Pharmaceutical Related Substances HPLC / UHPLC Method Development and Translations

Alan P McKeown
amckeown@ace-hplc.com

Business Development Director

Advanced Chromatography Technologies Ltd

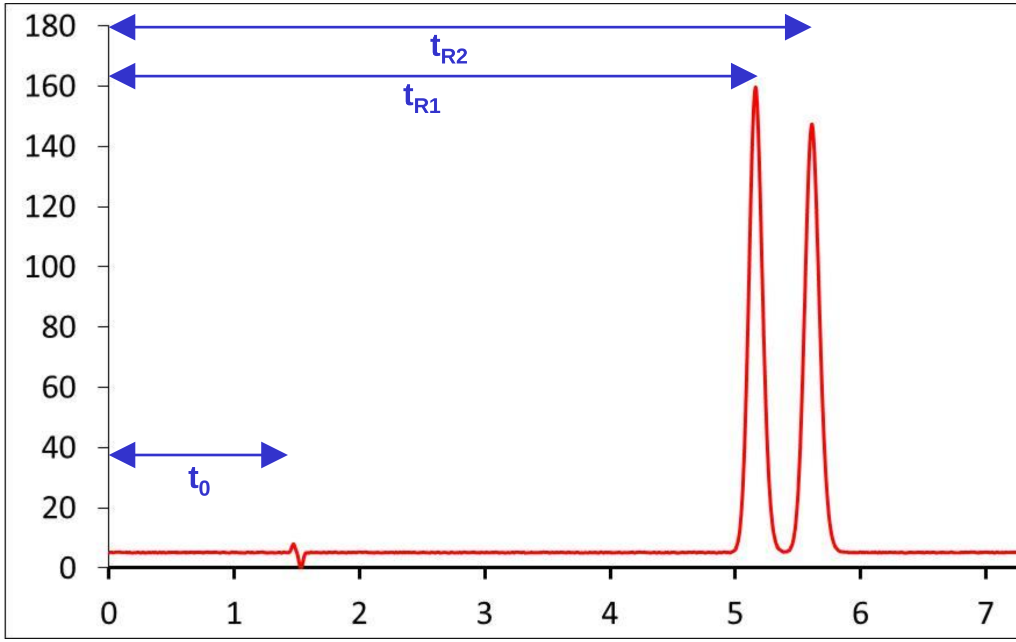
www.ace-hplc.com

Outline

- What is selectivity?
- Optimizing method development based on selectivity
- Example related substances method development
- Method translations – isocratic and gradient
- Maximizing resolution for complex samples

What is Selectivity?

- **Alpha (α) is the symbol used to denote the separation factor or separation selectivity between 2 adjacent peaks.**



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

$$\alpha = \frac{k_2}{k_1}$$

- **Selectivity can be thought of as 'peak spacing'.**
- **Selectivity values should be > 1.0**

Resolution, Selectivity, Efficiency & Retention

Particle size, column length, dispersion etc

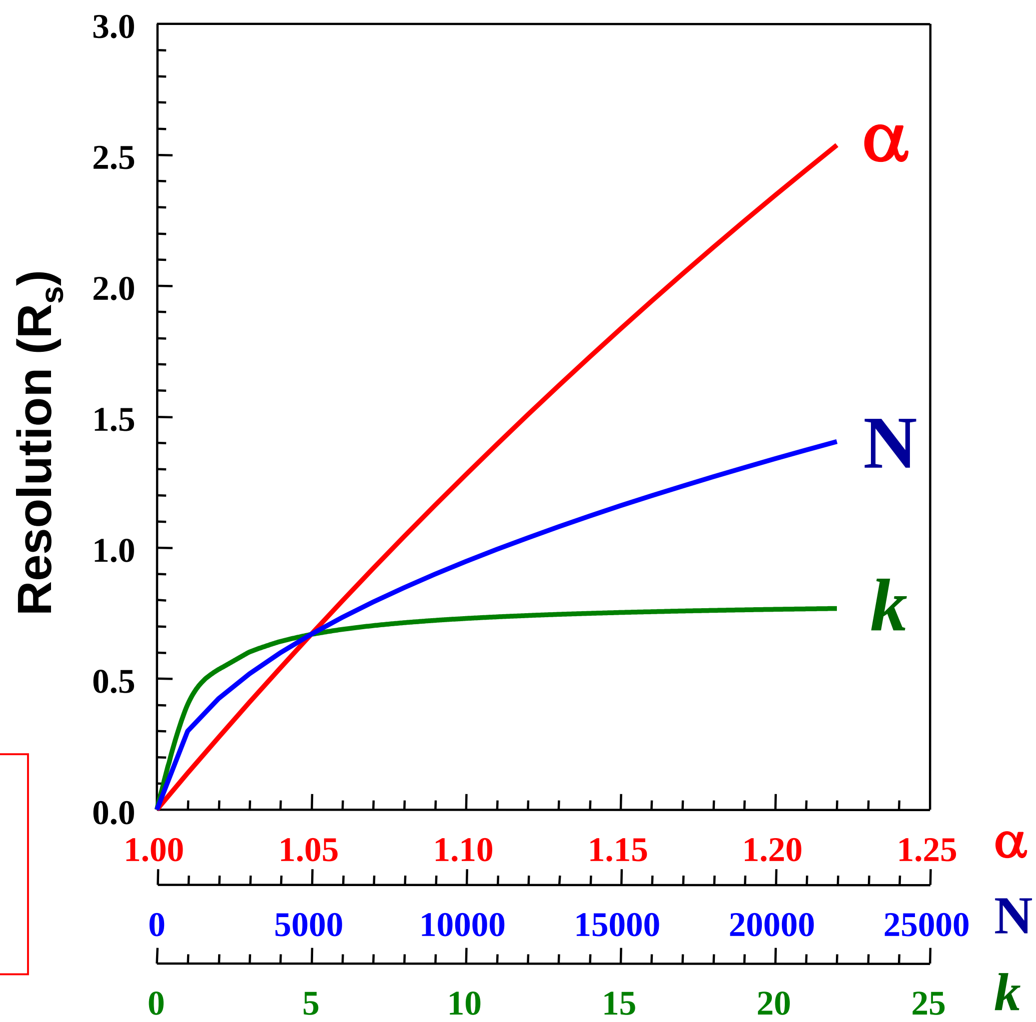
Phase design, eluent etc

Efficiency

Selectivity

Retention

$$R_s = \frac{\sqrt{N}}{4} \; \frac{\alpha-1}{\alpha} \; \frac{k}{1+k}$$



Zhao, J.H. and P.W. Carr. Analytical Chemistry, (1999) 71, 2623-2632

Selectivity (α) is the key to resolution and efficiency (N) boosts performance

Which Factors[1] Affect Selectivity?

- Strongly influenced by physicochemical properties of the analyte, stationary phase, eluent etc
- From a practical perspective:

Isocratic Separations

MOST Influence

- Column stationary phase type
- pH (ionisable analytes only)
- Organic modifier type
- % Organic modifier
- Buffer selection
- Column temperature
- Buffer concentration

LEAST Influence

Gradient Separations

All parameters for isocratic **PLUS**

Gradient steepness,

k^* (t_G, F, V_m, ΔΦ, M),

$$k^* = \frac{t_G F}{\Delta\Phi V_m M}$$

Dwell volume,

Column dimensions.

1 Adapted from 'Introduction to Modern Liquid Chromatography", 3rd Edition, Snyder, Kirkland, Dolan, 2010, p.29, Wiley & sons

Method Development Strategies: Overview

- **Selectivity is integral to method development**

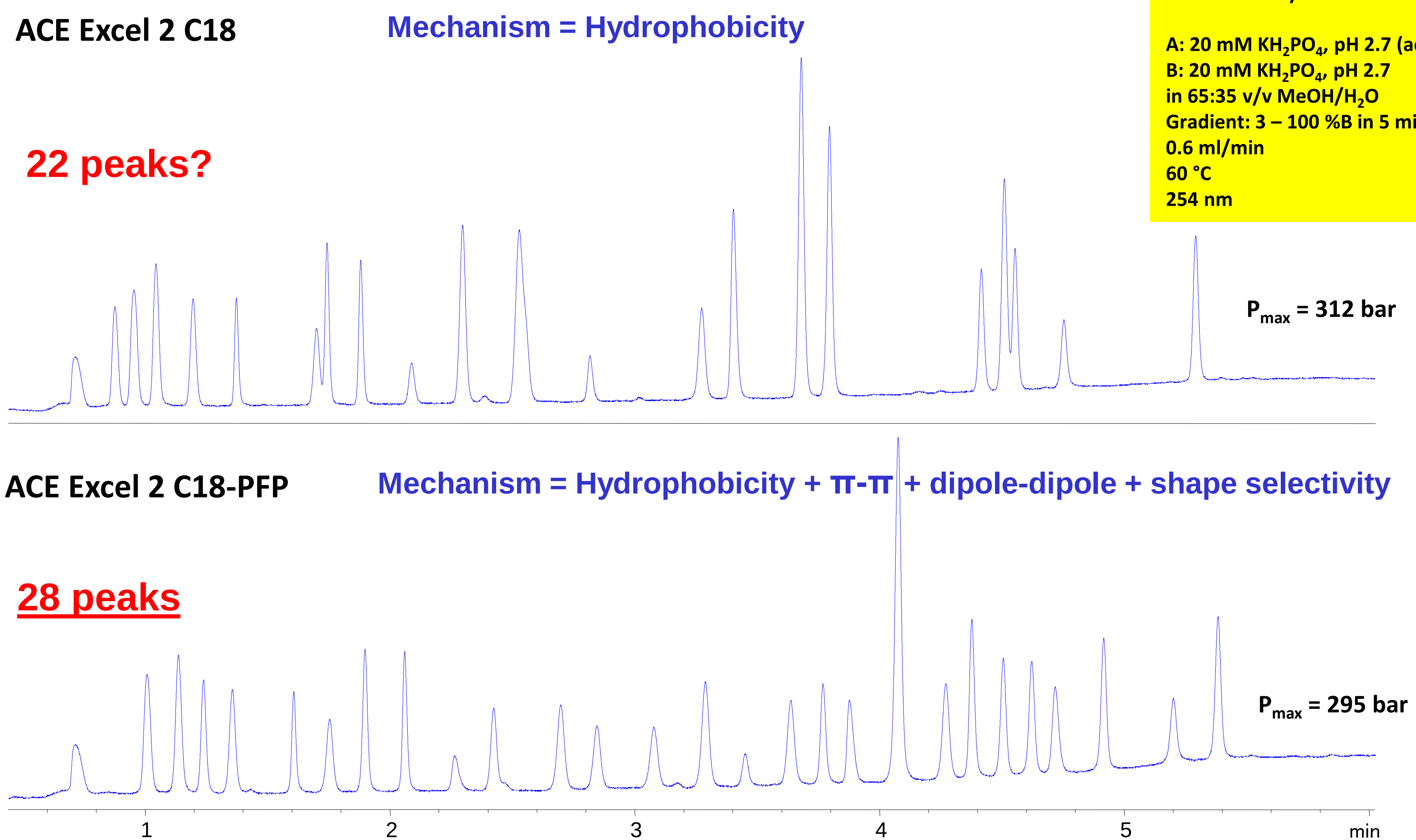
- 1 column - 1 temperature - 1 pH - 1 organic modifier - 1 x tG	- 1 column - 2 temperatures - 1 pH - 1 organic modifier - 2 x tG	- 1 column - 2 temperatures - 1 pH - 2 organic modifier - 2 x tG	- ≥ 2 columns - 2 temperatures - 1 pH - 2 organic modifier - 2 x tG	- ≥ 2 column - 2 temperatures - 2 or 3 pH - 2 organic modifier - 2 x tG
	20C & 60C	MeOH & MeCN	Alkyl chains eg SuperC18, C18, C8 Aromatic eg Phenyl, C18-AR or C18-PFP Polar eg C18-PFP, C18-Amide CN-ES	pH 2.5 pH 7 pH 10.7

INCREASING COMPLEXITY

Degraded Sample With Unknown Number of Peaks

50x2.1mm, 2um
Gradient analysis

A: 20 mM KH_2PO_4, pH 2.7 (aq)
B: 20 mM KH_2PO_4, pH 2.7
in 65:35 v/v MeOH/H_2O
Gradient: 3 – 100 %B in 5 mins
0.6 ml/min
60 °C
254 nm

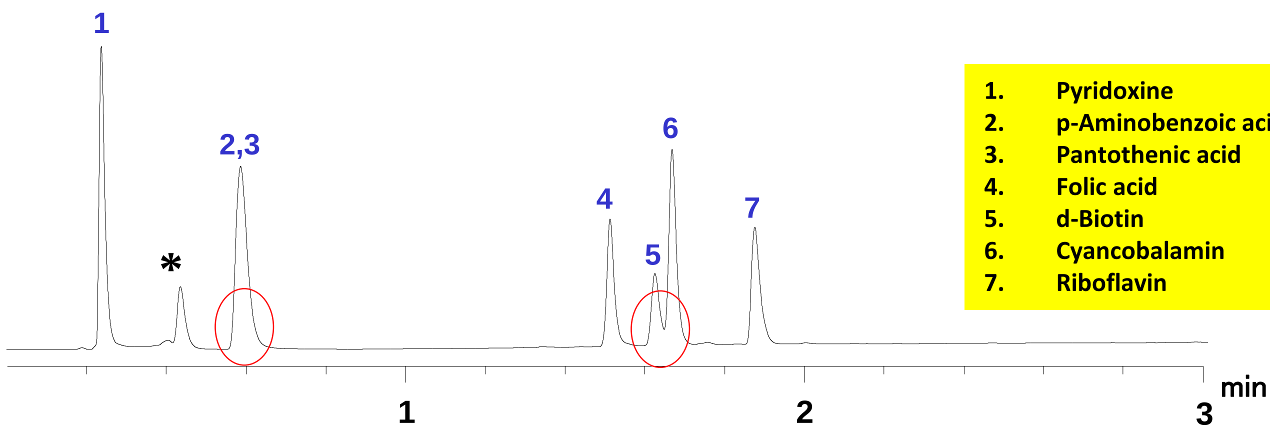


Stationary Phase Selectivity Is Powerful

ACE® UltraCore™: Bonded Phase Selectivity

♦ Exploring selectivity with stationary phase type:

ACE UltraCore 2.5 SuperC18



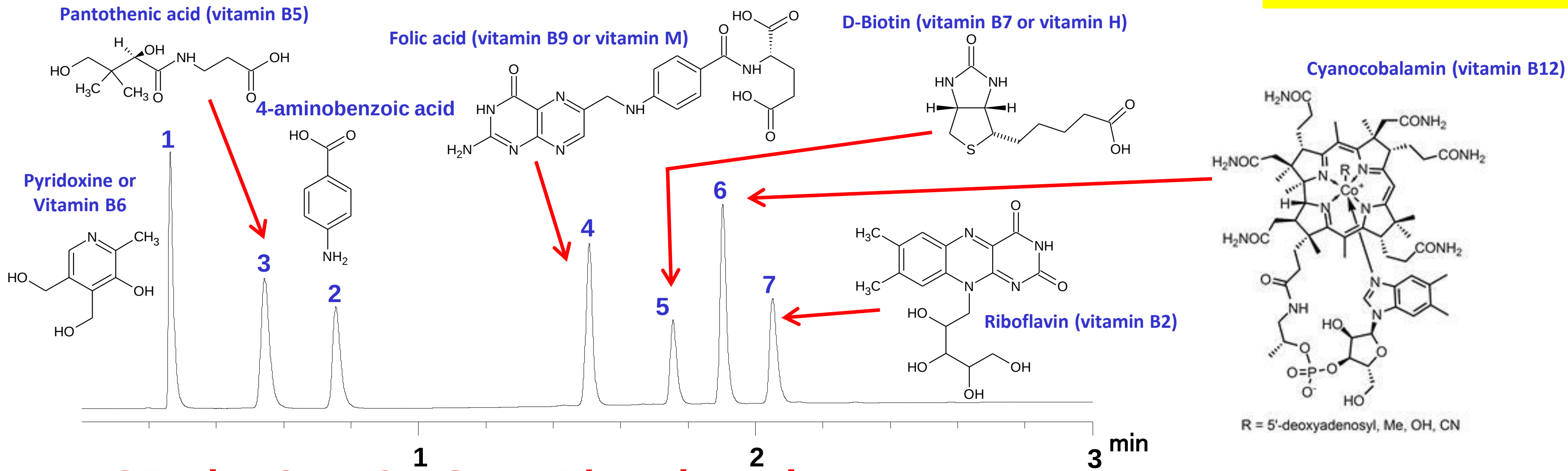
1. Pyridoxine
2. p-Aminobenzoic acid
3. Pantothenic acid
4. Folic acid
5. d-Biotin
6. Cyancobalamin
7. Riboflavin

50x2.1mm, 2.5µm
Gradient analysis

A: 20 mM KH_2PO_4 pH 2.7 (aq)
B: 20 mM KH_2PO_4 pH 2.7 in MeOH:Water 50:50 v/v

T	%B
0	20
1	60
2	70

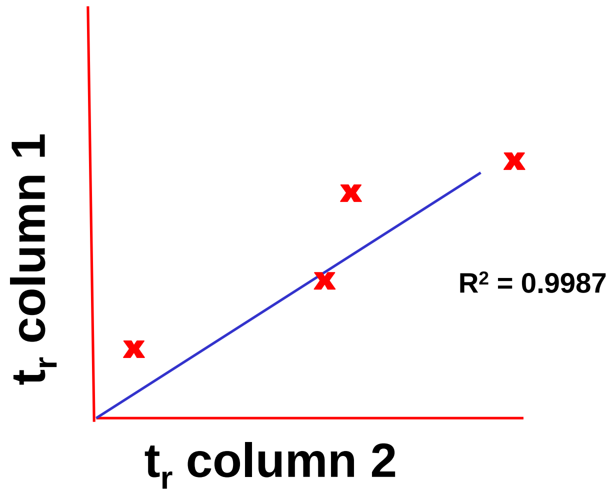
40 °C
0.60 mL/min
205 nm



ACE UltraCore 2.5 SuperPhenylHexyl

Selectivity Descriptor*

Selectivity = 100 x $\sqrt{(1 - R^2)}$

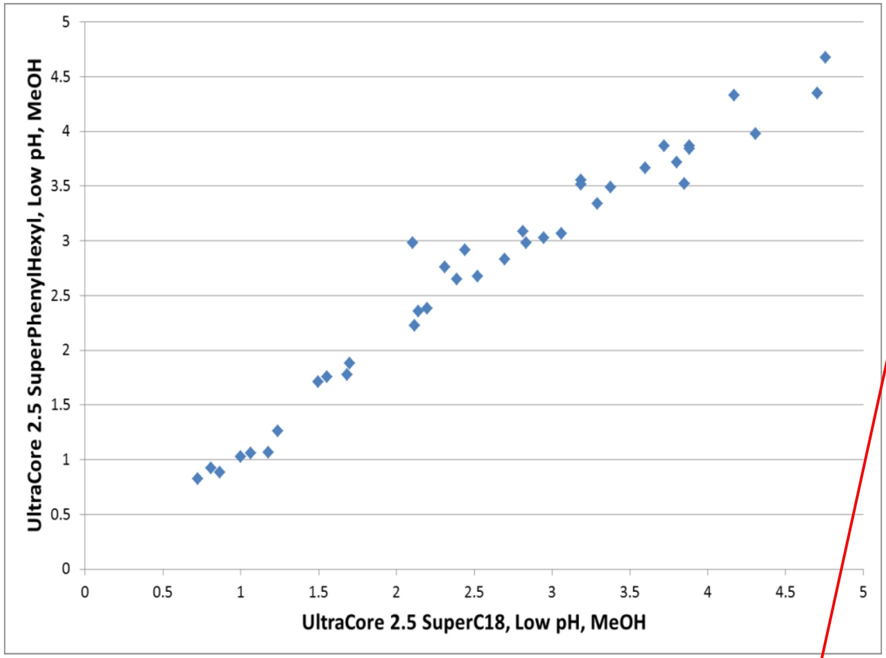


- **Selectivity values from ~8 upwards indicate suitable changes in selectivity for method development.**
- **Large Selectivity values can be achieved with multiple parameter changes.**

*** Neue, O'Gara, Méndez "Selectivity in Reversed-Phase Separations: Influence of the Stationary Phase", J. Chromatogr. A 1127 (2006), 161-174.**

Selectivity Plot: Exploring The Effect Of Solid Core Phase

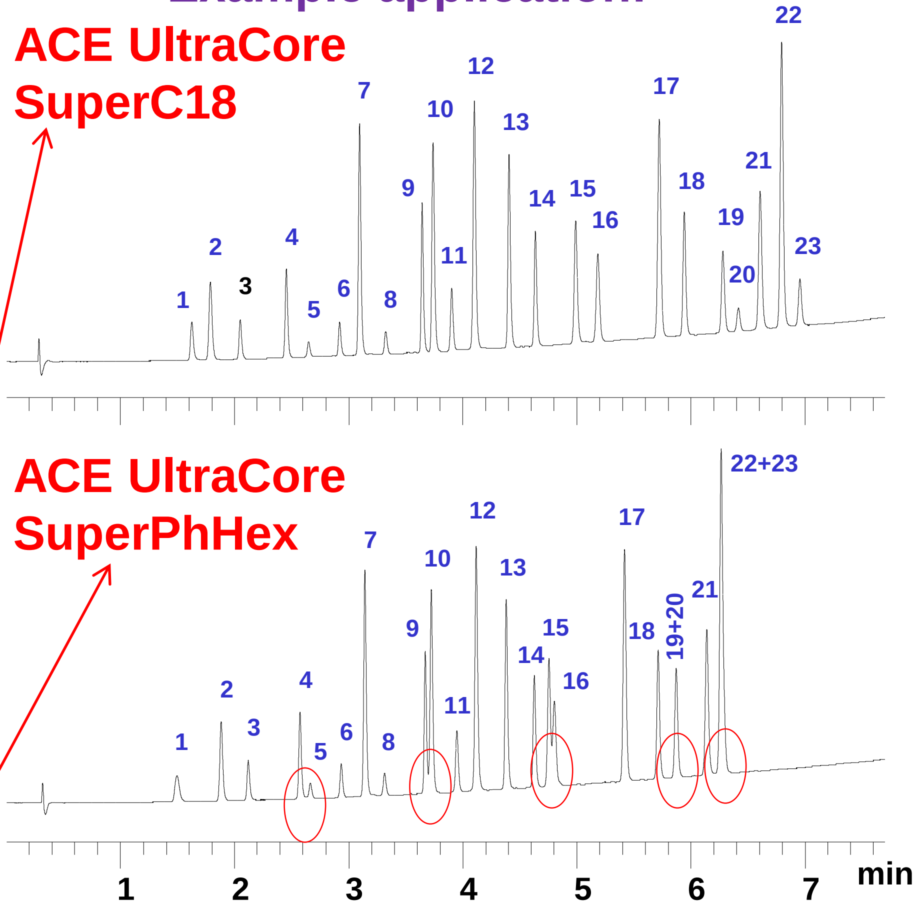
SuperC18, low pH, MeOH vs SuperPhenylHexyl, low pH, MeOH



Selectivity = 19

Changes in peak spacing noted

Example application:

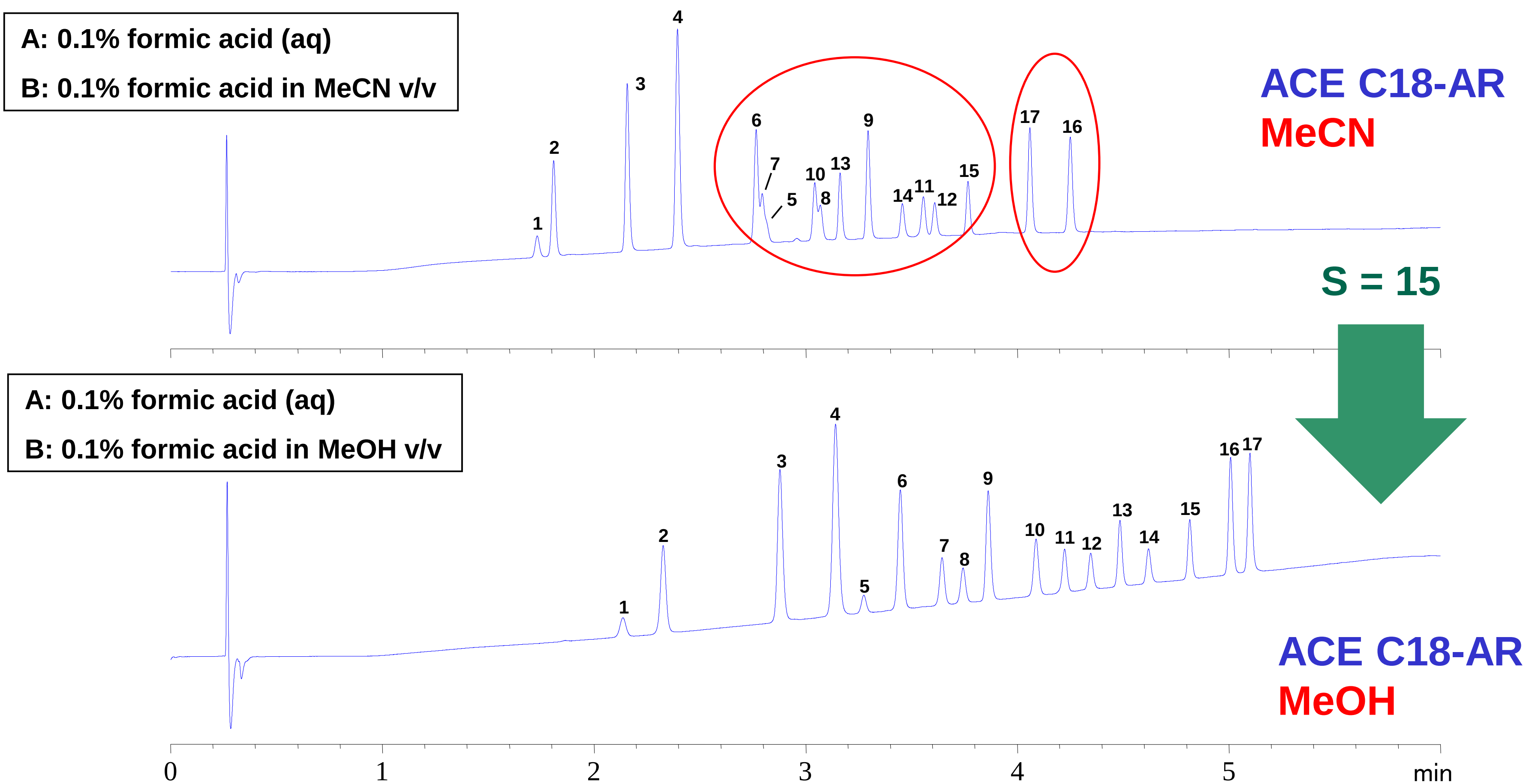


50x2.1mm, 2.5µm, gradient analysis, A= 20mM $HCOONH_4$, pH3 (aq), B= 20mM $HCOONH_4$, pH 3 in MeCN/water 9:1 v/v, 3-100%B in 7.5 mins, hold 100%B for 1.5 mins, 40°C, 0.40 mL/min, 254 nm.

1 amiloride, 2 benzamide, 3 3-hydroxybenzoic acid, 4 vanillin, 5 2-hydroxybenzoic acid, 6 benzoic acid, 7 methyl paraben, 8 p-cresol, 9 cortisone, 10 ethyl paraben, 11 dimethylpthalate, 12 piroxicam, 13 hydro cortisone-21-acetate, 14 ketoprofen, 15 ethylbenzoate, 16 toluene, 17 valerophenone, 18 mefenamic acid 19 hexanophenone, 20 propylbenzene, 21 phenanthrene, 22 heptaphenone, 23 butylbenzene

ACE® C18-AR: Solvent Selectivity

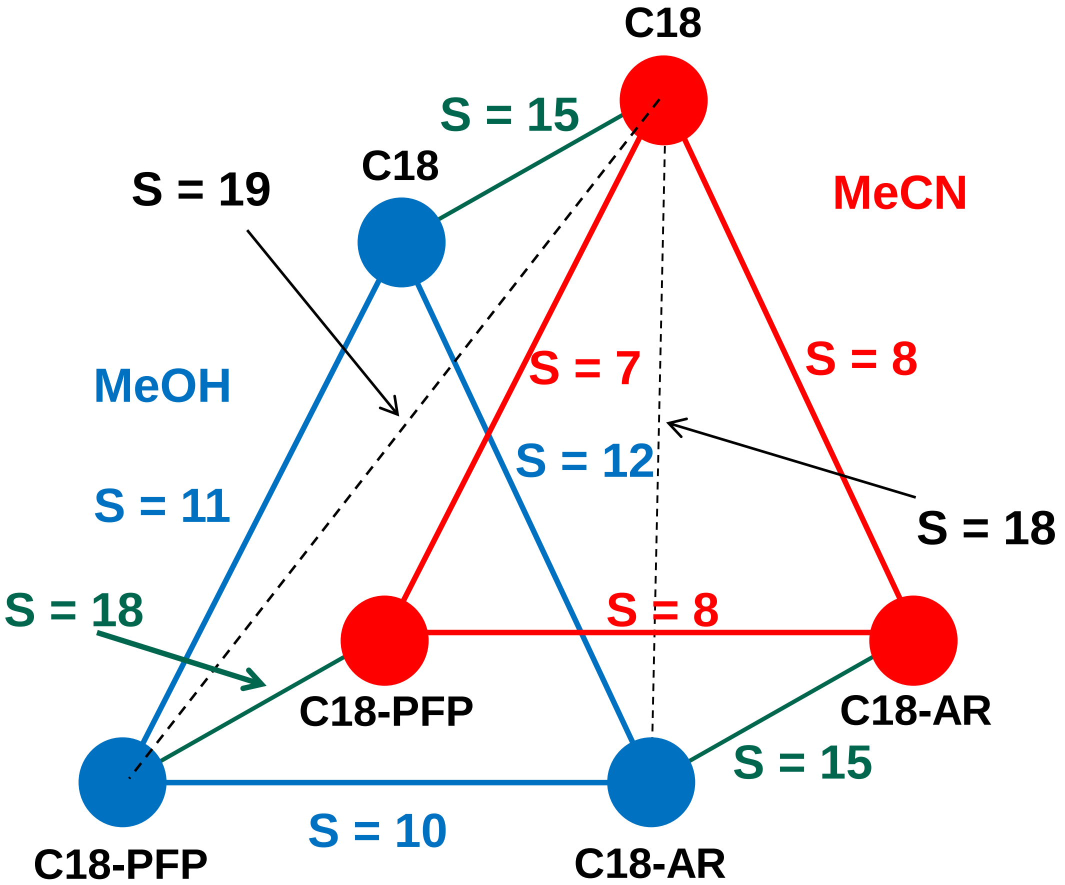
- **Exploring selectivity with solvent type:**



50x2.1mm, 3 µm, gradient analysis, 3-100%B in 5.0 mins, hold 100%B for 1.0 mins, 40°C, 0.60 mL/min, 254 nm.
1 3-hydroxybenzoic acid, 2 methylphenylsulfoxide, 3 quinoxaline, 4 salicylic acid, 5 benzylcyanide, 6 1,2-dimethoxybenzene, 7 ethyl paraben, 8 1,4-dimethoxybenzene, 9 bendroflumethiazide, 10 piroxicam, 11 benzylchloride, 12 thioanisole, 13 sulindac, 14 chrysin, 15 ibuprofen, 16 1,2,3-trichlorobenzene, 17 meclofenamic acid

ACE® 3 Phases, Exploring Selectivity, MD Approach

- 102 analytes, 3 stationary phases, 2 solvents



ACE C18

- Hydrophobic

ACE C18-AR

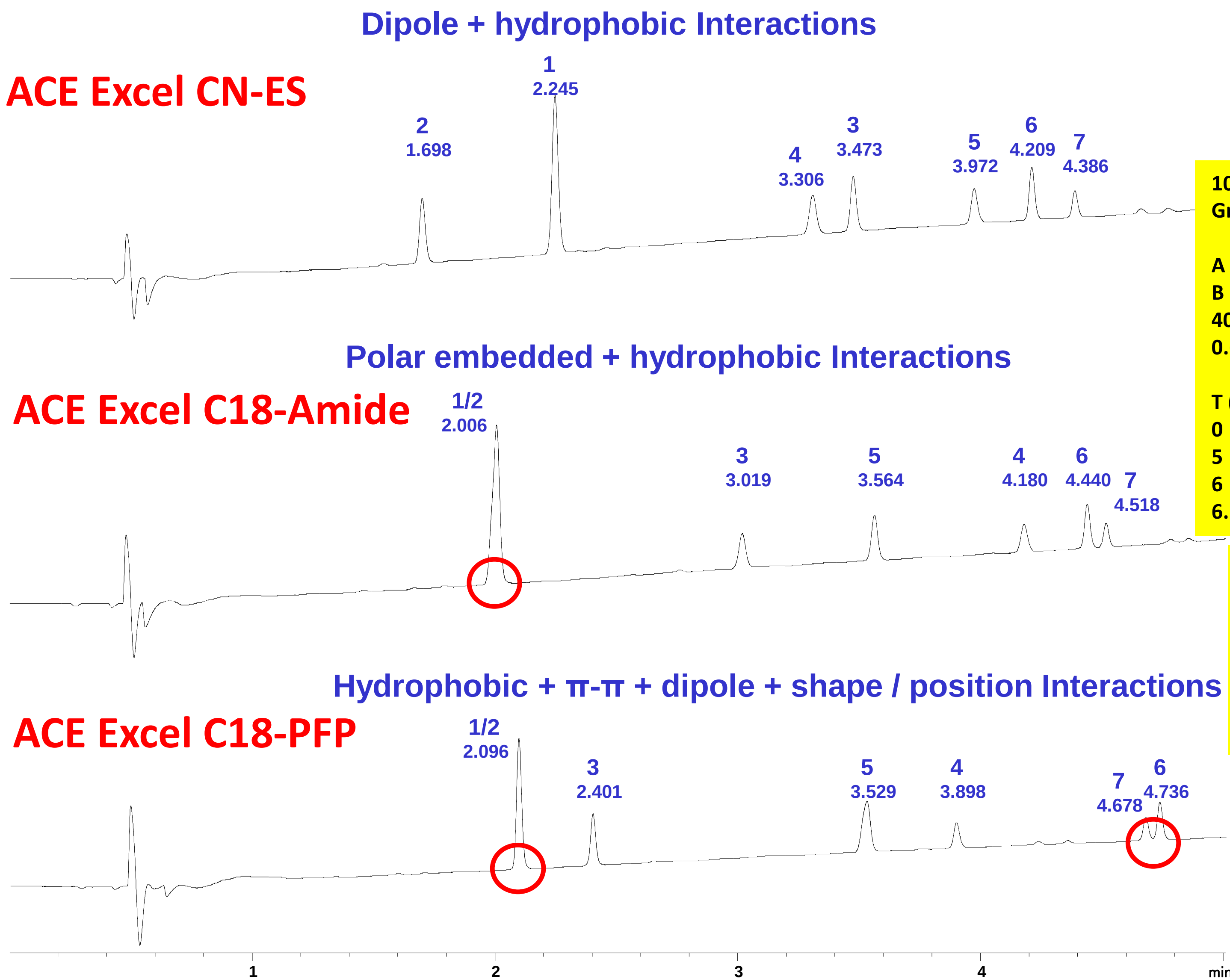
- Hydrophobic
- π-π interactions

ACE C18-PFP

- Hydrophobic
- π-π interactions
- Dipole-dipole
- Shape selectivity

Varied Mechanisms...Helpful For Selectivity And Method Development

Choose Your Phases To Maximize Selectivity



100x3mm, 3µm
Gradient analysis

A = 20mM ammonium formate (aq)
B = 20mM ammonium formate in MeOH
40C
0.6 mL/min

T (min)	%B
0	3
5	100
6	100
6.5	3

1. Hydrochlorothiazide
2. Methylphenylsulphoxide
3. 1,3,5 Trinitrobenzene
4. Myricetin
5. P-Cresol
6. Sulindac
7. Toluene

Selectivity Based Method Development Workflow

- 3 columns - 1 temperatures - 1 pH - 1 organic modifier - 1 x t_G	- 3 column - 1 temperatures - 1 pH - 2 organic modifier - 1 x t_G
1. C18, C18-AR, C18-PFP 2. SuperC18, C18-Amide & CN-ES	MeOH MeCN

Information Rich Data

3 columns, 2 solvents method development / screening approach based on selectivity data

Summary #1: Selectivity and Method Development Workflows

- Selectivity is the key to resolution
- Method and instrument parameters can affect selectivity. Understanding selectivity ensures the chromatographer focusses on the key variables
- Optimizing workflows to maintain retention information but reduce development time is possible
- A 3 phase, 2 solvent optimized method development platform based on selectivity has been described

General LC Method Development Approach

Overview of method development steps

- **Step 1**: Scouting runs with general starting conditions
- **Step 2**: Optimize for peak shape, run time etc
- **Step 3**: Validate according to local guidance
- **Step 4**: Transfer / Implement

General Method Development Initial Conditions

- Perform a **broad scouting gradient** run on the samples at **acidic eluent** pH

- How do you calculate your starting conditions?

For a <u>100 x 3mm</u> column:

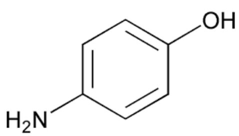
t_G	= 5 minutes
F	= 1.2 mL/min
$\Delta\phi$	= 0.95
V_m	= 0.459 mL
M	= 5

$$k^* = \frac{t_G F}{\Delta\Phi V_m M} = \sim 3$$

- Ideally retention (or *k** in gradient elution) should be >2 and <20 for initial method development

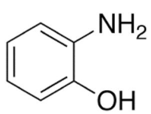
Selected Acetaminophen Impurities For Method Development

1.	4-aminophenol	synthesis/degradation product
2.	Hydroquinone	deg product of 4-amino phenol
3.	2-aminophenol	Included as would be a synthesis impurity of (8) if not fully removed
4.	Acetaminophen	API (a.k.a. Paracetamol)
5.	2-acetamidophenol	Specified in USP
6.	Phenol	Included as extra compound
7.	4-nitrophenol	Ph. Eur. related substance
8.	2-nitrophenol	Synthesis impurity of 4-amino phenol (39% yield, normally removed by steam distillation)
9.	4-chloroacetanilide	Eu. Ph. Related substance
10.	4-chlorophenol	Potential low level impurity

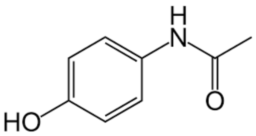


4-aminophenol
pKa = 5.28, 10.17
LogP = -0.29
LogD 5.5 = -0.04
LogD 7.4 = 0.16

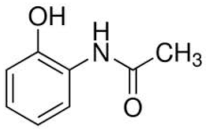
Hydroquinone
pKa = 10.33
LogP = 0.64
LogD 5.5 = 0.53
LogD 7.4 = 0.53



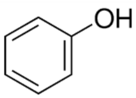
2-aminophenol
pKa = 4.84, 10.01
LogP = 0.44
LogD 5.5 = 0.58
LogD 7.4 = 0.64



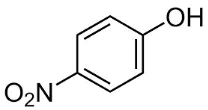
Acetaminophen
(Paracetamol)
pKa = 9.86
LogP = 0.34
LogD 5.5 = 0.4
LogD 7.4 = 0.4



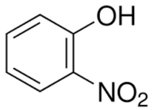
2-acetamidophenol
pKa = 8.79
LogP = 0.72
LogD 5.5 = 0.79
LogD 7.4 = 0.79



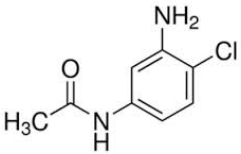
Phenol
pKa = 9.95
LogP = 1.48
LogD 5.5 = 1.63
LogD 7.4 = 1.63



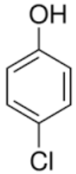
4-nitrophenol
pKa = 7.23
LogP = 1.57
LogD 5.5 = 1.7
LogD 7.4 = 1.31



2-nitrophenol
pKa = 7.23
LogP = 1.71
LogD 5.5 = 1.7
LogD 7.4 = 1.26



4-chloroacetanilide
pKa = -1.97/14.25
LogP = 2.05
LogD 5.5 = 2.14
LogD 7.4 = 2.14

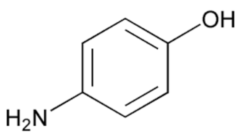


4-chlorophenol
pka = 9.3
LogP = 2.43
LogD 5.5 = 2.43
LogD 7.4 = 2.42

Initial Acetaminophen UHPLC Screening Chromatograms

Conditions

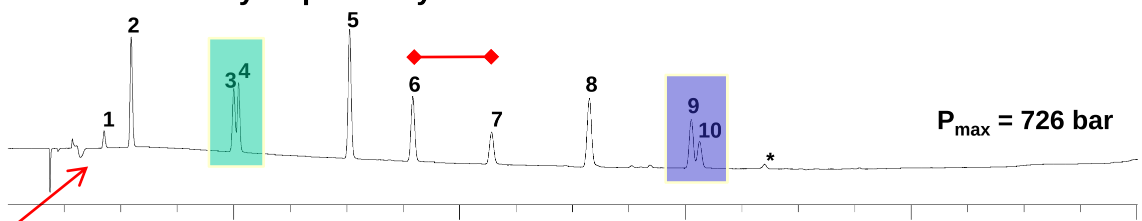
A: 20 mM ammonium acetate pH 6.0
B: 20 mM ammonium acetate pH 6.0 in MeCN:water 9:1 *v/v*
Gradient: 0-95% B in 10 mins., hold 2.5 mins, ramp down 0.5 mins.
Post time: 5 mins
Inj. Vol.: 2 µL
Temp: 40 °C
Flow rate: 1.2 mL/min



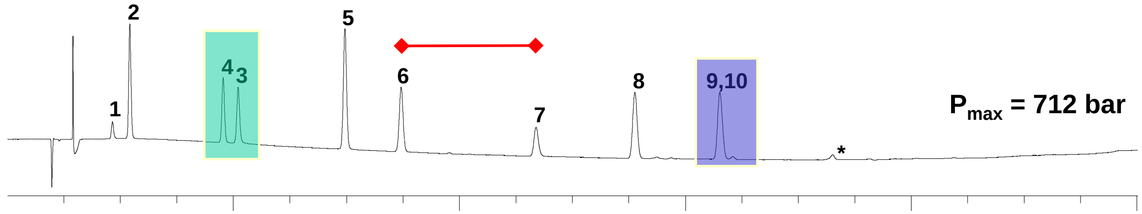
4-aminophenol
pKa = 5.28, 10.17
LogP = -0.29
LogD 5.5 = -0.04
LogD 7.4 = 0.16

Hence pH 6 eluent chosen

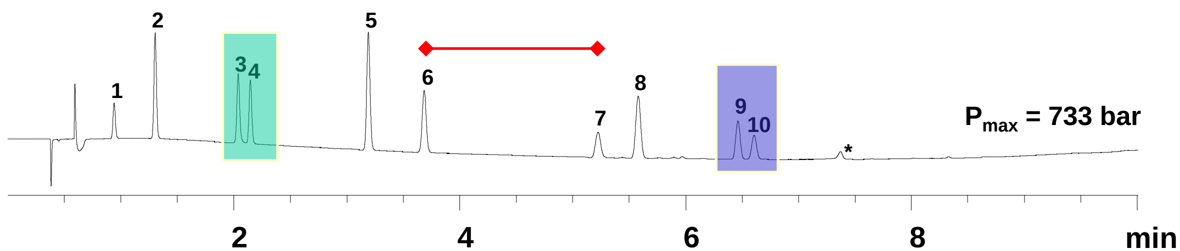
ACE Excel 2µm C18 100 x 3.0mm
Mechanism = Hydrophobicity



ACE Excel 2µm C18-AR 100 x 3.0mm
Mechanism = Hydrophobicity + π-π



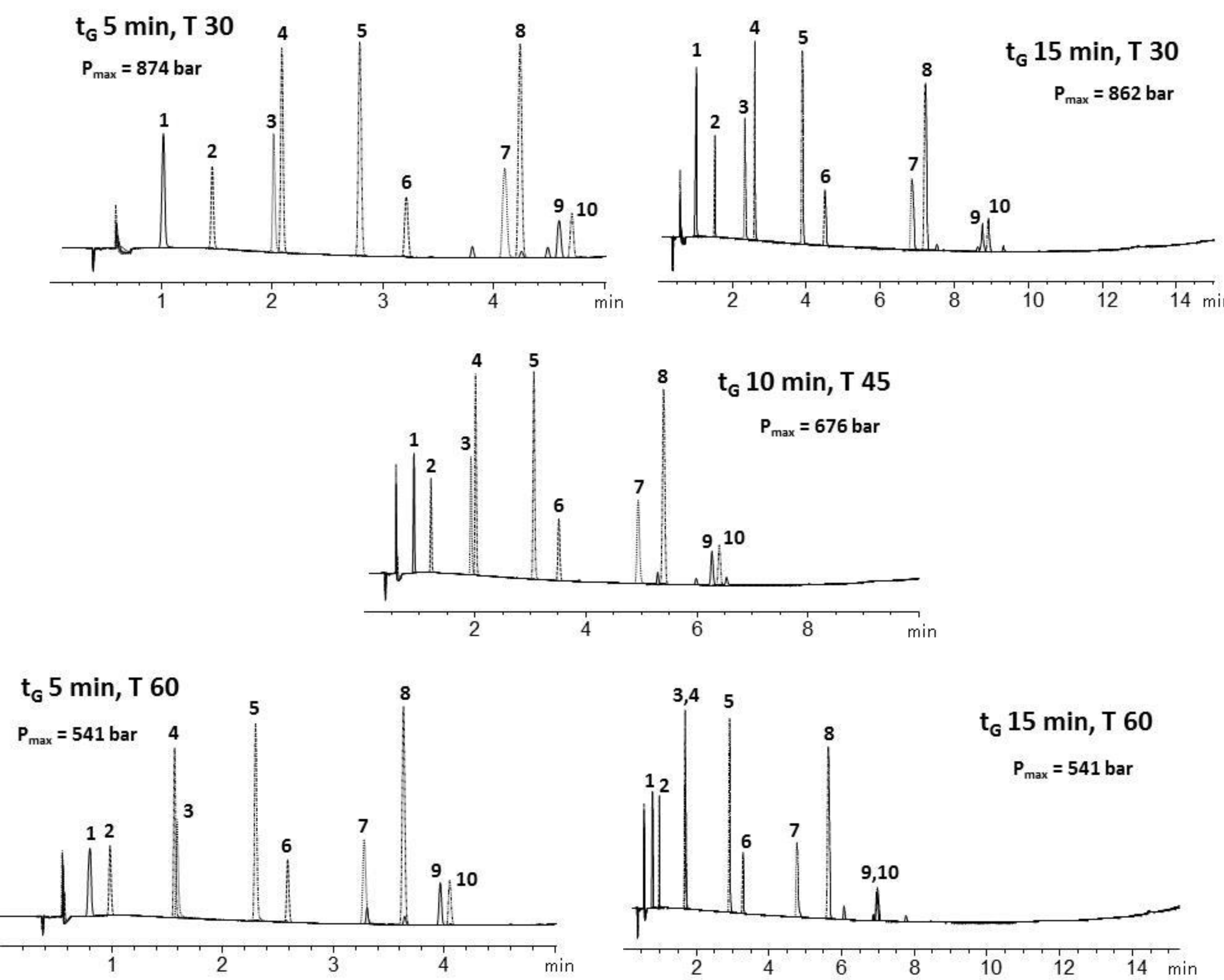
ACE Excel 2µm C18-PFP 100 x 3.0mm
Mechanism = Hydrophobicity + π-π + dipole-dipole + shape selectivity



Chosen for modeling

Modelling Software Can Be Powerful If Available

ACE Excel 2μm C18-PFP 100 x 3.0mm
Input runs for retention modeling (t_G vs Temp)

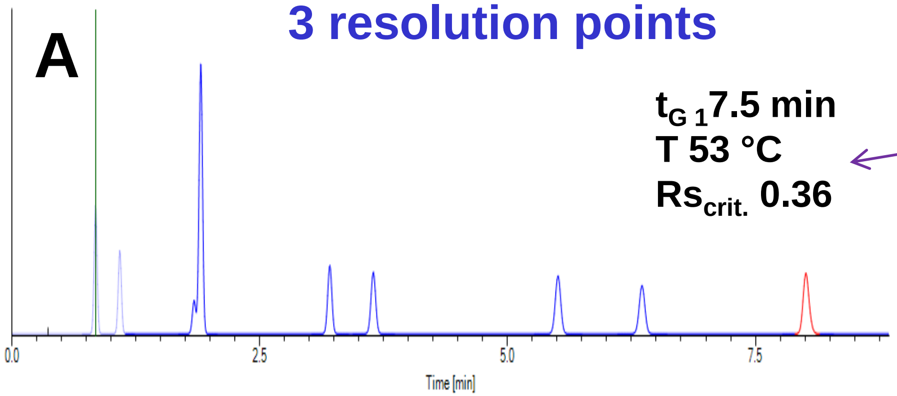


Conditions:
ACE Excel 2μm C18-PFP, 100x3mm
A: 10 mM ammonium acetate pH 6.0
B: 10 mM ammonium acetate pH 6.0 in MeOH:water 9:1 *v/v*
Flow rate: 1.2 mL/min
5-95% B scouting gradients

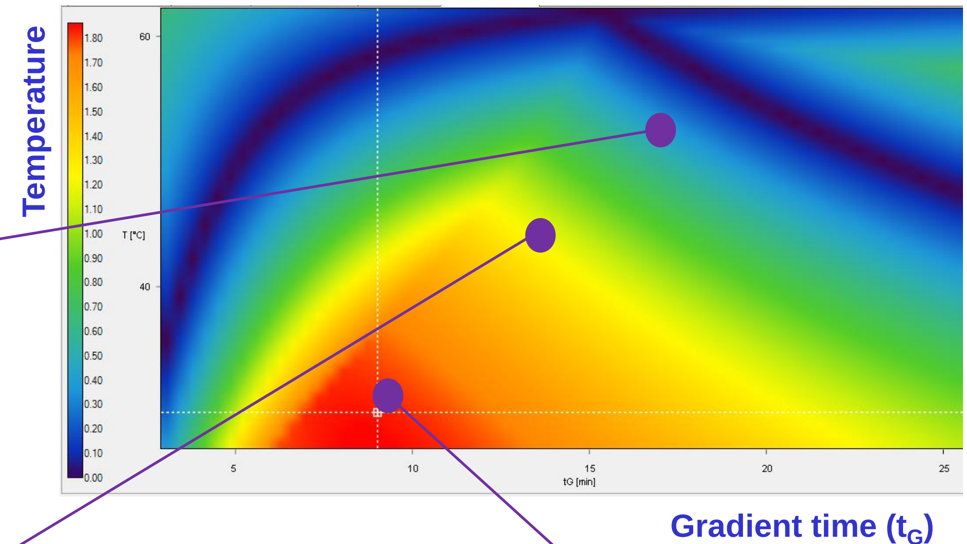
Retention Modelling
5, 15 minute (and 10 minute) gradient times
30 and 60 °C (and 45°C) temperatures

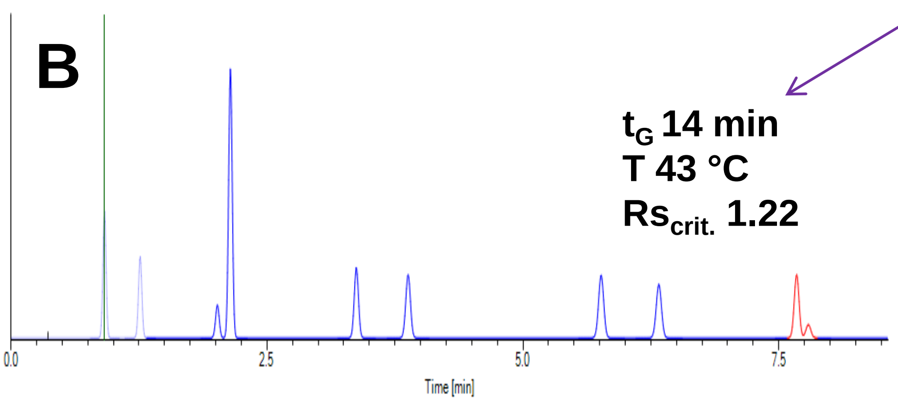
Resolution Map Assists Elution Order / Separation Interpretation

Chromatograms from 3 resolution points

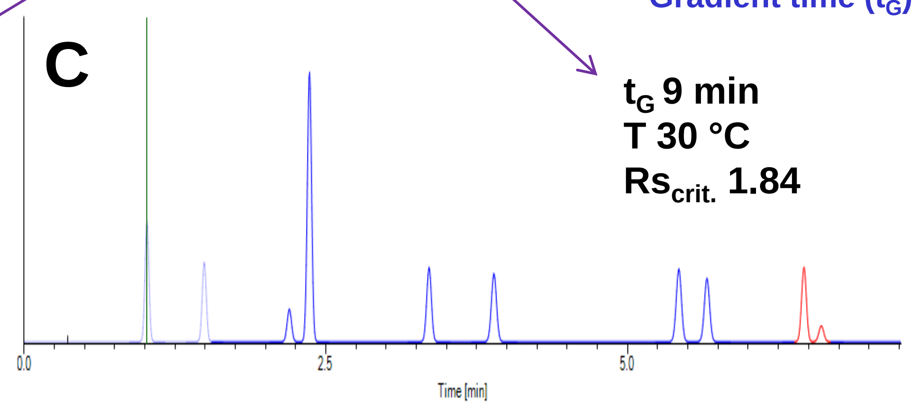


$t_{G\,1}$7.5 min
T 53 °C
$Rs_{crit.}$ 0.36





t_G 14 min
T 43 °C
$Rs_{crit.}$ 1.22



t_G 9 min
T 30 °C
$Rs_{crit.}$ 1.84

Over the entire resolution map, 3 peak pairs are critical at various points:

2-amino phenol / acetaminophen
2-nitrophenol / 4-nitrophenol
4-chloroacetanilide / 4-chlorophenol

ACE Excel 2µm C18-PFP

Predicted vs Actual Chromatograms

Center of predicted robust region from resolution map

Name	tR[min]	Area	Width	Rs	Time	Area	Width	Rs
4-aminophenol	0.96	657.4	0.06	6.92	1.018	249.4	0.0281	10.49
Hydroquinone	1.4	449.6	0.06	10.57	1.487	210	0.027	14.59
2-aminophenol	2.11	199.7	0.07	2.11	2.183	236.5	0.0309	3.01
Paracetamol	2.25	1585.3	0.07	13.8	2.331	335.1	0.0288	17.37
2-acetamidophenol	3.22	482.1	0.07	6.92	3.283	258.4	0.0364	7.97
Phenol	3.76	484.9	0.08	18.03	3.82	141	0.0434	16.70
4-nitrophenol	5.22	526.1	0.08	2.74	5.271	363.1	0.0614	2.39
2-nitrophenol	5.44	460.1	0.08	9.54	5.494	453	0.0514	8.64
4-chloroacetanilide	6.19	498.1	0.08	1.85	6.223	113.8	0.0485	1.76
4-chlorophenol	6.34	109.4	0.08		6.37	254.9	0.0504	

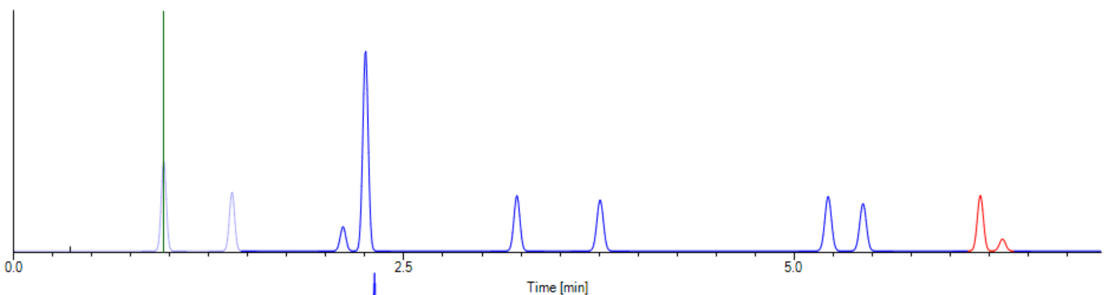
Gradient Table

Time [min]	%B	Rate [%B/min]
0.00	5.00	
8.50	95.00	10.59

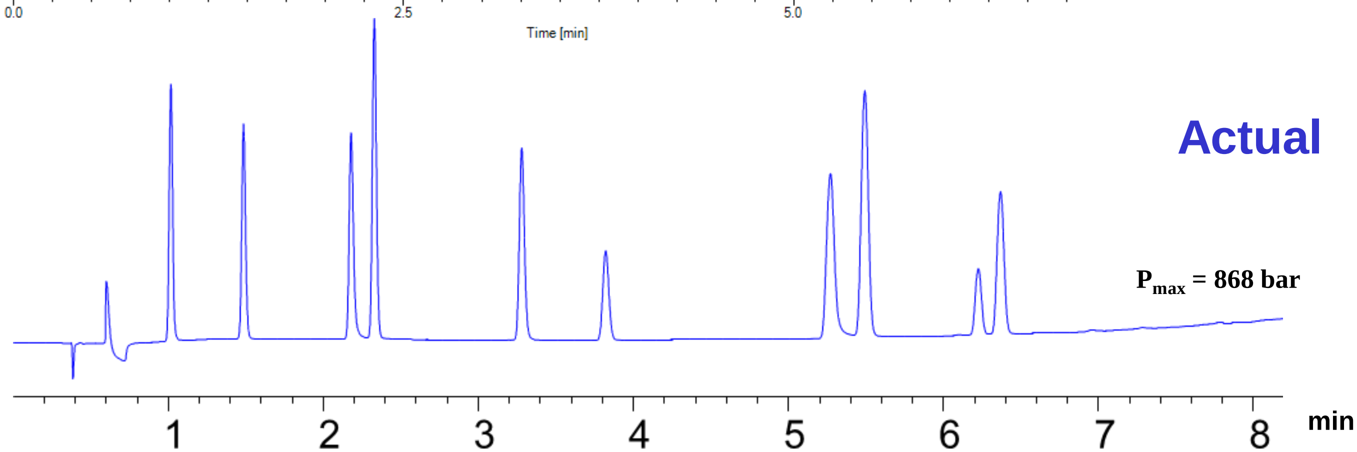
Status

8.50	tG [min]
30.00	T [°C]

Pressure [psi] : 12770
Plate Number : 14371 (calculated)
Rs,crit : 1.85
Crit. Peak Pair : 2, 10
Run Time [min] : 7.00
Eluent Used [mL] : 8.40



Predicted



Actual

ACE Excel 2µm C18-PFP

Rapid Predicted vs Actual Data Chromatograms

Name	tR[min]	Area	Width	Rs	Time	Area	Width	
4-aminophenol	0.88	657.4	0.06	5.34	0.9	243.7	0.0264	7.94
Hydroquinone	1.21	449.6	0.06	11.6	1.246	210.8	0.0263	17.07
2-aminophenol	2	199.7	0.07	1.98	2.032	234.7	0.0303	2.11
Paracetamol	2.14	1585.3	0.07	6.32	2.12	338.6	0.0221	13.32
2-acetamidophenol	2.54	482.1	0.06	2.55	2.54	270.7	0.0161	5.70
Phenol	2.69	484.9	0.06	5.48	2.712	151.8	0.019	9.22
4-nitrophenol	3.01	526.1	0.06	2.02	3.08	369.7	0.0287	2.94
2-nitrophenol	3.14	460.1	0.06	1.82	3.214	462.6	0.0263	2.88
4-chloroacetanilide	3.25	498.1	0.06	1.83	3.343	109.4	0.027	1.76
4-chlorophenol	3.36	109.4	0.06	0	3.427	276.9	0.0301	

Now includes a step gradient

Gradient Table

Time [min]	%B	Rate [%B/min]
0.00	6.00	
1.00	11.00	4.99
1.50	60.00	98.98
2.50	75.00	14.91

Status

2.50 tG [min]

41.00 T [°C]

Pressure [psi] : 10308

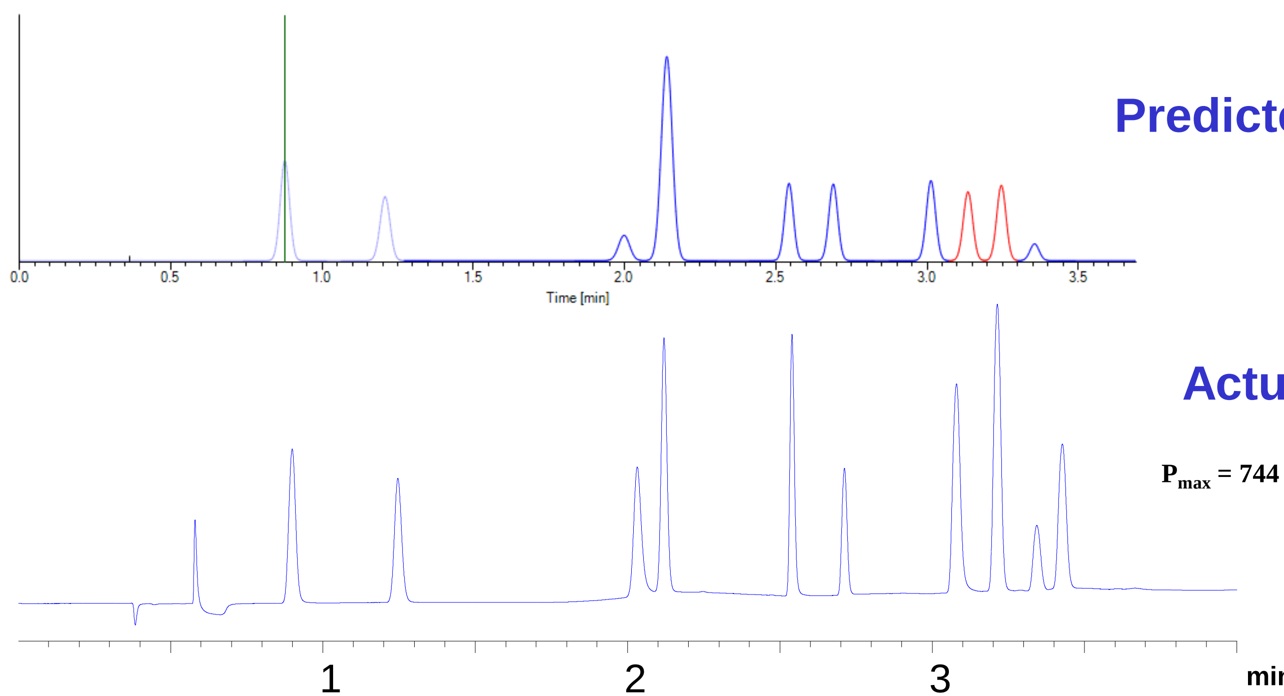
Plate Number : 7642 (calculated)

Rs,crit : 1.82

Crit. Peak Pair : 8, 2

Run Time [min] : 4.00

Eluent Used [mL] : 4.80



Predicted

Actual

P_{max} = 744 bar

ACE Excel 2µm C18-PFP

Method Translations: What Are You Trying to Achieve?

- **Faster separations with the same performance?**
  - **eg converting HPLC → UHPLC methods for increased productivity / sample throughput with similar efficiency, selectivity or resolution?**
- **Converting UHPLC → HPLC methods?**
  - **eg for offshoring or third party labs or manufacturing?**
- **Porous particle → solid core particle method change?**
  - **Take advantage of solid core efficiency, speed or low backpressure?**
- **Faster method development?**
  - **eg reduce overall cycle time?**
- **Higher resolution / peak capacity?**
  - **eg for related substances or complex samples?**

Isocratic Method Translations: Pharmacopeial Methods

- New guidance on allowable parameter changes recently issued for USP <621> chapter*
  - Significant update to the general chapter – reading recommended
  - Isocratic monograph LC methods can be -25% to +50% of the original L/d_p according to the USP guidance
  - Also, L/d_p ratios outside this range can be -25% to +50% of the original peak efficiency, N ← large flexibility
- Very few changes / flexibility for gradient monograph methods
- When translating to smaller columns dimensions (and therefore smaller column volume), system dispersion effects are important

* www.usp.org. New guidance in general chapter USP <621> issued August 2014.

Isocratic Method Translations: General Principles

- Maintain a **constant length to particle size ratio, L/d_p** (for the same phase type and phase vendor)

$$N = \frac{L}{HETP}$$

  - Will give ~ **similar** performance i.e. efficiency (selectivity, resolution)*

- Thus, 300 x 3.9 mm, 10 µm ≈ 150 x 4.6 mm, 5 µm = 30,000

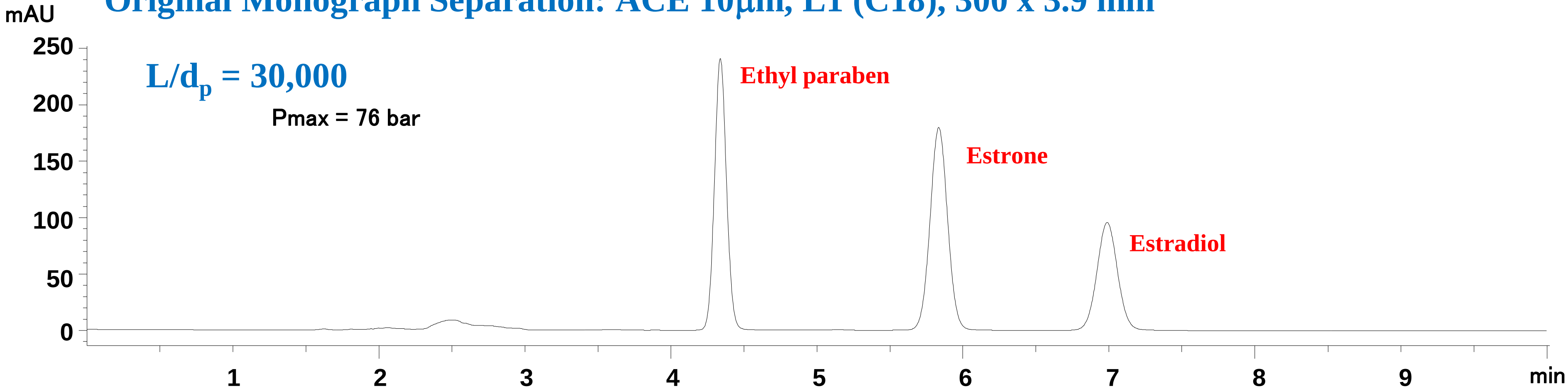
Particle Size (µm)	Column Length (mm) 50	75	100	150	250	300
1.7	29,412	44,118	58,824			
1.8	27,778	41,667	55,556			
1.9	26,316	39,474	52,632			
2	25,000	37,500	50,000	75,000		
2.5	20,000	30,000	40,000	60,000	100,000	
2.6	19,231	28,846	38,462	57,692	96,154	
2.7	18,519	27,778	37,037	55,556	92,593	
3	16,667	25,000	33,333	50,000	83,333	
5	10,000	15,000	20,000	30,000	50,000	
10	5,000	7,500	10,000	15,000	25,000	30,000

Columns meeting L/dp of 30,000

* Neue et al., Stimuli to the revision process: USP <621>. *Pharm Forum*. 2009; 35(6): 1622-1626.

Method Translations: Isocratic Estradiol Porous → Porous (I)

Original Monograph Separation: ACE 10μm, L1 (C18), 300 x 3.9 mm



Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1 mL/min, 25 μL injection

1. Find suitable 'new' column: 300x3.9mm, 10μm = L/dp of 30,000 = 150x4.6, 5μm

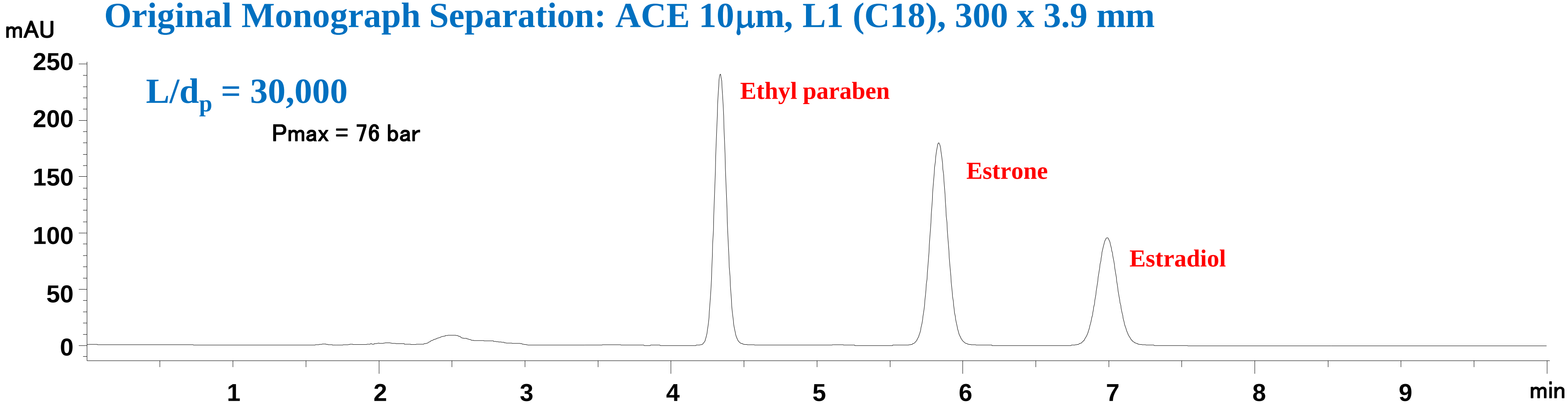
2. Geometrically scale flow rate: $F_2 = F_1 \times \frac{{d_{c_2}}^2}{{d_{c_1}}^2}$ = 1 x 4.6^2 / 3.9^2 = 1.39 mL/min

3. Volumetrically scale injection: $Inj_2 = Inj_1 \times \frac{V_{m_2}}{V_{m_1}}$ = 25 x 1.620 / 2.329 = 17.4 μL

$$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$

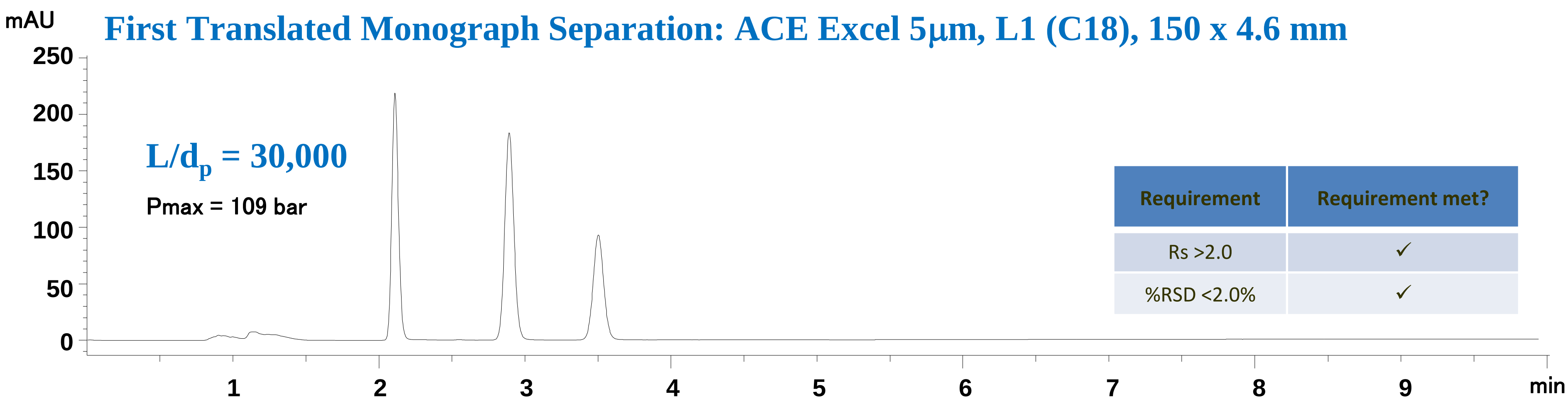
Method Translations: Isocratic Estradiol Porous → Porous (II)

Original Monograph Separation: ACE 10µm, L1 (C18), 300 x 3.9 mm



Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1 mL/min, 25 µL injection

First Translated Monograph Separation: ACE Excel 5µm, L1 (C18), 150 x 4.6 mm



Requirement	Requirement met?
Rs >2.0	✓
%RSD <2.0%	✓

Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1.39 mL/min, 17.4 µL injection

Further examples in A P McKeown, article in Chromatography Today March/April 2015, in press

Method Translations: Isocratic Methods

- Isocratic monograph LC methods can be **-25% to +50% of the original L/d_p** according to the USP guidance*

Column Length (mm)	300
Particle Size (um)	10
Lower L/dp (-25%)	22,500
L/dp	30,000
Upper L/dp (+50%)	45,000

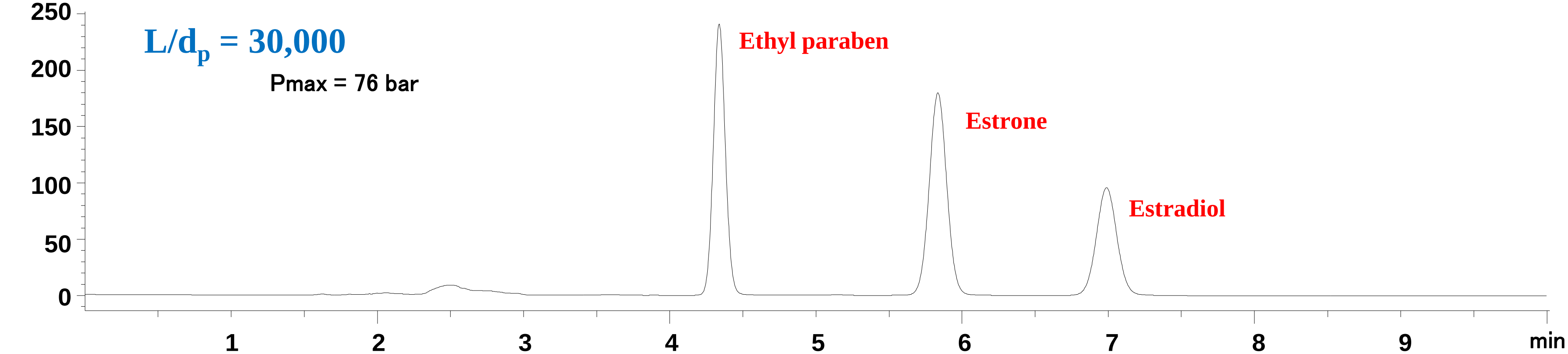
Particle Size (µm)	Column Length (mm) 50	75	100	150	250	300
1.7	29,412	44,118	58,824			
1.8	27,778	41,667	55,556			
1.9	26,316	39,474	52,632			
2	25,000	37,500	50,000	75,000		
2.5	20,000	30,000	40,000	60,000	100,000	
2.6	19,231	28,846	38,462	57,692	96,154	
2.7	18,519	27,778	37,037	55,556	92,593	
3	16,667	25,000	33,333	50,000	83,333	
5	10,000	15,000	20,000	30,000	50,000	
10	5,000	7,500	10,000	15,000	25,000	30,000

Columns meeting L/dp of 22,400 to 45,000

* www.usp.org. New guidance in general chapter USP <621> issued August 2014.

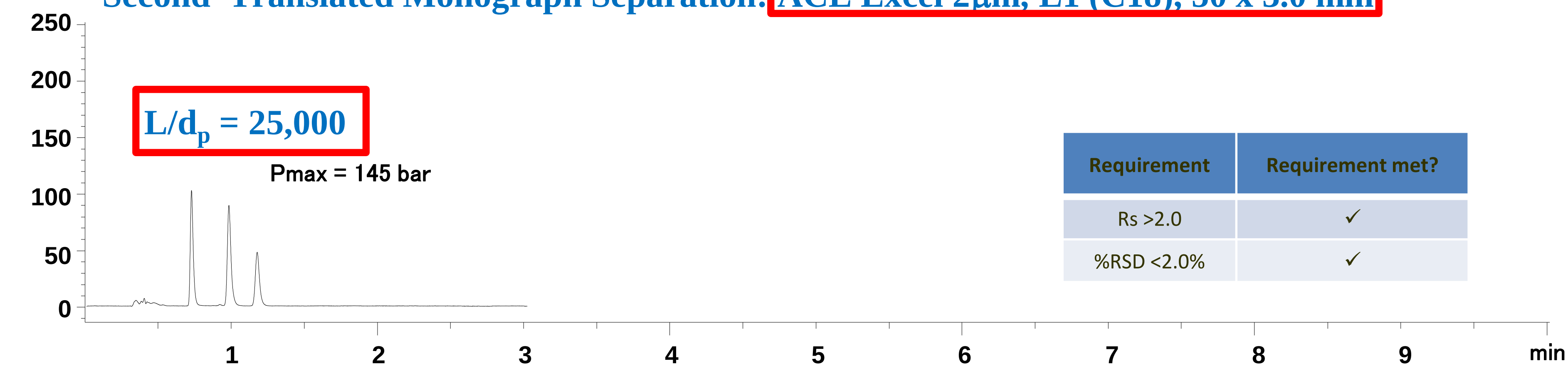
Method Translations: Isocratic Estradiol Porous → Porous (III)

Original Monograph Separation: ACE 10µm, L1 (C18), 300 x 3.9 mm



Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1 mL/min, 25 µL injection

Second Translated Monograph Separation: ACE Excel 2µm, L1 (C18), 50 x 3.0 mm



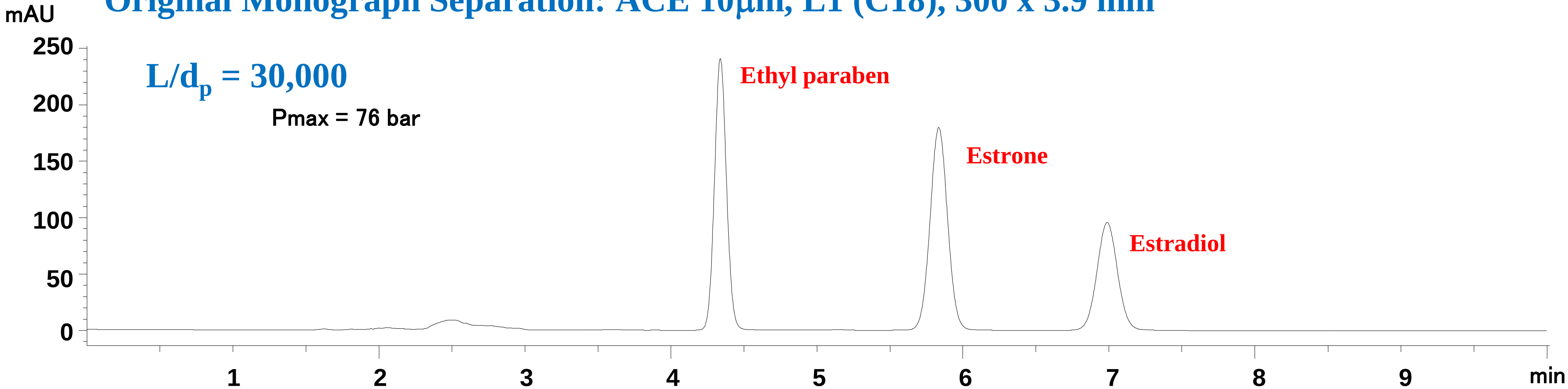
Requirement	Requirement met?
Rs >2.0	✓
%RSD <2.0%	✓

Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 0.62 mL/min, 2.4 µL injection

Further examples in A P McKeown, article in Chromatography Today March/April 2015, in press

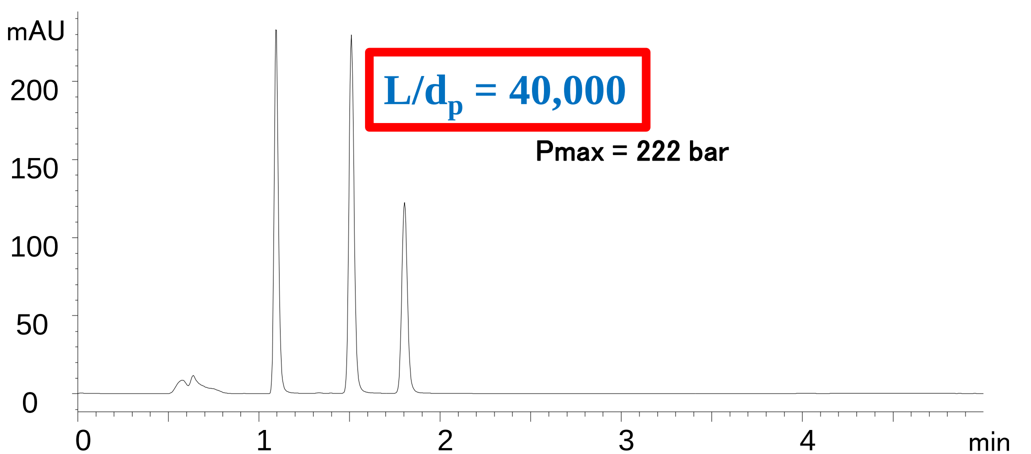
Method Translations: Isocratic Estradiol Porous → Solid Core

Original Monograph Separation: ACE 10µm, L1 (C18), 300 x 3.9 mm



Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1 mL/min, 25 µL injection

Third Translated Monograph Separation: ACE UltraCore 2.5µm, L1 (SuperC18), 100 x 4.6 mm



Conditions: 45:55 v/v water:acetonitrile, 205 nm, 22°C, 1.39 mL/min, 10.1 µL injection

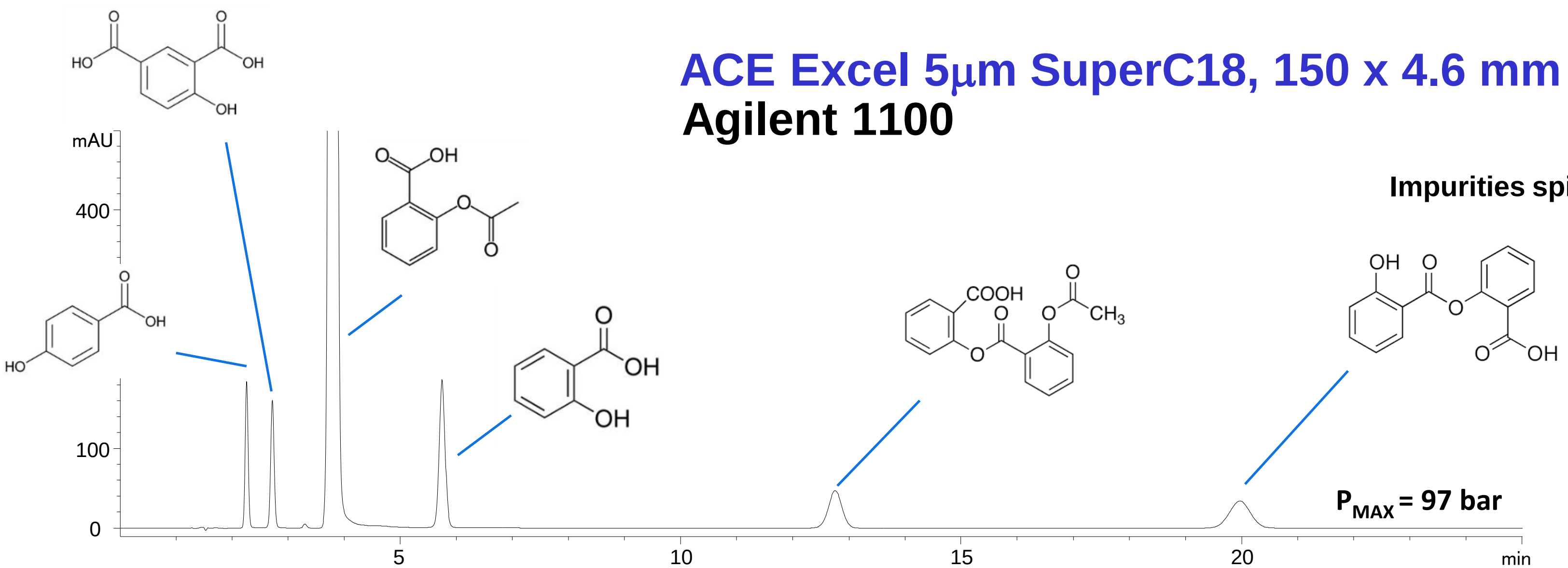
Requirement	Requirement met?
Rs >2.0	✓
%RSD <2.0%	✓

- **Use appropriate solid core porosity values to calculate V_m!**
- **Look out for negative effects of extra-column band broadening**

$$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$

Further examples in A P McKeown, article in Chromatography Today March/April 2015, in press

Isocratic Method Translations: Aspirin Porous → Solid Core (I)



Translation to ACE UltraCore 5μm SuperC18, 150 x 4.6 mm:

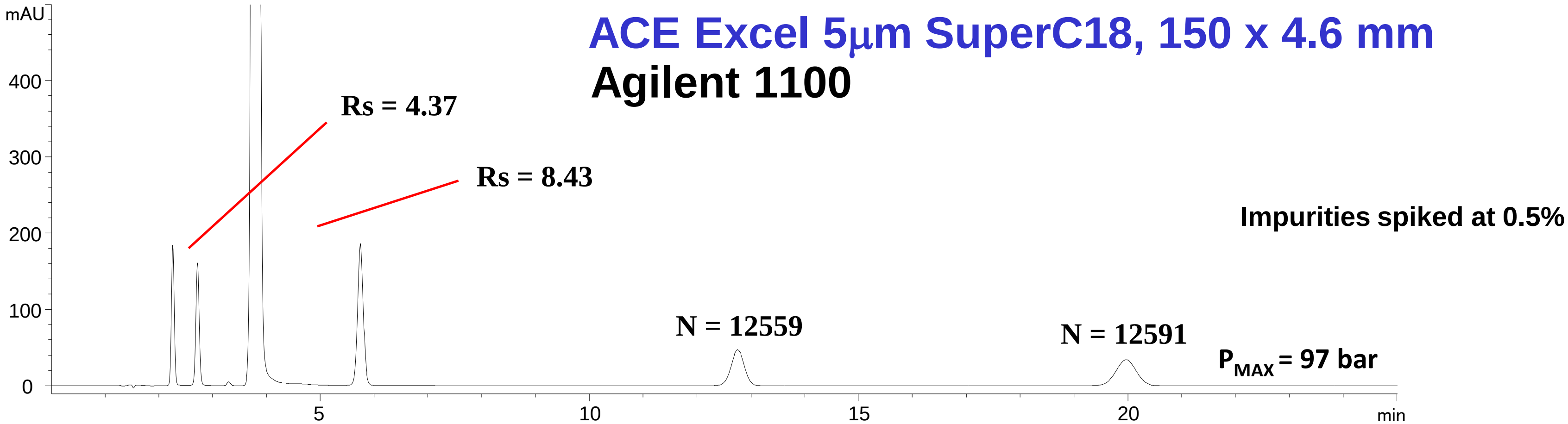
1. Column dimensions and particle size similar so same flow rate used
2. Determine column volumes and scale injection volume

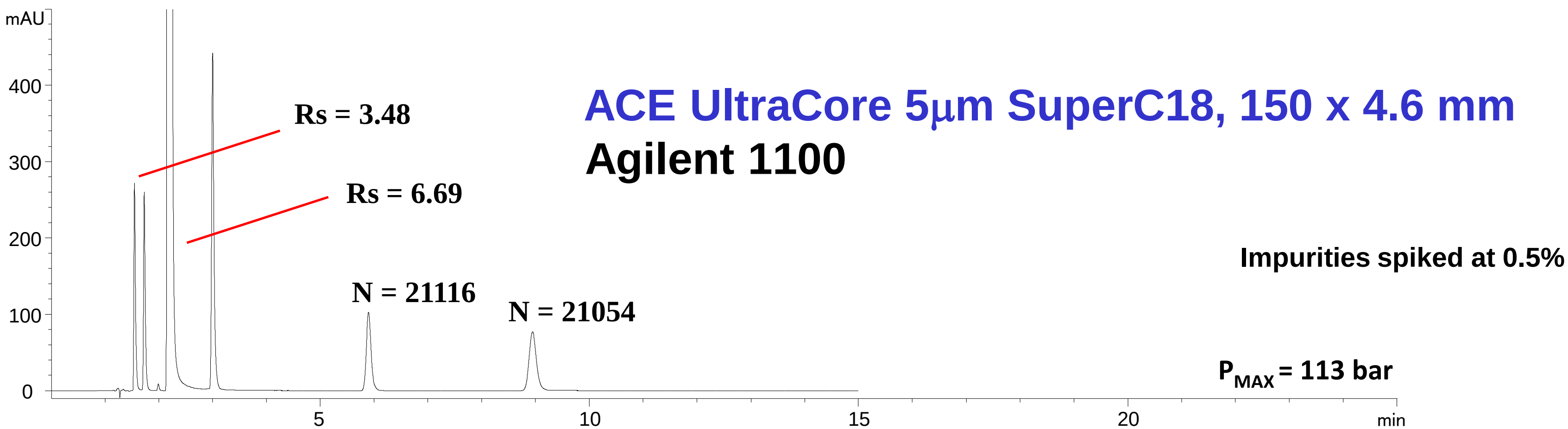
$$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$

$$Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$$

Conditions: 60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm, 25°C, 1 mL/min, 5 μL injection

Isocratic Method Translations: Aspirin Porous → Solid Core (II)

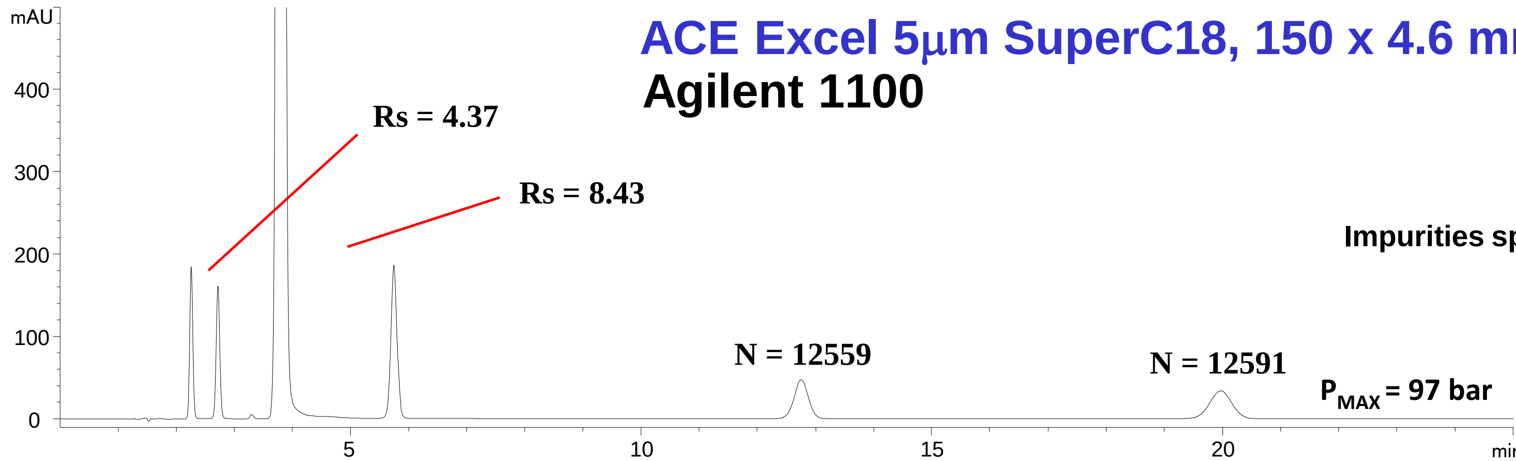




- **Reduced hydrophobicity of solid core particles leads to 'faster' analysis**
- **Can we go faster...?**

Conditions: (Top): 60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (2.5 Hz), 25°C, 1 mL/min, 5 µL injection
(Bottom): 60:35:5:0.2 v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (20 Hz), 25°C, 1 mL/min, 3.9 µL injection

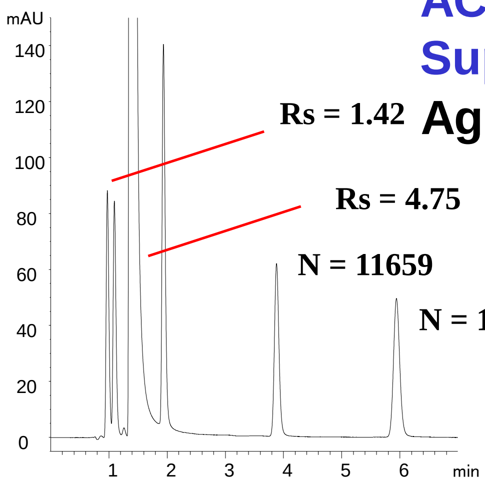
Isocratic Method Translations: Aspirin Porous → Solid Core (III)



ACE Excel 5µm SuperC18, 150 x 4.6 mm
Agilent 1100

Impurities spiked at 0.5%

P_{MAX} = 97 bar



ACE UltraCore 2.5µm SuperC18, 75 x 3.0 mm
Agilent 1100

P_{MAX} = 132 bar

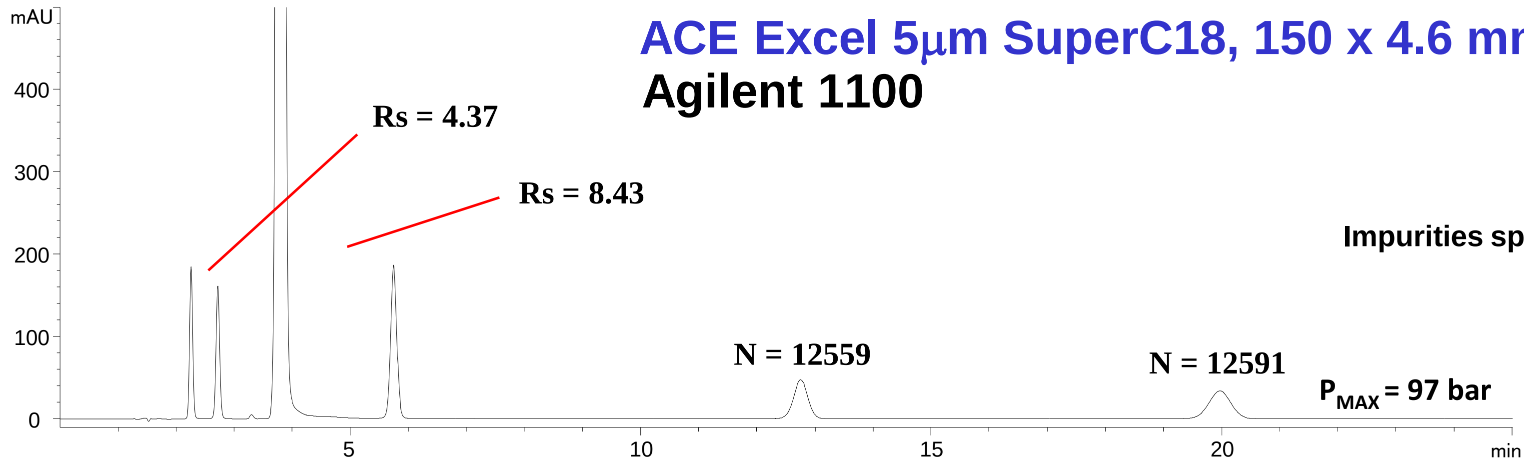
- **N and Rs not maintained. Impact of extra column band broadening of LC system** ☹
- **Increasing i.d. to 4.6 mm (with associated increase in flow and inj. vol.) should help.** ?

Conditions (Top): 60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm, 25°C, 1 mL/min, 5 µL injection
(Bottom): 60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (20 Hz), 25°C, 0.43 mL/min, 0.9 µL injection

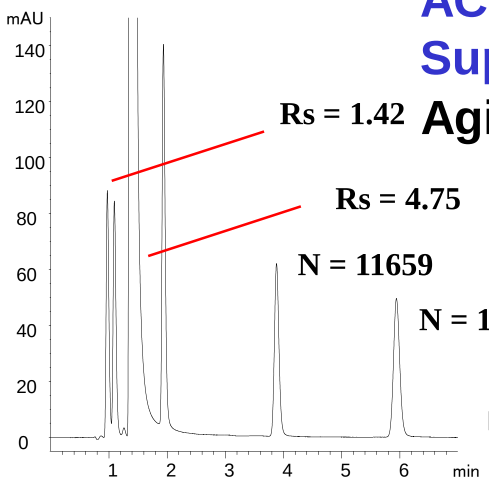
Isocratic Method Translations: Aspirin Porous → Solid Core (IV)

ACE Excel 5µm SuperC18, 150 x 4.6 mm
Agilent 1100

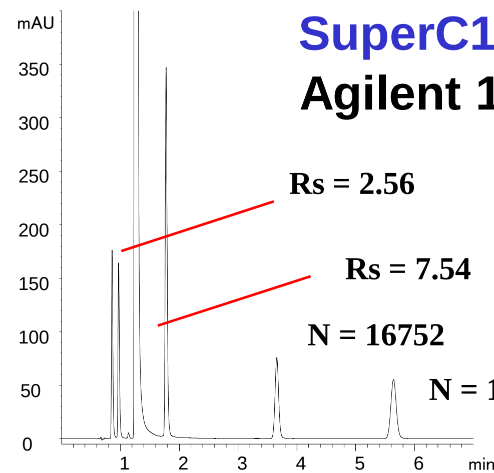
Impurities spiked at 0.5%



ACE UltraCore 2.5µm SuperC18, 75 x 3.0 mm
Agilent 1100



ACE UltraCore 2.5µm SuperC18, 75 x 4.6 mm
Agilent 1100

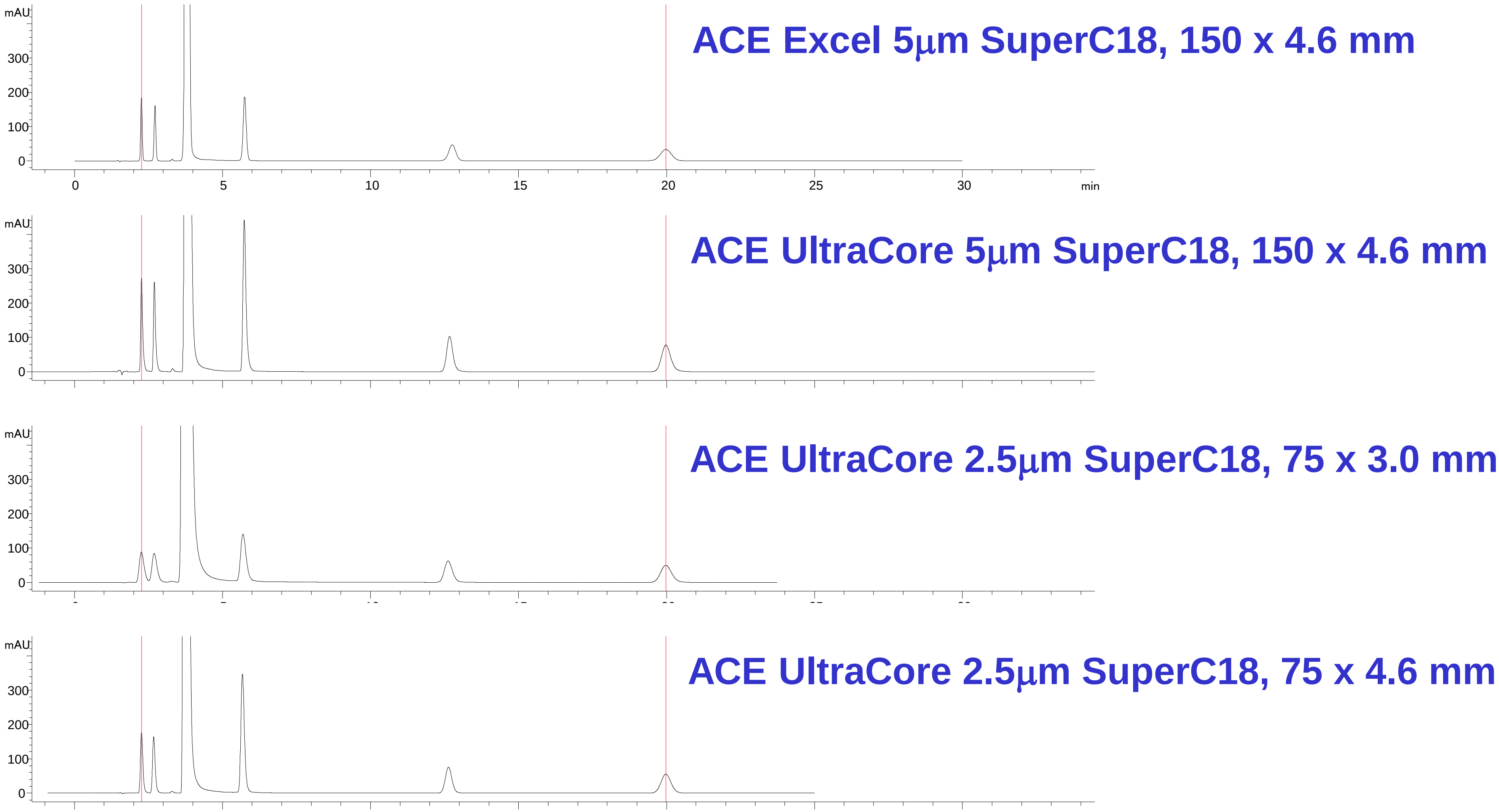


☺

Conditions ACE UltraCore 2.5 Super C18 75 x 4.6 mm:
60:35:5:0.2 v/v/v/v water:acetonitrile:methanol:85% phosphoric acid, 237 nm (20 Hz), 25°C, 1.0 mL/min, 2 µL injection

Isocratic Method Translations: Aspirin Porous → Solid Core (V)

Aligning first & last peaks



Selectivity Maintained Across ACE Column / Particle Formats

Translations Steps For Gradient Methods

1. **Calculate column volumes**
   - **Better to experimentally determine porosity for accuracy**

$$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$

Experimentally determined

2. **Translate gradient time**
   - **To maintain constant k***

$$\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$$

3. **Translate flow rate**
   - **Constant linear velocity**

$$F_2 = F_1 \times \frac{{d_{c_2}}^2}{{d_{c_1}}^2}$$

4. **Translate injection volume**
   - **To give similar response**

$$Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$$

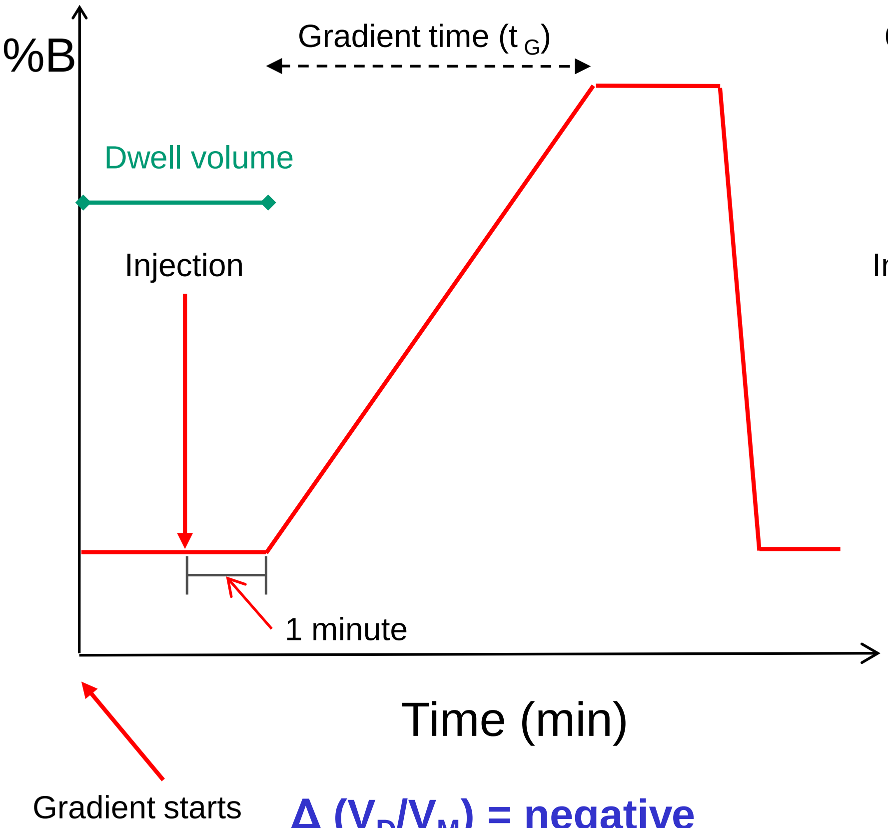
5. **Calculate whether an injection hold or pre-injection is needed**
   - **For the most accurate translations and if possible on your instrument**

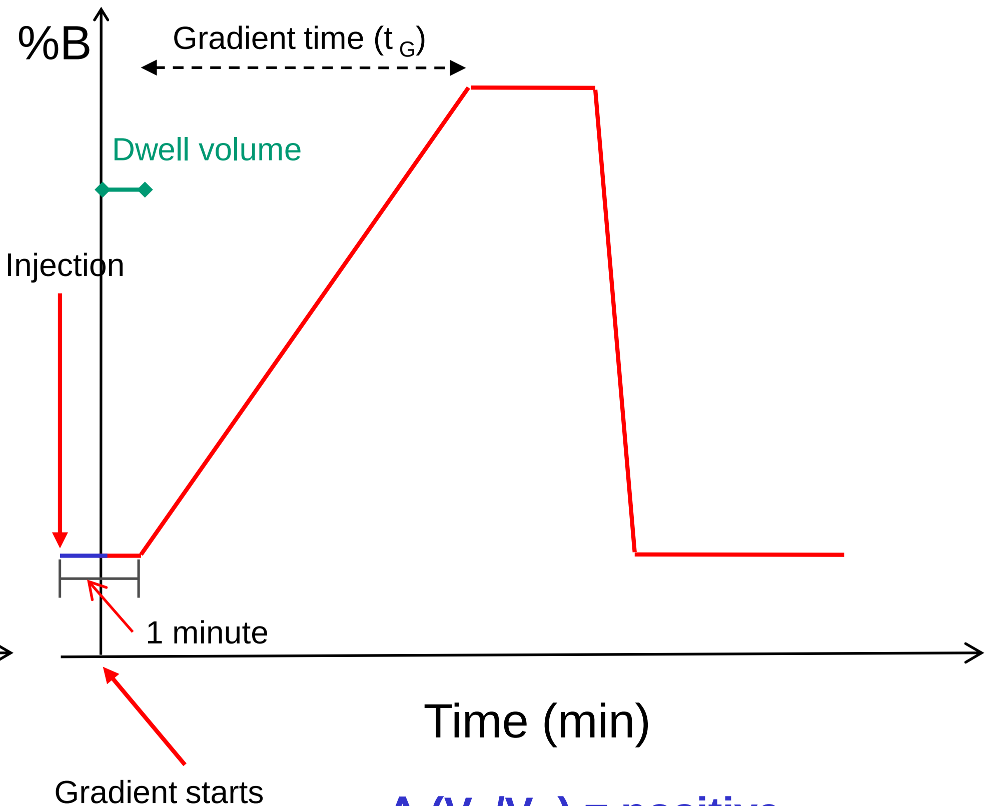
Experimentally determined

$$\Delta = \left(\frac{V_D}{V_M}\right)_{original} - \left(\frac{V_D}{V_M}\right)_{new} \quad must\ approach\ zero$$

V_D = dwell volume

Method Translations: Dwell Volumes / Injection Times





Δ (V_D/V_M) = negative

e.g. small column on a high dwell volume system. The dwell time is artificially long.

Injection is delayed until after the start of gradient

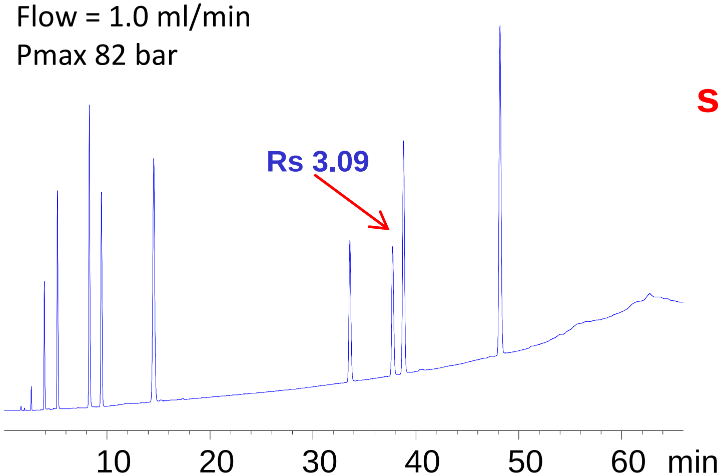
Δ (V_D/V_M) = positive

e.g. large column on a low dwell volume system. The dwell time is artificially short.

Pre-gradient hold is added. Effectively extends dwell time

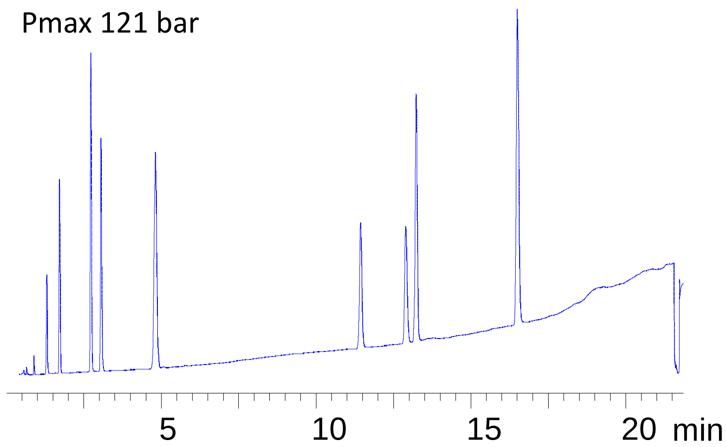
Gradient Method Translations: Porous → Porous (II)

HPLC
C18-PFP: 150 x 4.6 mm 5µm
tG = 60 min
Flow = 1.0 ml/min
Pmax 82 bar



Translate to smaller column and dp →

(U)HPLC
C18-PFP: 50 x 3.0 mm 2µm
tG = 19.79 min
Flow = 0.43 ml/min
Pmax 121 bar

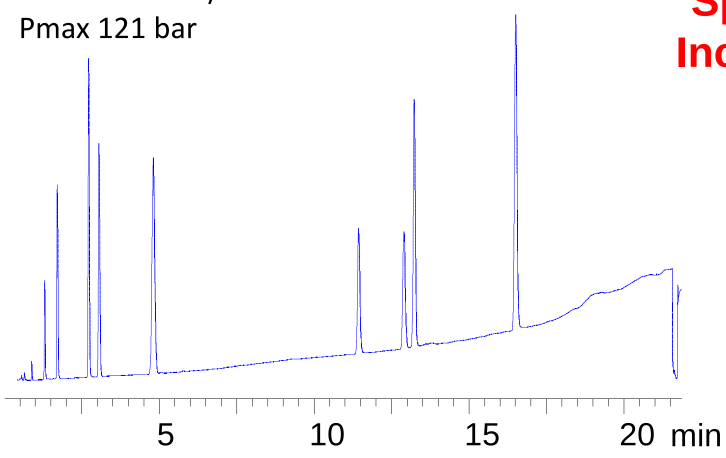


1. **Calculate column volumes** $V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$
2. **Translate gradient time** $\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$
3. **Translate flow rate** $F_2 = F_1 \times \frac{{d_{c_2}}^2}{{d_{c_1}}^2}$
4. **Translate injection volume** $Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$
5. **Calculate whether an injection hold or pre-injection is needed**

$$\Delta = \left(\frac{V_D}{V_M}\right)_{original} - \left(\frac{V_D}{V_M}\right)_{new}$$

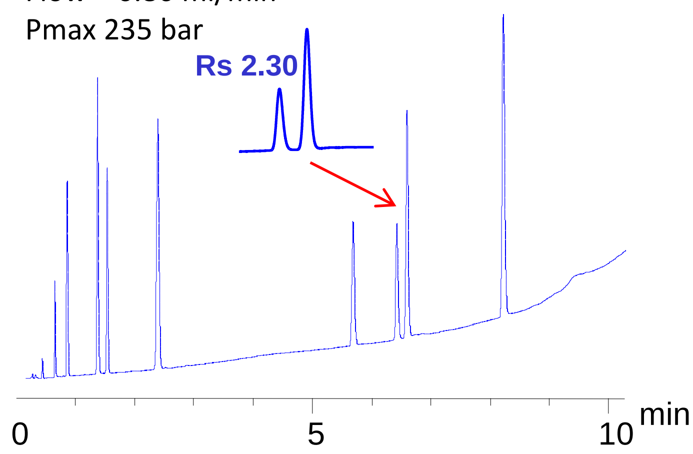
Gradient Method Translations: Porous → Porous (III)

(U)HPLC
C18-PFP: 50 x 3.0 mm 2µm
tG = 19.79 min
Flow = 0.43 ml/min
Pmax 121 bar



Speed up further: Increase Flow rate, Reduce t_G

(U)HPLC
C18-PFP: 50 x 3.0 mm 2µm
tG = 9.90 min
Flow = 0.86 ml/min
Pmax 235 bar



2. **Translate gradient time**

$$\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$$

3. **Translate flow rate**

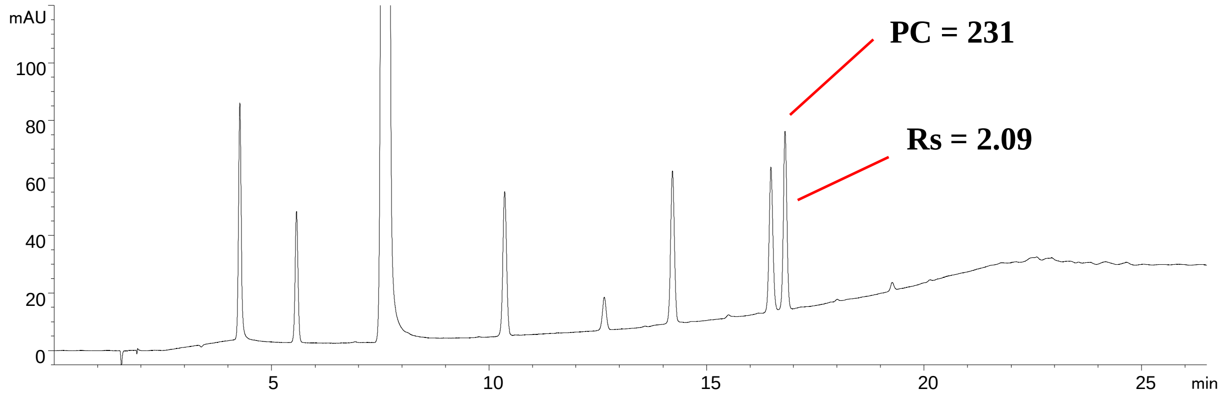
$$F_2 = F_1 \times \frac{{d_{c_2}}^2}{{d_{c_1}}^2}$$

5. **Calculate whether an injection hold or pre-injection is needed**

$$\Delta = \left(\frac{V_D}{V_M}\right)_{original} - \left(\frac{V_D}{V_M}\right)_{new}$$

Gradient Method Translations: Acetaminophen Porous → Solid Core

ACE Excel 5µm SuperC18 150 x 4.6 mm



t_G	20 min
Flow:	1 mL/min
Inj. Vol.	5 µL
P_{MAX}	138 bar

$$Peak\ Capacity = 1 + \frac{t_G}{W_{0.5}}$$

<u>Translation to ACE UltraCore 5µm Super C18 150 x 4.6 mm:</u>

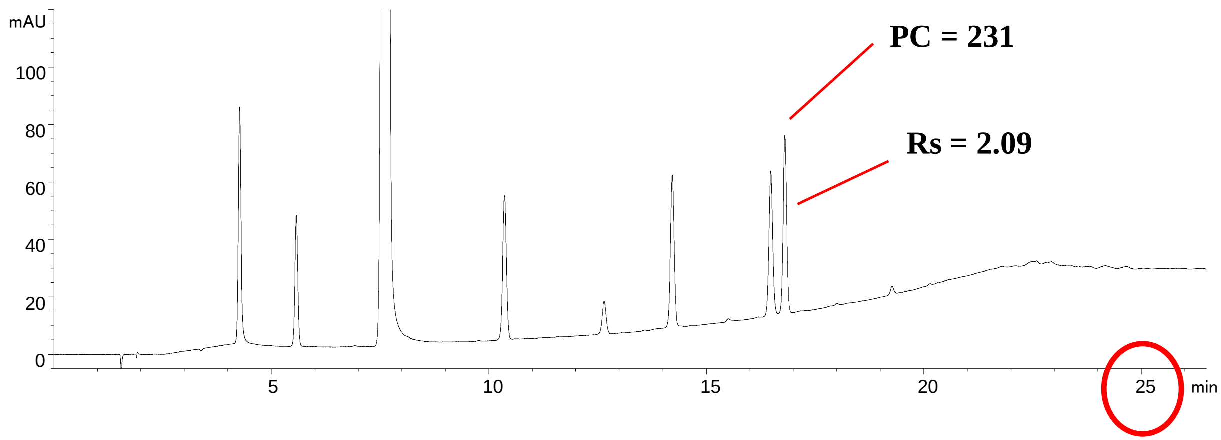
1. Calculate column volumes...solid core porosity differences $$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$
2. Translate gradient time $$\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$$
3. No flow rate change needed $$F_2 = F_1 \times \frac{{d_{c_2}}^2}{{d_{c_1}}^2}$$
4. Translate injection volume $$Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$$
5. Calculate whether an injection hold or pre-injection is needed

$$\Delta = \left(\frac{V_D}{V_M}\right)_{original} - \left(\frac{V_D}{V_M}\right)_{new}$$

150x4.6mm, 5 µm, gradient analysis, A= 20mM ammonium acetate pH 6.0 (aq), B= 20mM ammonium acetate pH 6.0 in MeCN:water 9:1 v/v, 5-95%B in 20.0 mins, hold 95%B for 5.0 mins, 30°C, 1.0 mL/min, 230 nm.
1. 4-aminophenol 2. Hydroquinone 3. acetaminophen 4. 4-acetamidophenol 5. phenol 6. 4-nitrophenol 7. 2-nitrophenol 8. 4-chloroacetanilide

Gradient Method Translations: Acetaminophen Porous → Solid Core

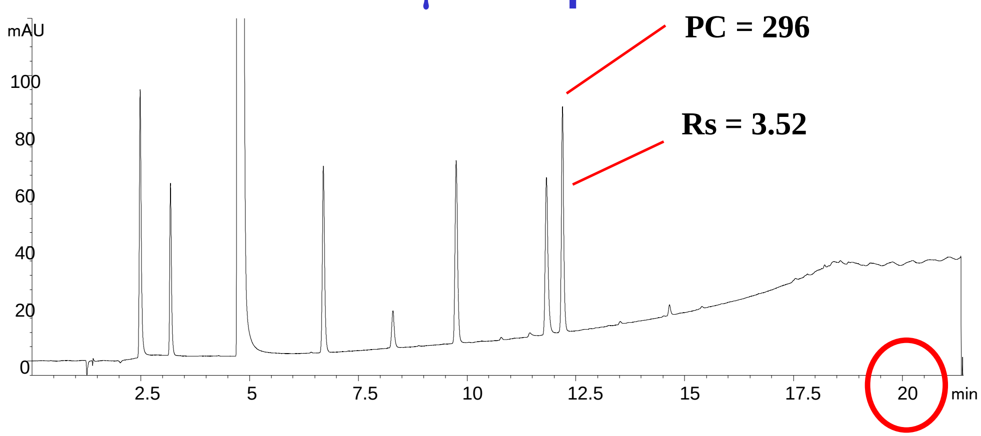
ACE Excel 5µm Super C18 150 x 4.6 mm



t_G	20 min
Flow:	1 mL/min
Inj. Vol.	5 µL
P_{MAX}	138 bar

$$Peak\ Capacity = 1 + \frac{t_G}{W_{0.5}}$$

ACE UltraCore 5µm Super C18 150 x 4.6 mm



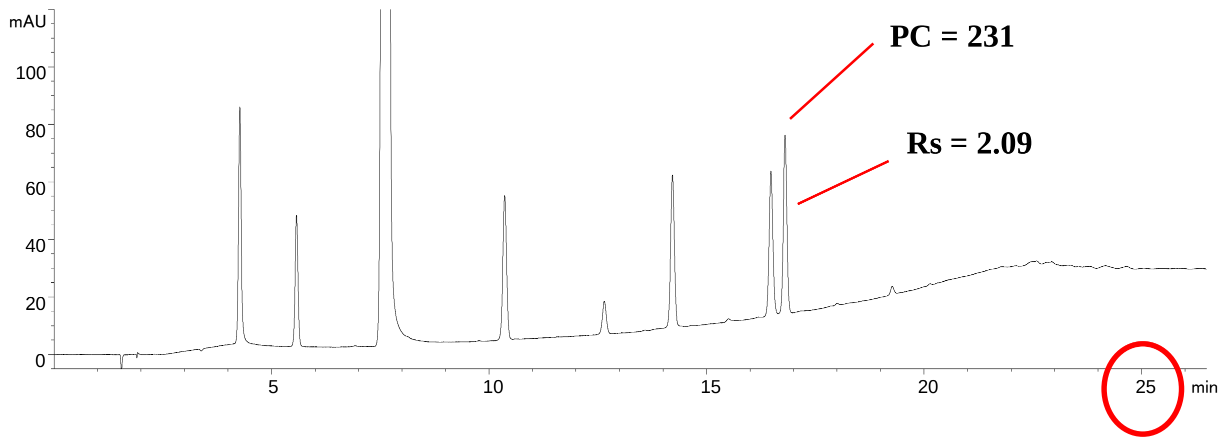
t_G	16.4 min
Flow:	1 mL/min
Inj. Vol.	3.9 µL
P_{MAX}	159 bar

Corrected
- **Vm (porosity)**
- **tG adjusted for constant k***
- **Injection volume adjusted**

Run time reduced whilst peak capacity and resolution are increased

Gradient Method Translations: Acetaminophen Porous → Solid Core

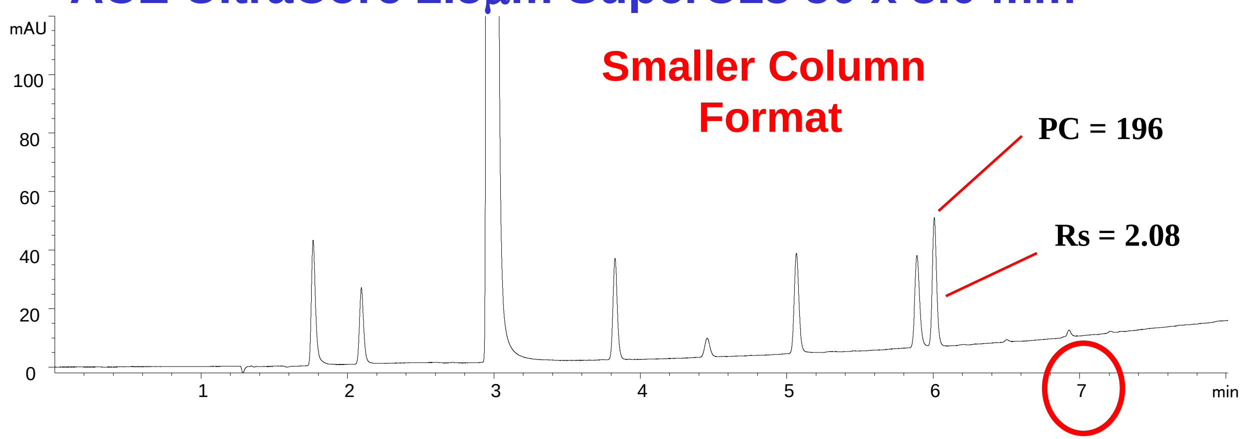
ACE Excel 5µm SuperC18 150 x 4.6 mm



t_G	20 min
Flow:	1 mL/min
Inj. Vol.	5 µL
P_{MAX}	138 bar

$$Peak\ Capacity = 1 + \frac{t_G}{W_{0.5}}$$

ACE UltraCore 2.5µm SuperC18 50 x 3.0 mm



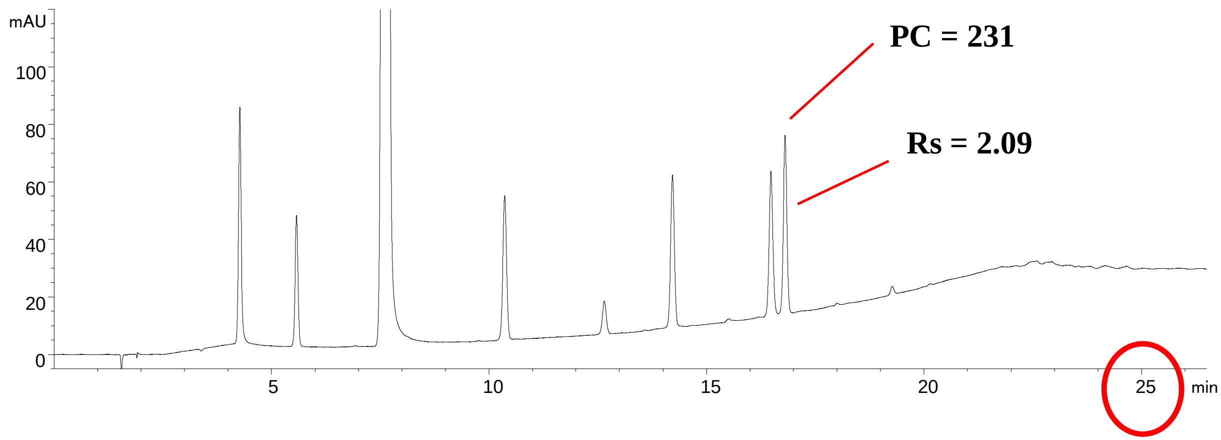
t_G	5.92 min
Flow:	0.43 mL/min
Inj. Vol.	0.63 µL
P_{MAX}	162 bar

Corrected
- Vm (porosity)
- Constant k* (tG & F)
- Injection volume (Vm)

Significant reduction in run time whilst maintaining resolution

Gradient Method Translations: Acetaminophen Porous → Solid Core

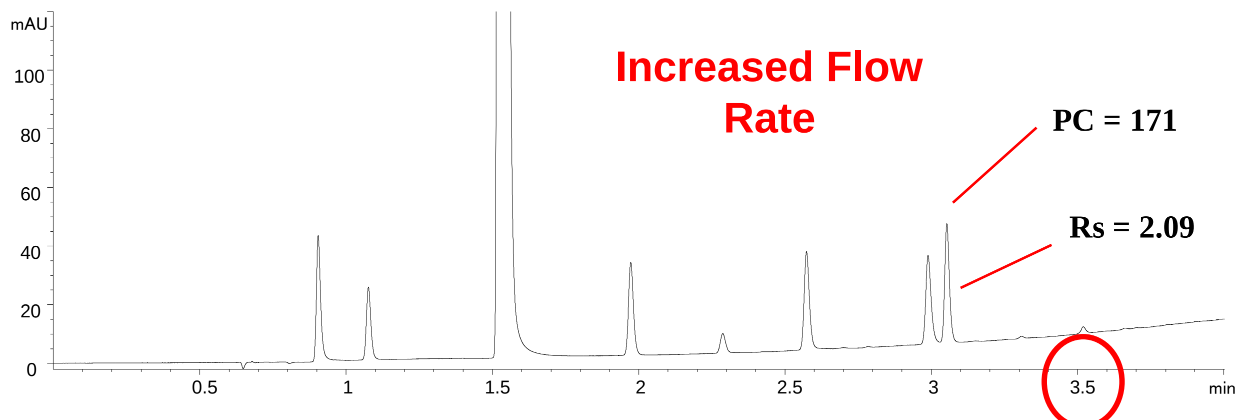
ACE Excel 5µm SuperC18 150 x 4.6 mm



t_G	20 min
Flow:	1 mL/min
Inj. Vol.	5 µL
P_{MAX}	138 bar

$$Peak\ Capacity = 1 + \frac{t_G}{W_{0.5}}$$

ACE UltraCore 2.5µm SuperC18 50 x 3.0 mm



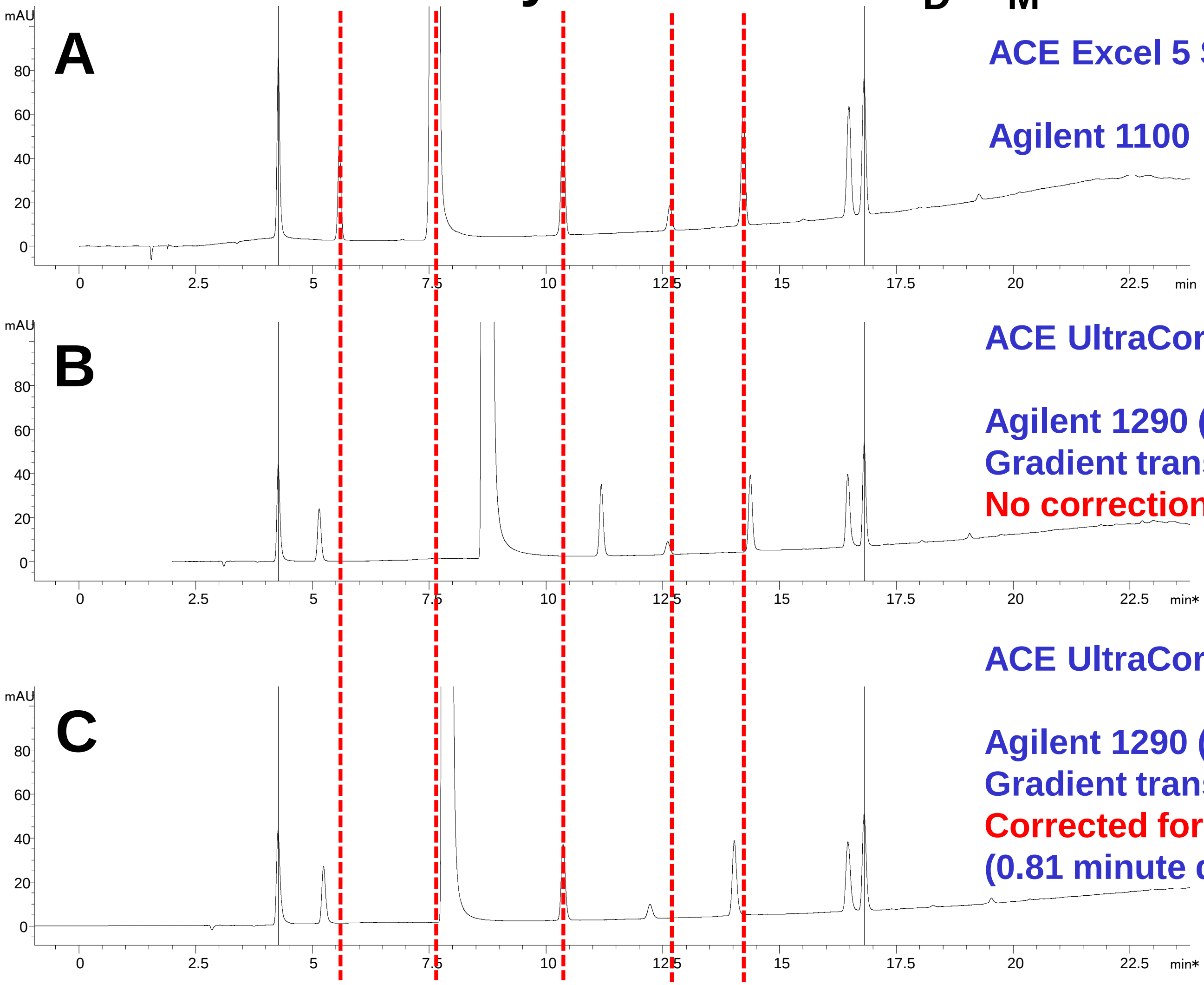
t_G	2.99 min
Flow:	0.85 mL/min
Inj. Vol.	0.63 µL
P_{MAX}	315 bar

Corrected
- Constant k* (tG & F)

Significant reduction in run time whilst still maintaining resolution

Gradient Method Translations: Acetaminophen Porous → Solid Core

Why correct for V_D/V_M?



ACE Excel 5 Super C18 150 x 4.6 mm

Agilent 1100

ACE UltraCore 2.5 Super C18 50 x 3.0 mm

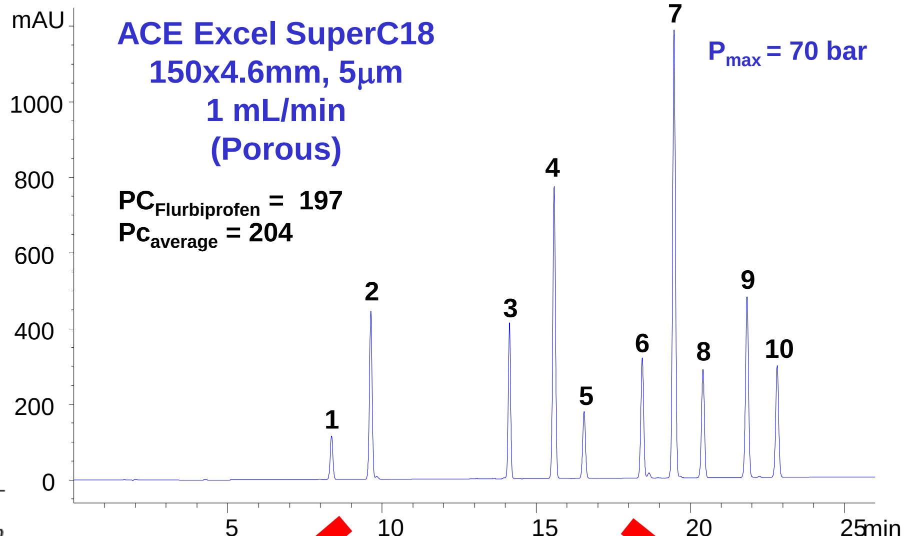
Agilent 1290 (Quaternary)
Gradient translated correctly
No correction for V_D/V_M

ACE UltraCore 2.5 Super C18 50 x 3.0 mm

Agilent 1290 (Quaternary)
Gradient translated correctly
Corrected for V_D/V_M
(0.81 minute delayed injection)

Aligning first & last peaks shows increased selectivity changes without V_D/V_M correction

Gradient Method Translations: Speed Or Resolution?



Gradient

	R_s		
Peak pair	150 x 4.6	50 x 3.0	100 x 3.0
1,2	7.9	7.0	9.5
2,3	26.6	25.4	36.8
3,4	9.6	8.0	11.7
4,5	5.9	5.1	7.2
5,6	11.2	9.5	13.2
6,7	6.1	5.4	7.1
7,8	5.4	4.7	6.5
8,9	8.0	7.0	9.6
9,10	5.4	4.6	6.4

$$P_x = 1 + \frac{t_G}{W_x}$$

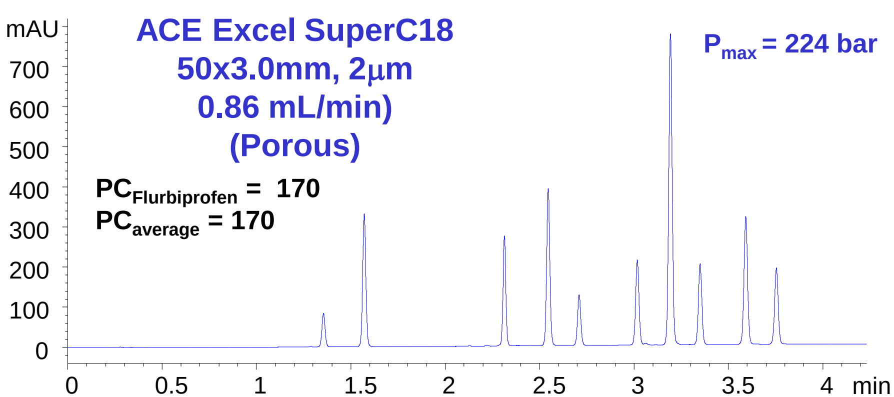
$$P_{Average} = 1 + \frac{t_G}{\frac{1}{10}\sum_1^{10} W_p}$$

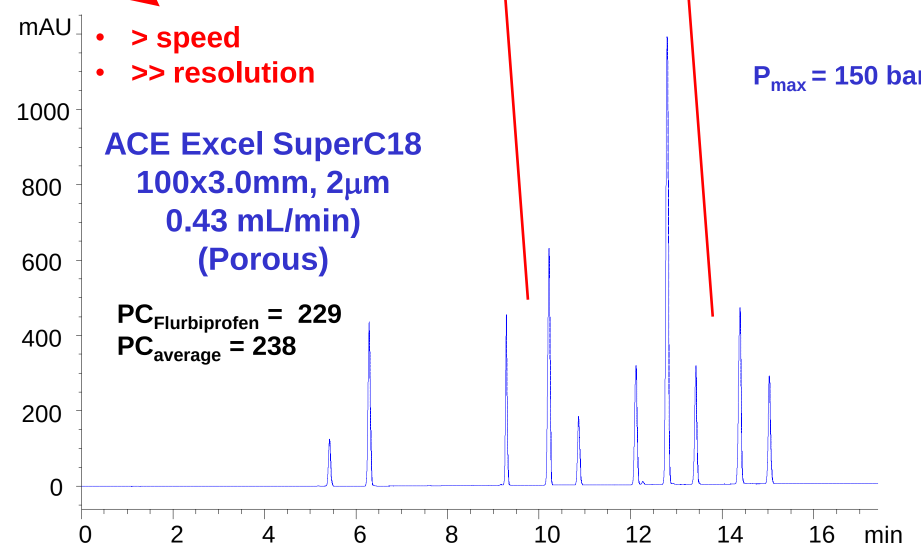
Speed

- >> speed
- ≈ resolution

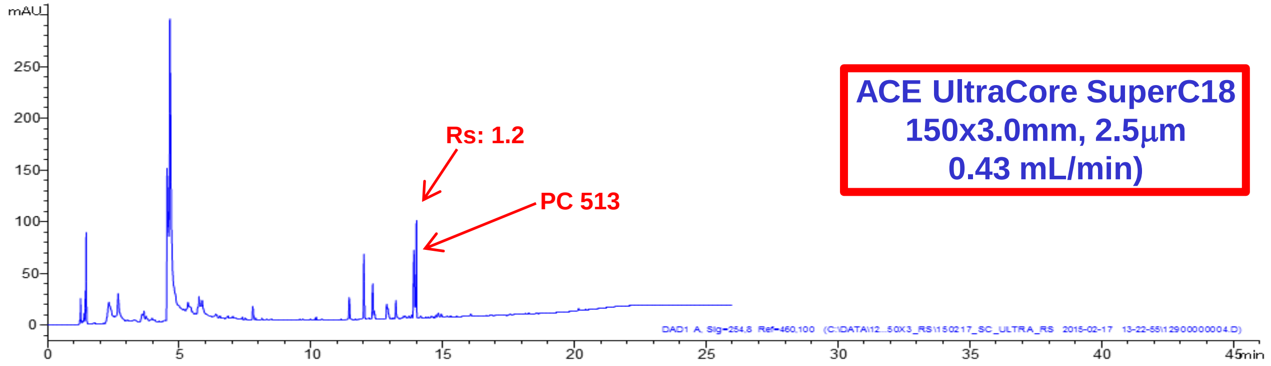
Resolution

- > speed
- >> resolution





High Resolution: Echinacea, Constant Flow / Longer Column



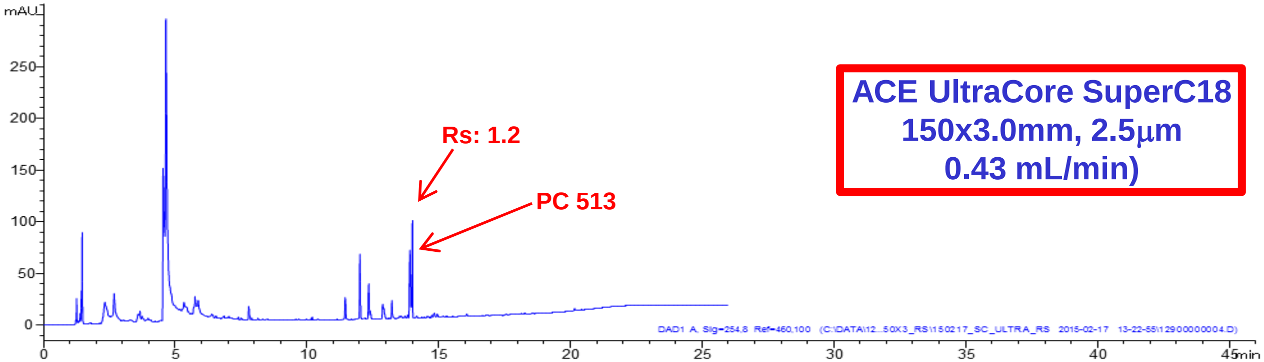
ACE UltraCore SuperC18
150x3.0mm, 2.5µm
0.43 mL/min)

Column	150 x 3.0 mm
t_G	20 min
Flow:	0.43 mL/min
Inj. Vol.	5 µL
PMAX	130 bar

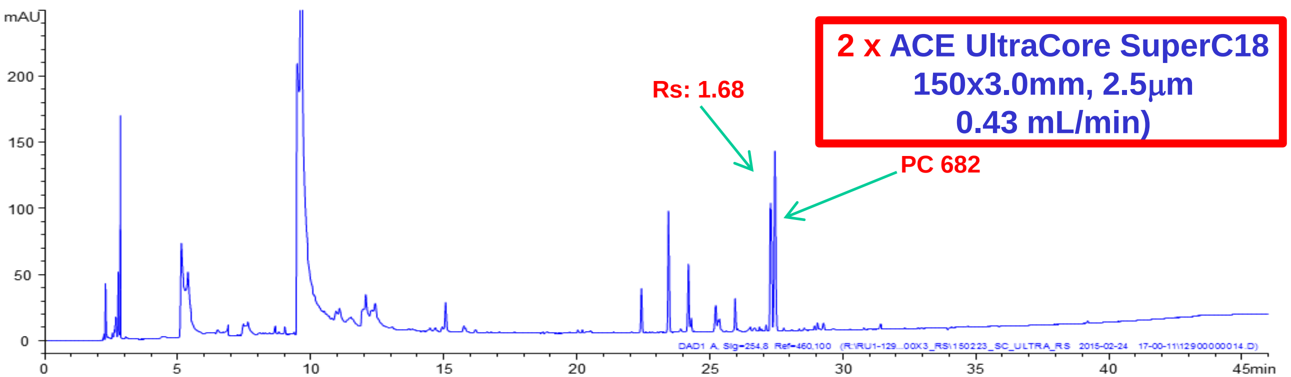
Translation to Longer Solid Core Columns For Ultra Resolution:

1. Calculate column volumes $$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$
2. Translate gradient time $$\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$$
3. No flow rate change needed
4. Modify injection volume $$Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$$
5. Calculate whether an injection hold or pre-injection is needed

High Resolution: Echinacea, Constant Flow / Longer Column



Column	150 x 3.0 mm
t_G	20 min
Flow:	0.43 mL/min
Inj. Vol.	5 µL
PMAX	130 bar

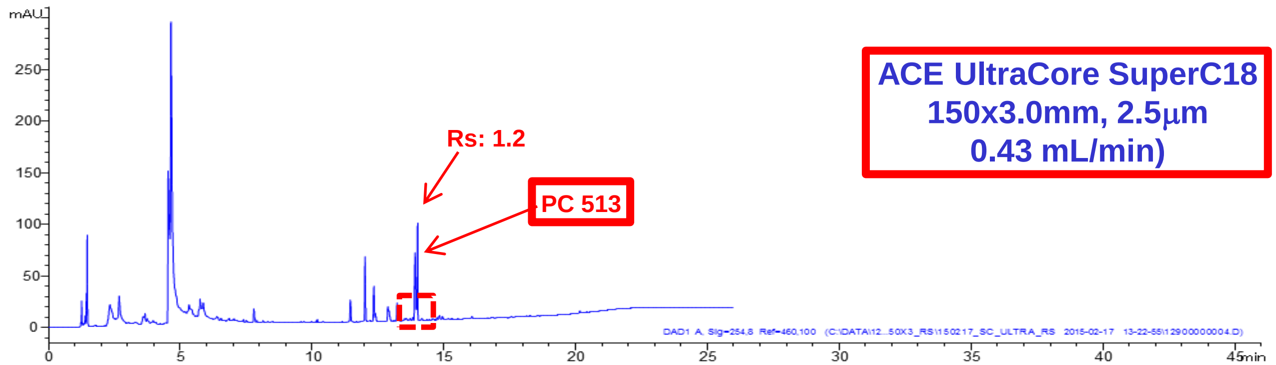


Column	300 x 3.0 mm
t_G	40 min
Flow:	0.43 mL/min
Inj. Vol.	10 µL
PMAX	223 bar

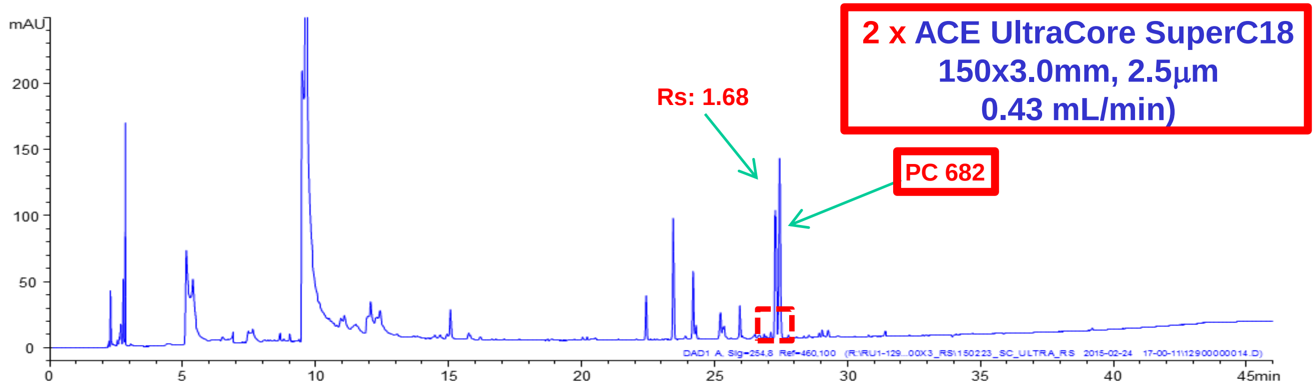
Translation to Longer Solid Core Columns For Ultra Resolution:

1. **Calculate column volumes** $$V_M \approx \pi \left(\frac{d}{2}\right)^2 L\varepsilon$$
2. **Translate gradient time** $$\frac{t_{G1}F_1}{V_{M1}} = \frac{t_{G2}F_2}{V_{M2}}$$
3. **No flow rate change needed**
4. **Modify injection volume** $$Inj_2 = Inj_1 \times \left(\frac{V_{m2}}{V_{m1}}\right)$$
5. **Calculate whether an injection hold or pre-injection is needed**

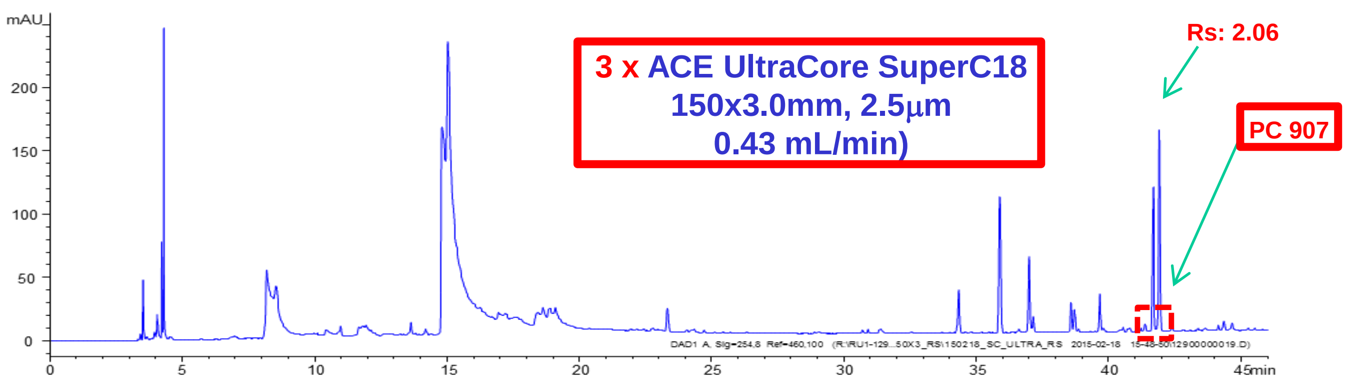
High Resolution: Echinacea, Constant Flow / Longer Column



Column	150 x 3.0 mm
t_G	20 min
Flow:	0.43 mL/min
Inj. Vol.	5 µL
PMAX	130 bar

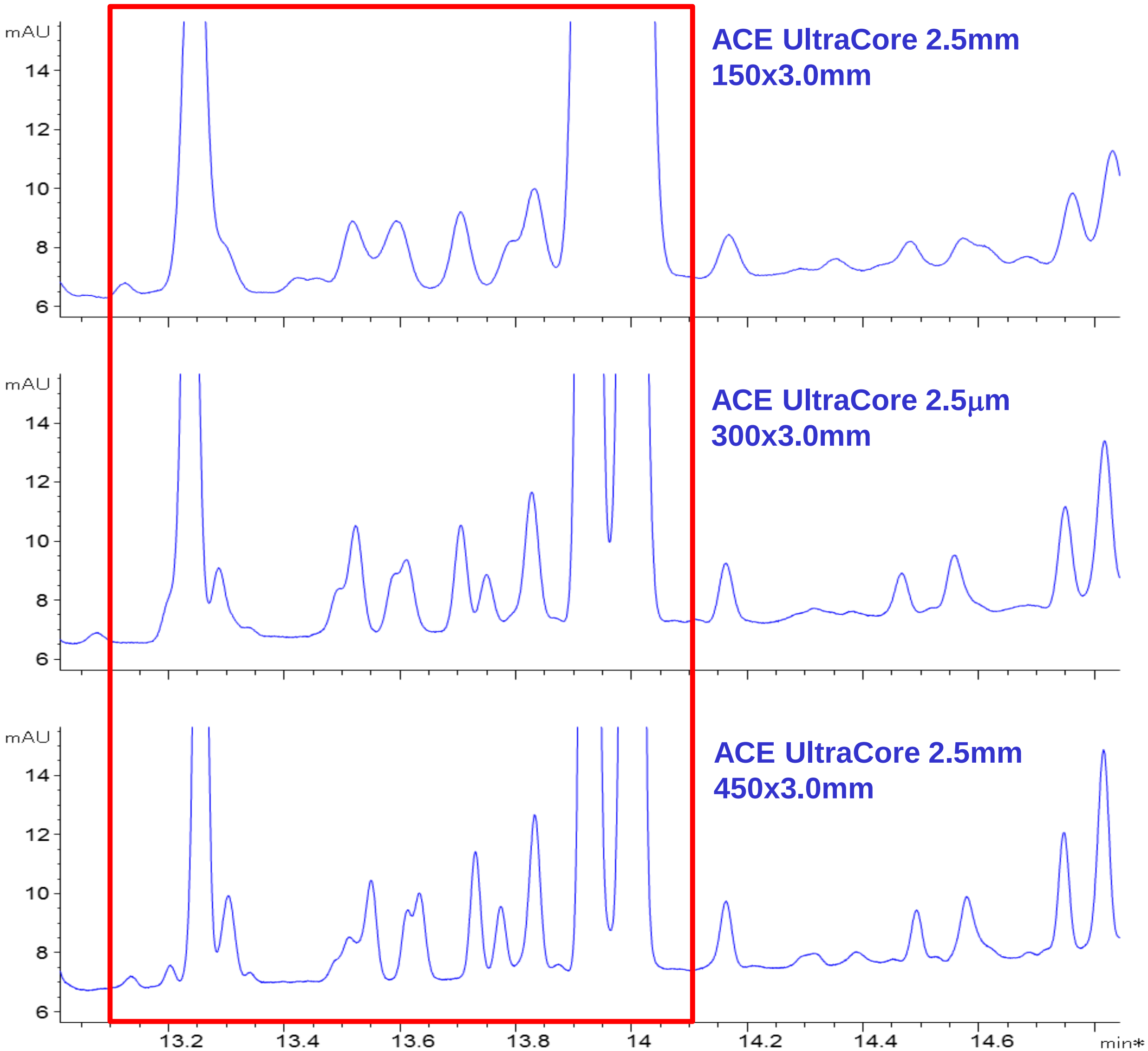


Column	300 x 3.0 mm
t_G	40 min
Flow:	0.43 mL/min
Inj. Vol.	10 µL
PMAX	223 bar



Column	450 x 3.0 mm
t_G	59.96 min
Flow:	0.43 mL/min
Inj. Vol.	15 µL
PMAX	338 bar

High Resolution: Echinacea, Longer Columns Aligned



Gradient analysis
A= 0.1% formic acid (aq)
B = 0.1% formic acid in MeCN
5-100%B in 20 mins, hold 100%B for 5 mins
80°C, 0.43 mL/min, 254 nm.

Sample: 1.0 mL echinacea extract added to 9.0 mL MeCN:water 1:1 v/v and filtered through 0.43 µm Whatman Mini UniPrep filter vial

Summary #2: Method Translations

- **Relatively Accurate method translations are achievable**
- **Isocratic translations**
  - **Use L/dp ratio translations**
  - **Translate to columns that have scalable bonded phases**
  - **Be aware of system dispersion effects on smaller columns**
- **Gradient translations**
  - **More complex but easy calculations described**
  - **Consider V_D/V_m impact upon translation accuracy ← few online calculators currently offer this**
- **Improved sample detail / high peak capacities for complex samples are possible using column coupling**

Overall Conclusions

- **Selectivity is a principal concept in chromatography**
- **Understanding selectivity aids method development by focussing efforts on high impact variables**
- **Screening columns with differing retention mechanisms is a useful first step for method development**
- **Accurate method translations are possible using the simple appraoches describes**
- **Consider system dispersion effects with smaller columns**
- **Method translations also help achieve high resolution**

Thank You For Your Attention

info@ace-hplc.com