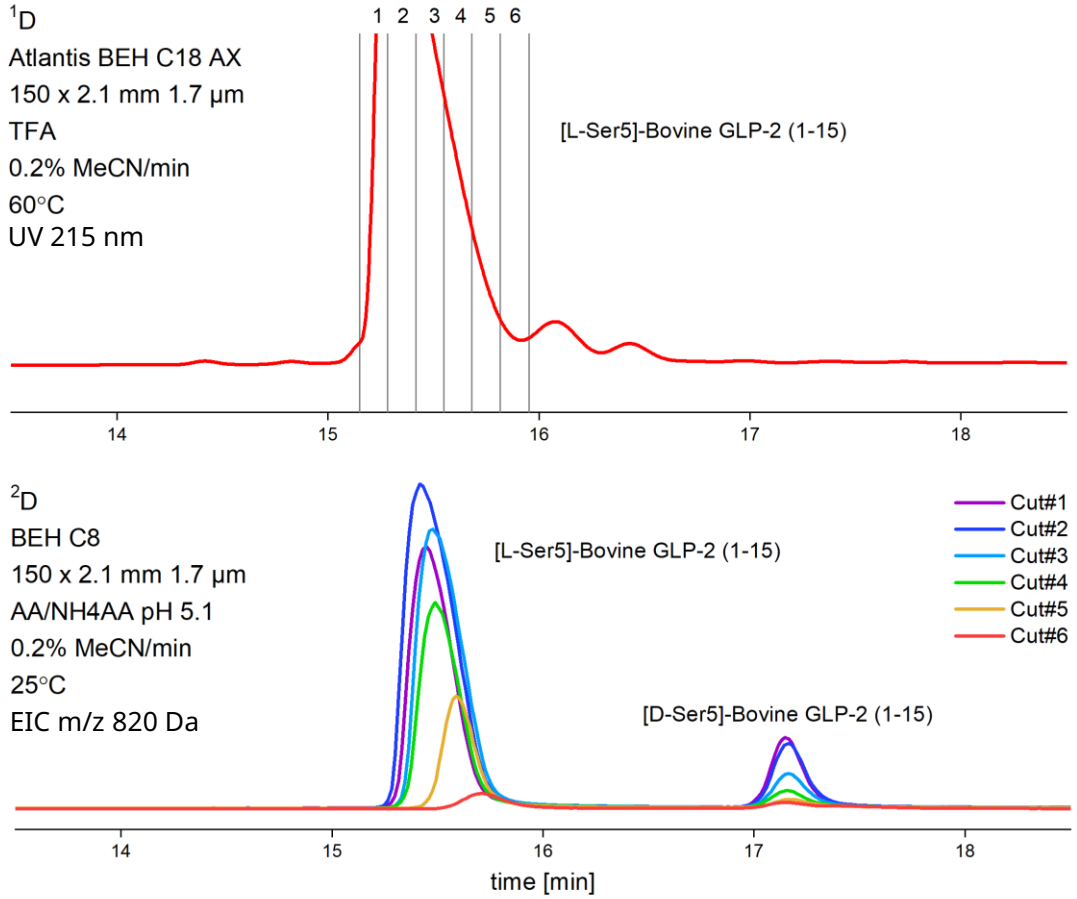
Yes

5a) Method complete and we can perform the peak purity analysis with *n* cuts over the ^{1}D main peak.

Typically in target window directly or after one update of the model

Peak purity analysis: Proof of concept

- Peak purity analysis of a purity method for GLP-2 (1-15)
- Isomer elutes directly under the main peak – racemization of Ser in position 5



Wandering first-dimension peaks in 2D-LC

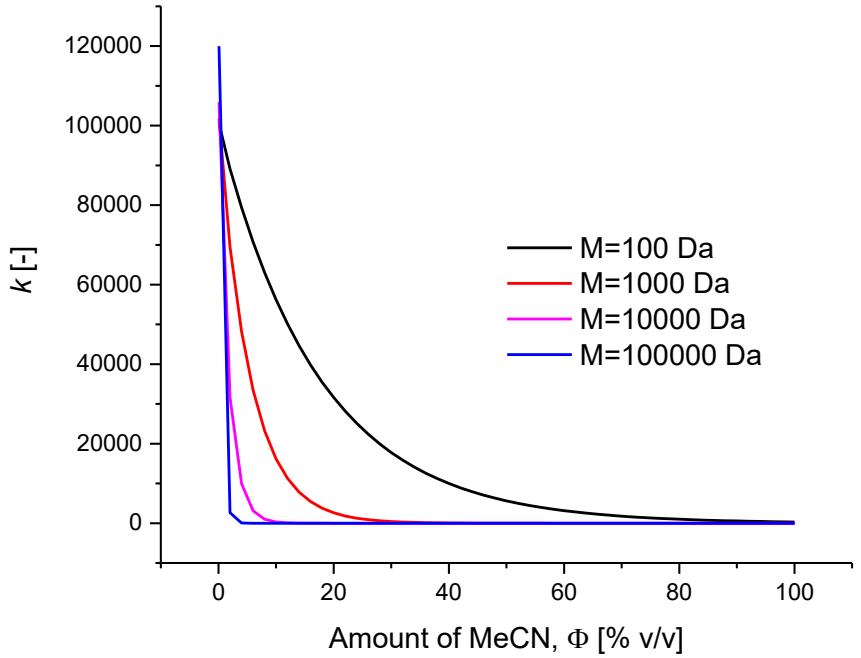
Dealing with Wandering First-Dimension Peaks in 2D-LC Separations, S. Buckenmaier, P. Petersson, LCGC North America, May 2022, Volume 40, Issue 5, pages 201–206

Wandering first-dimension peaks in 2D-LC

- Shallow peptide/protein gradients a challenging for any LC system
- Large molecules respond strongly to small changes in %B and T [10]
- Small fluctuations in %B and temperature result in wandering peaks – a moving target

$$\log k \approx \log k_0 - 0.25\sqrt{M}\Phi$$

Assuming the same $\log k_0$=5

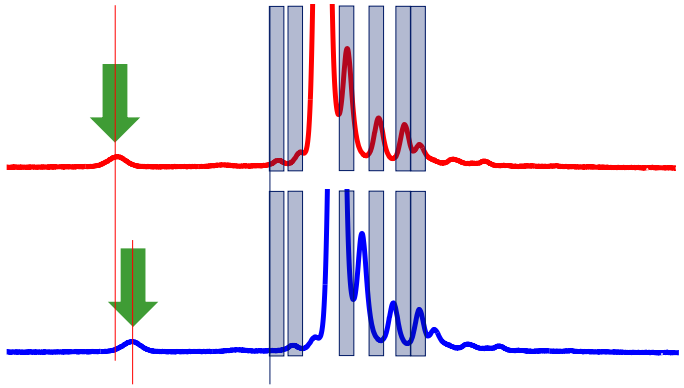


[10] L.R. Snyder, and J.W. Dolan, High performance gradient elution: The practical application of the liner-solvent strength model, John Wiley & Sons, Hoboken, New Jersey, 2007, p 230.

Wandering first-dimension peaks in 2D-LC

- Shallow peptide/protein gradients a challenging for any LC system
- Small fluctuations in %B and temperature result in a moving target

- 1st run definition of cuts
- 2nd run



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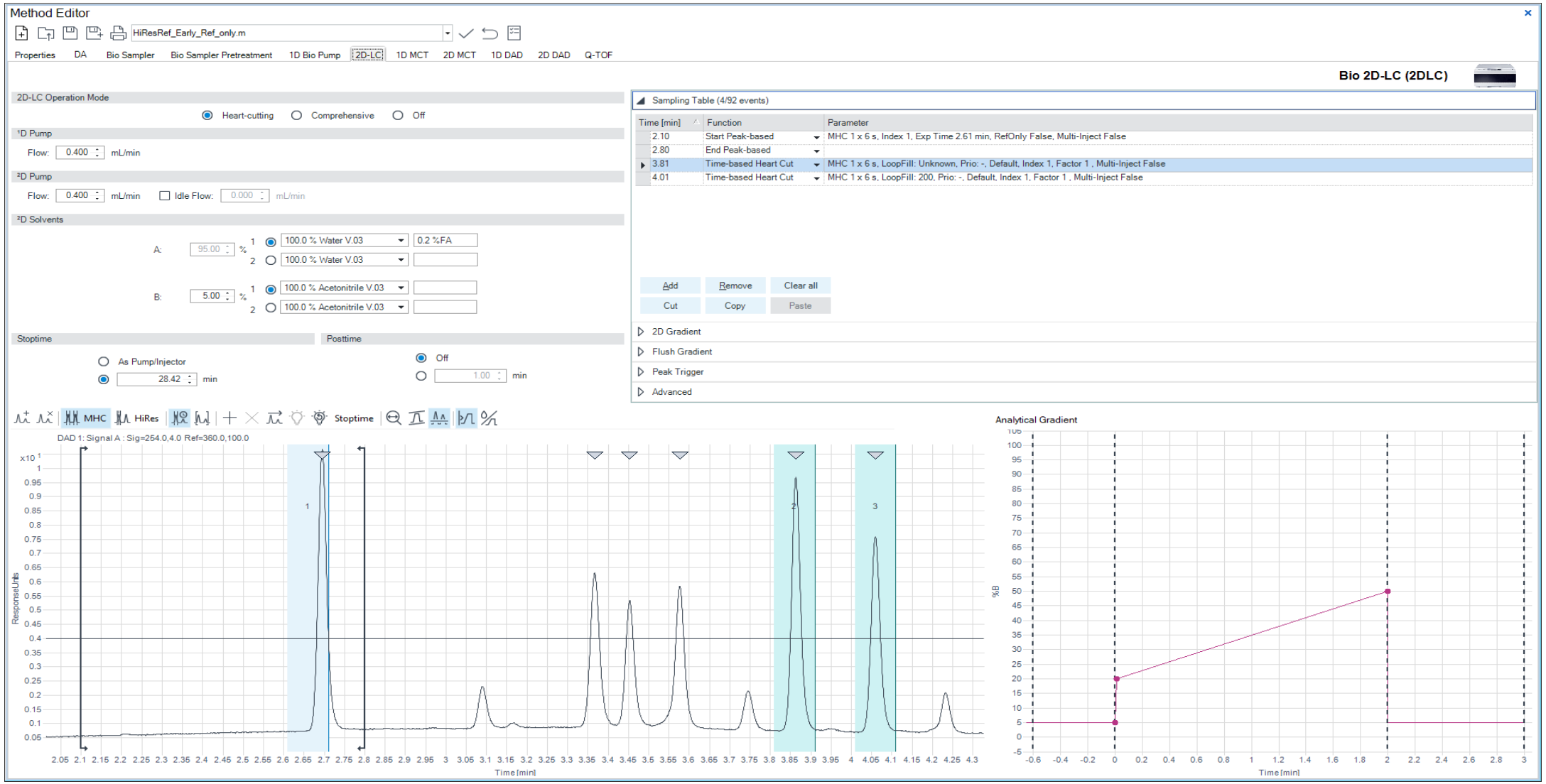
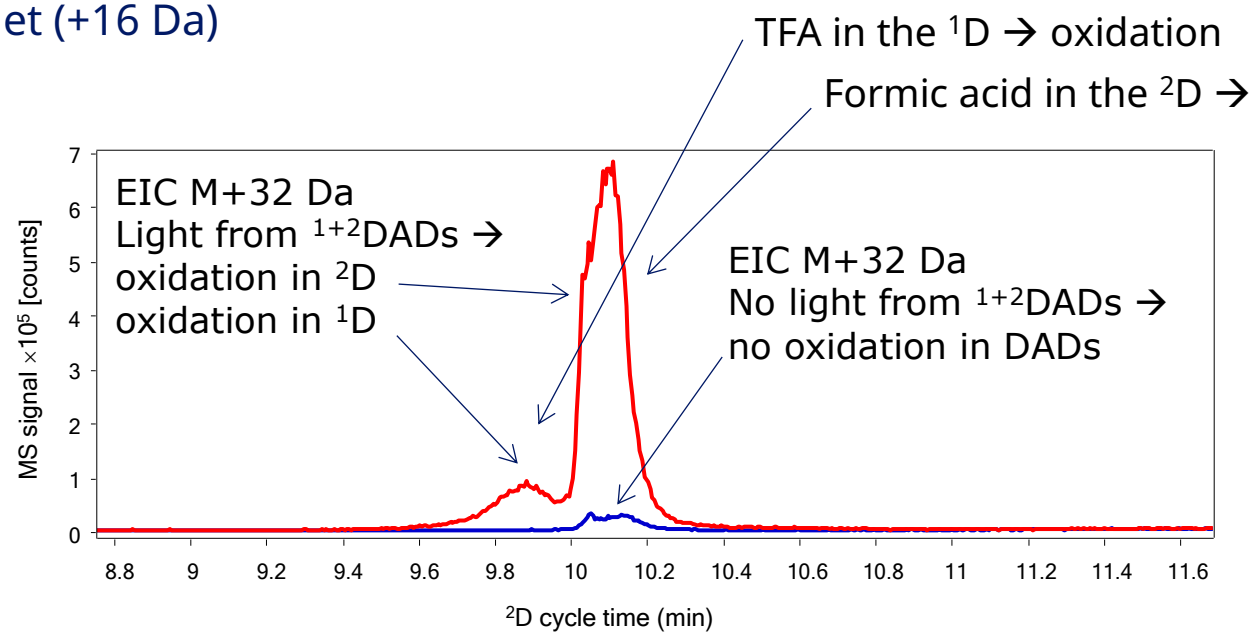
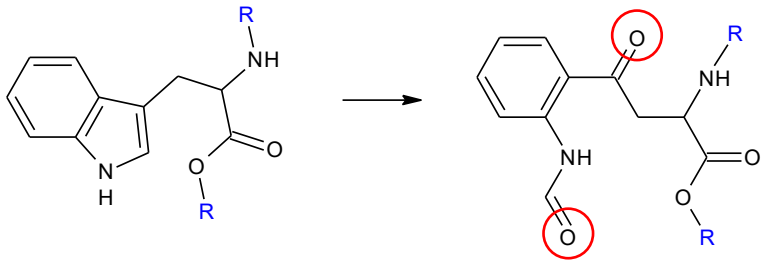


Post column photo oxidation a potential problem in 2D-LC and fraction collection

Post column photo oxidation a potential problem …

- Most of us probably have a diode array detector in front of our MS
- Photo oxidation of peptides/proteins observed at Novo Nordisk during 2D-LC-MS
  - Trp (+32 Da)
  - Met (+16 Da)



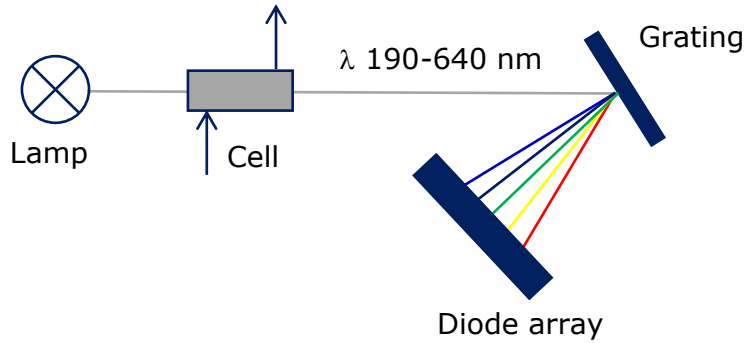


Trp

Trp +2O

Post column photo oxidation a potential problem …

- Observed for DADs from several vendors



Conclusions

- Mutiple heart-cutting 2D-LC-MS is probably the best tool available for peak purity analysis
- A neutral character C8/C18 and AA/NH_4AA pH 5 appears to be a good combination for the ^{2}D separation of isomers in peptide peak purity analysis
- Poor peak shape try 0.1% TFA (typically >10 kDa)
- Retention modelling facilitates the definition of the initial %B needed to elute the isomers in the middle of the very shallow ^{2}D gradient required for peak purity analysis
- Wandering first-dimension peaks is a problem in 2D-LC of large molecules but there is a software solution
- Post column photo oxidation a potential problem for 2D-LC-DAD but there are hardware solutions
- Salt based eluents often provide better peak shape and selectivity than MS compatible eluents like TFA (also less harmful for the environment)