QUOI DE NEUF EN CHROMATOGRAPHIE AU XXI ÈME SIÈCLE?

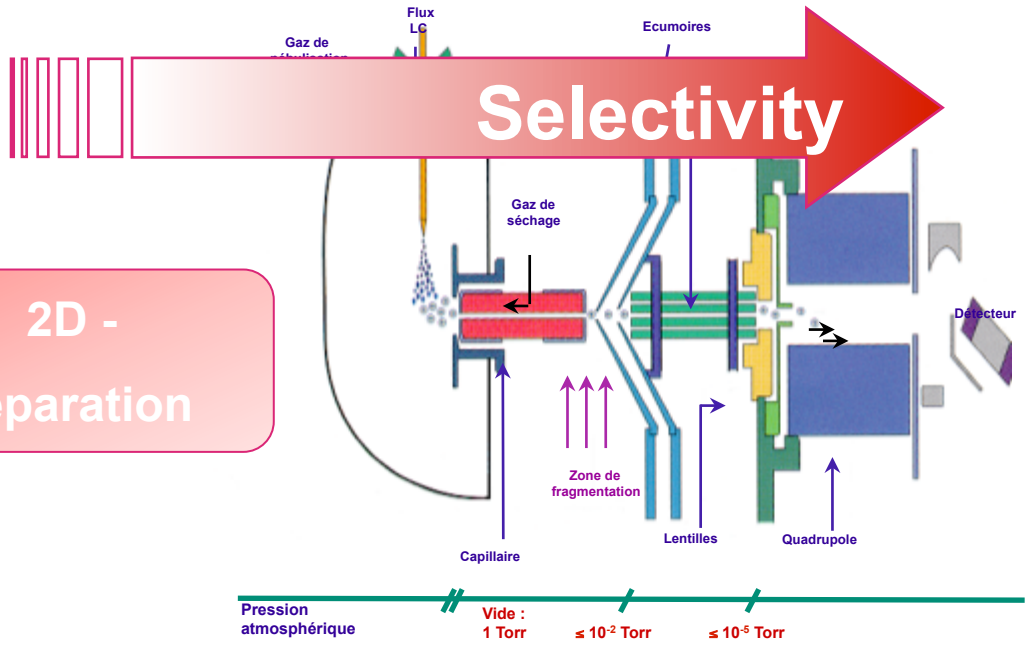
J-L. Veuthey, D. Guillarme

SFTA, Paris, 22 Janvier 2016

LC-MS | the gold standard

Analysis

Sample preparation → Separation → MS Detection → Data handling



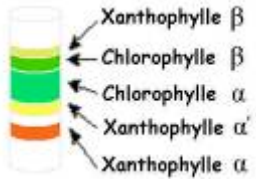
Selectivity

2D - separation

Automation
Rapidity
Simplicity

Evolution of liquid chromatography

1903 — Discovery of chromatography



1965 — First HPLC systems

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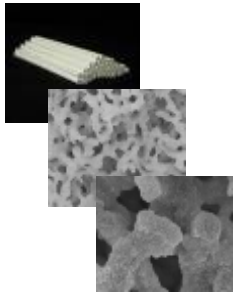
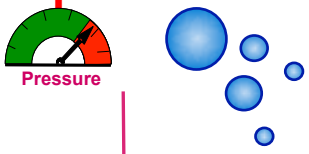
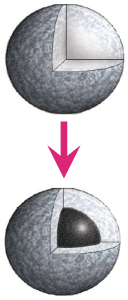


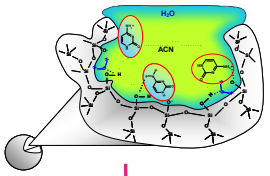
2004 — First UHPLC systems and sub-2µm columns



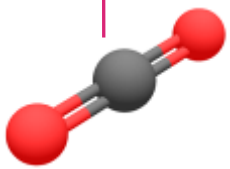
2007 — Core-shell technology



2008 — HILIC



2010 — Green LC and SFC



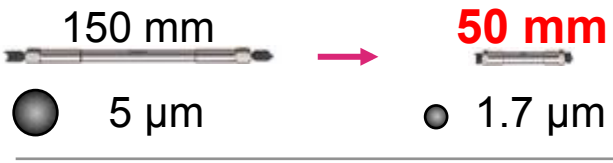
Biomolecules

Fast and ultra-fast analysis in UHPLC

High Throughput

N ≈ 10’000 plates

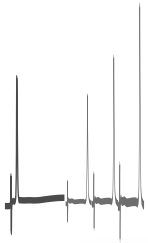
t_{ana} < 2 min (k=10)



N ≈cst

T_r÷9

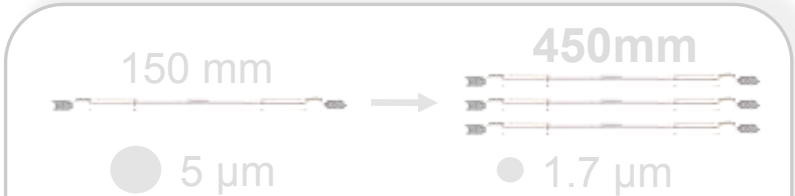
Reduced extra column volume



High Resolution

N ≈ 90’000 plates

t_{ana} < 20 min (k=10)

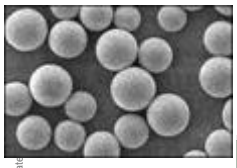


N ×9

T_r ≈ cst

High pressure required

Method transfer | HPLC towards UHPLC

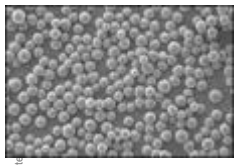


5µm

4.6 x 150 mm

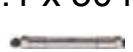
HPLC calculator Version 3

http://www.unige.ch/sciences/pharm/



1.7µm

2.1 x 50 mm



- **Analysis time : proportional to column dead time**

÷ 9

$$\frac{t_{ana2}}{t_{ana1}} = \frac{dp_2}{dp_1} \times \frac{L_2}{L_1}$$

- **Backpressure : inversely proportional to d_p^3 (at u_{opt}) and column length**

x 9

$$\frac{\Delta P_2}{\Delta P_1} = \frac{L_2}{L_1} \times \frac{dp_1^3}{dp_2^3}$$

- **Solvent consumption : proportional to internal diameter and column length**

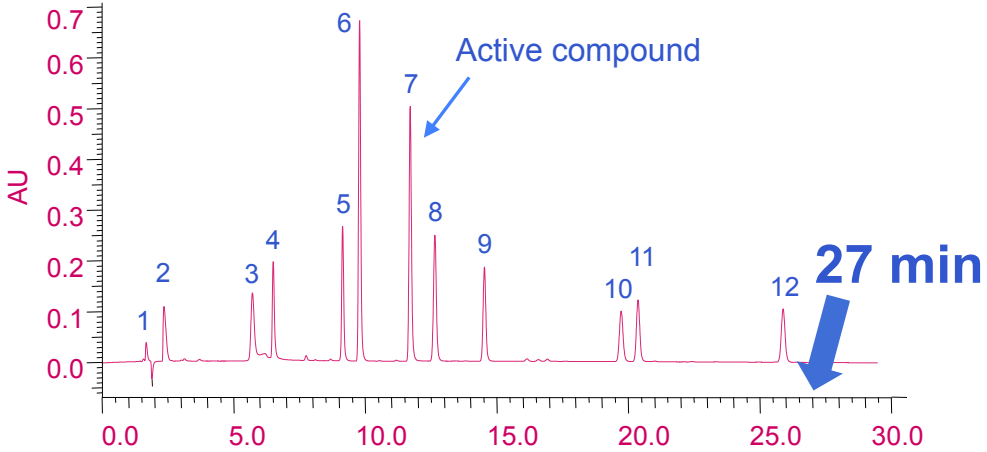
÷ 14

$$\frac{V_2}{V_1} = \frac{dc_2^2}{dc_1^2} \times \frac{L_2}{L_1}$$

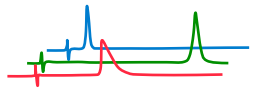
Separation of drugs and impurities

ORIGINAL METHOD

HPLC
4.6 x 150 mm, 5 µm
F = 1000 µL/min
ΔP = 80 bars

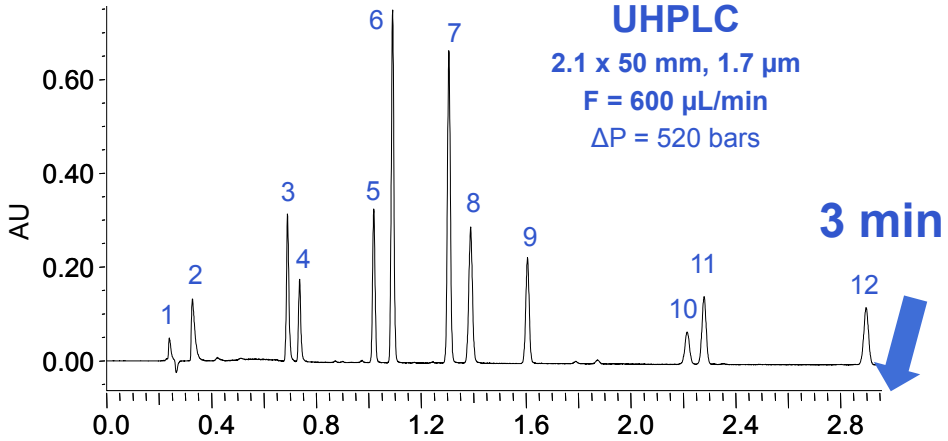


Impurity profiling

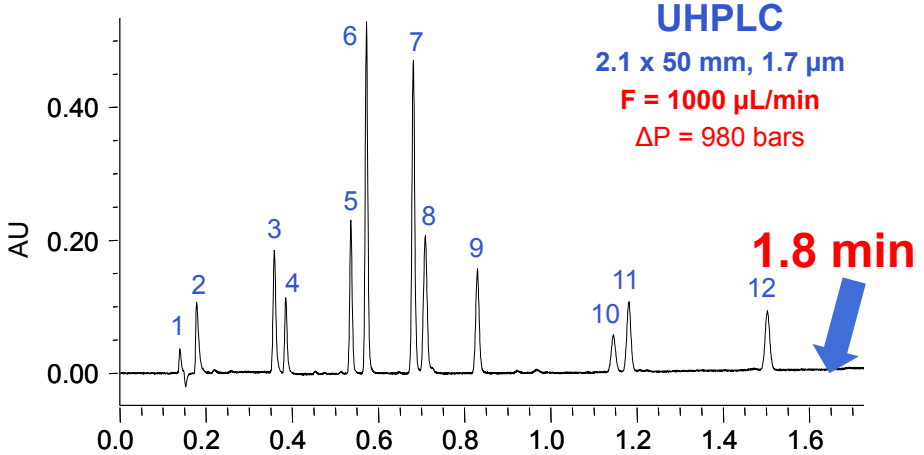


TRANSFERRED METHOD

UHPLC
2.1 x 50 mm, 1.7 µm
F = 600 µL/min
ΔP = 520 bars



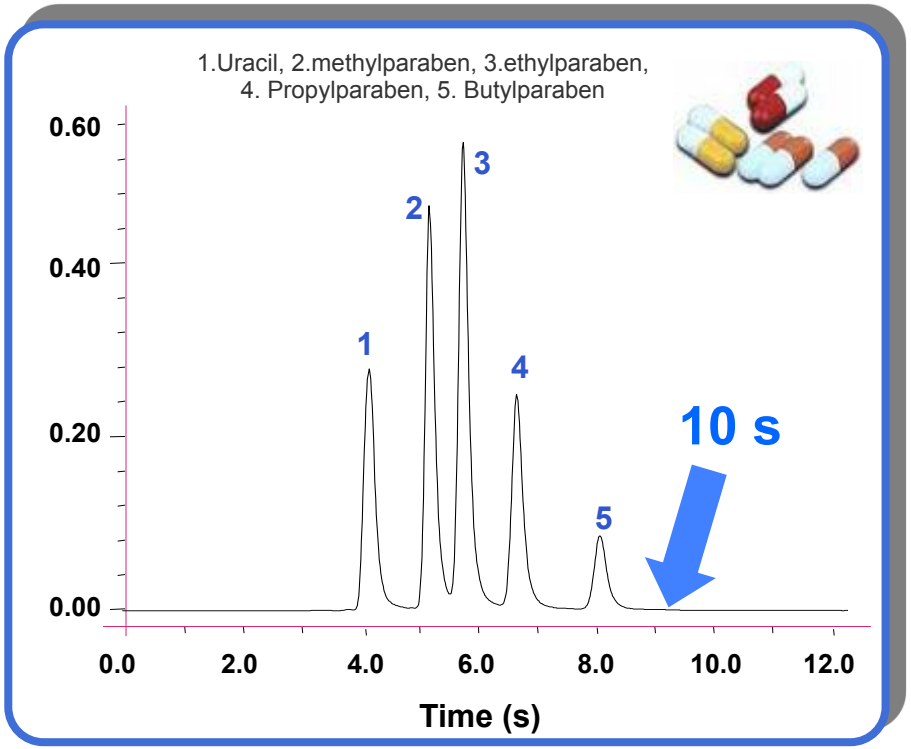
UHPLC
2.1 x 50 mm, 1.7 µm
F = 1000 µL/min
ΔP = 980 bars

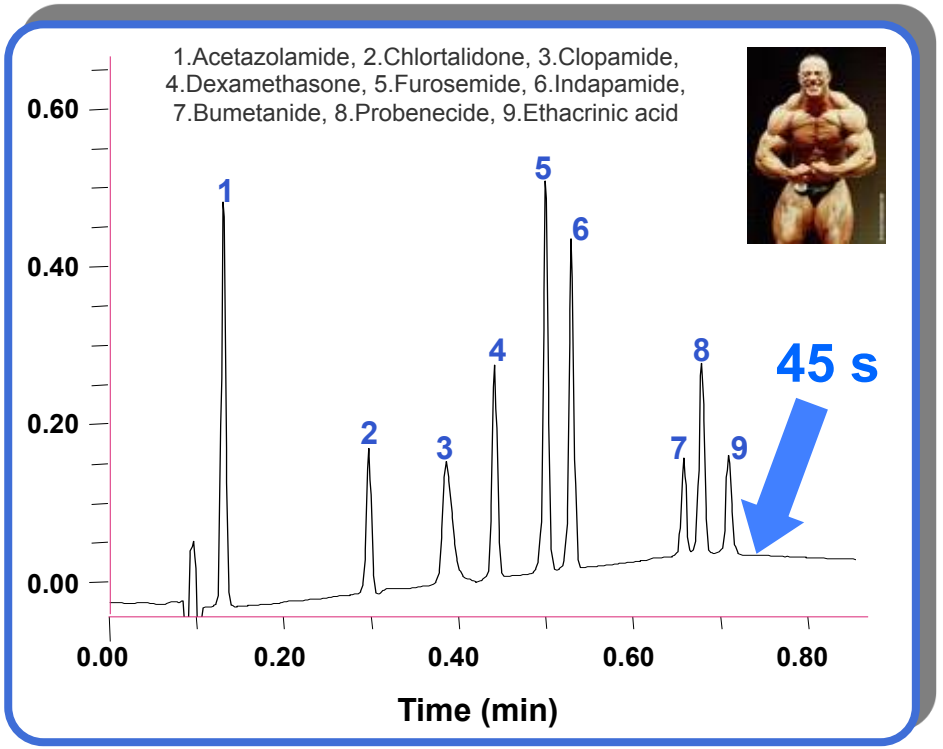


Guillarme et al., Eur. J. Pharm. Biopharm., 68 (2008) 430-440

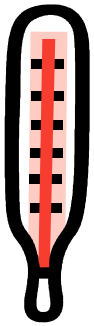
High throughput analysis in HT-UHPLC

Column C_{18} 2.1 x 50mm, 1.7µm, **Flow rate** 1.7 mL/min, **Oven temperature** 90 C, **UV Detection** 25 ms, 80 Hz





90 C



- **Important to minimize extra-column band broadening**
- **Need to increase acquisition rate**
- **Short injection cycle time**

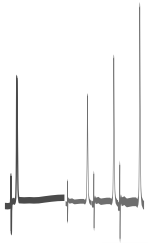
D.T.T. Nguyen et al., J. Chromatogr. A, 1167 (2007) 76–84

High resolution analysis in HPLC

High Throughput

N ≈ 10’000 plates

t_{ana} < 2 min (k=10)



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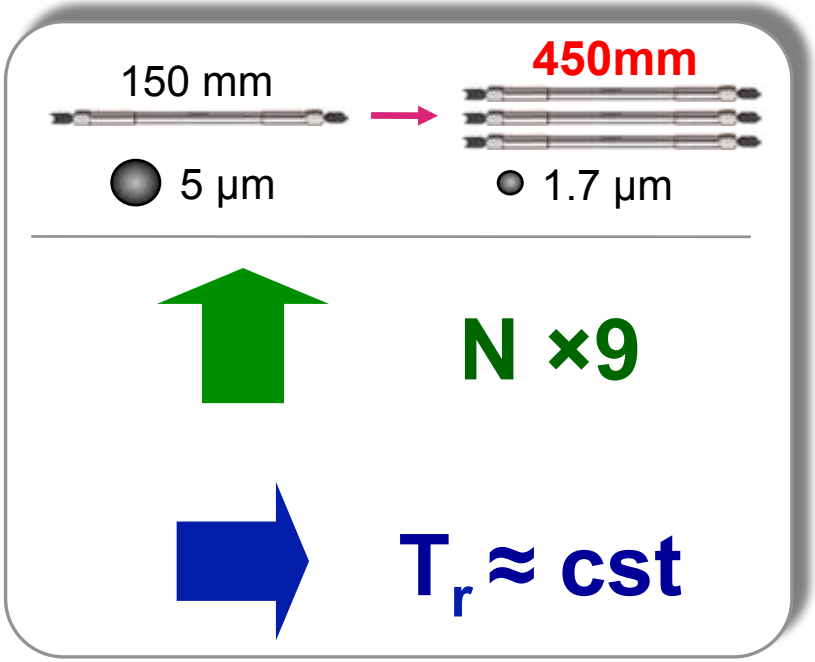
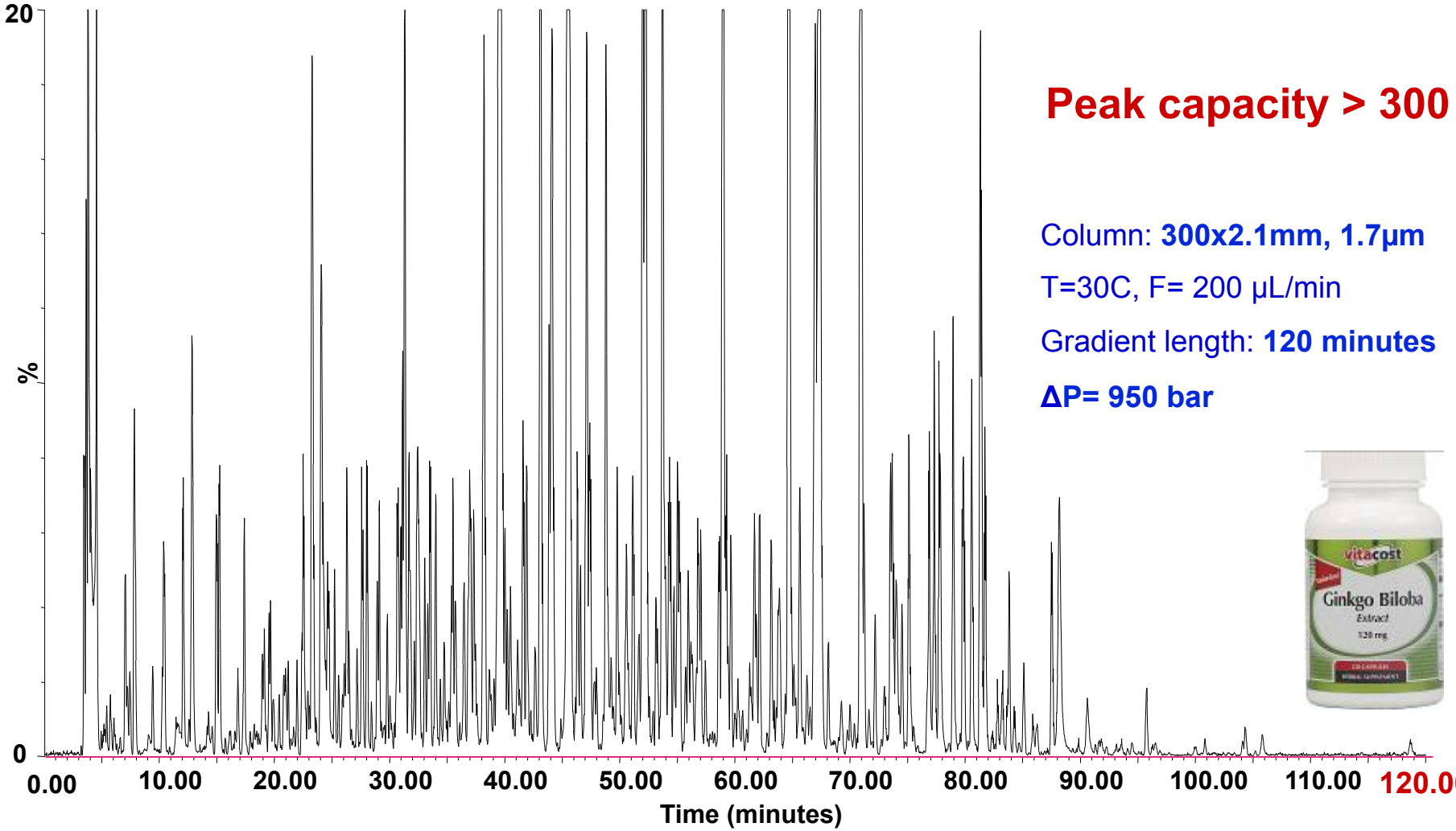


Reduced extra column volume

High pressure required

Metabolite profiling of complex plant extract

Analysis of a standardized extract of *Ginkgo Biloba*



Peak capacity > 300

Column: **300x2.1mm, 1.7µm**

T=30C, F= 200 µL/min

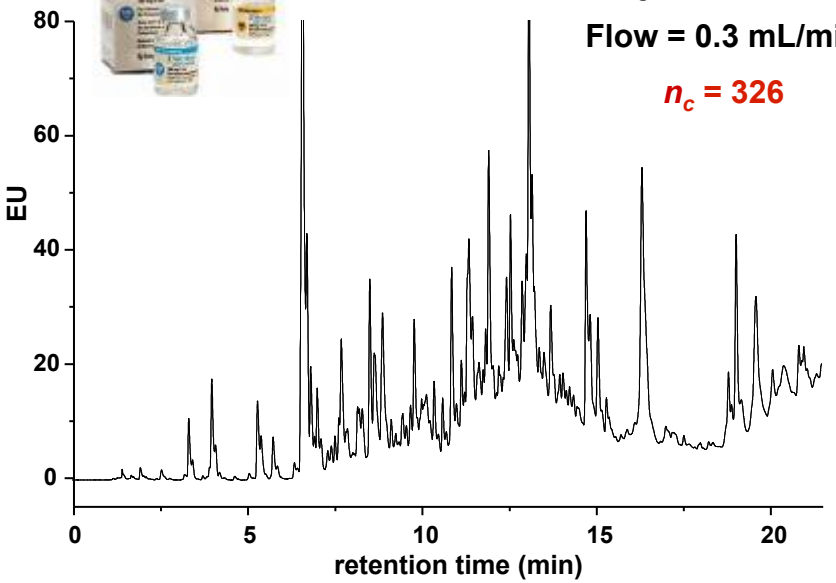
Gradient length: **120 minutes**

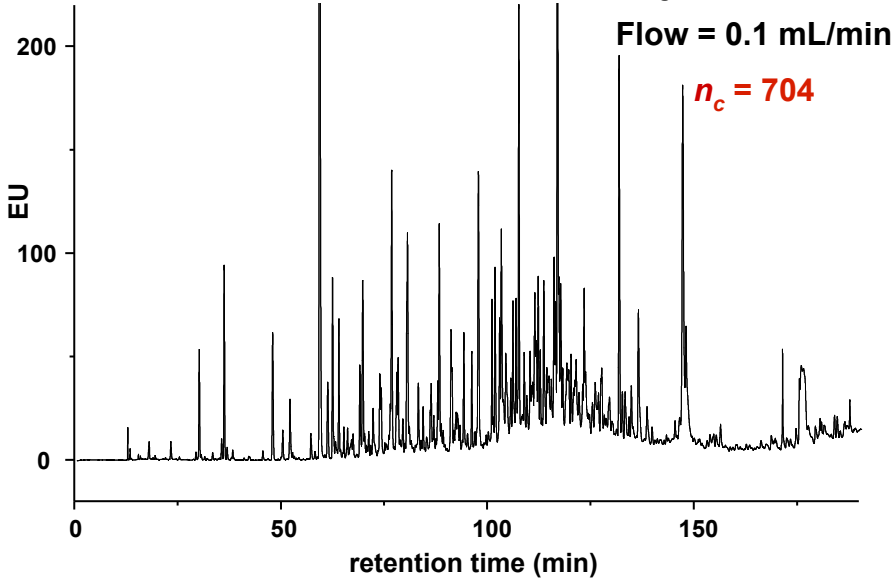
ΔP= 950 bar

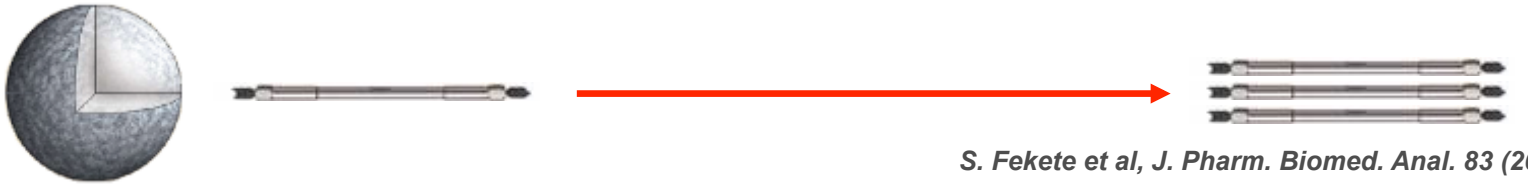
E. Grata et al., J. Chromatogr. A, 1216 (2009) 5660–5668

Peptide mapping of therapeutic mAb

Tryptic digest (peptide mapping) of **panitumumab**. Columns: Acquity BEH C18 300A - **150 mm x 2.1 mm, 1.7 µm** (1 and 3 columns in series). Mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 10-55 %B, flow: 0.30 and 0.10 mL/min for the 150 and 450 mm long columns, respectively. **Temperature: 50C**, Detection FL: 280-360 nm. Injected volume: 5 and 15 µL for the 150 and 450 mm long columns, respectively.

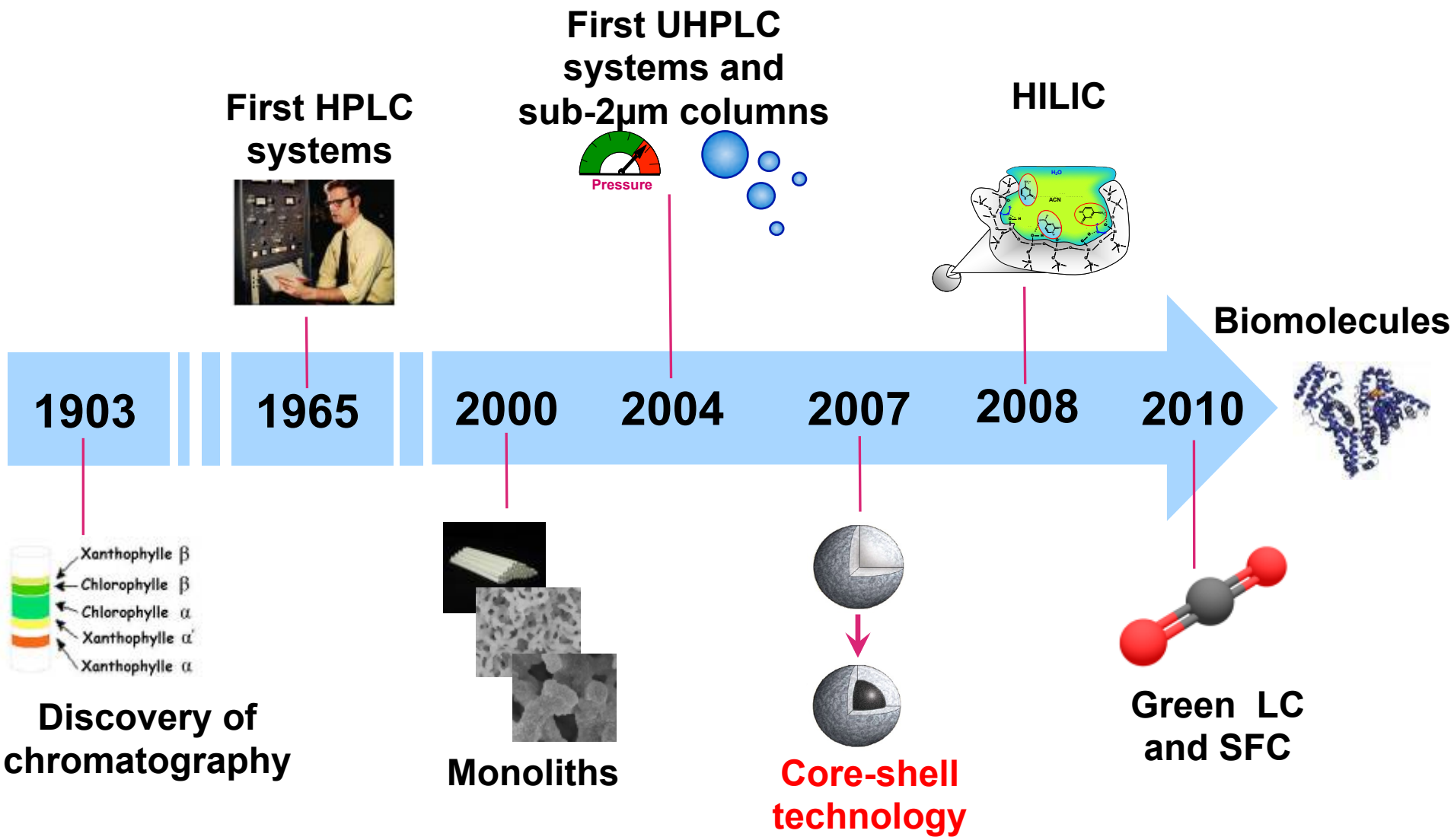




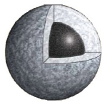


S. Fekete et al, J. Pharm. Biomed. Anal. 83 (2013) 273

Evolution of liquid chromatography



Superficially porous particles : SPP



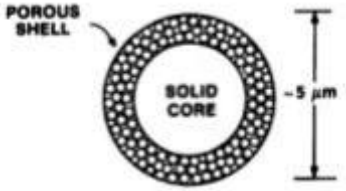
SPP = superficially porous particles = core-shell = fused-core

1st generation: Pellicular particles originally imagined by Horvath et al. in the 60's (particle size of 50 – 100 µm, shell thickness of 1 µm).

→ **Analysis of proteins by ion exchange chromatography**

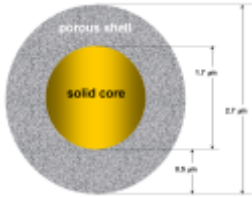
2nd generation: Technology developed by J.J. Kirkland and commercialized by Agilent in the 90's under the trademark Poroshell (particle size of 5 µm, shell thickness of 0.25 µm).

→ **Analysis of peptides/proteins by RPLC**



3rd generation: Technology updated by J.J. Kirkland and commercialized by various companies since 2007 (particle size of 2.6 - 2.7 µm, shell thickness of 0.35 - 0.5 µm).

→ **Analysis of small and large molecules by RPLC**



2.5-2.7 µm SPP phases on the market

2007
- Advanced Material Technologies **HALO** 2.7 µm (core 1.7 µm) 
- Supelco **ASCENTIS** 2.7 µm (core 1.7 µm) 

2009
- Phenomenex **KINETEX** 2.6 µm (core 1.9 µm) et 1.7 µm (1.25 µm) 

2010
- Agilent **POROSHELL** 2.7 µm (core 1.7 µm) 

2011
- Thermo **ACCUCORE** 2.6 µm (core 1.6 µm) 
- Macherey-Nagel **NUCLEOSHELL** 2.6 µm (core 1.6 µm) 
- ChromaNik **SUNSHELL** 2.6 µm (core 1.6 µm) 
- Perkin-Elmer **BROWNLEE SPP** 2.7 µm (core 1.7 µm) 

2012
- Fortis **SPEEDCORE** 2.6 µm (core 1.8 µm) 
- Nacalai **COSMOCORE** 2.6 µm (core 1.6 µm) 
- Restex **RAPTOR** 2.7 µm (core 1.7 µm) 

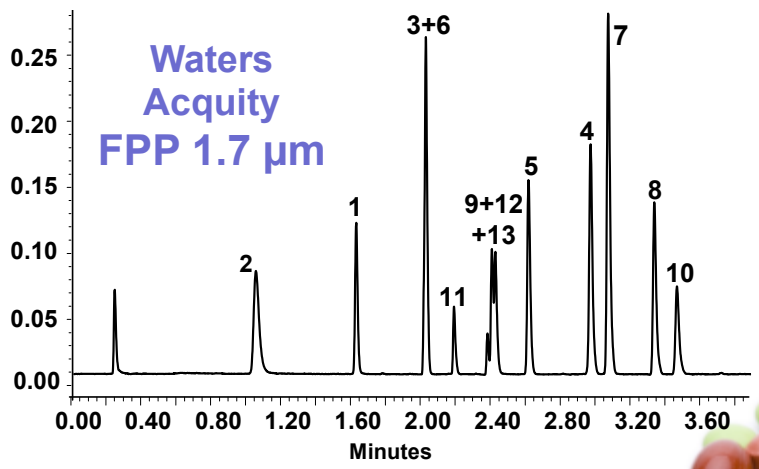
2013
- Ace **ACEULTRACORE** 2.5 µm (core 1.6 µm) 

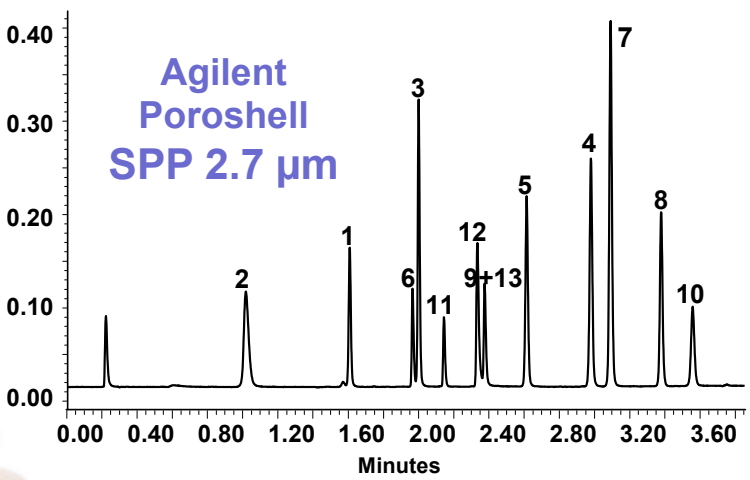
2014
- Waters **CORTECS** 2.7 µm (core 1.9 µm) 

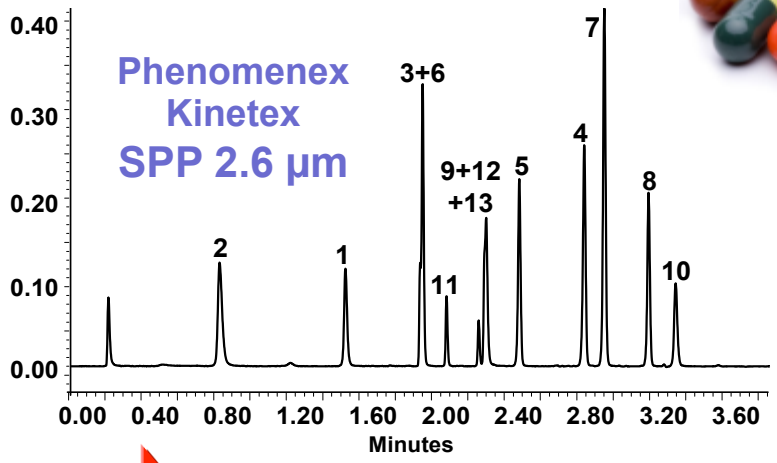
Analysis of drugs: core-shell *vs* porous particles

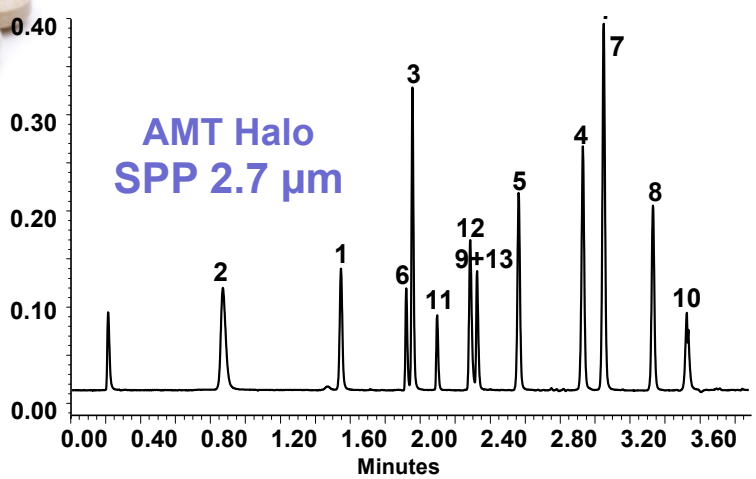
Columns of 50x2.1mm; gradient: 1 min at 5%ACN, then 5-95%ACN in 3 min @ 500 µL/min; 0.1%FA in both ACN and water.

1 – Morphine, 2 – atenolol, 3 - codeine, 4 – lidocaine, 5 – prilocaine, 6 – acebutolol, 7 – bupropion, 8 – bupivacaine, 9 - propranolol, 10 - trimipramine, 11 - ketoprofen, 12 - flurbiprofen, 13 - Ibuprofen







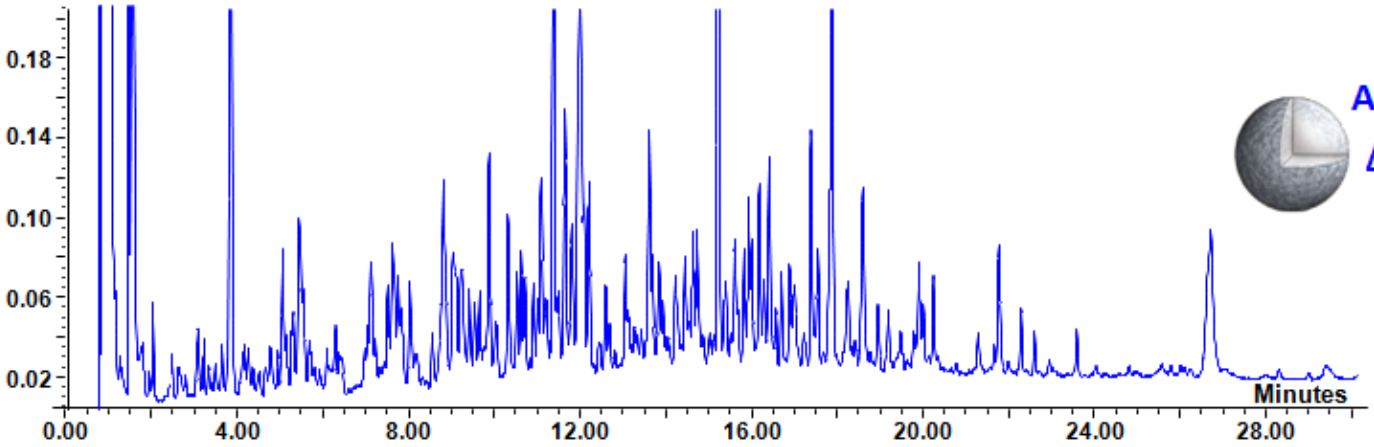


Similar performance, no major change in retention/selectivity
Pressure is strongly reduced

J. Ruta et al., J. Chromatogr. A, 2012, 1228, 221

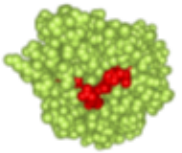
High resolution separation of a tryptic digest

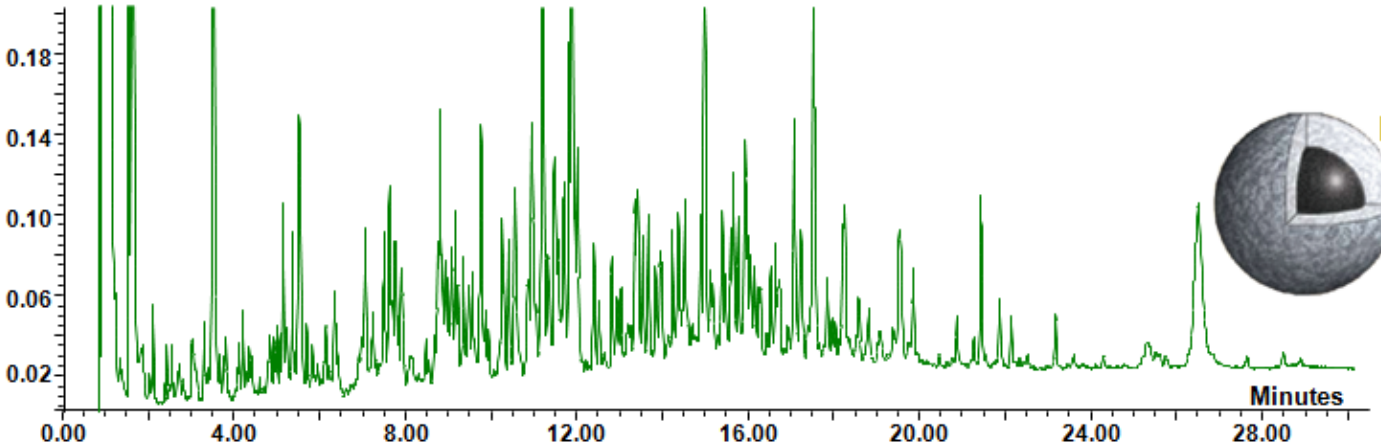
Tryptic digest of four proteins (myoglobin, BSA, ovalbumin, β-lactoglobulin) – gradient 2-50%ACN in **30 min** @ 500µL/min with 0.1%TFA in water and ACN – T=50 C – λ=214nm, column: **150x2.1mm**



ACQUITY 1.7 µm

ΔP= 760 bar





POROSHELL 2.7 µm

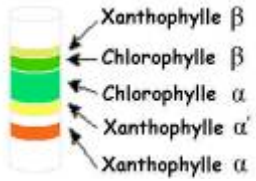
ΔP= 460 bar

High peak capacity with peptides of 0.5 to 2 kDa on any modern RPLC phases

A. Staub et al., J. Chromatogr. A, 2011, 1218, 8903

Evolution of liquid chromatography

1903 — Discovery of chromatography



1965 — First HPLC systems

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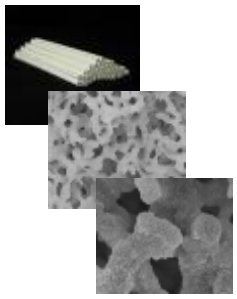
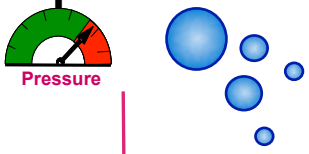


2004 — First UHPLC systems and sub-2µm columns



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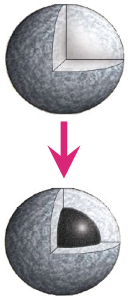

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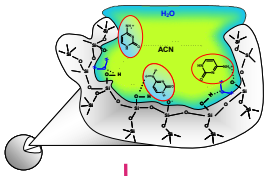

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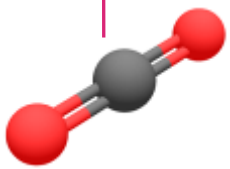
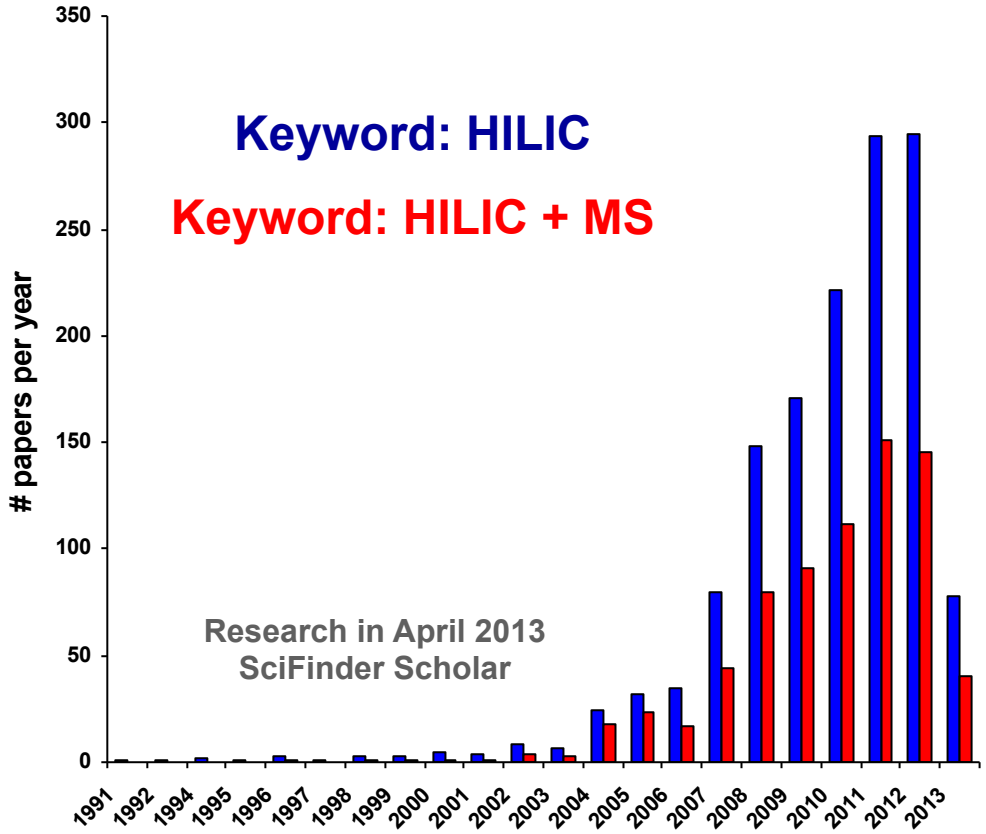
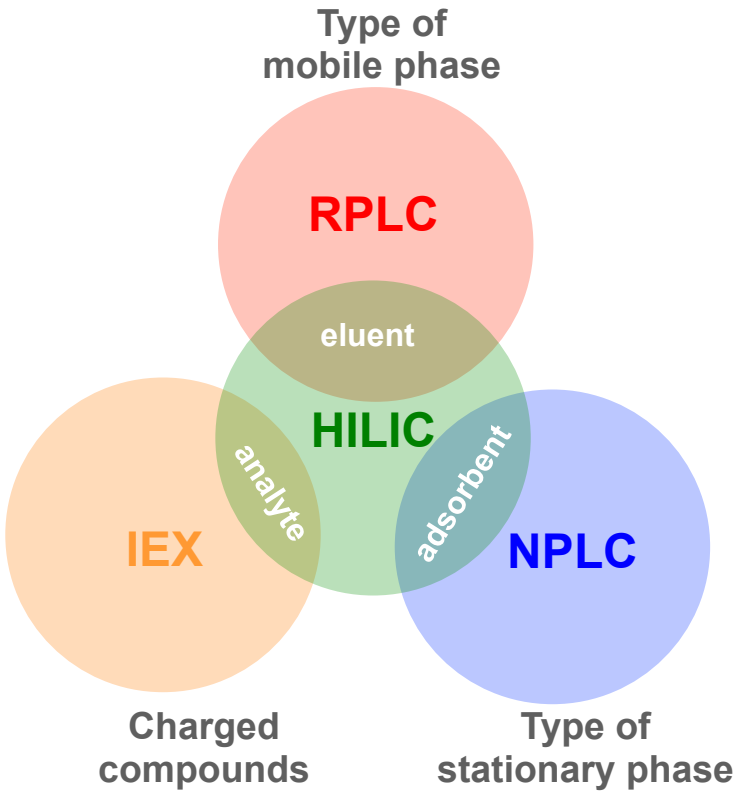


Biomolecules

HILIC: an alternative to RPLC

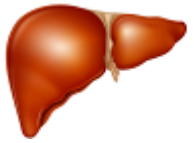
1990: Alpert coined the term « HILIC » to describe the hydrophilic partitioning between a water enriched layer at the surface of the stationary phase and the mobile phase.

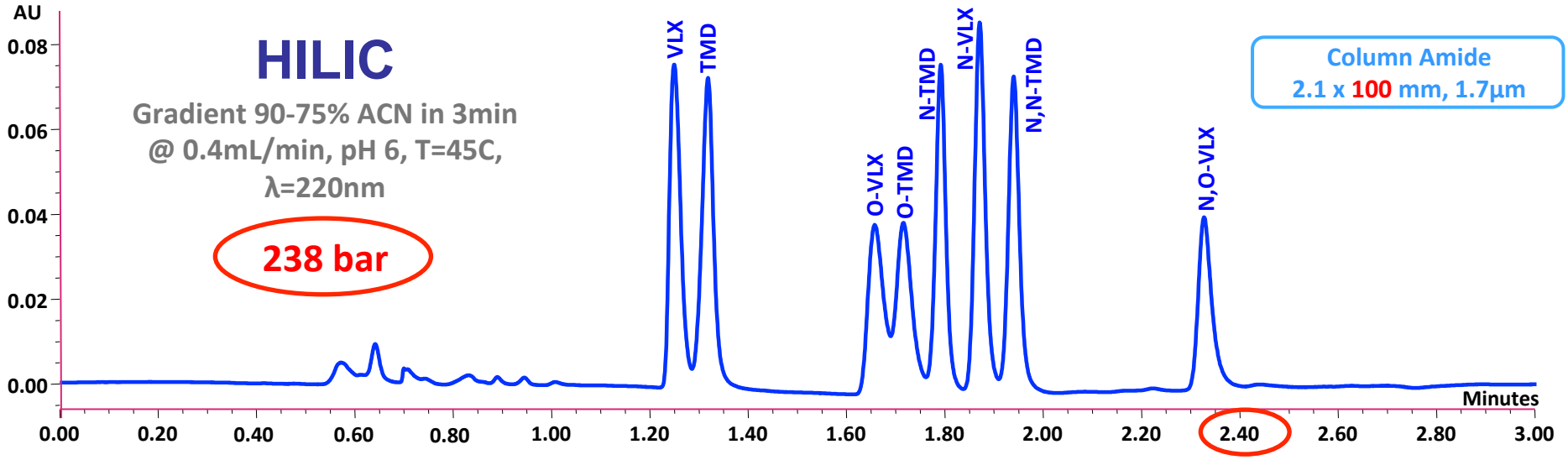


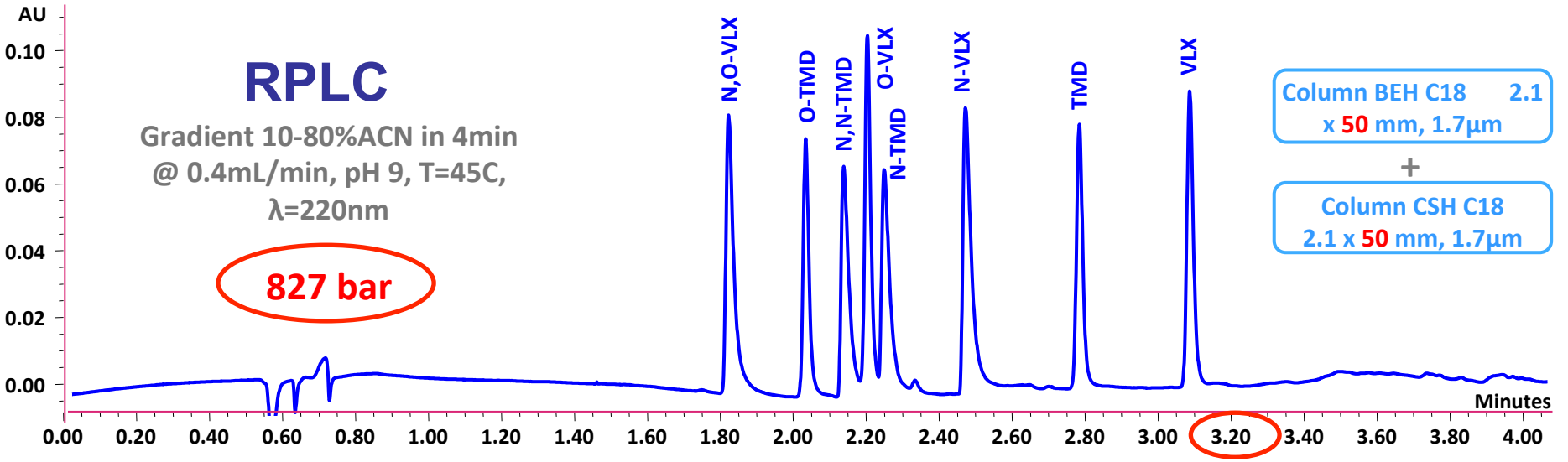


B. Buszewski et al., Anal. Bioanal. Chem., 402 (2012) 231-247

HILIC for drugs and metabolites



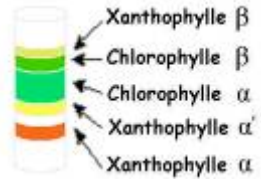




J. Ruta et al., J. Pharm. Biomed. Anal. 63 (2012) 95

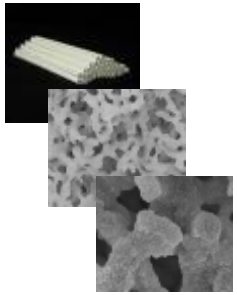
Evolution of liquid chromatography

1903 — Discovery of chromatography

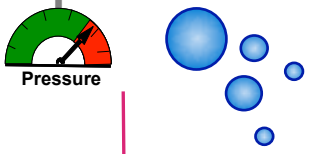


1965 — First HPLC systems

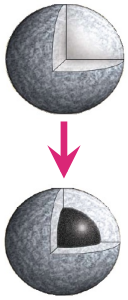
2000 — Monoliths



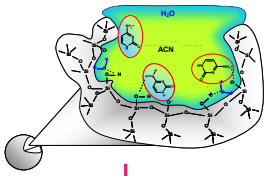
2004 — First UHPLC systems and sub-2µm columns



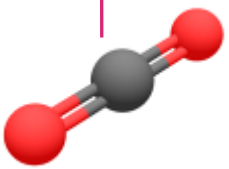
2007 — SPP technology



2008 — HILIC

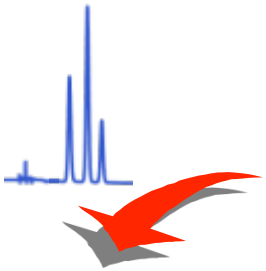


2010 — Green LC and SFC



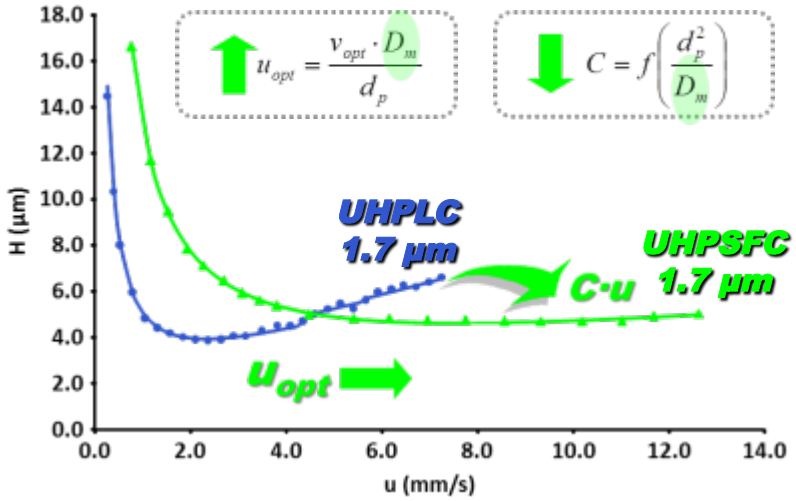
Biomolecules

Modern SFC: a viable alternative to RPLC?

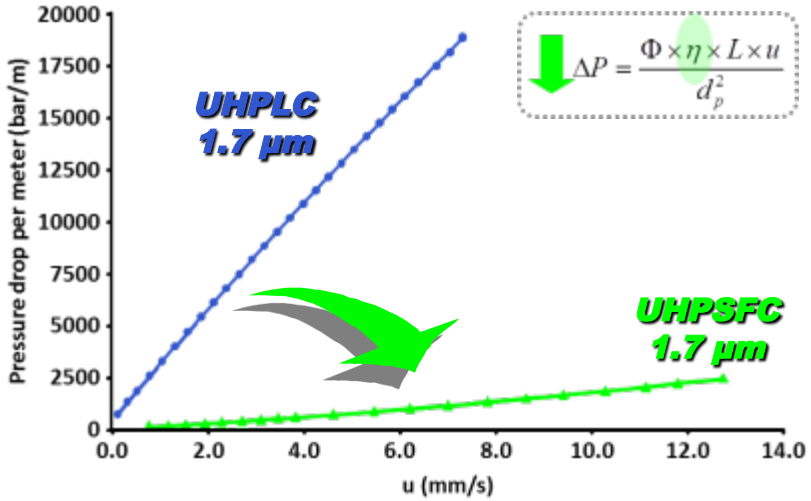


Diffusion coefficient D_M [m²/s]		Viscosity η [cP]
(0.2 – 2.0) x 10⁻⁹	Liquids (1 atm ; 15 – 30 °C)	0.2 – 3.0
0.7 x 10⁻⁷	Supercritical fluids (T_c ; P_c)	(1.0 – 3.0) x 10⁻²
(1.0 – 4.0) x 10⁻⁵	Gas (1 atm ; 15 – 30 °C)	(1.0 – 3.0) x 10⁻²

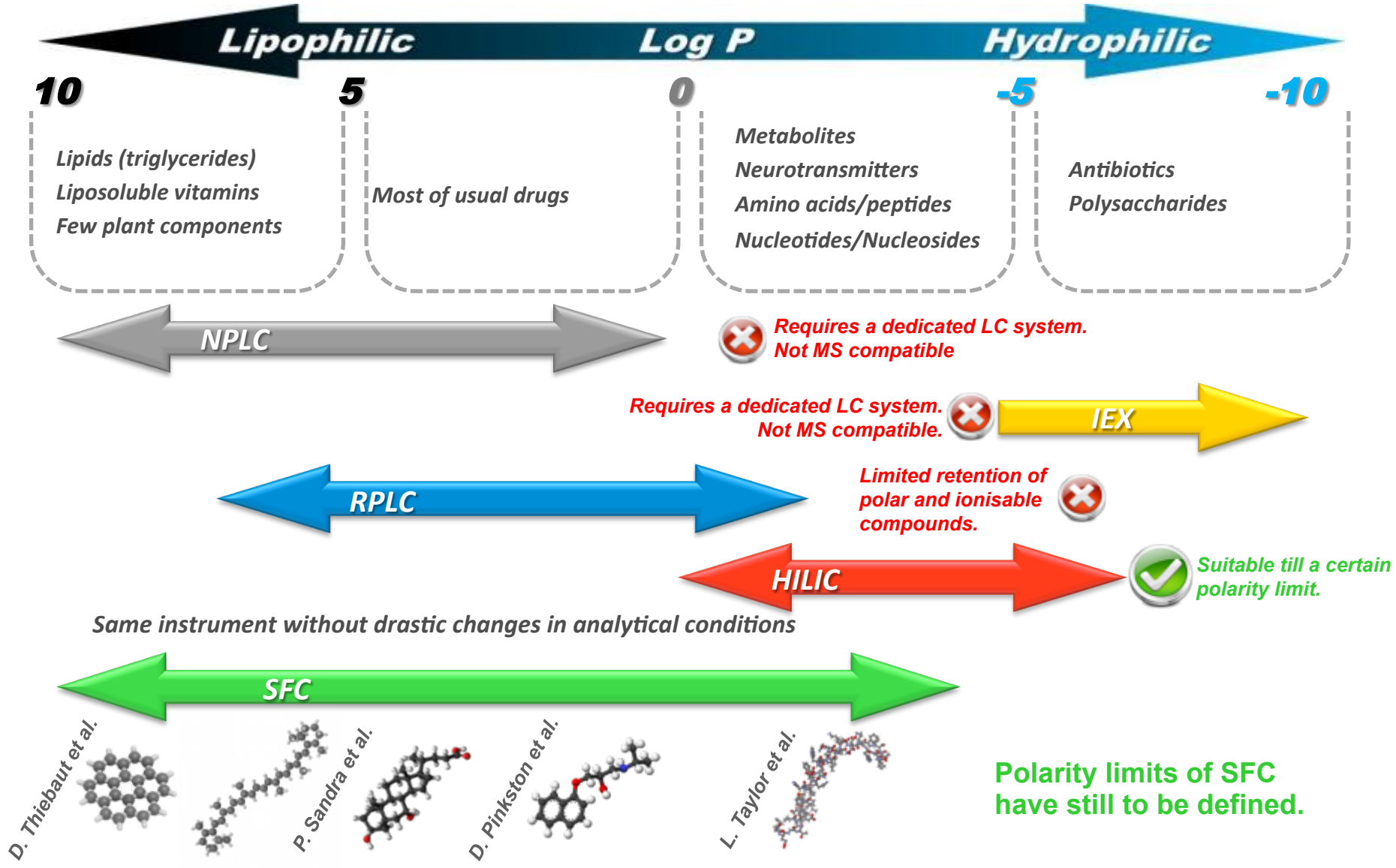
Kinetic performance



Pressure drop



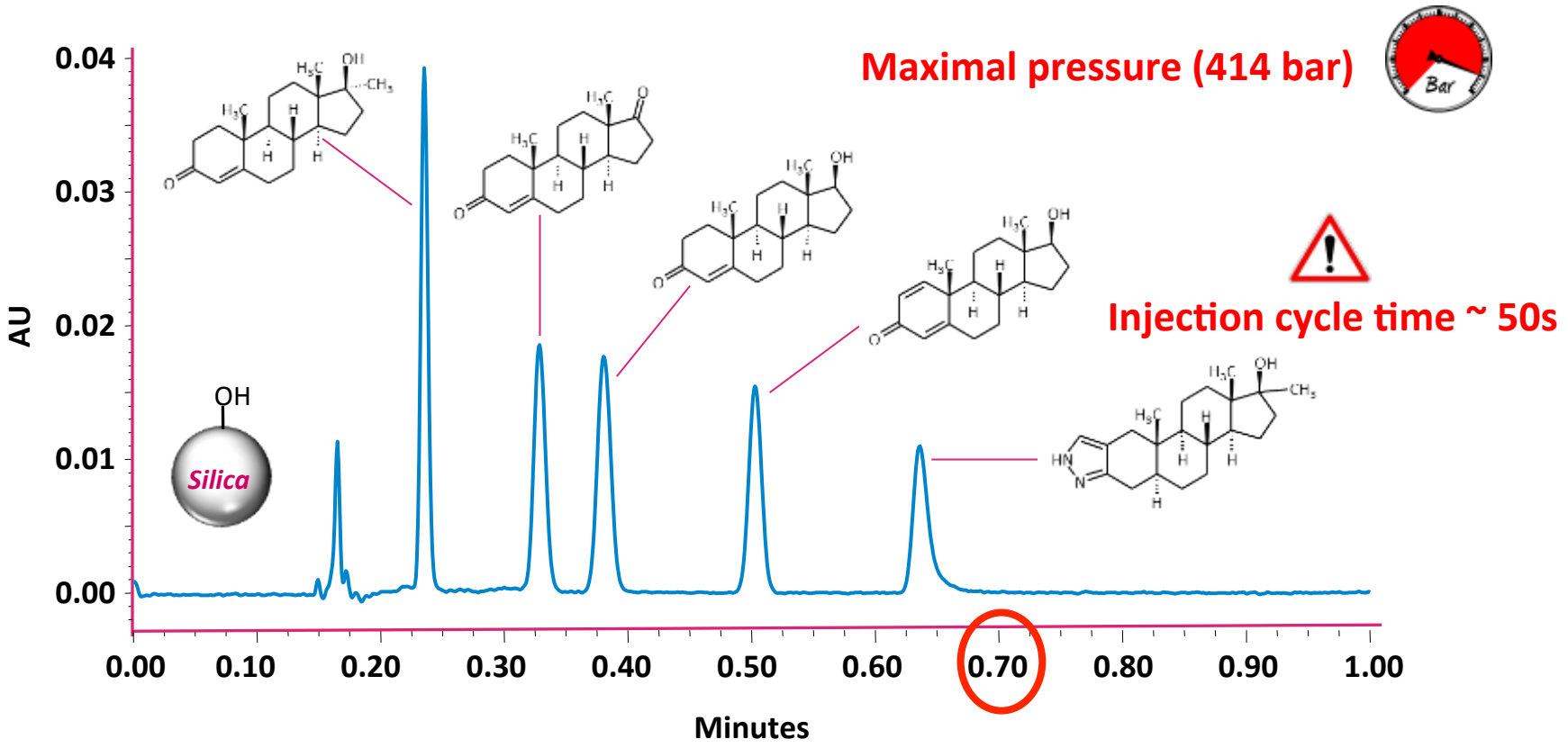
High versatility of SFC *vs* other techniques



Polarity limits of SFC have still to be defined.

Ultra-fast analysis of steroids

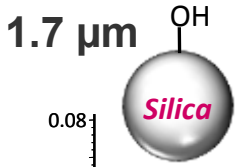
Mixture of 5 structurally related steroids. Column UPC² BEH (3.0 x 100mm, 1.7µm). Gradient from 10% to 12% MeOH in 0.6min followed by an isocratic step of 0.4 min. Back pressure regulator and oven temperature were set at 145 bar and 40°C, respectively.

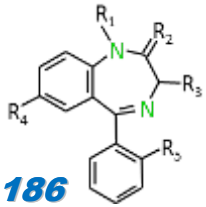


For 10 successive injections, RSD on t_r were between 0.35 and 0.59%

High resolution analysis of benzodiazepines

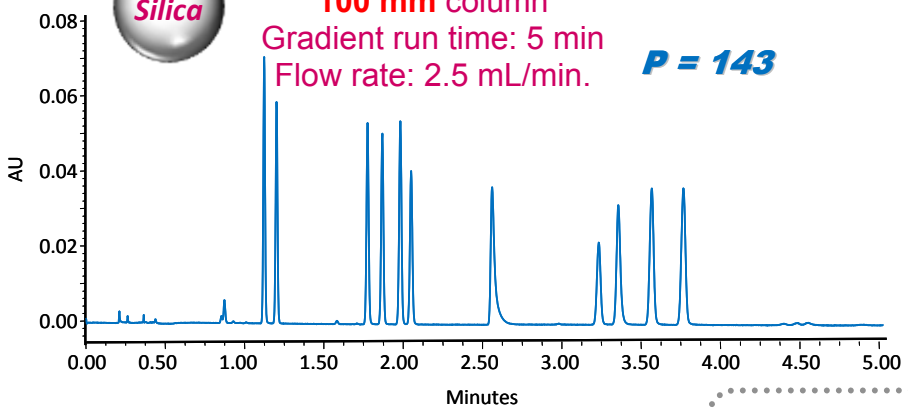
Mixture of 11 structurally related benzodiazepines (elution order: prazepam, flunitrazepam, clorazepate, desmethylflunitrazepam, nitrazepam, clonazepam, midazolam, brotizolam, 7-aminoflunitrazepam, alprazolam and triazolam).





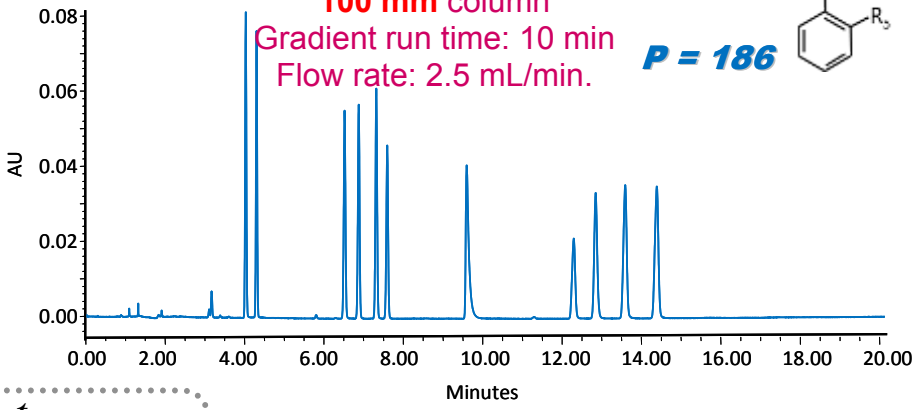
100 mm column
Gradient run time: 5 min
Flow rate: 2.5 mL/min.

P = 143



100 mm column
Gradient run time: 10 min
Flow rate: 2.5 mL/min.

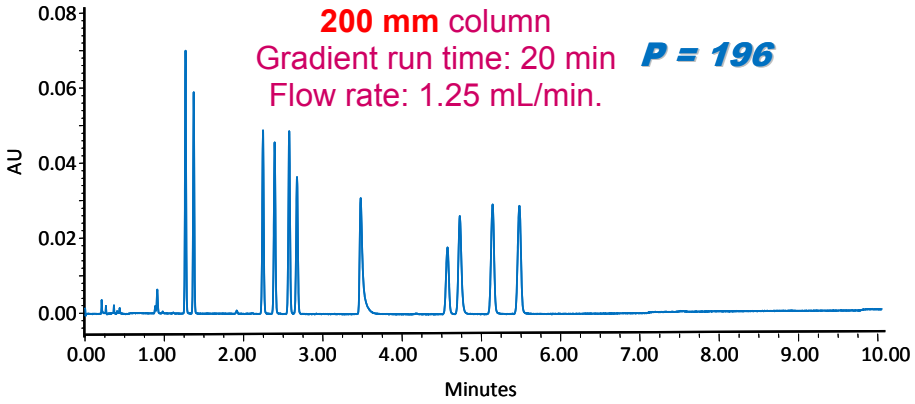
P = 186



$$P = 1 + \frac{t_{grad}}{(1/n)\sum_{1}^{n} w_{13.4\%}}$$

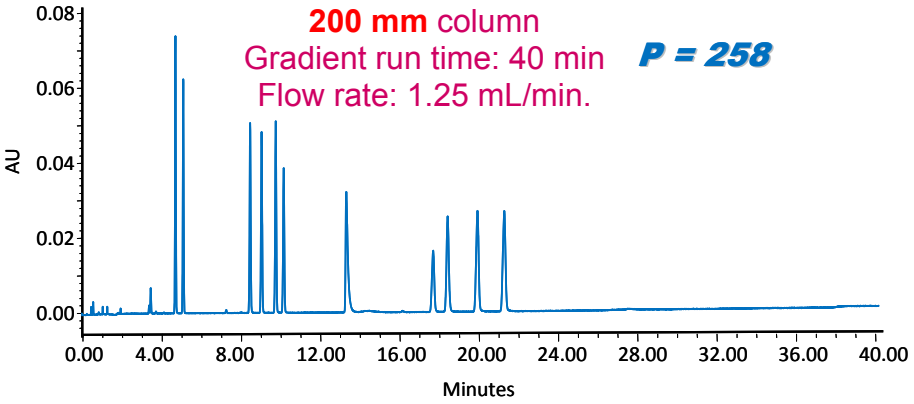
200 mm column
Gradient run time: 20 min
Flow rate: 1.25 mL/min.

P = 196

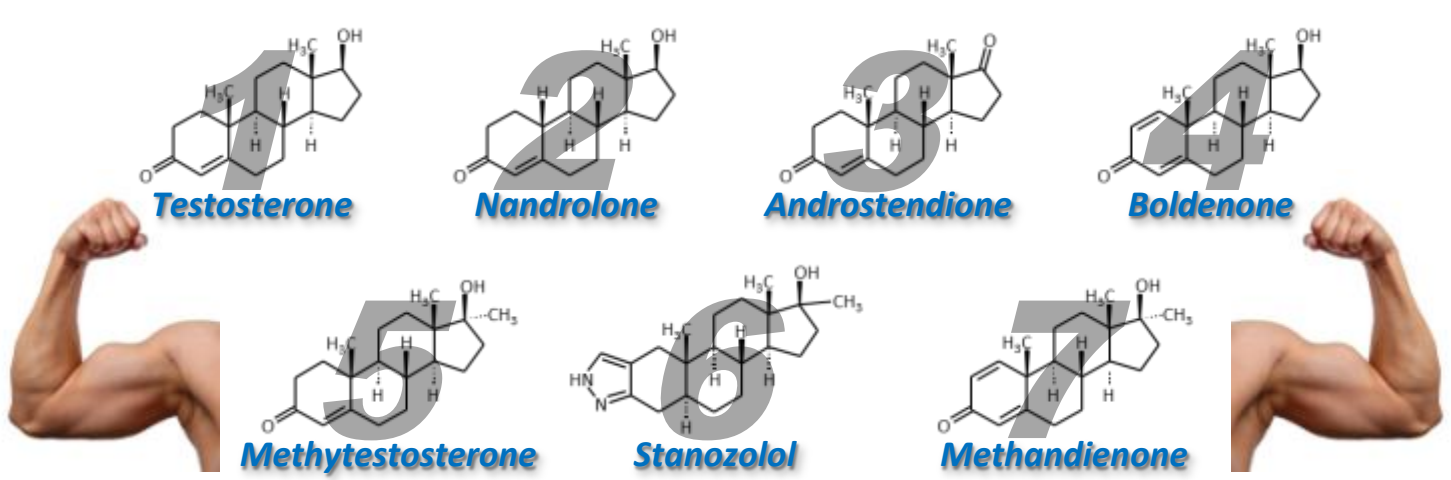


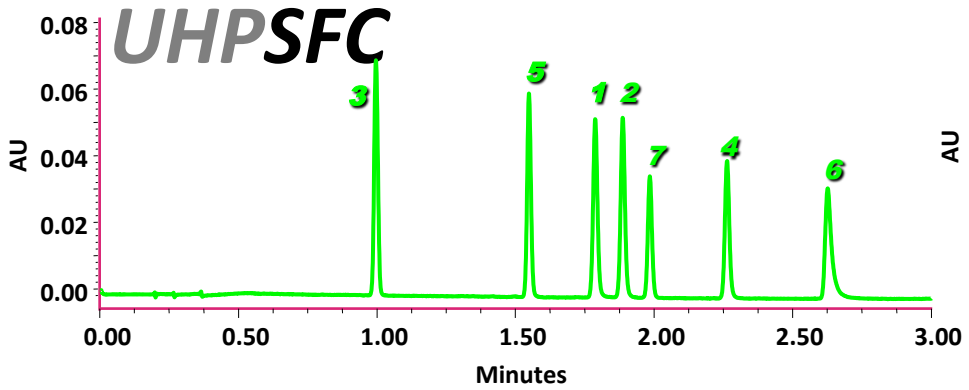
200 mm column
Gradient run time: 40 min
Flow rate: 1.25 mL/min.

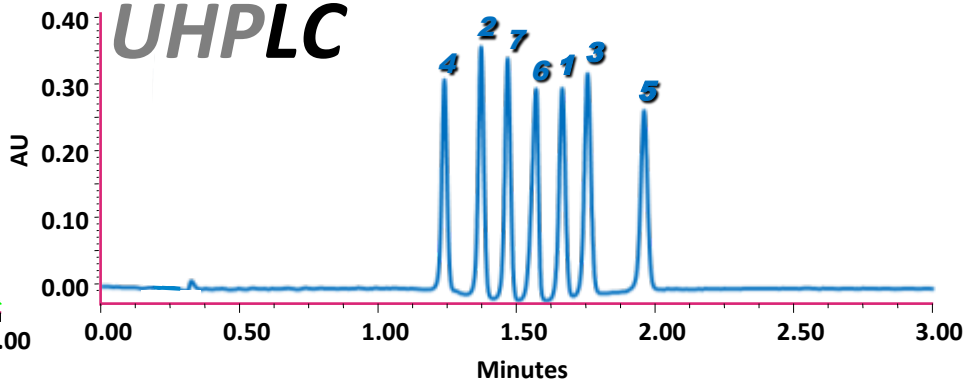
P = 258



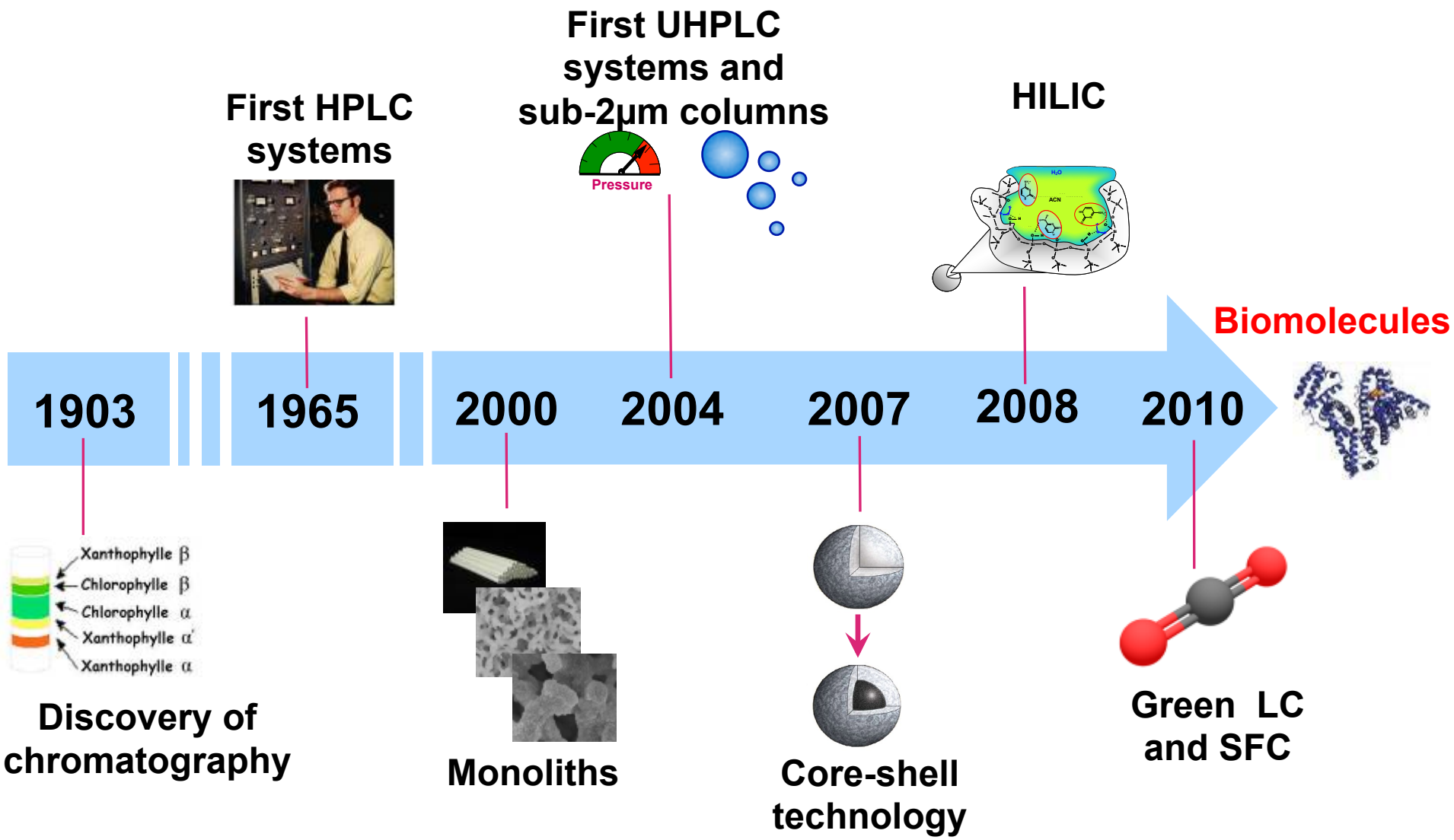
Selectivity on structurally related compounds



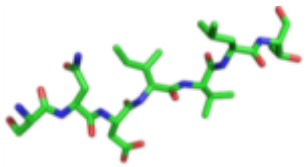
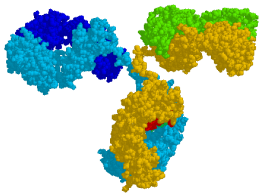
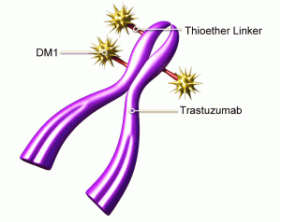




Evolution of liquid chromatography



Analytical characterization of molecules

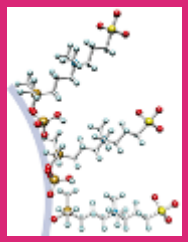
Small molecules	Peptides	Proteins	mAbs	ADC
				
salicylic acid MW 138 Da	triptorelin MW 1,311 Da	human growth hormone MW 22,125 Da	Rituximab MW ~150,000 Da	Brenduximab-vedotin MW ~150,000 Da

Analytical complexity

Important micro-heterogeneity during the production of biologics

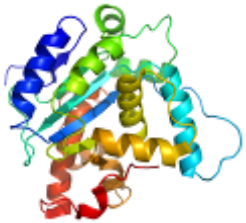
Analytical challenge !

Analytical methods for macromolecules



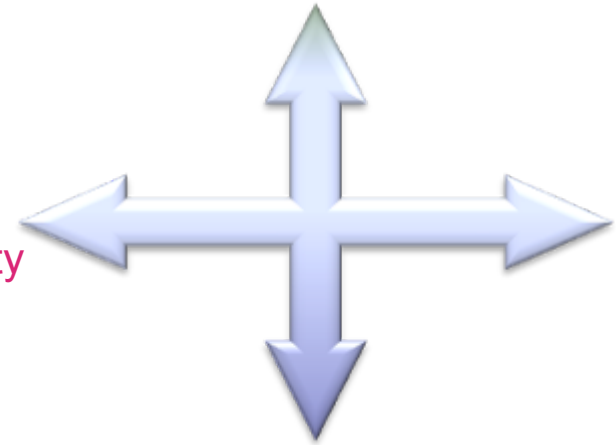
RPLC

- Quality control
- Characterization of biosimilars

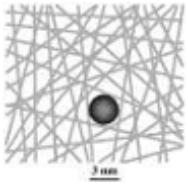


IEX

- Charge heterogeneity
- PEGylations



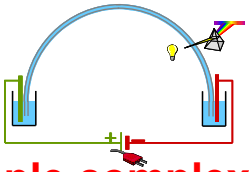
SEC



- MW determination
- Aggregation

Electrophoresis

- MW determination
- Charge heterogeneity



Need for high resolution because of sample complexity

Importance to combine methods with MS detection

A. Staub et al., JPBA, 2011, 55, 810-822

Analysis of intact mAbs

Columns of 150x2.1mm, T = **80°C** (Waters Acquity C18, 1.7µm, 300A) and **90°C** (Phenomenex Aeris C18, 3.6 µm SPP, widepore)

Bevacizumab (IgG1)

Cancer

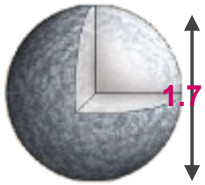
Rituximab (IgG1)

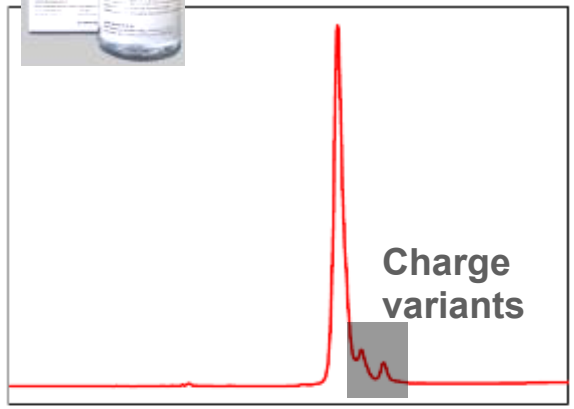
Leukemia

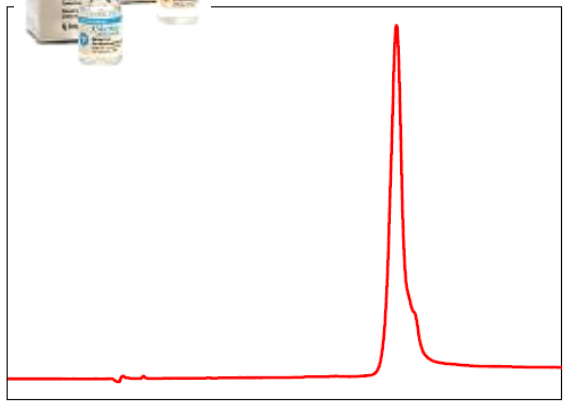
Panitumumab (IgG2)

Cancer

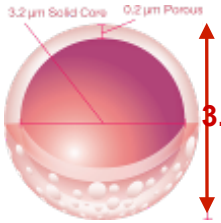
C18 - 1.7 µm

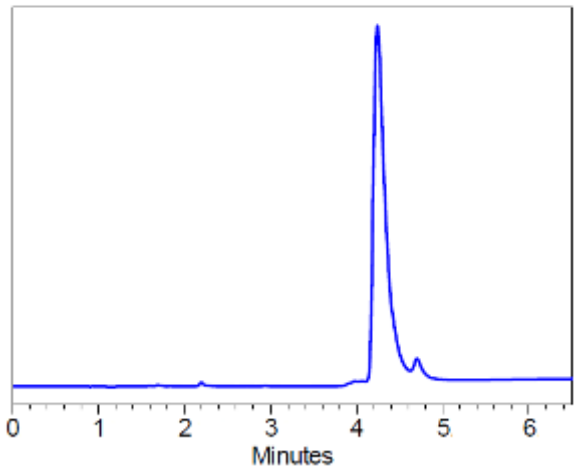


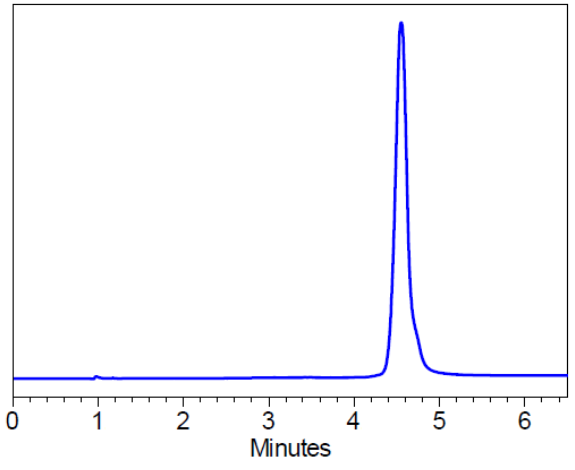




C18 - 3.6 µm SPP



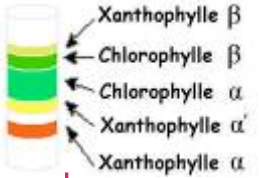




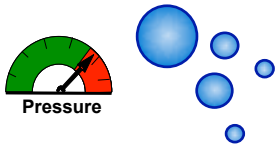
Impressive peak shapes for RPLC of an intact 150 kDa antibody

Evolution of liquid chromatography

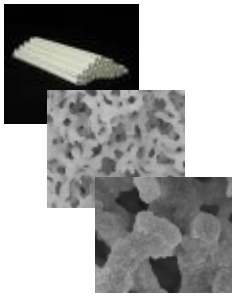
Discovery of chromatography



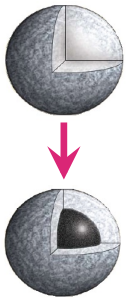
UHPLC and sub-2µm columns



1903 | 2000 | 2004 | 2007 | 2015 | 2030



Monoliths



SPP

- Instrumentation – robustness
- Miniaturization (3D printed micro devices)
- New stationary phases: monoliths, pillar array, Sphere-on sphere particles, silica-colloidal particles, PLOT, parallel-segmented flow chromatography
- 2D or 3D chromatography
- New MS or detection systems

Acknowledgments

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Aurélie Periat

Serge Rudaz

Jean-Luc Wolfender

Alain Beck

Thank you for your attention !

Réponses aux questions

1. La chromatographie en phase supercritique

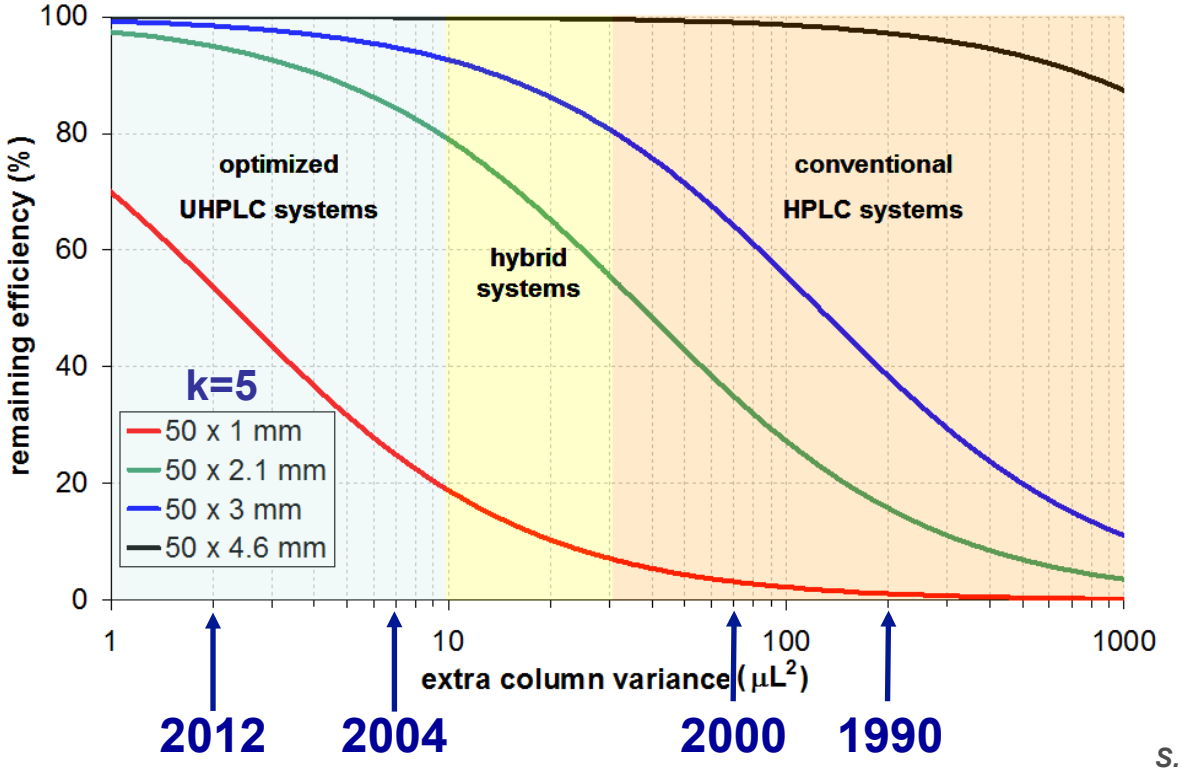
A : est réservée aux composés apolaires	NON
B : permet des séparations rapides	OUI
C : est adaptée aux biomolécules de grande taille	NON
D : utilise une phase mobile constituée d'un mélange de CO_2 et de méthanol	OUI
E : peut-être couplée à la spectrométrie de masse	OUI

Instrumental constraints

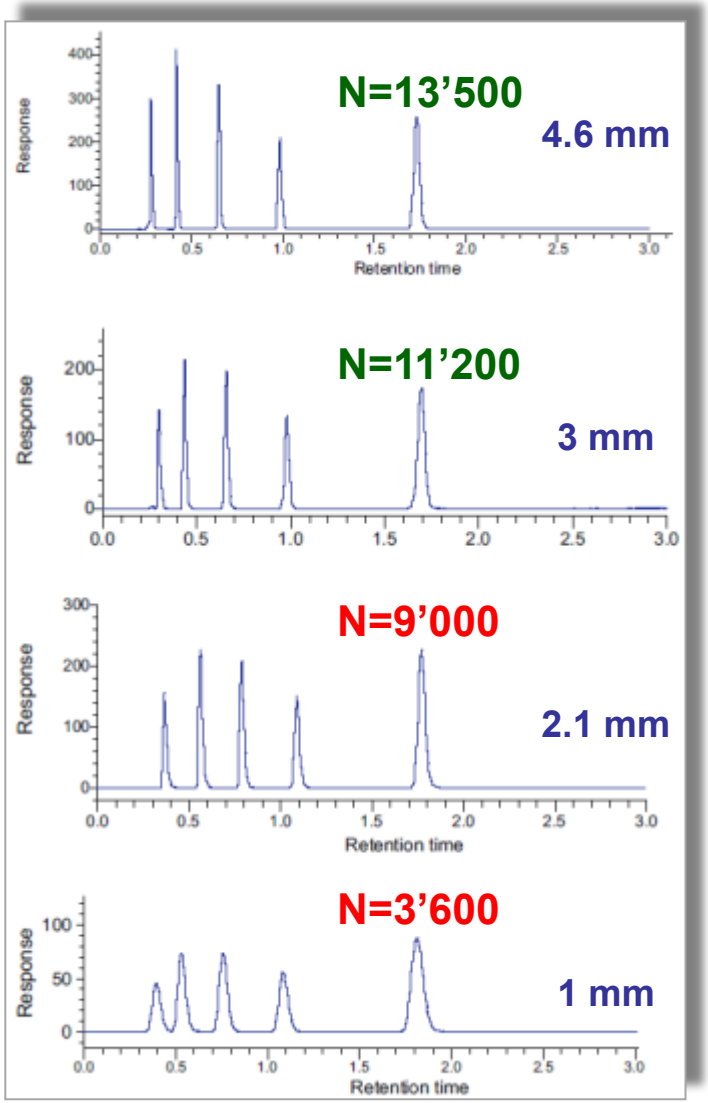
$$\sigma^2_{col} = \frac{V_r^2}{N_{col}} = \frac{(V_0 \cdot (1+k))^2}{N_{col}}$$

$$\sigma^2_{ext} = K_{inj}\frac{V^2_{inj}}{12} + K_{cell}\frac{V^2_{cell}}{12} + \tau^2 F^2 + \frac{r_c^4 \times l_c \times F}{7.6 \times D_m}$$

injector detector tubing

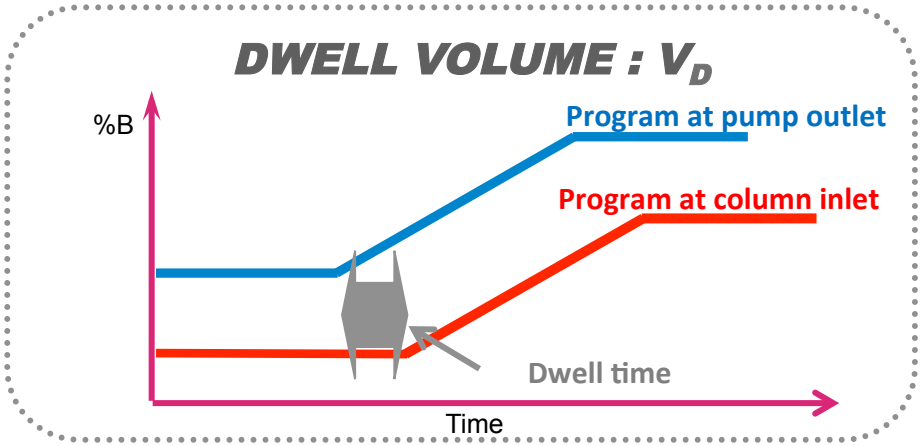


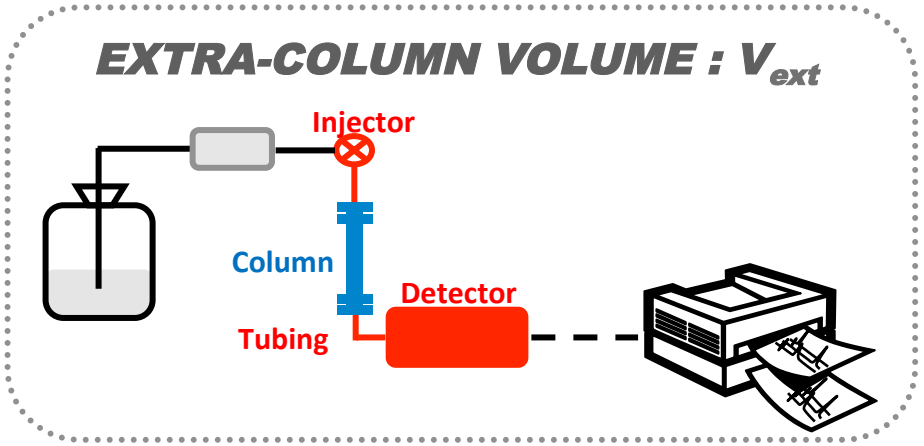
σ^2_{ext} = 20 µL²



S. Fekete et al. J. Pharm. Biomed. Anal., 87, 2014, 105-119

A new generation of UHPSFC systems

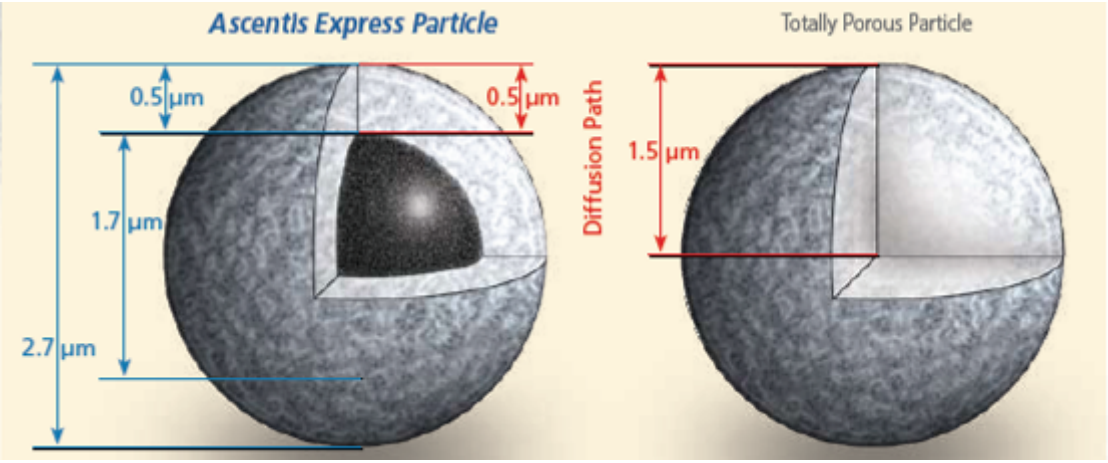




	 SFC	 UHPLC	 UPC²
Dwell volume (V_D)	2270 µL	90 µL	440 µL
Isocratic step for generic conditions	1.13 min (F = 2 mL/min)	0.15 min (F = 0.6 mL/min)	0.22 min (F = 2 mL/min)
Extra column volume (V_{ext})	118 µL	13 µL	60 µL
Suitable column dimensions	150 x 4.6 mm (volume ~ 1750 µL)	50 x 2.1 mm (volume ~ 120 µL)	100 x 3 mm (volume ~ 490 µL)

Superficially porous particles : SPP

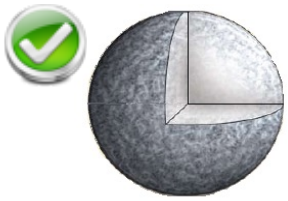
❖ A small layer (0.5µm) of uniform porous particles is grown around a solid core of non porous silica material (1.7µm).



www.sigmaaldrich.com

- **Lower mass transfer resistance, longitudinal and eddy diffusion:** better chromatographic performance
- Compatible with **fast analysis and high resolution chromatography**
- Initially developed for **high molecular weight compounds** (MW>600g.mol^{-1}) such as peptides and proteins (low D_m) but today applied to all molecules

SPP: a good compromise…



Fully porous particles (FPP)

Good kinetic performance

Good mechanical stability

Large specific surface area

- **High loading capacity**
- **Good retention**

High backpressure with small particles

Superficially porous particles (SPP)

Diameter: 2.6-2.7 µm

Porous layer: 0.35-0.5 µm

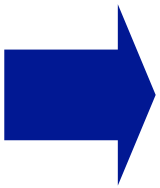
Non-porous particles (NPP)

Low specific surface area

- **Poor loading capacity**
- **Limited retention**

High kinetic performance

Huge mechanical stability

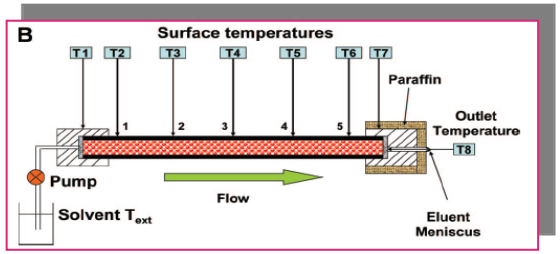


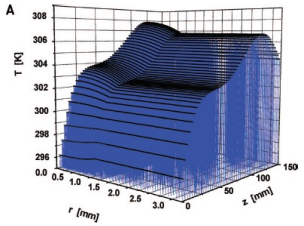
Good loading capacity and retention

Large choice of column dimensions and chemistries/providers

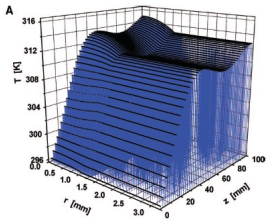
Frictional heating in UHPLC ?

Theoretical approach

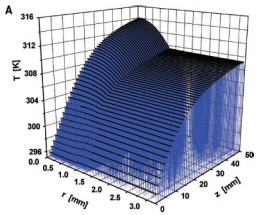




150x3mm I.D., 1.7µm



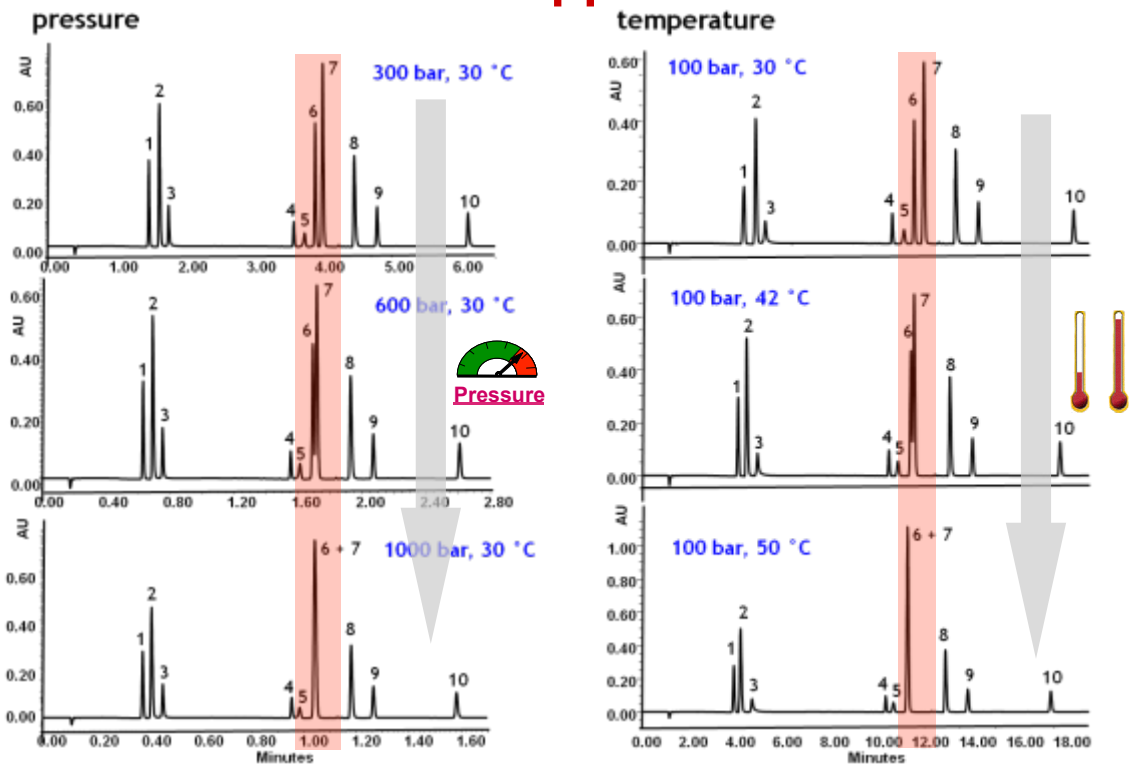
100x3mm I.D., 1.7µm



50x3mm I.D., 1.7µm

F. Gritti, G. Guiochon, Anal. Chem., 2008

Practical approach



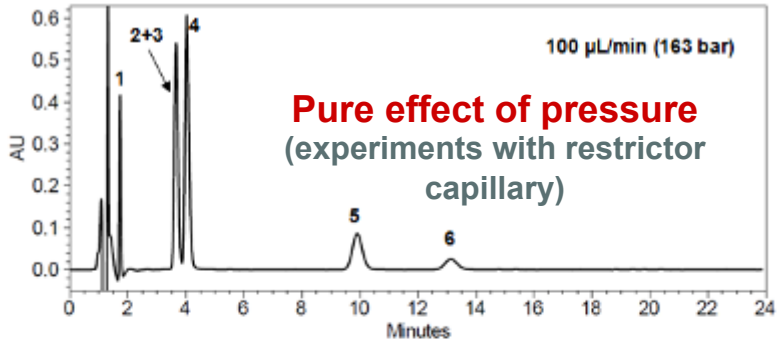
Analytical column:	2.1 mm I.D. 1.7 µm		1.0 mm I.D. 1.7 µm	
	ΔT [°C] pH 3.0	ΔT [°C] pH 9.0	ΔT [°C] pH 3.0	ΔT [°C] pH 9.0
100 bar	+ 0	+ 0	+ 0	+ 0
300 bar	+ 4	+ 4	+ 3	+ 3
600 bar	+ 8	+ 8	+ 6	+ 6
1000 bar	+ 16	+ 16	+ 12/+13	+ 12/+13

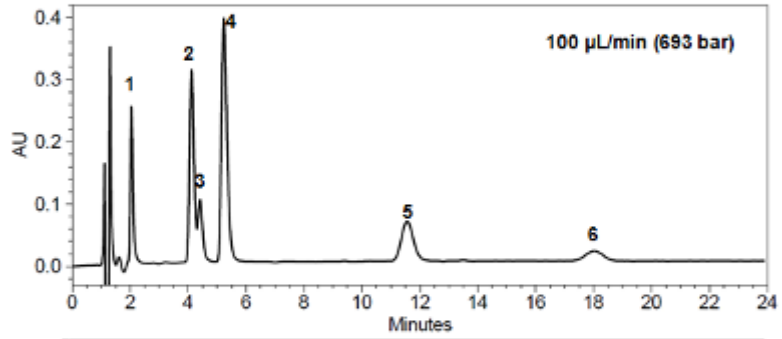
L. Novakova, J.L. Veuthey, D. Guillarme, J. Chromatogr. A, (2011), 1218, 7971-7981

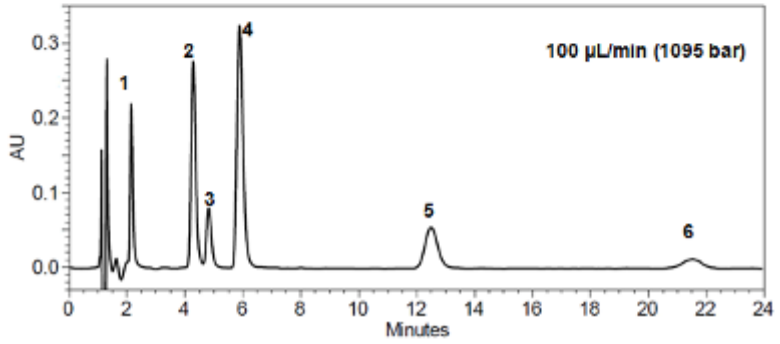
Effect of high pressure on retention

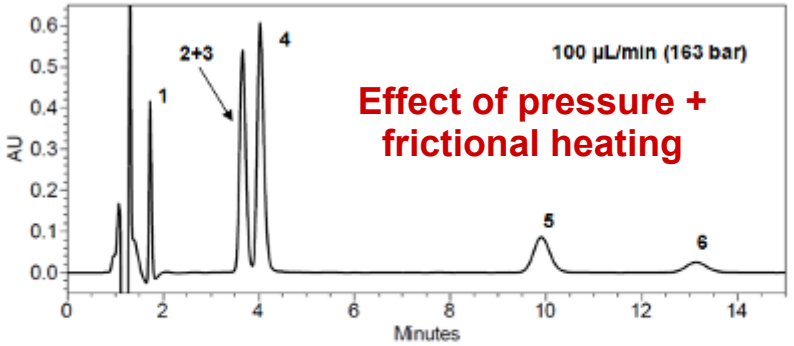
Case of small analytes

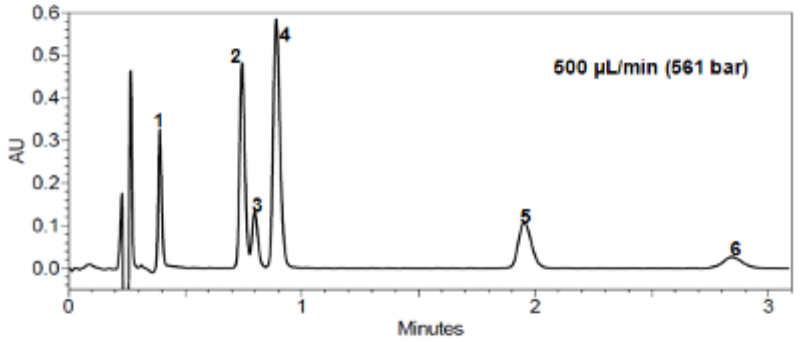
Column: C18 (50 mm x 2.1 mm, 1.7µm), mobile phase: water (0.1% TFA) + acetonitrile (0.1% TFA) : 70 + 30 v/v, flow-rate: varied between 100, 500 and 900 µL/min, temperature: 30 °C, injected volume: 0.5 µL, detection: 210 nm. Peaks: lidocaine (1), salicylic acid (2), bupivacaine (3), propranolol (4), propylparaben (5) and testosterone (6).

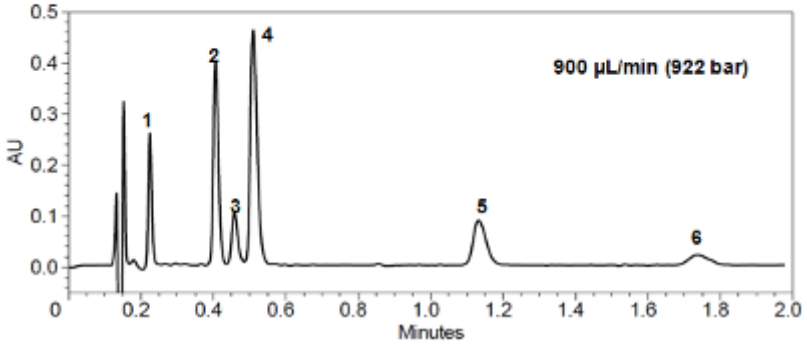








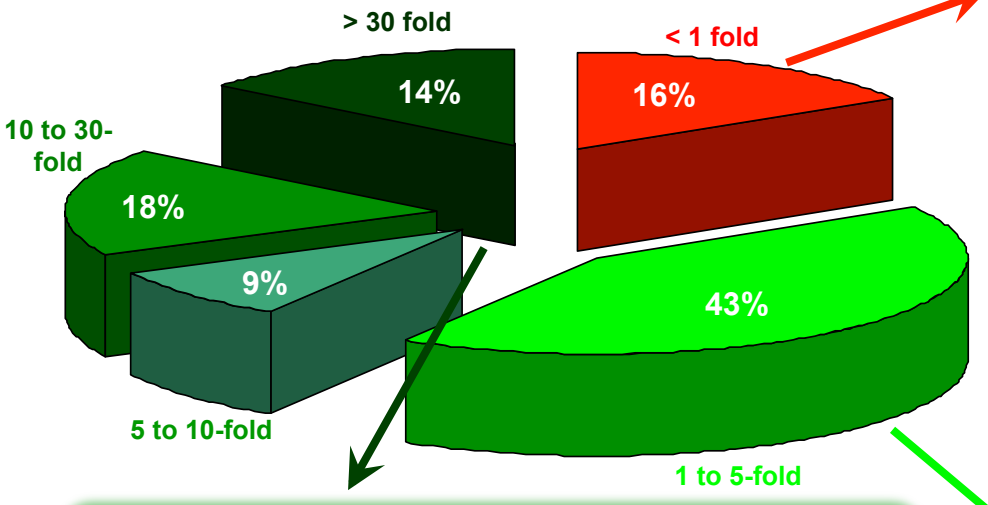


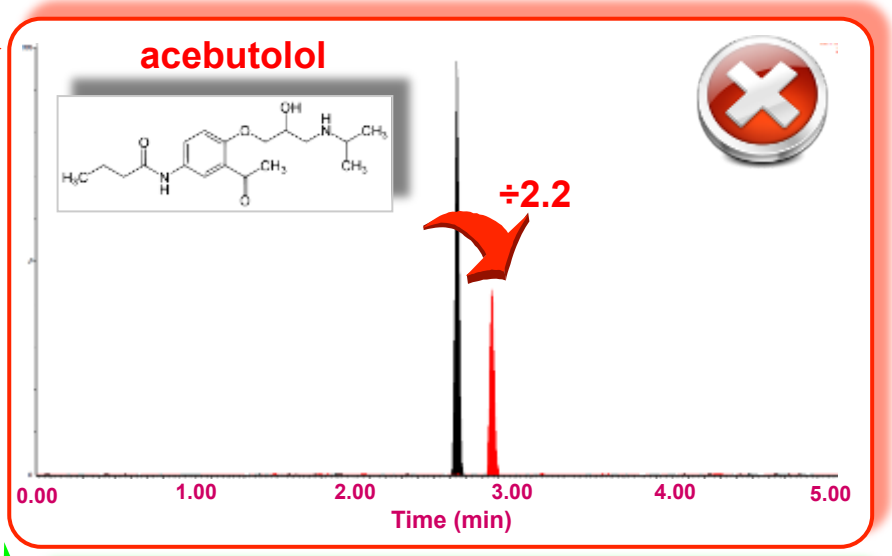


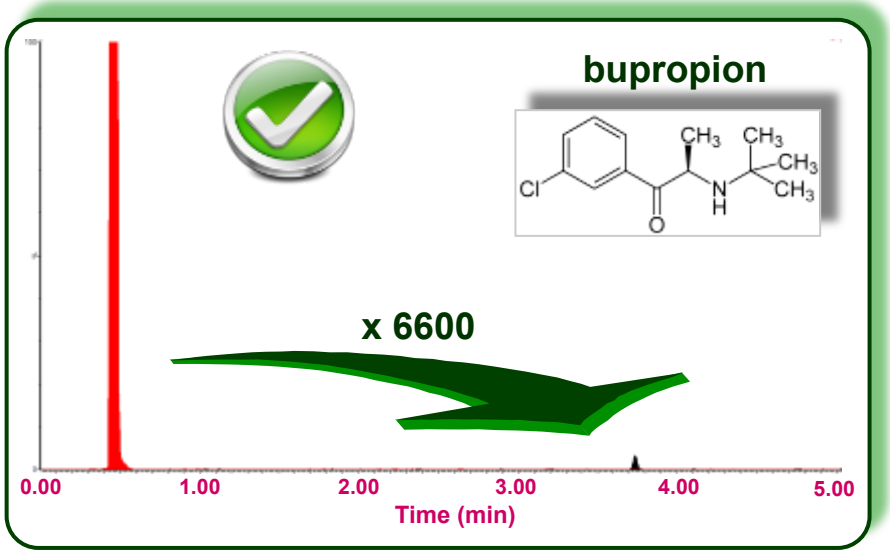
Sensitivity in HILIC-MS *vs.* RPLC-MS ?

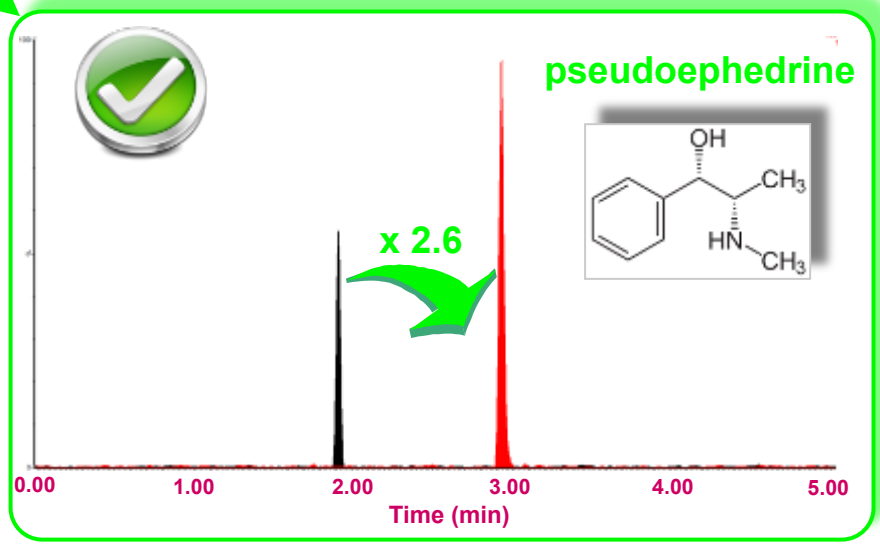
56 basic drugs of diverse polarity were tested on modern UHPLC-ESI-MS/MS instrument

Gain in sensitivity in HILIC vs. RPLC @ 600µL/min



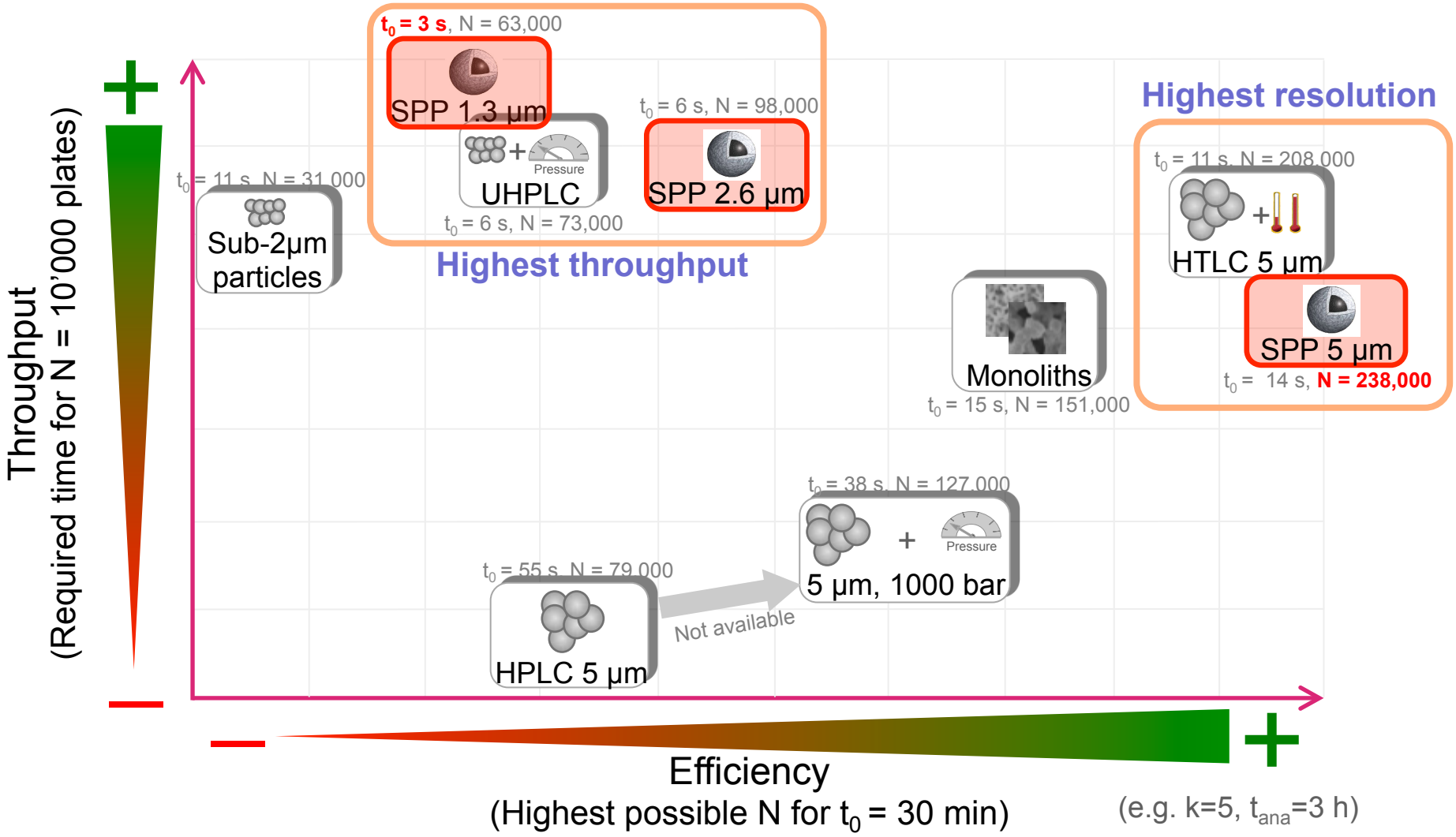






Comparison of current LC technologies

Comparison made for butylparaben (MW=200 g.mol^{-1}) with η=0.89 cp @ 30°C and 0.41 cp @ 90°C. Pressure was equal to 400 bar in all cases except those cited at 1000 bar and UHPLC and SPP 1.3µm, monoliths at 200 bar and SPP 2.6 µm at 600 bar.



D. Guillarme et al., Anal. Bioanal. Chem., 2010, 397, 1069