How to optimize your (U)HPLC separation for optimal coupling with mass spectrometry: "do's and do-not-do's" Part - 2

About the need to separate in LC-MS, Stationary Phases for LC-MS, Column Diameter to Use, System Requirements, Chip HPLC Front-End LC-MS

Strategies for Efficient Coupling of (U)HPLC with MS

Part 2 (10:30-11:10 hrs.)

Optimize (U)HPLC Column Technology and Systems for LC-MS

- Is separation prior to MS needed?
- What column technology to use
- (U)HPLC instrumental factors
- Recommendations for sample preparation

Quote

"Chromatographic separation is not required when using MS. Extract individual m/z values, do SIM or choose precursor ions for MS/MS."

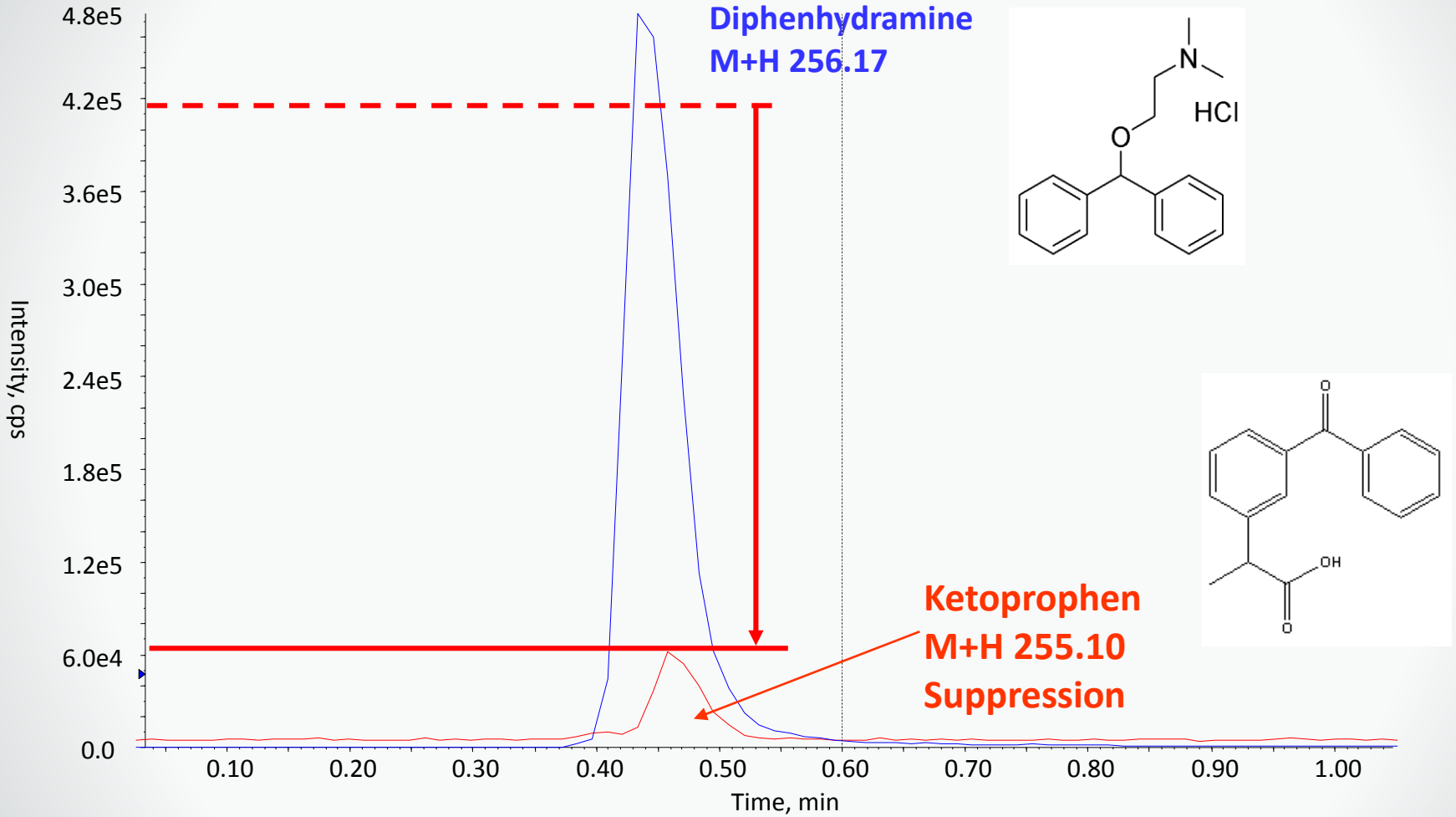
• • •

Is separation prior to MS needed?

Why is Separation Needed?

- MS will not or poorly differentiate isomeric substances
  (same MW but different structure of stereoisomers, e.g. leucine, isoleucine)
- MS will not or barely differentiate isobaric substances
  (same molecular formula but different molecules)
- Separation will reduce ion suppression/matrix effect

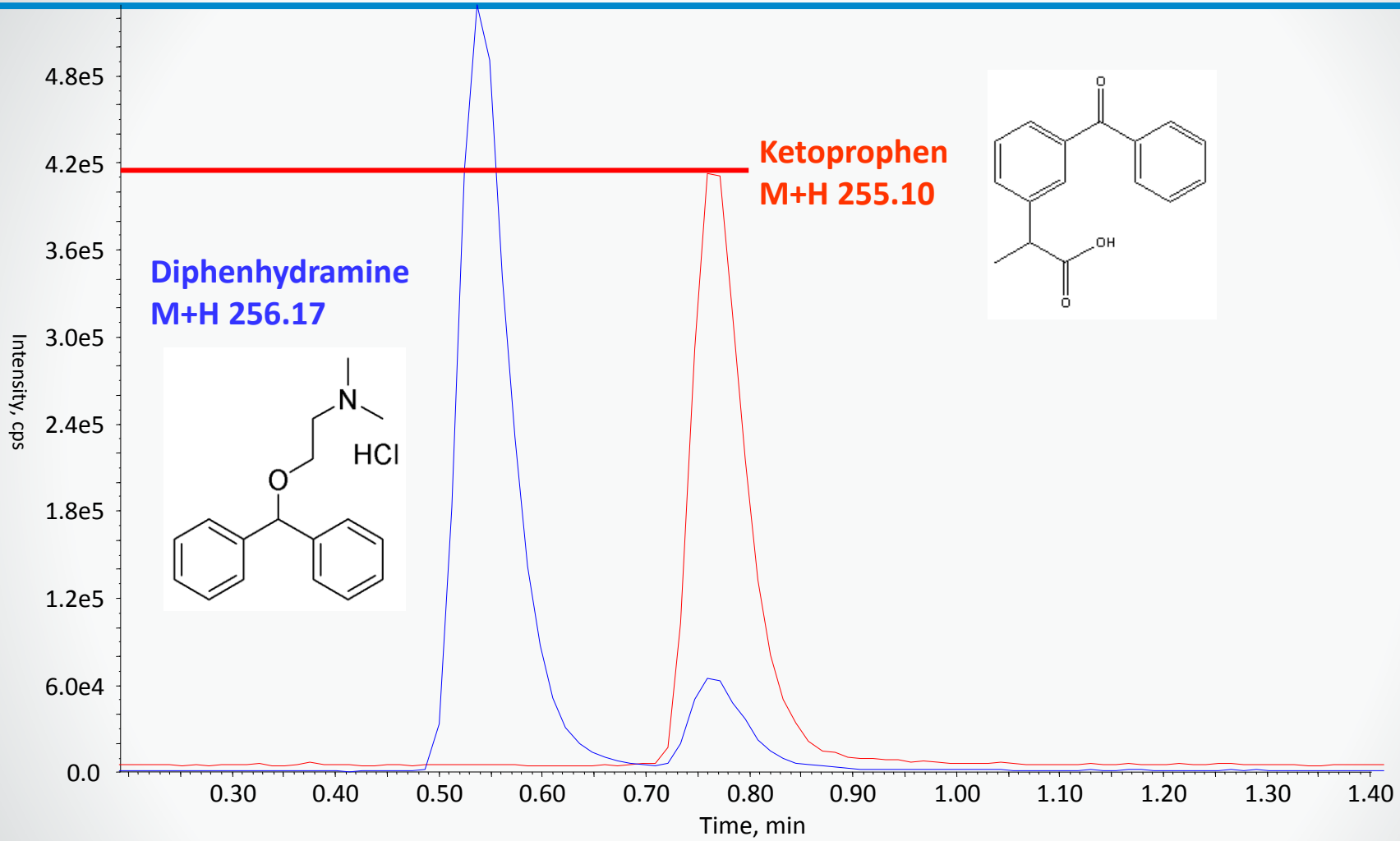
Without Separation Prior to MS Detection



5:95 Water/Methanol, 0.2 % Acetic Acid

Courtesy Udo Huber, Agilent Technologies Waldbronn

With Separation Prior to MS Detection



30:70 Water/Methanol, 0.2 % Acetic Acid

Courtesy Udo Huber, Agilent Technologies Waldbronn

Part 2 (10:30-11:10 hrs.)

Optimize (U)HPLC Column Technology and Systems for LC-MS

- Is separation prior to MS needed?
- What column technology to use?
  - Requirements?
  - What stationary phase to use?
  - What column diameter to use?
  - What particle size to use?
  - HPLC Chip column technologies for LC-MS?
- (U)HPLC instrumental factors
- Recommendations for sample preparation

Stationary Phases for LC-MS

Requirements:

- High plate count (UHPLC!)
- Low stationary phase bleed
- Quick re-equilibration
- No compromise peak shape while using MS compliant mobile phases in the separation

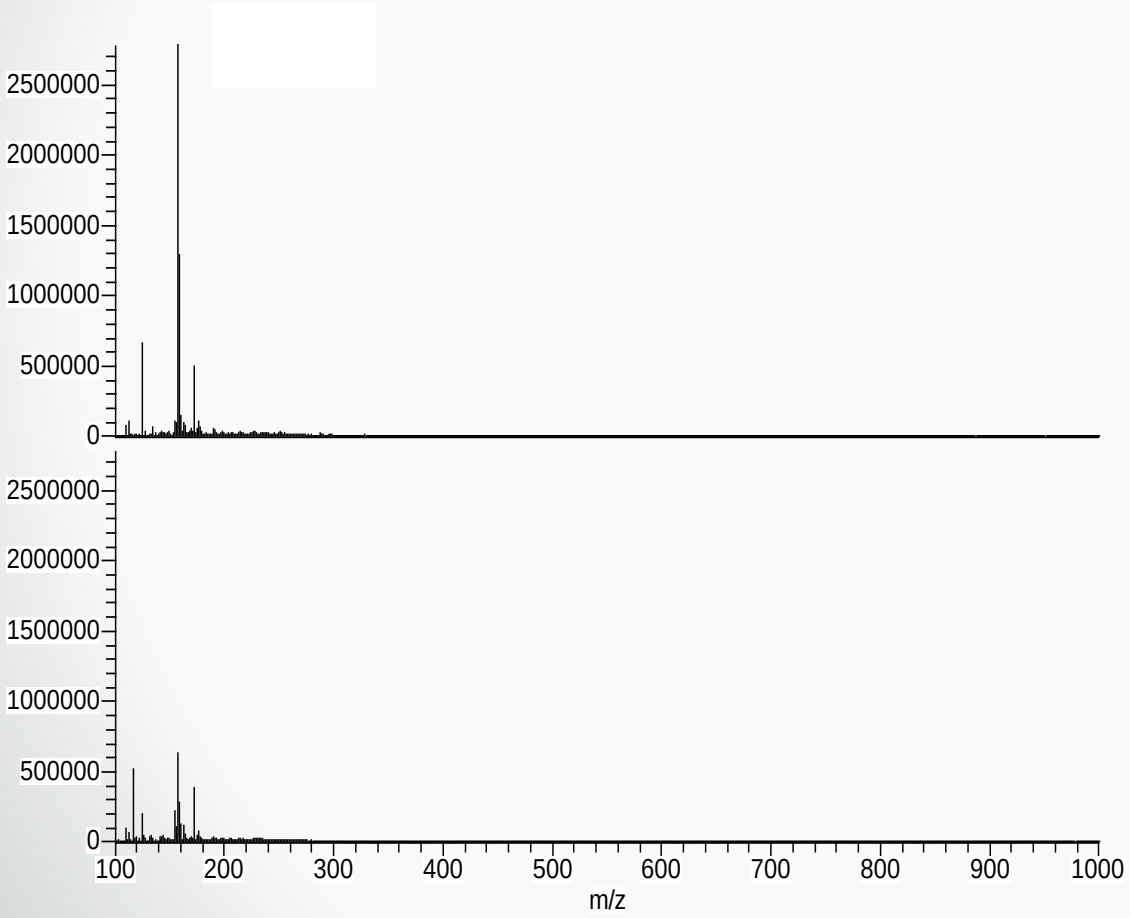
Stationary Phases for LC-MS

Low Bleed RP-MS Columns*

Columns: Accucore RP-MS 2.6□m, 100x2.1mm Mobile phase:
A – H2O + 0.1%formic acid, B – ACN + 0.1%formic acid

Gradient: Time (min) 5 -`100 %B

Blank run is acquired with no column in the system



There is no evidence of column bleed on the Accucore RP-MS

Slide courtesy of Luisa Pereira, ThermoScientific

TFA Containing Solvents for Separation of Tryptic Peptides

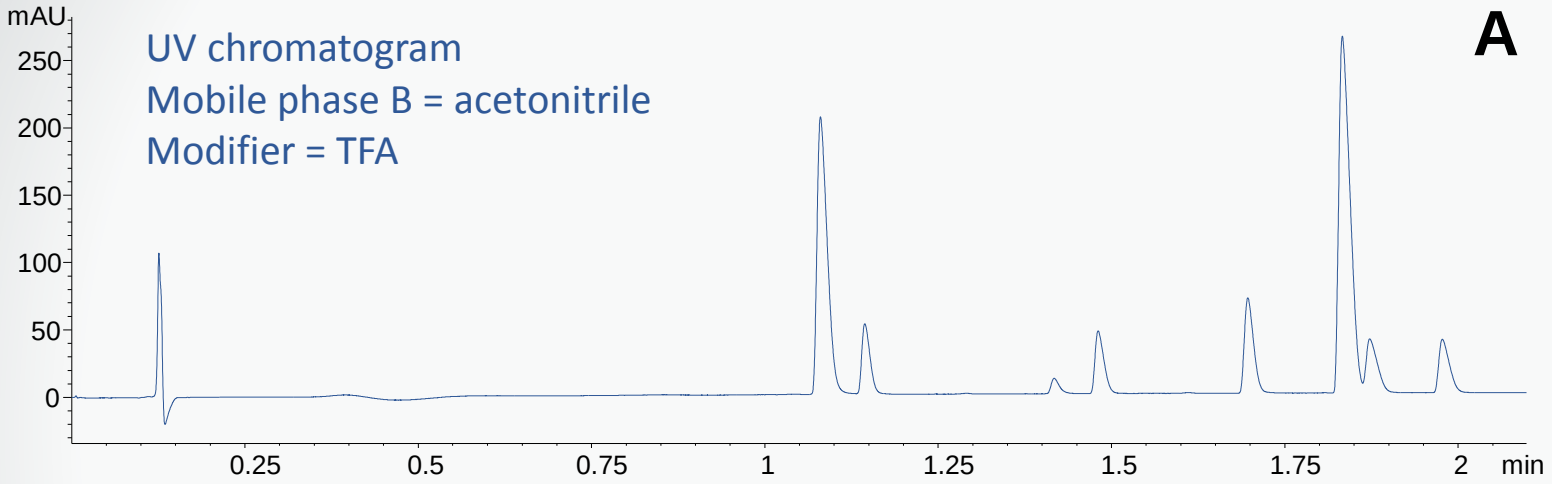
- Ideally suited for RP LC since trypsin cleaves proteins at lysine or arginine leaving a basic peptide. TFA forms an ion-pair with basic sites of the peptides.
- The low pH renders the carboxylic sites neutral which increase retention
- The neutral ion-pair separates well on RP column
- TFA neutralizes “hot” sites on the silica matrix surface
- TFA is volatile

but……

Avoid Sensitivity Loss with TFA Containing Eluents

- Post-column addition of a“TFA-fix” (propionic acid)
  - No compromise on chromatography
  - Additional hardware required (cost, reliability, mixing efficiency)
- Do not use TFA for pH adjustment!

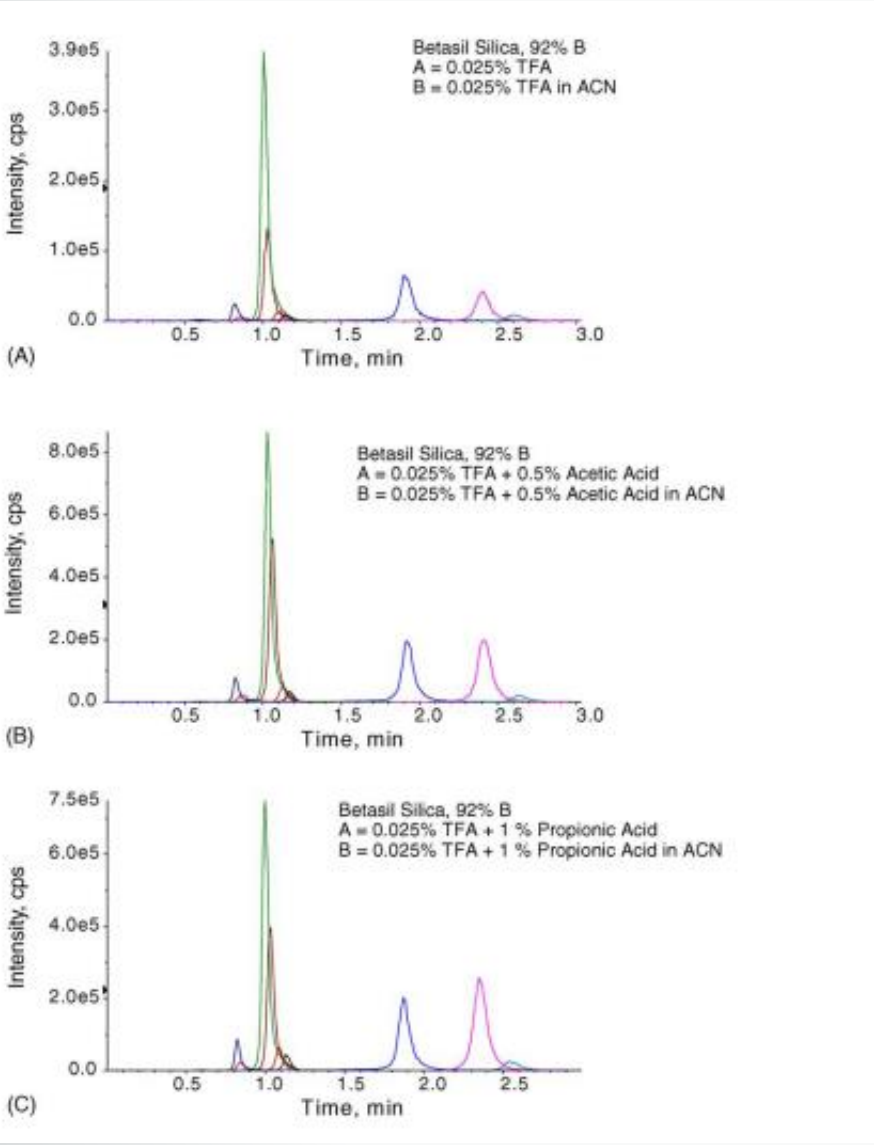
HPLC-UV with 1% Formic acid/0.2% TFA*



Avoid Sensitivity Loss with TFA Containing Eluents

- Post-column addition of a“TFA-fix” (propionic acid)
  - No compromise on chromatography
  - Additional hardware required (cost, reliability, mixing efficiency)
- Do not use TFA for pH adjustment!
- Use mixtures of TFA and formic acid

Avoid Sensitivity Loss with TFA Containing Eluents*



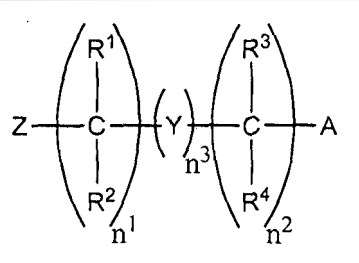
*W.Z. Shou, W Naidong J. Chrom. B, 825 (2005) 186–192

Avoid Sensitivity Loss with TFA Containing Eluents

- Post-column addition of a“TFA-fix” (propionic acid)
  - No compromise on chromatography
  - Additional hardware required (cost, reliability, mixing efficiency)
- Do not use TFA for pH adjustment
- Use dedicated stationary phases that have low silanophilic interactions allowing good peptide separations without compromising chromatograph by using formic acid etc.
  - Waters CSH130 and Cortecs series
  - Thermo BioBasic columns
  - Agilent AdvanceBio Peptide Mapping columns
  - Dionex Acclaim Pepmap

Optimal Stat. Phase for LC-MS of Basic Solutes

- Charged Surface Hybrid (CSH) Technology*



Z = silica backbone, Y = polar and/or chargeable group, A = functional group govern retention and selectivity

Two silica backbones:

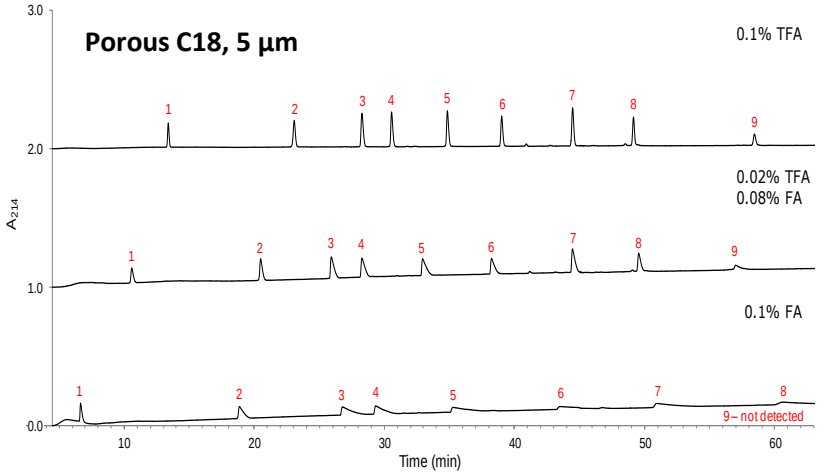
- Totally porous: BEH-CSH130, 1.7, 3.5 and 5 µm
- Superficially porous: CORTECS-CSH, 1.7 and 2.7 µm

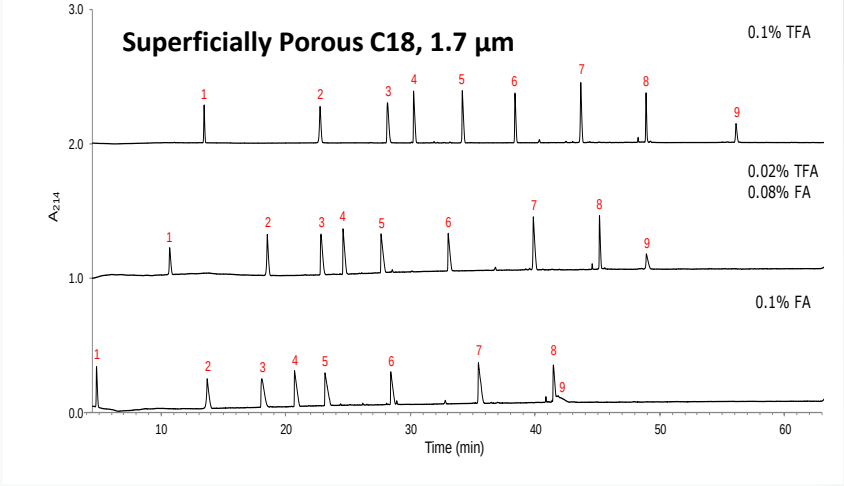
- Reversed phase and HILIC

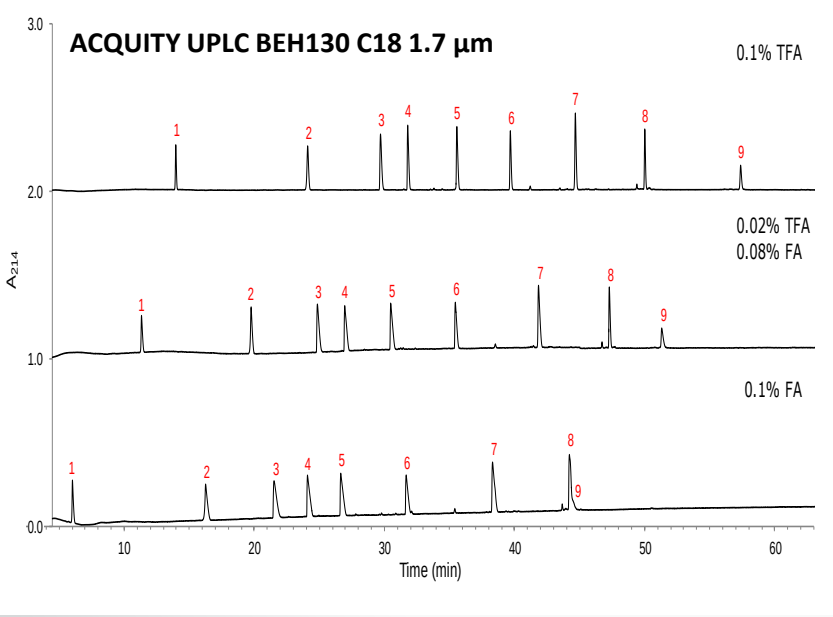
*K.D. Wyndham et al., Patent Application WO 2013173494 A4
& M.A. Lauber et al., Anal. Chem., 85, 6936 (2013)

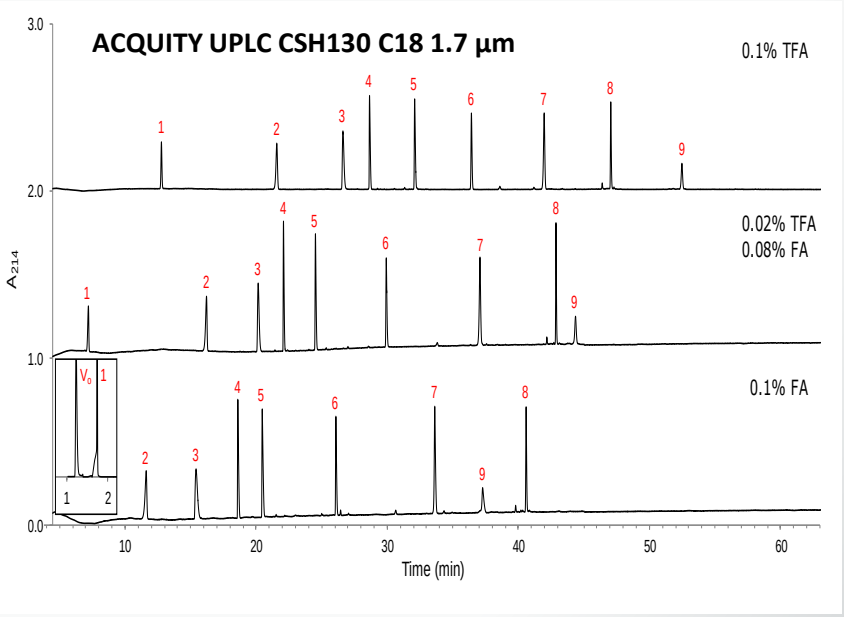
Charged Surface Hybrid Phases for LC-MS

Sample: Waters MassPREP Peptide Mixture

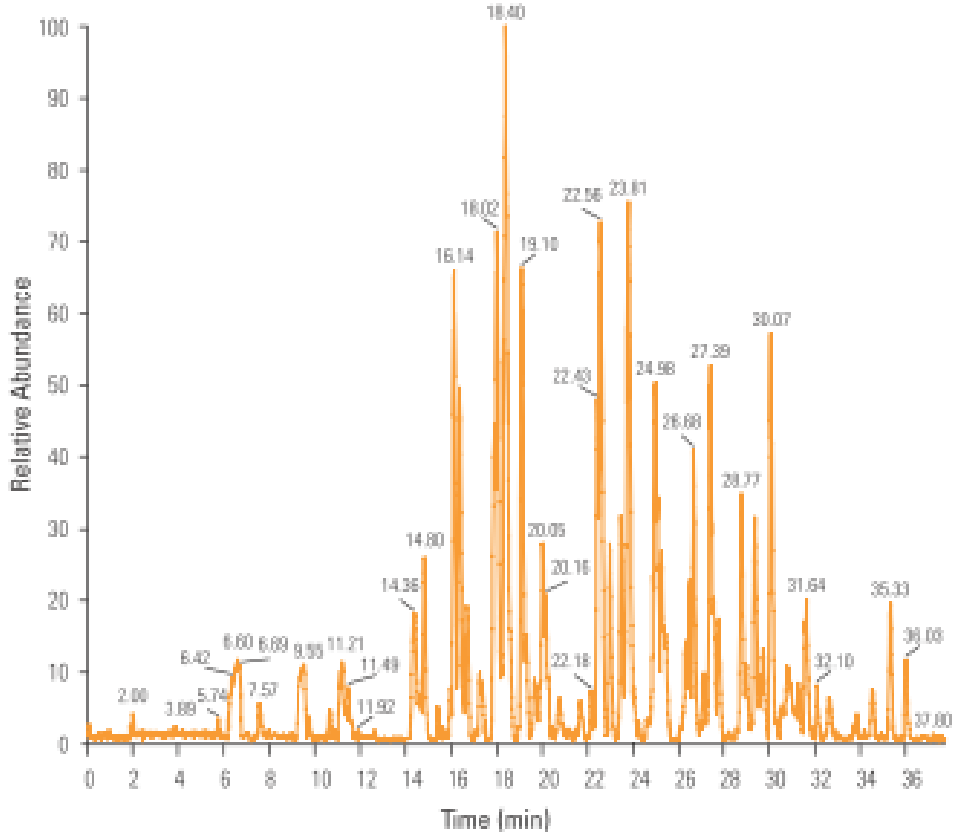








Accucore RP-MS



Mobile phase A:	0.1 % formic acid in water
Mobile phase B:	90 % acetonitrile in water
Gradient:	4-40 % B over 30 mins
	40-95 % B over 2 mins
	95 % B hold for 2 mins
Flow:	300 nL/min
Temperature:	not controlled
Column:	Accucore 150-C18, 2.6 µm, 150 x 75 µm
Backpressure:	198 bar (100 % A)
Injection:	Direct on-column loading of 1 µL of BSA digest, 50 fmol/µL in water + 0.1 % formic acid
Detection:	Thermo Scientific LTQ Orbitrap XL Mass Spectrometer coupled with a Proxeon Nano Spray Flex Ion Source

Hydrophilic Interaction Liquid Chromatography*

- In HILIC mobile phases acetonitrile rich
- HILIC well suited for polar metabolites and peptides which are lowly retained in RP chromatography**
- Excellent 1st dimension in two-dimensional chromatography

*P. Jandera, Anal. Chim. Acta 692 (2011) 1–25
**A. Heck et al. Anal. Bio-anal. Chem. (2008) 391:151 – 159

What Column Diameter to Use?

$$c_{i,max} = \frac{m_i}{\sqrt{2\pi}\sigma_{V_{i,tot}}} = \frac{c_{i,0}V_{inj.}}{\sqrt{2\pi}\sigma_{V_{i,tot}}}$$

$$\sigma^2_{V_{i,tot}} = \sigma^2_{V_i} + \sigma^2_{V_{ext}}$$

$c_{i,max}$	Concentration of solute *i* at peak maximum
m_i	Mass of the injected solute *i*
$V_{inj.}$	Injection volume
$\sigma_{V_{i,tot}}$	Total peak width of solute *i* in volume units
σ_{V_i}	Peak width of i in volume units on column
$\sigma_{V_{ext}}$	External band spreading

*What Column Diameter to Use?

With $\sigma_{V_i}^2 = \frac{V_{R_i}^2}{N_i} = \frac{\varepsilon_T V_0^2 (1 + k_i)}{N_i}$ the previous equation results in $c_{i,\max} \propto \frac{m_i}{d_c^2} N_i$

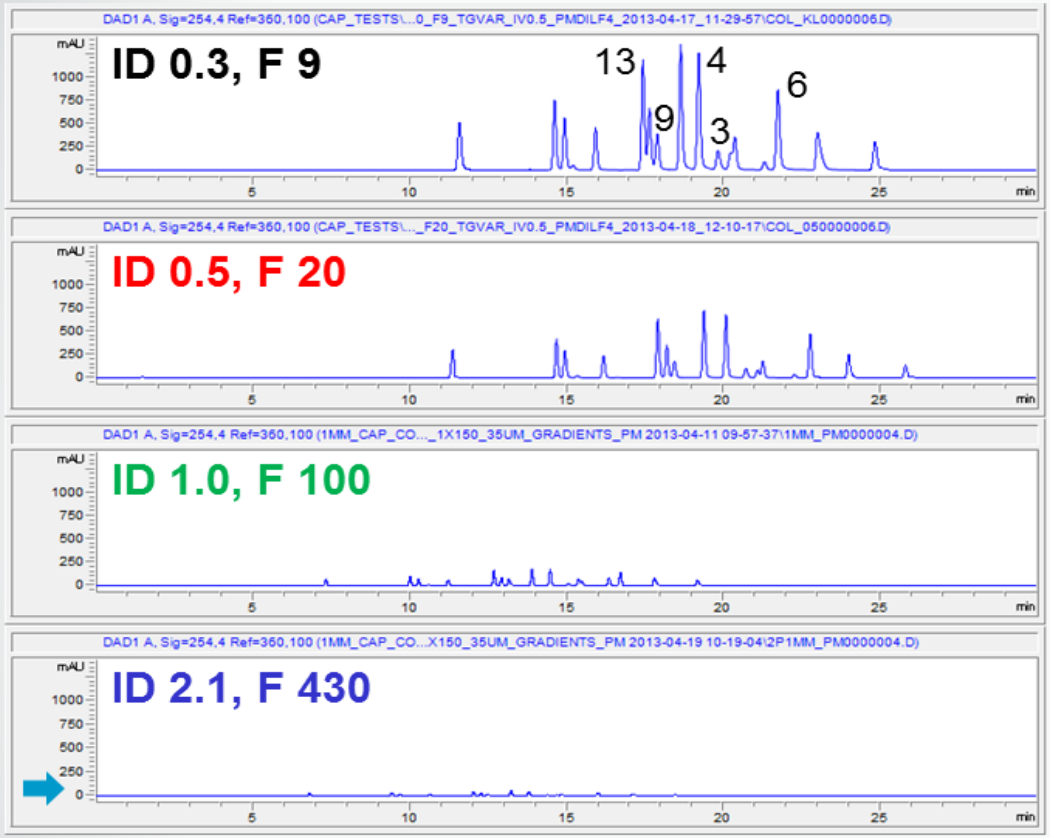
V_{R_i}	Retention volume solute *i*
N_i	Plate number for solute *i*
ε_T	Total porosity
V_0	Volume of empty column
k_i	Retention factor for solute *i*

In case a concentration sensitive detector is used:
when one reduces the column diameter but keeps the amount of injected analyte constant, detector response (here peak height) increases with the square of the column diameter ratio, while assuming the plate number is not deteriorated by external bandspreading and mass overload of the column.

*see also slides 18-21 in part 1

Influence of Column Diameter

– Conc. Sensitive Detector



Mobile phase: Water/ACN, 0.1% FA
gradients from 5 – 90 %B in 15’
Inj. vol. 0.5 µL (constant amount)

Peak height increase

**Slide courtesy Stephan Buckenmaier,
Agilent Technologies, Waldbronn**

Influence of Column Diameter

– MS response with Agilent Jetstream IF and Ion Funnel

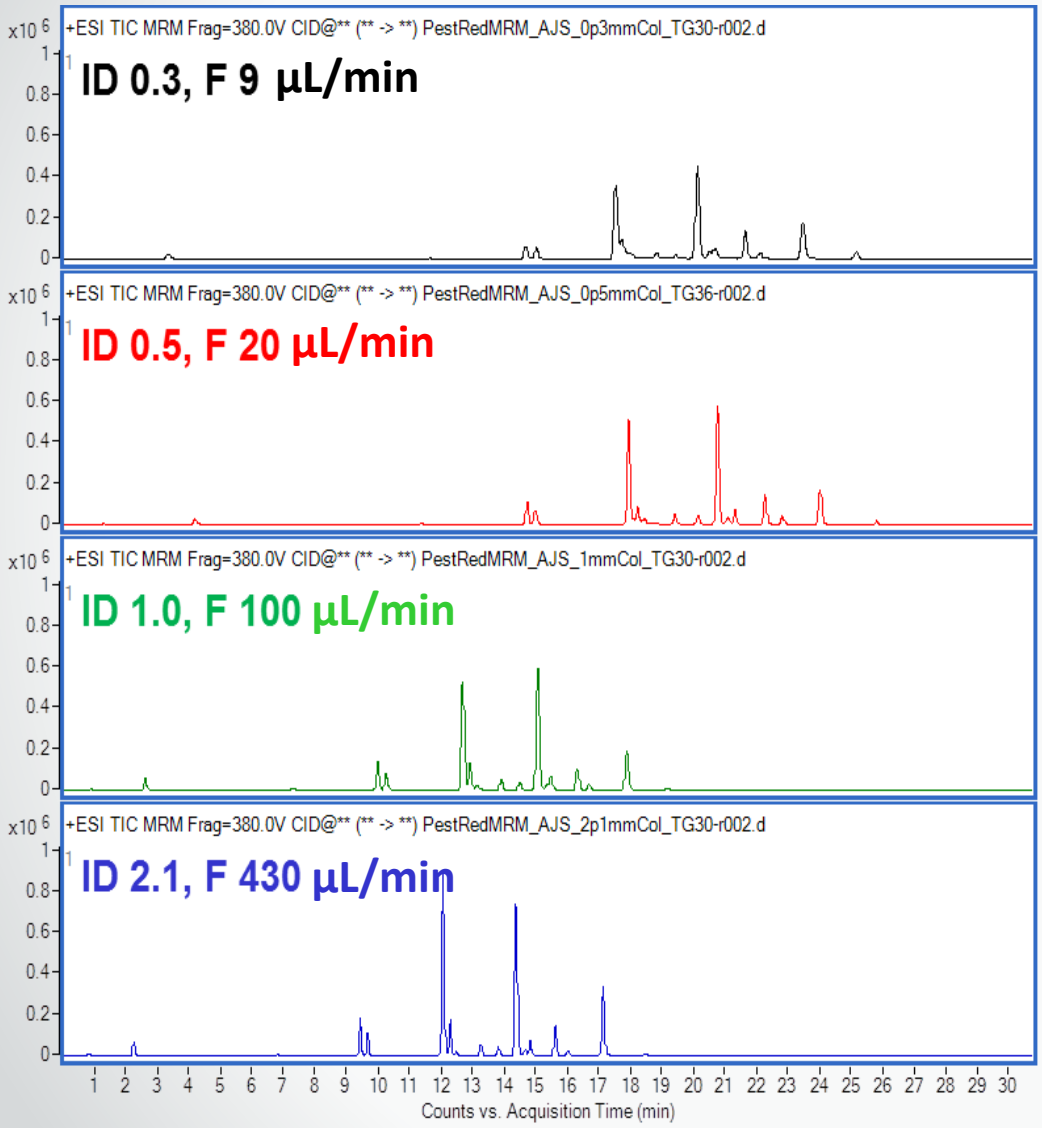


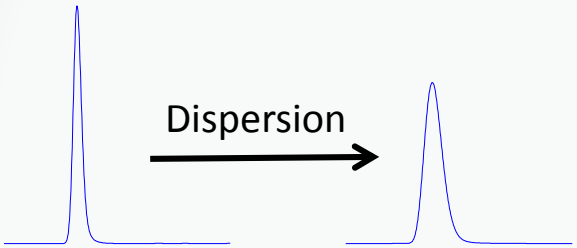
Table-2 Cpd	*H*-ratio *(0.3/2.1)*	*A*-ratio *(0.3/2.1)*
#3	0.6	1.0
#4	0.4	0.8
#6	1.5	2.7
#9	0.6	1.0
#13	0.4	0.7

Advances in ionization and sampling efficiency peak abundances allows ESI to act as a mass sensitive detector!!

Slide courtesy Stephan Buckenmaier, Agilent Technologies, Waldbronn

HPLC Instrumental Factors : Extra Column Dispersion

- *"Dispersion is the sample bandspreading or dilution which occurs in connecting tubing, sample valves, flow cells and in column end-fittings."*



Peak height: Reduced sensitivity

Peak width: Resolution loss

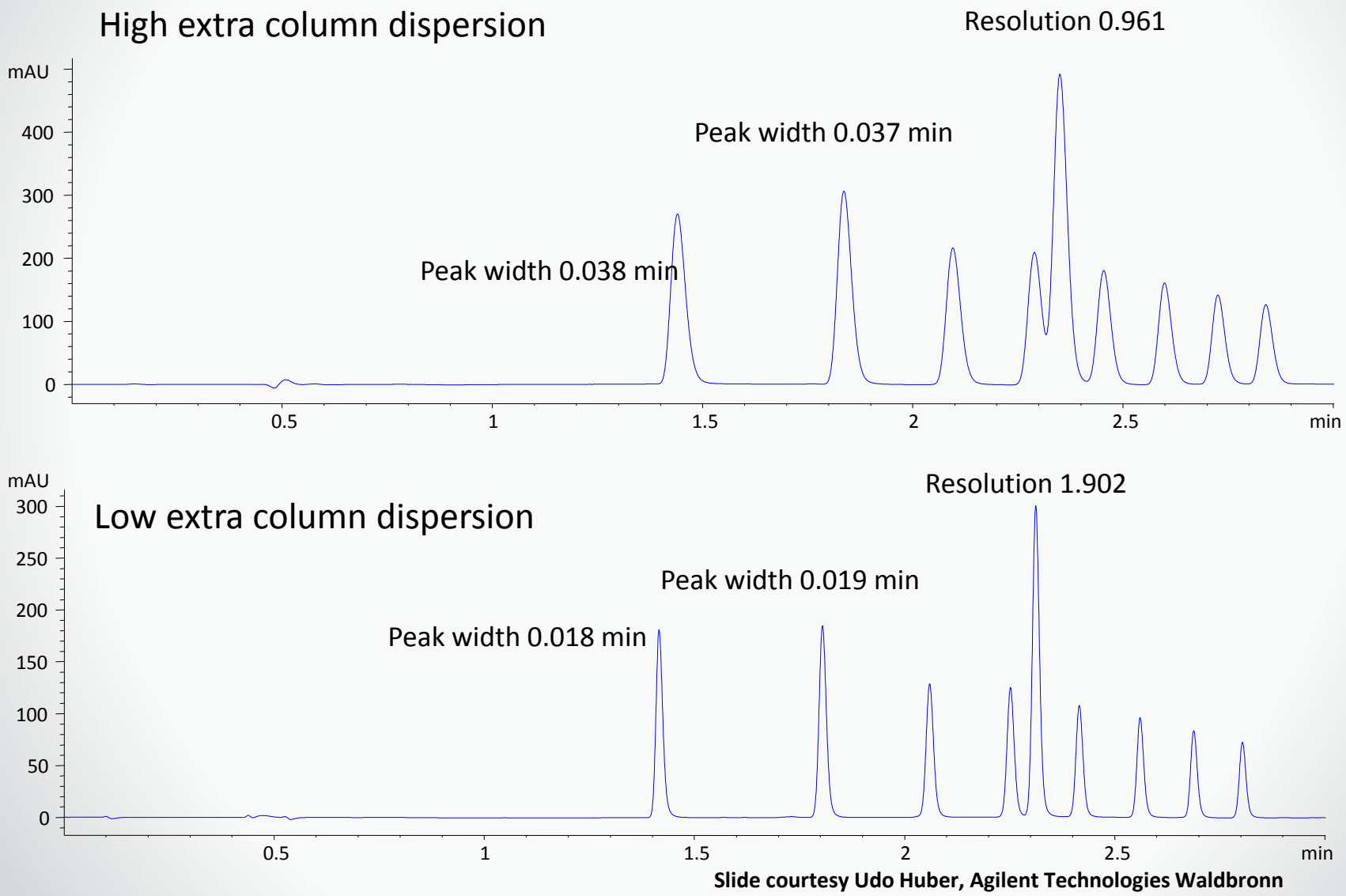
- Connection capillaries (I.D. Length)

$$\sigma_v^2 = \frac{\pi \cdot d^4 \cdot F \cdot L}{96 \cdot D_m}$$

Aris-Taylor Equation

Slide Courtesy Udo Huber, Agilent Technologies Waldbronn

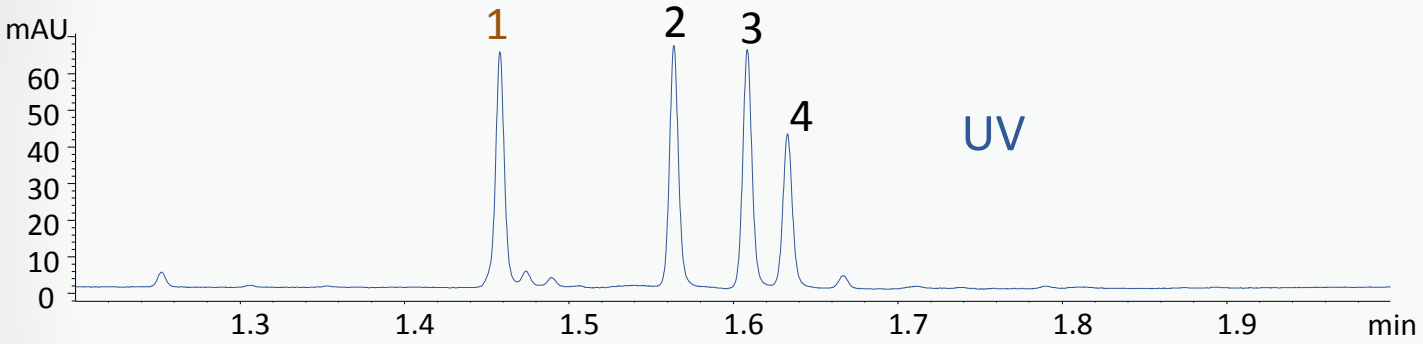
HPLC Instrumental Factors : Extra Column Dispersion

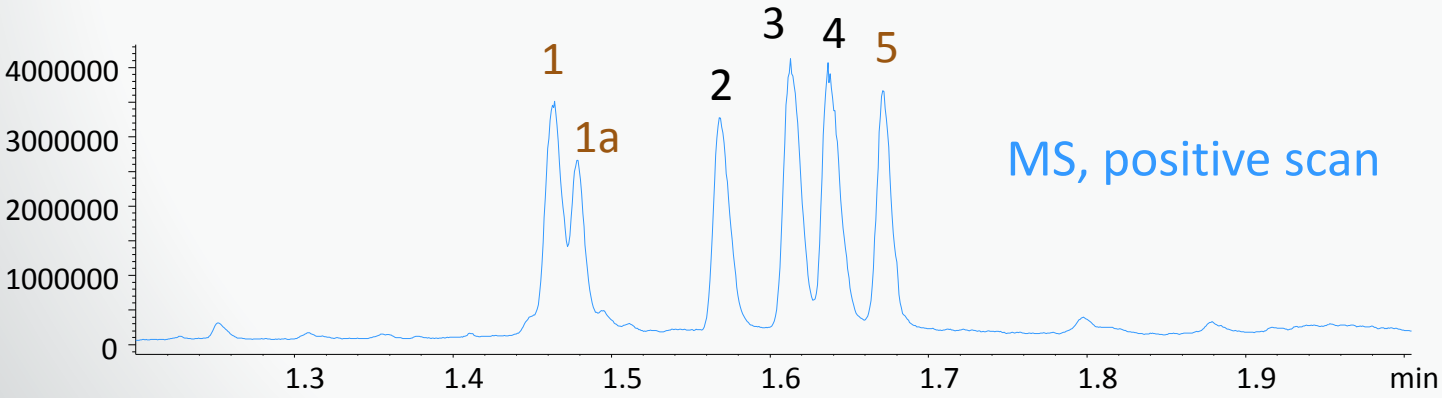


Slide courtesy Udo Huber, Agilent Technologies Waldbronn

Connection HPLC with MS

- Short capillaries with low internal diameter (mind pressure drop)
- Avoid unnecessary unions
- Matching connections





Slide courtesy Udo Huber, Agilent Technologies Waldbronn

Columns for NanoESI/MS

$$d_c = \sqrt{\frac{4.F}{\varepsilon_T . \pi . u_0}}$$

Nano-electrospray MS mandates flow rates 50 – 500 nL/min.
For (U)HPLC to work properly the solvent has to move with a velocity of 1-10 mm/s

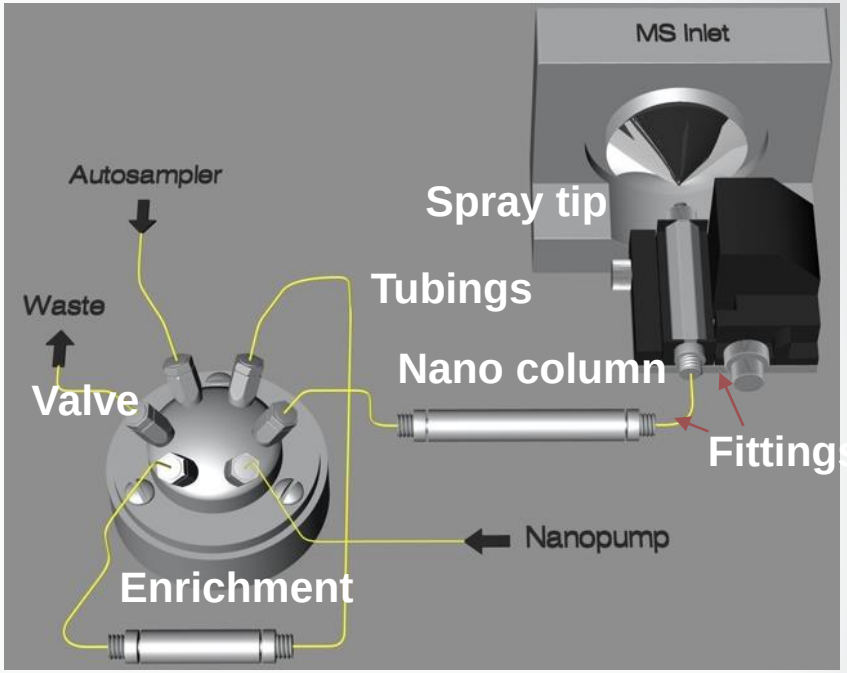
Column I.D. must be between 0.05 and 0.15 mm

Sensitivity of NanoESI/MS increases dramatically at flow rates <50 nL/min

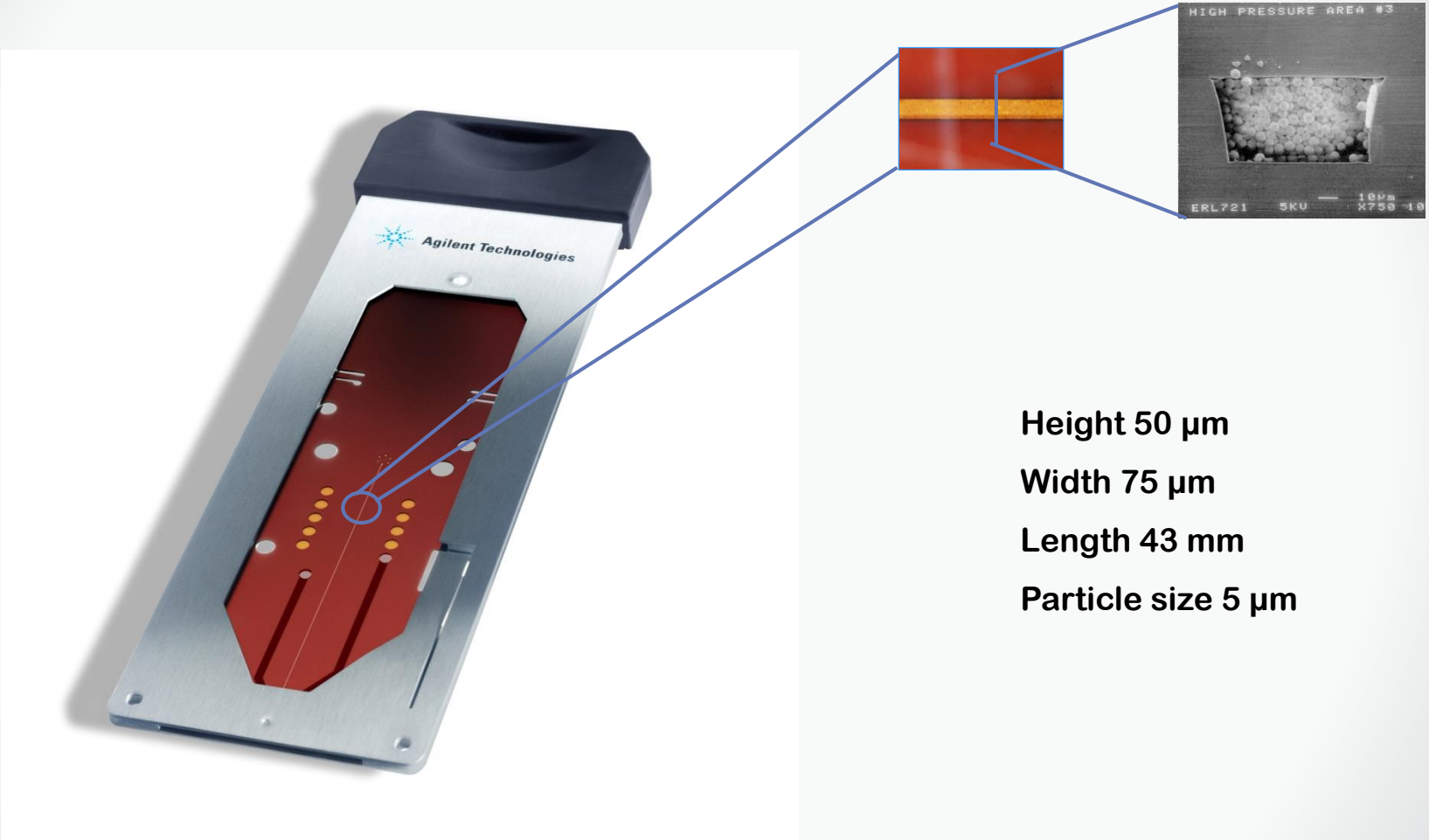
Proteomics research
➔ Ultra small samples
➔ Ultra high sensitivity mandated

Typical Set-up of Nanoflow HPLC Electro-Spray Ionization MS System

- Sensitivity
  - 75µm ID analytical column
- Challenging to Set-up & Maintain
  - Multiple Parts
  - Possible leaks, misalignments
- Robustness & Ease-of-use
  - Clogging of spray needle
  - After part replacement system can take hours to stabilize
- Chromatographic Fidelity
  - Rel. large extra column volume leads to band broadening
  - Limited to peptide separation



Agilent HPLC-Chip/MS



Height 50 µm

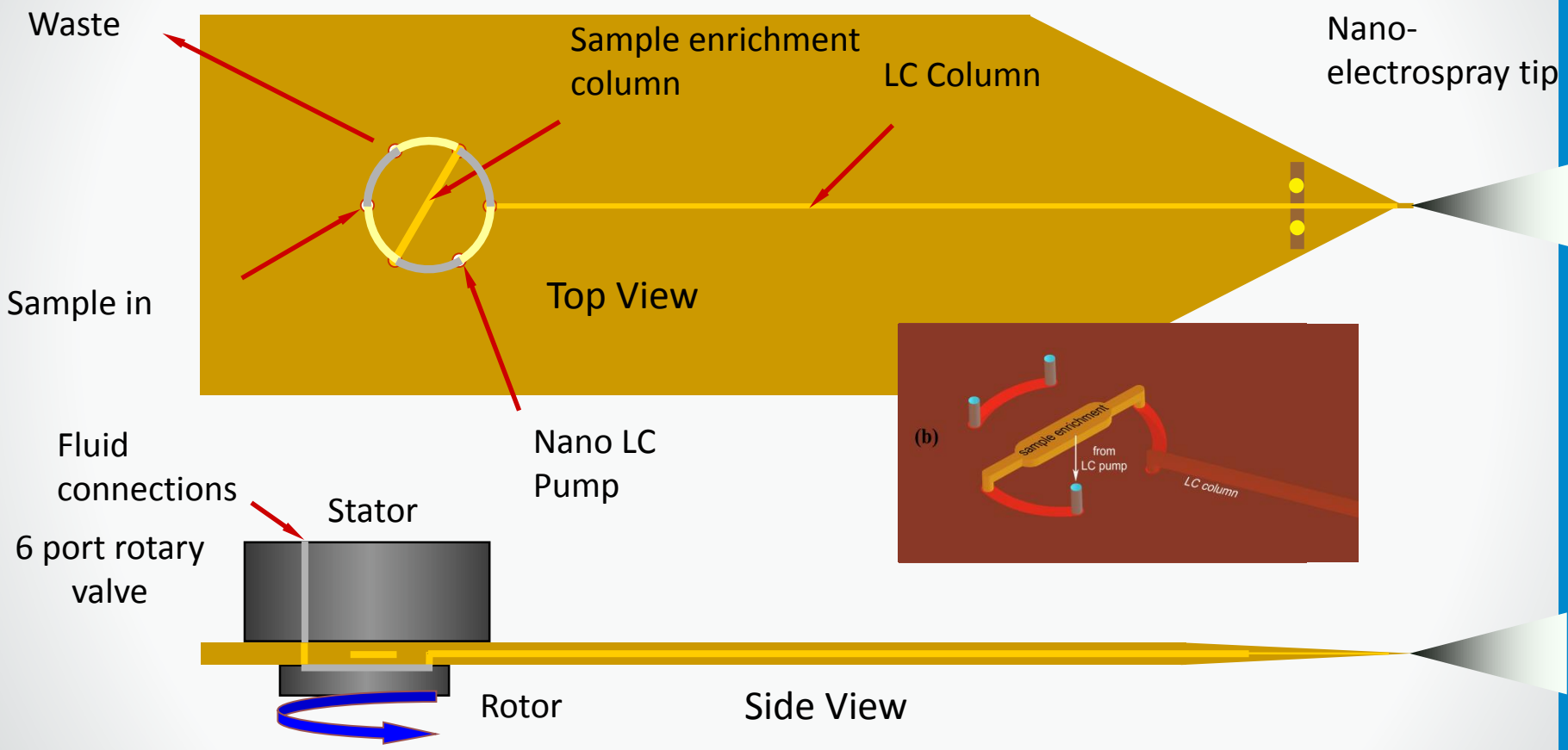
Width 75 µm

Length 43 mm

Particle size 5 µm

Agilent HPLC-Chip/MS:

Sample Enrichment, RP HPLC Separation, Electrospray



cHiPLC® System – Eksigent

A platform for using chip-based nanoLC columns and trap columns

Addresses two problem areas of nanoLC in proteomics

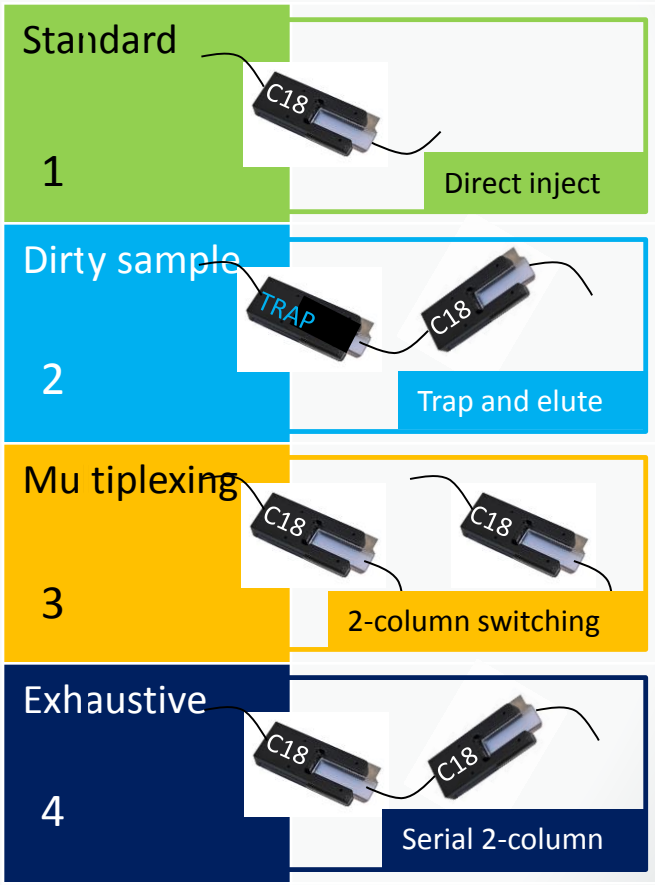
1. Connecting traps and columns without introducing dead-volume
2. Reproducibility of nanoLC columns

Courtesy Remco van Soest, Eksigent

Expert results for non-experts - cHiPLC® system

Four workflow modes

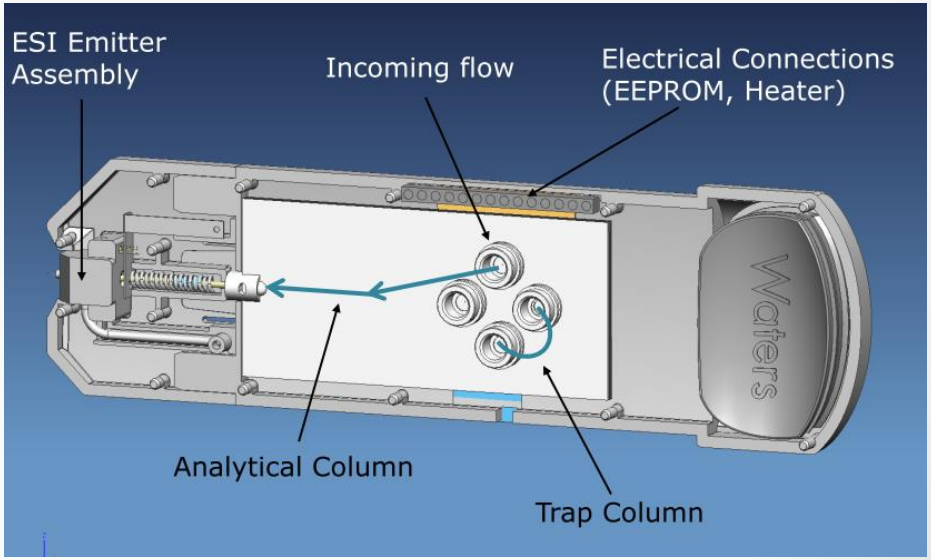
- Change the chips – change the workflow
- Combine traps and columns in several ways to carry out very different experiments
- Four workflows currently supported:
  - Direct injection - standard
  - Trap and Elute – for dirty samples
  - Dual column multiplexing – for throughput
  - Serial 2-column – for higher capacity with standard products



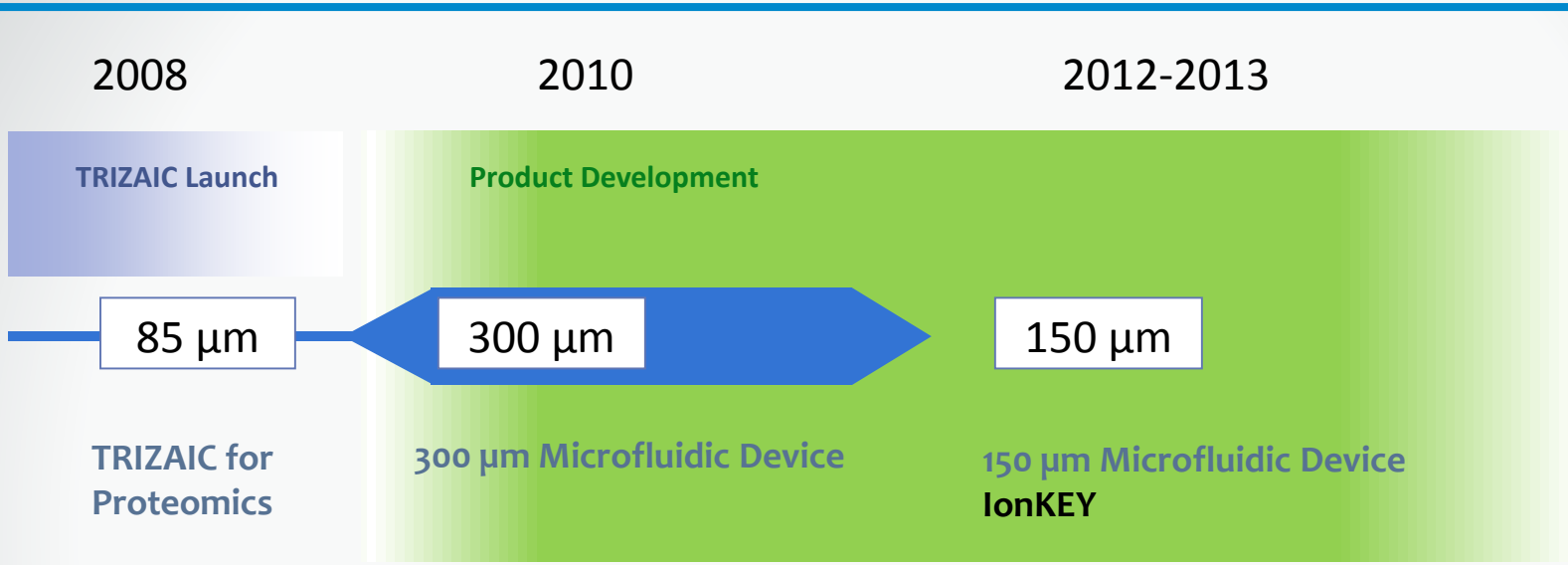
Courtesy Remco van Soest, Eksigent

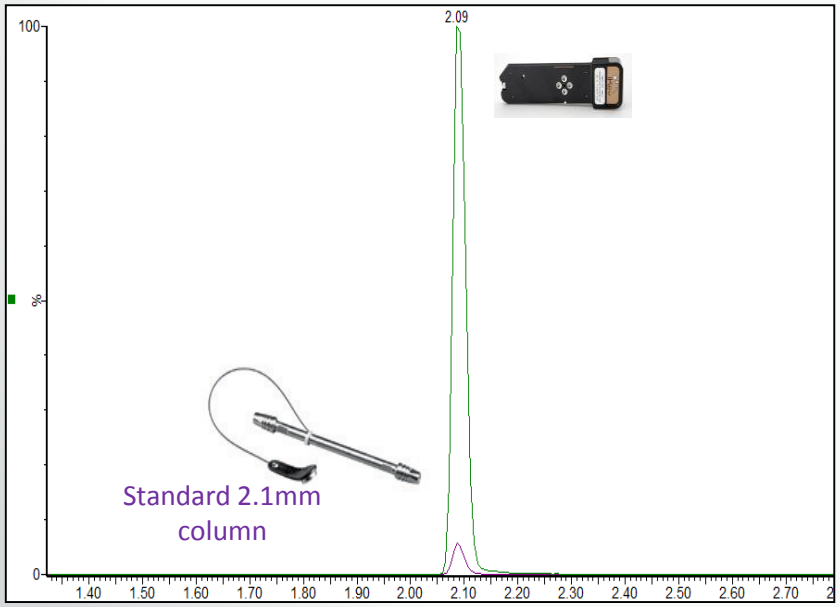
TRIZAIC UPLC System with nanoTile Technology

- Ceramic chip with 85 µm i.d.
- High pressure range allowing the use of sub-2-µm particles
- Trapping column for pre-concentration and/or salt removal
- Separation column can be thermostated
- Exchangeable sprayer tip



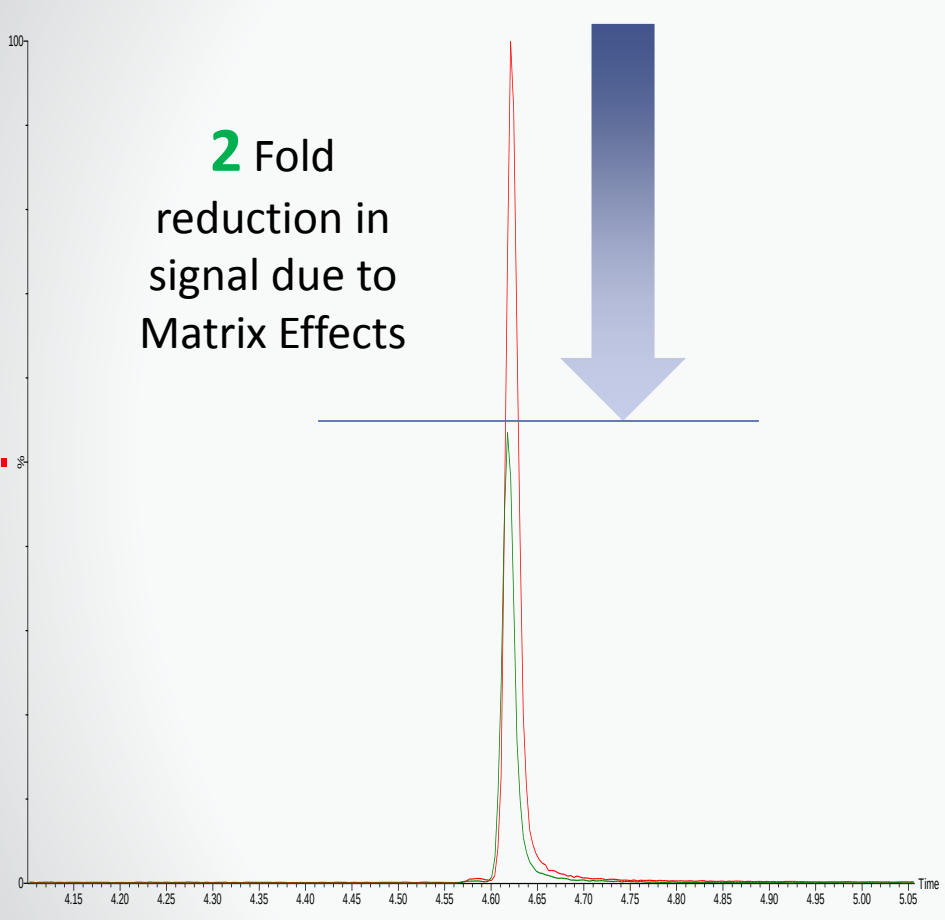
Waters HPLC Chip Technology Evolution (reborn)





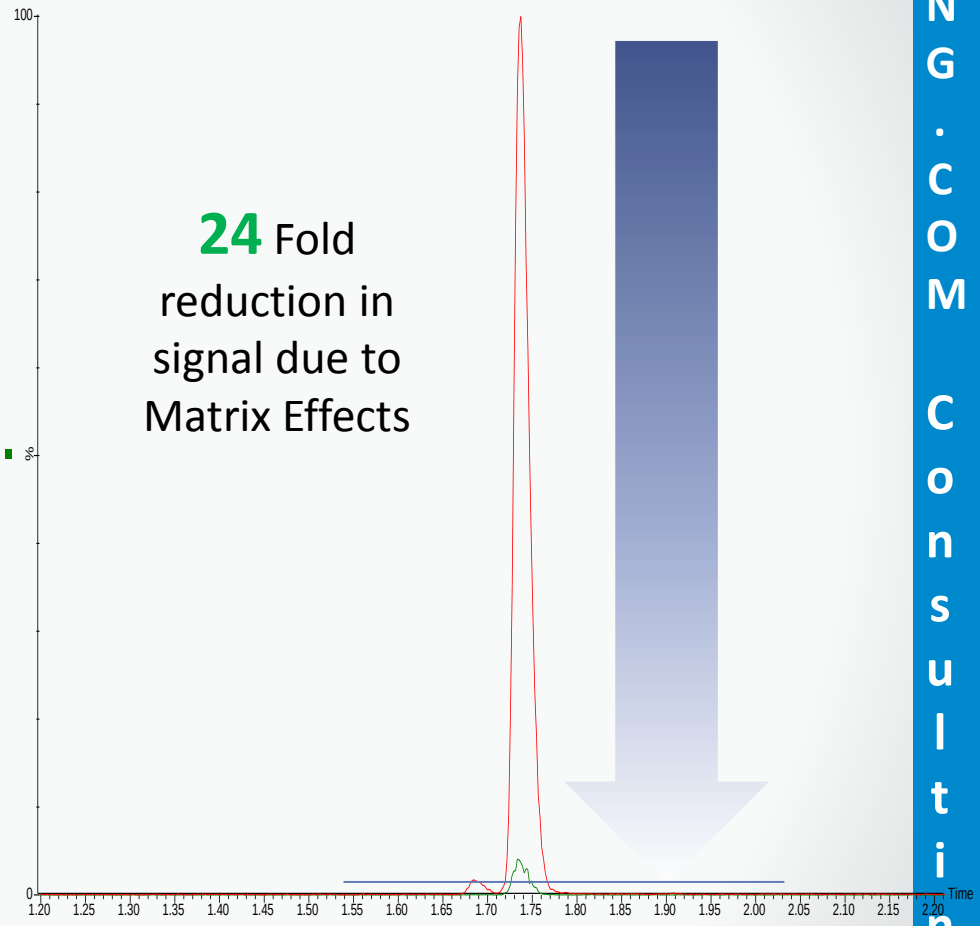
Increased peak height when the same amount is injected

Reduction of Matrix Effect with lower ID



2 Fold reduction in signal due to Matrix Effects

150 µm ID



24 Fold reduction in signal due to Matrix Effects

2.1 mm ID

Is there a future for Chip HPLC?

- Agilent HPLC Chip is proven in multiple years of reliable usage and has high functionality.
- Eksigent HPLC Chip has nano- and capillary column dimensions but less functionality
- Waters HPLC Chip is making a re-entry in the market

But…

- Highly proprietary solutions lock user with vendor regarding HPLC and MS
- Especially in proteomics method flexibility and ultra high efficiency of nanoHPLC columns is demanded

Recommendations for Sample Preparation

Positive ion ESI

- Dissolve samples in acid
- Basic sites (N and O) bind to proton to give the molecule a positive charge
- Other cat ions (Na+, or K+) may also be used to form positive ions
- Anions (Cl-) may be removed from a molecule to allow the formation of positive ions

Negative ion ESI

- Dissolve samples in base
- Acidic sites (acids) give up a proton to form a negative ion.

- ESI works best when the samples are free of salt
- Samples that contain salt can be desalted in many ways (divert valve)

Acknowledgements

- Monika Dittmann, Stephan Buckenmaier, Udo Huber, Christian Scholz, Konstantin Choikhet all at Agilent Technologies, Waldbronn, Germany
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- Remco van Soest, Eksigent part of AB Sciex, Dublin, USA
- Martin Gilar, Matt Lauber, Ken Fountain, Waters Inc.

Thanks for your attention

谢谢

Reprints will be available soon on:

http://www.rozing.com

(Registration required!)