Exploiting the Powerful Advantage of Chromatographic Selectivity in HPLC Method Development

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HPLC Method Development Trends

Objectives

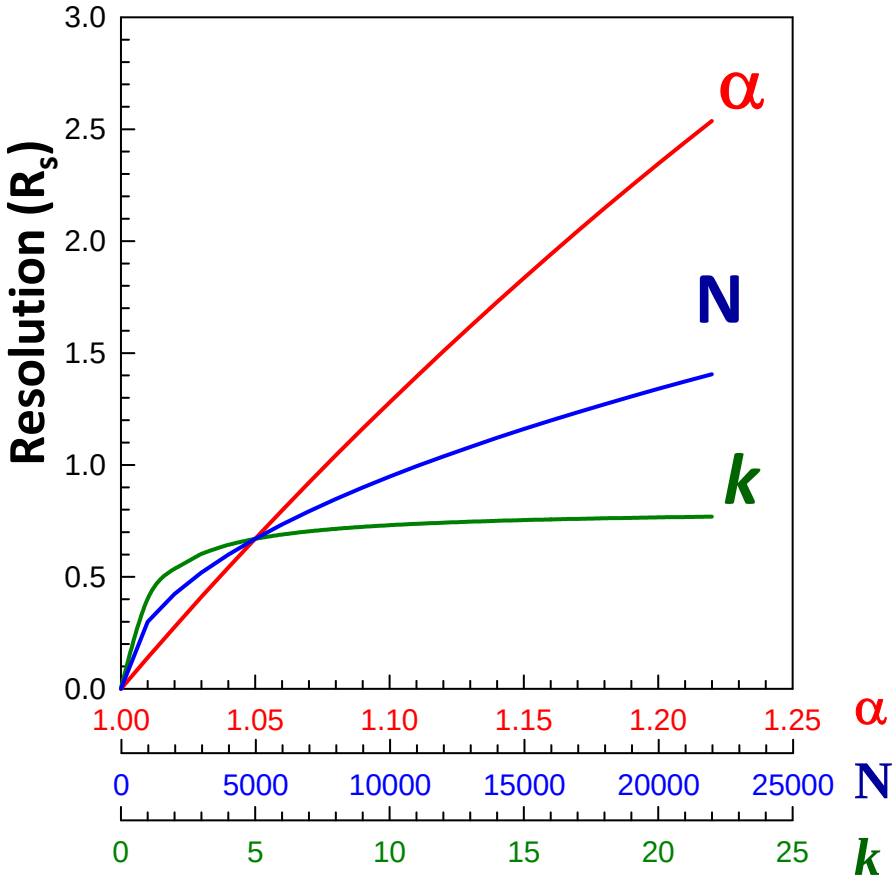
- **Improved Productivity**
  - Faster method development
  - Get on to method validation more quickly
  - More robust methods makes validation easier
  - Shorter final methods = higher instrument and analyst productivity
- **Better Quality**
  - Quality by Design principles and practices
  - Easier method transfer and implementation
  - Less rework and less effort when changes are needed
  - Less surprises…such as impurities found later
  - Easier acceptance by regulatory agencies and reviewers

Setting Goals for Method Performance Before Beginning Method Development

Criterion	Measure	Target
Analysis Speed	Run time	≤ 10 min. assay ≤ 20 min. impurity
Resolution	Minimum R_s critical pair	≥ 2.0
Peak Shape	USP Tailing Factor	≤ 1.5, as close to 1.0 as possible
Robustness	Δ Resolution	± 10% relative with small change in parameter
Instrument Limitation	Maximum backpressure	70-80% Instrument Maximum
Column Performance	Reproducibility and Lifetime	Meet Sys. Suitability Criteria Consistent for 250-1000 runs?

Resolution, peak shape and robustness: keys to accuracy and precision

Ultimate Goal of RPLC Separation: Resolution



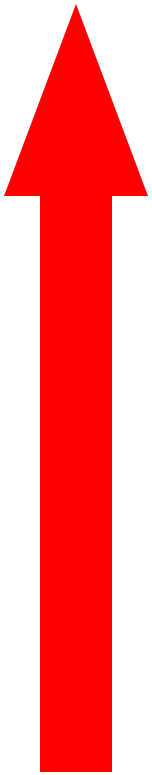
Efficiency **Selectivity** **Retention**

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

∴ **Small changes in selectivity (α) have a big impact on resolution.**

Which RPLC Parameters Affect Selectivity?

MOST Effective



LEAST Effective

Isocratic separations

- Column stationary phase
- Organic modifier
  - ACN, MeOH, ACN/MeOH blend
- pH (ionizable compounds only)
- % organic modifier
- "Buffer" choice
  - HCOOH, HOAc, phosphate, TFA, HCOOH/ NH_4COO, NH_4COO, NH_4OAc, NH_4HCO_3
- Column temperature
- "Buffer" concentration

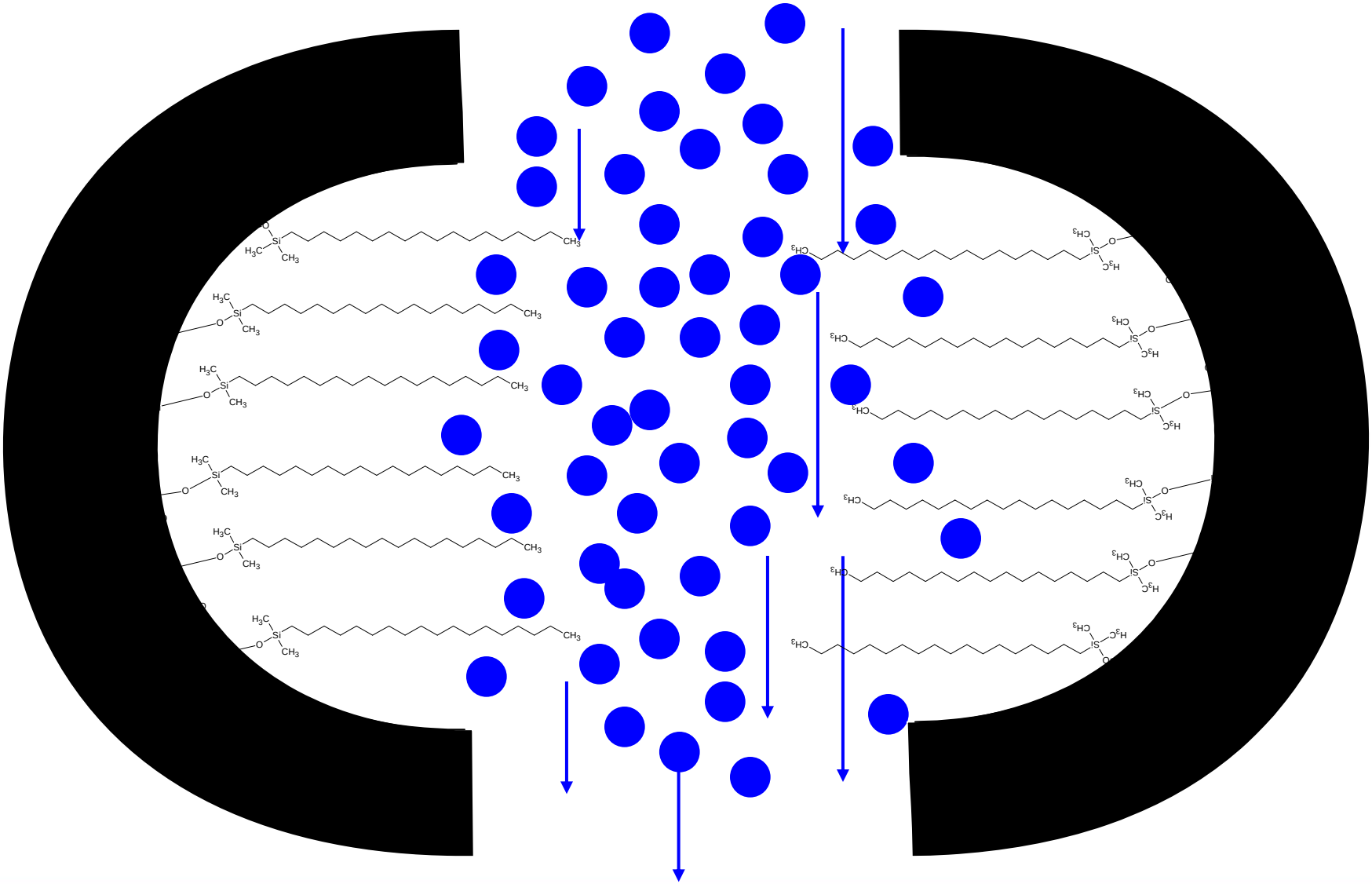
Gradient separations

- All parameters for isocratic separations
- Gradient steepness ("b" or k*)
- Delay volume
- Ratio of gradient volume to column volume

Selectivity is function of chemistry and thermodynamics – relative retention

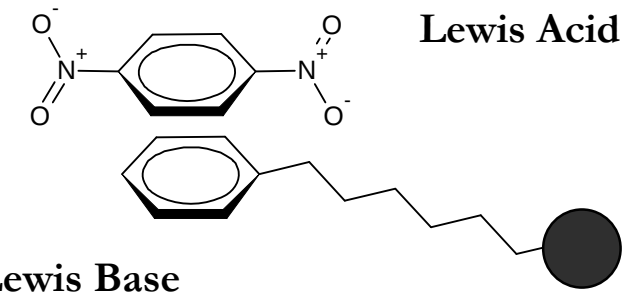
[1]adapted from "Introduction to Modern Liquid Chromatography", 3rd Edition, L. R. Snyder, J. J. Kirkland, J. W. Dolan; p. 29, 2010, John Wiley & Sons, Inc.

Hydrophobic Interactions: Partitioning and Adsorption

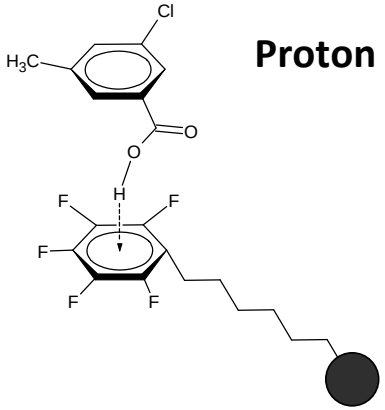


Interactions: Analytes and Stationary Phase

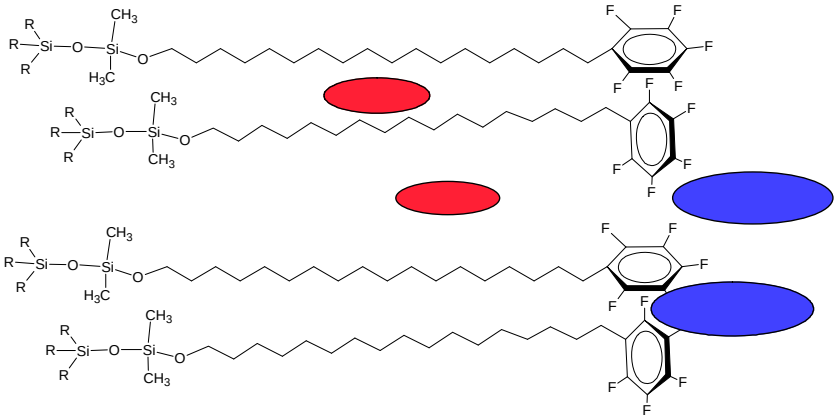
π–π Interactions



Hydrogen Bonding Interactions

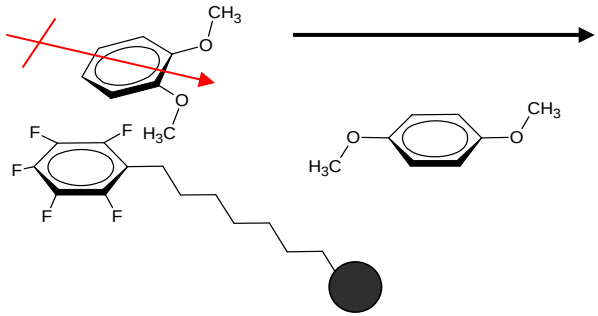


Shape-Selective Interactions



ligand rigidity contributes to steric selectivity

Dipole-Dipole Interactions

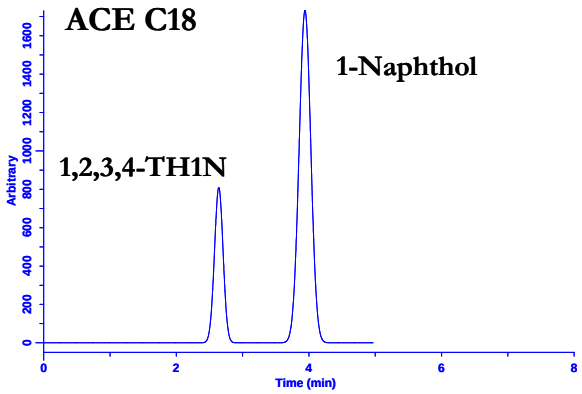


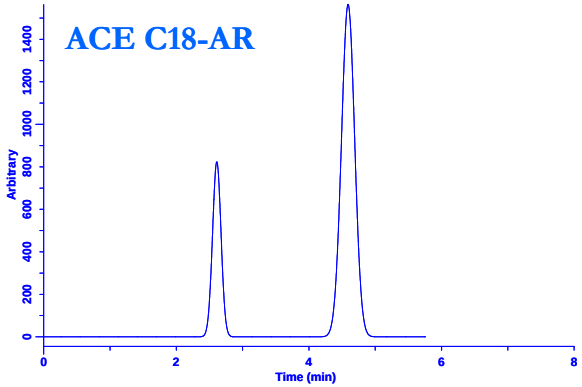
o-isomer has larger dipole than p-isomer

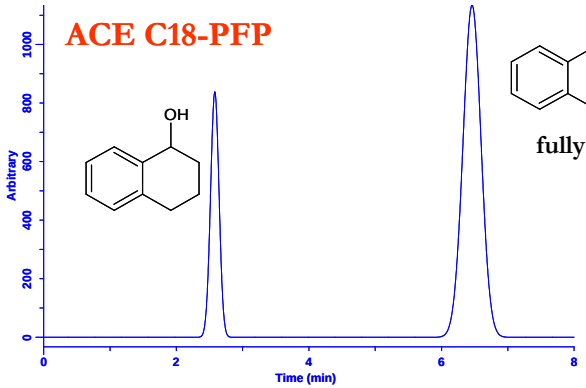
Types and Strengths of Analyte Interactions

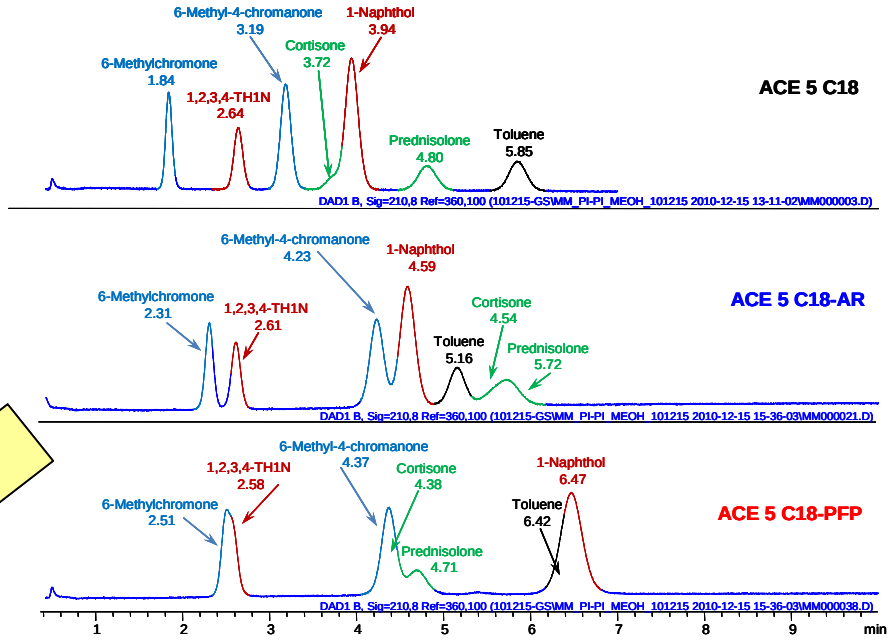
Types of Interactions	ACE C18	ACE C18-AR	ACE C18-PFP
Hydrophobic	++++	++++	++++
π–π	–	++++ electron-poor analytes	++++ electron-rich analytes
Dipole-Dipole	–	++	++++
Hydrogen Bonding	–	++	+++
Shape Selectivity	+	++	+++

π–π Interactions: Model Compounds









Retention Factors (k)

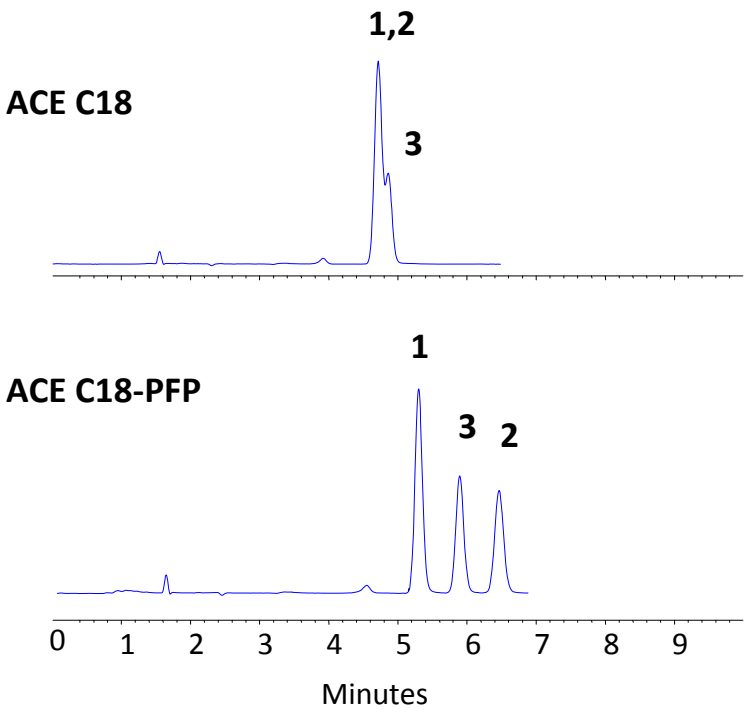
	ACE C18	ACE C18-AR	ACE C18-PFP
1,2,3,4 Tetrahydro-1-naphthol	10.0	9.7	9.6
1 Naphthol	15.4	17.8	25.5
$\alpha_{naph/thin}$	1.54	1.83	2.66
R_s	4.4	6.2	10.0

⬆40%

⬆130%

Note: Chromatograms simulated exactly from actual chromatograms for clarity using DryLab 2010

π–π Interactions: Methoxybenzene isomers



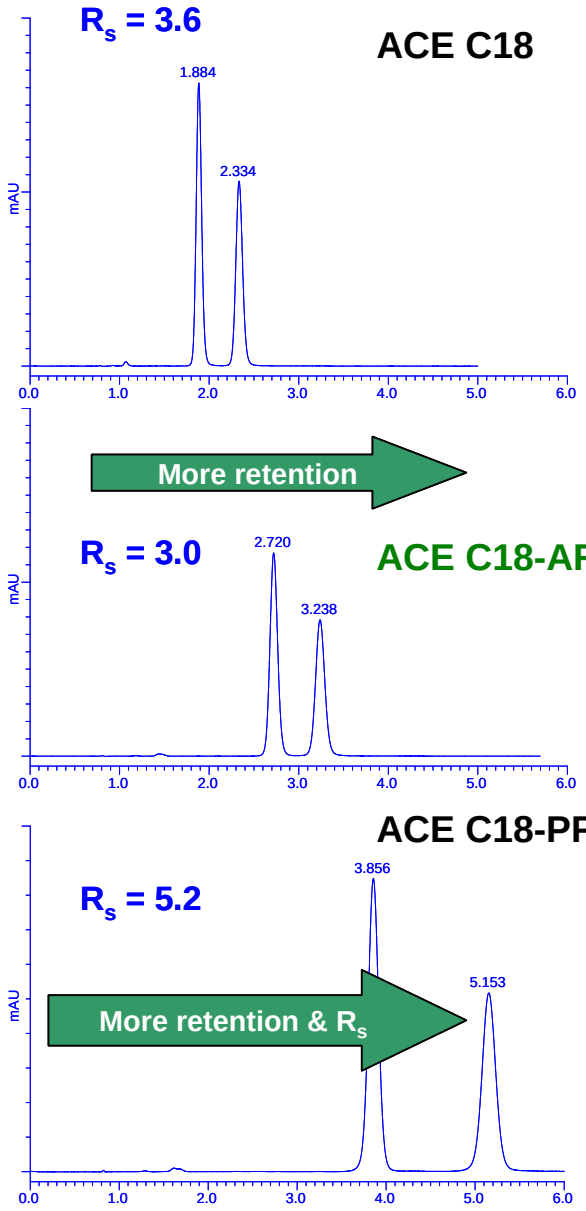
ACE C18-PFP shows additional separation mechanism compared to C18 columns

Compounds:

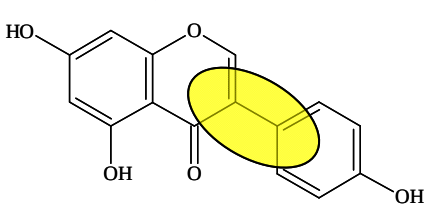
1. **1,2,3-trimethoxybenzene**
2. **1,2,4-trimethoxybenzene**
3. **1,2-dimethoxybenzene**

Columns:	**4.6 x 150 mm, 5 µm**
Mobile phase:	**1:1 (v/v) MeOH/water**
Flow rate:	**1 mL/min**
Temperature:	**40°C**
Detection:	**UV 254 nm**

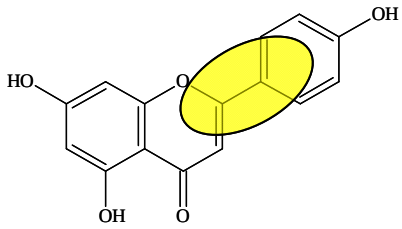
Shape-selective Interactions: Flavonoids



Ring 3 on different location on double bond of Ring 2



Genistein

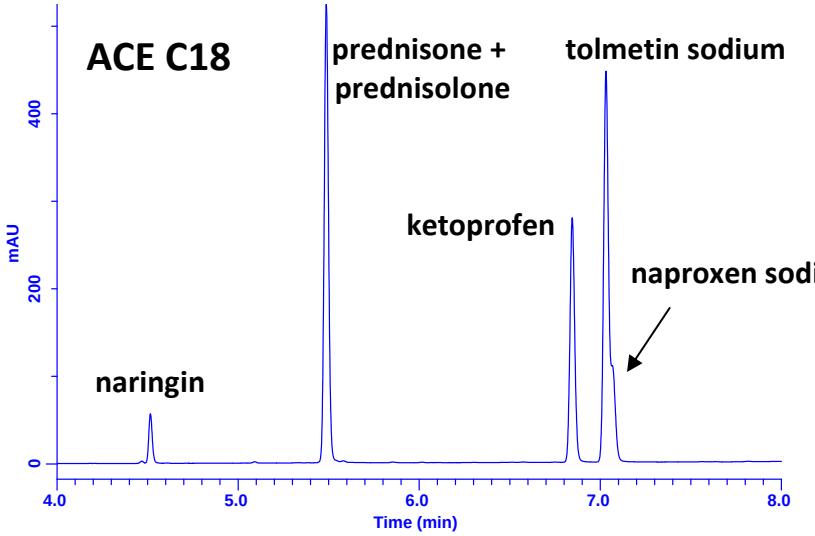


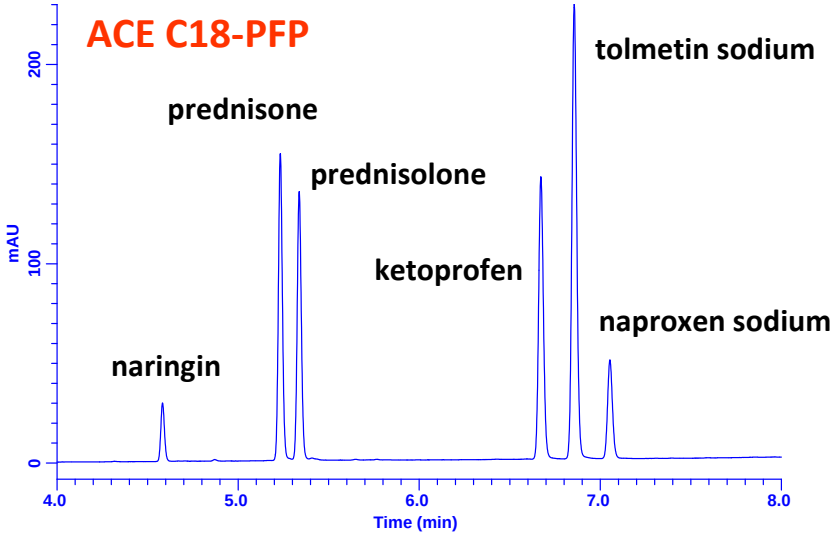
Apigenin

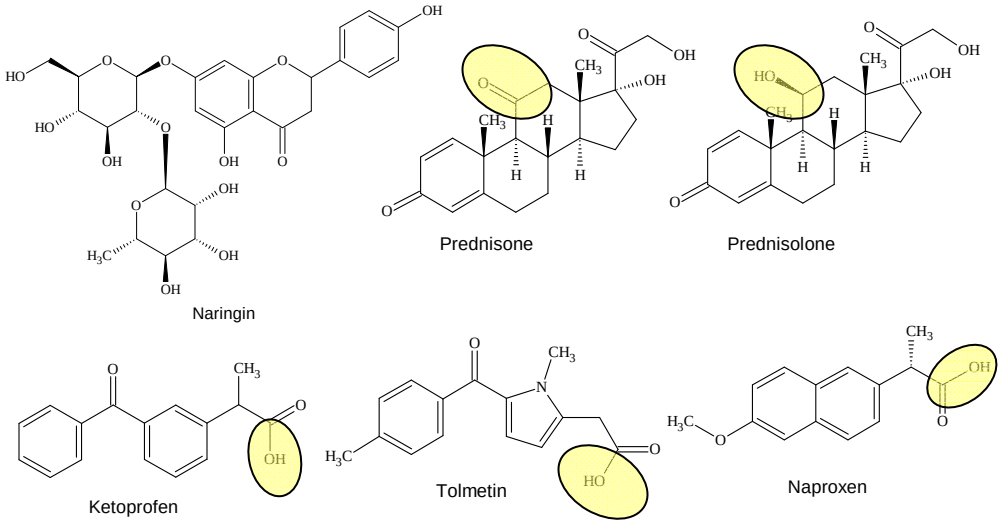
4.6 x 75 mm, 5 µm columns
60:40 MeOH/water
Flow Rate: 1.0 mL/min
Temperature: 30°C
1 µL injections

Shape Selectivity is one of the key retention mechanisms for C18-PFP phase

Hydrogen-bonding Interactions: C18-PFP



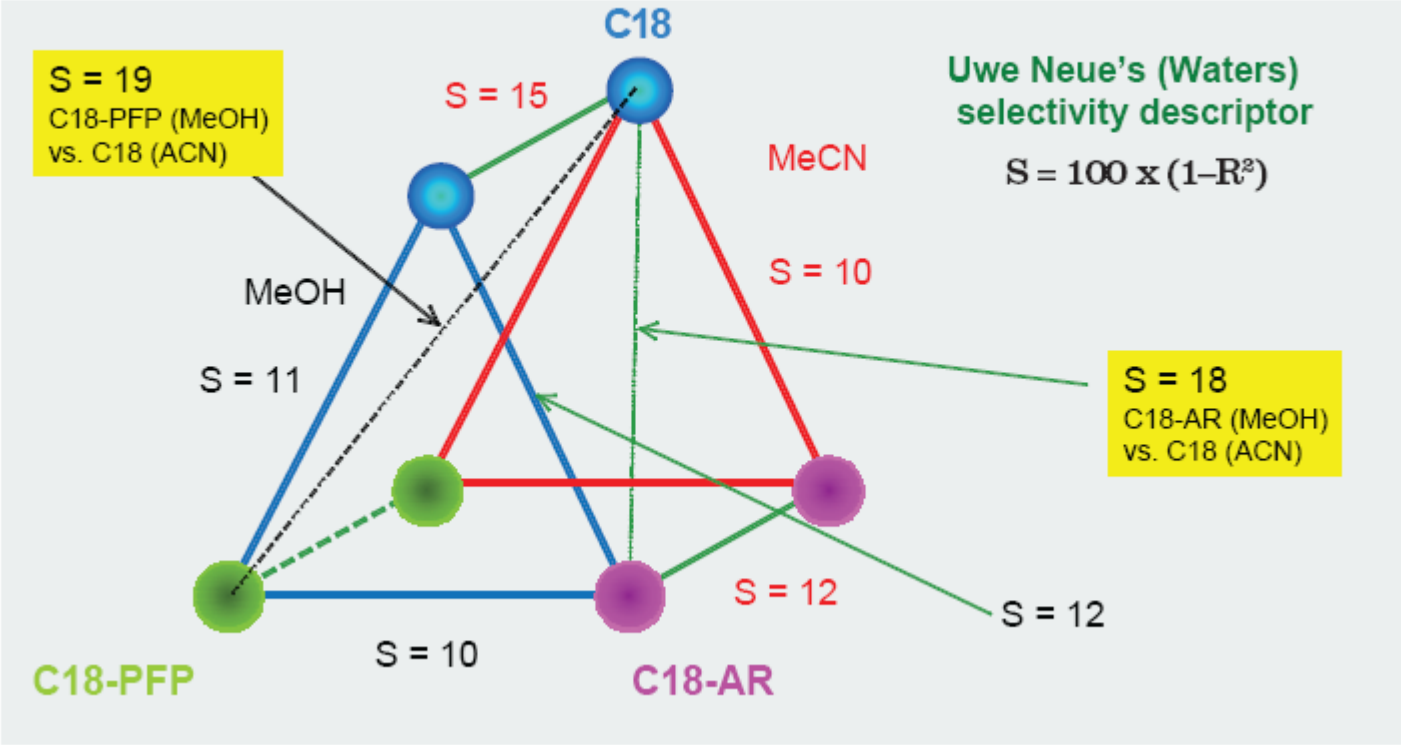




Columns: 3 x 50 mm, 3 µm columns
Flow Rate: 0.75 mL/min
Gradient: 5–100% ACN/water (0.1% HCOOH)
Temperature: 40°C
Injection volume: 1 µL

Construct a prism from S values: ACE phases

Orthogonality Prism Comparing ACE C18, C18-AR, C18-PFP with ACN and MeOH Mobile Phases

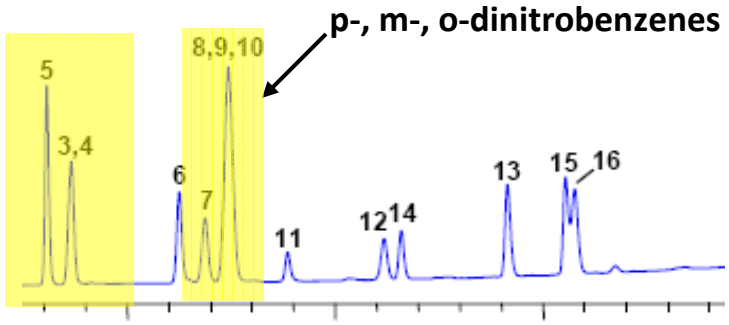


Most Orthogonal Combinations (> 100 analytes tested)

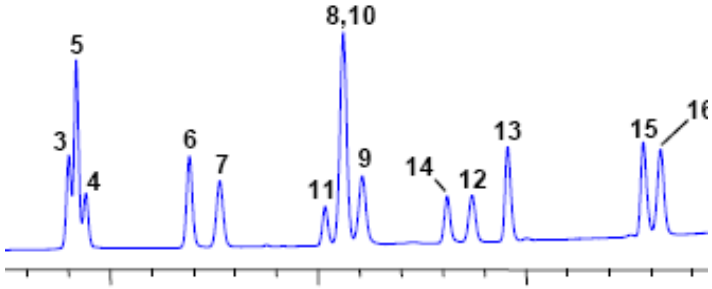
C18 (ACN) vs. C18-AR (MeOH) and C18-PFP (MeOH)

Changing Phases May Improve Resolution or just Change Selectivity

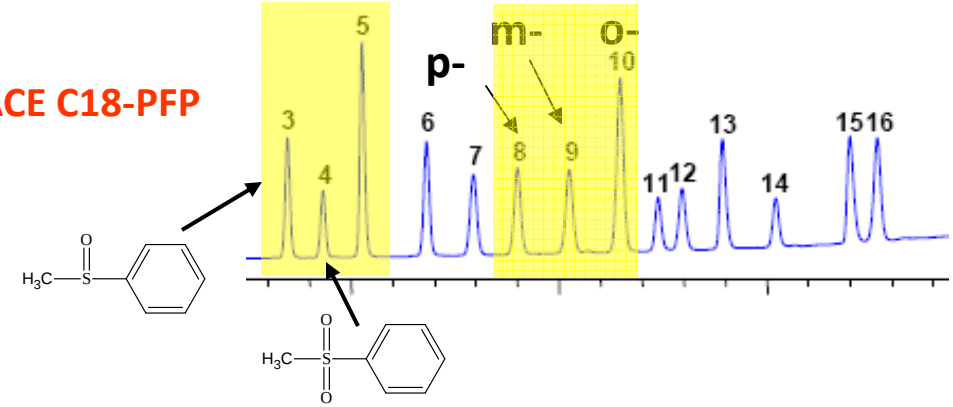
ACE C18



ACE C18-AR



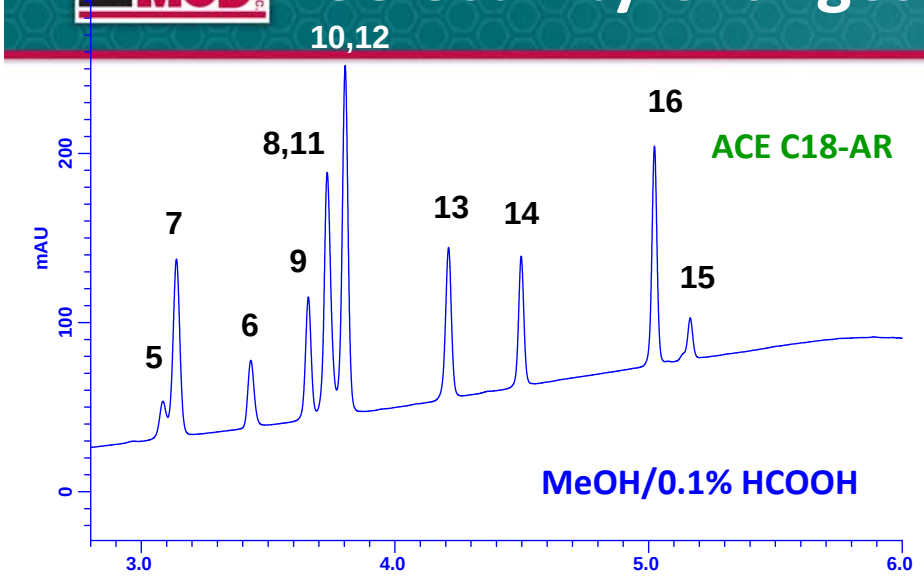
ACE C18-PFP



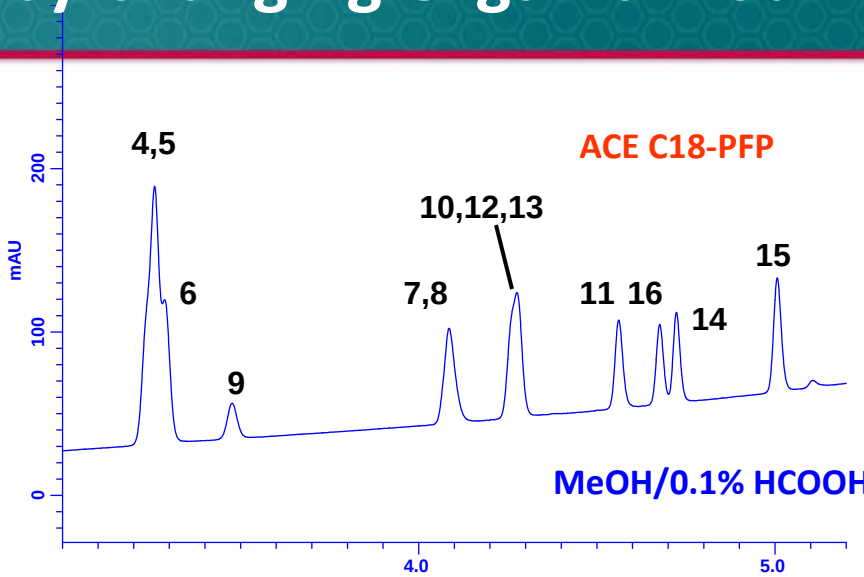
Columns: 2.1 x 50 mm, 3 µm
Flow rate: 0.6 mL/min, 60°C, 254 nm
A: 20 mM potassium phosphate, pH 2.7
B: 65:35 v/v MeOH/mobile phase A
Gradient: 3 to 100%B in 5 min., hold for 1 min

3) methylphenylsulfoxide
4) methylphenylsulfone
5) pindolol
6) quinoxaline
7) salicylaldehyde
8) 1,4-dinitrobenzene
9) 1,3-dinitrobenzene
10) 1,2-dinitrobenzene
11) myrecetin
12) juglone
13) remacemide
14) quercetin
15) methdilazine
16) plumbagin

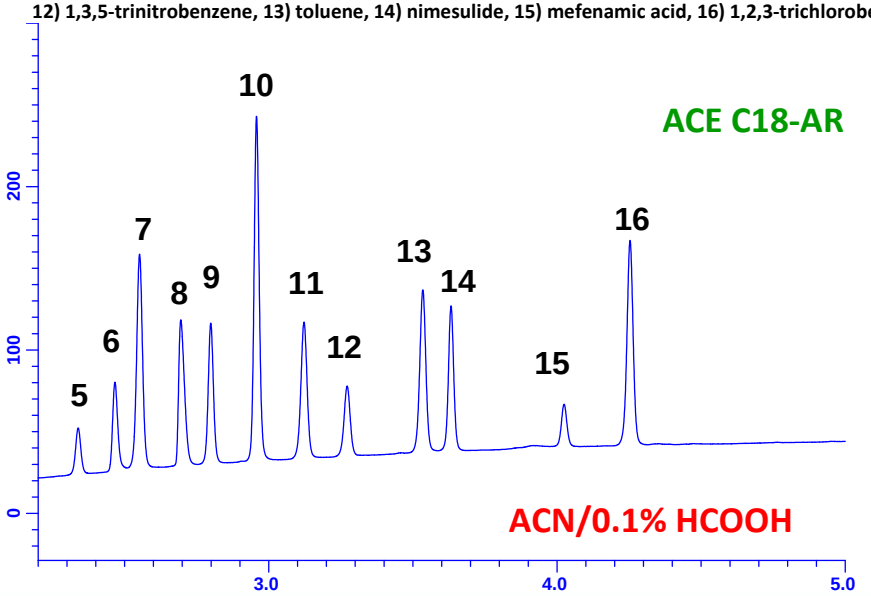
Selectivity Changes by Changing Organic Modifier

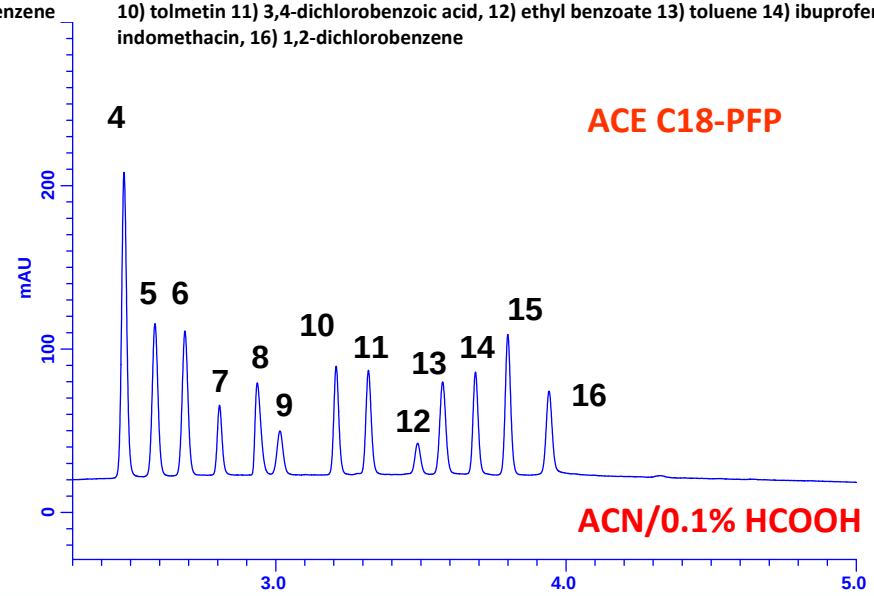


5) benzoic acid, 6) myrecetin, 7) p-cresol, 8) propranolol, 9) ethylparaben, 10) furosemide, 11) anisole, 12) 1,3,5-trinitrobenzene, 13) toluene, 14) nimesulide, 15) mefenamic acid, 16) 1,2,3-trichlorobenzene

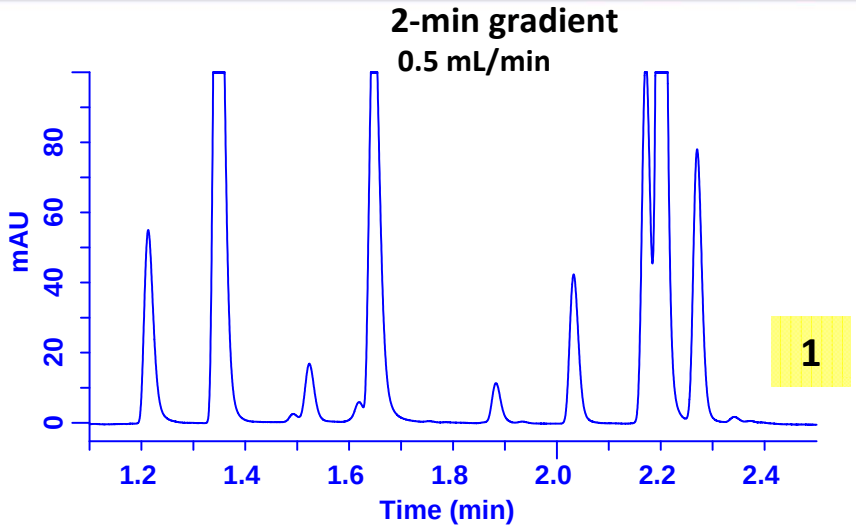


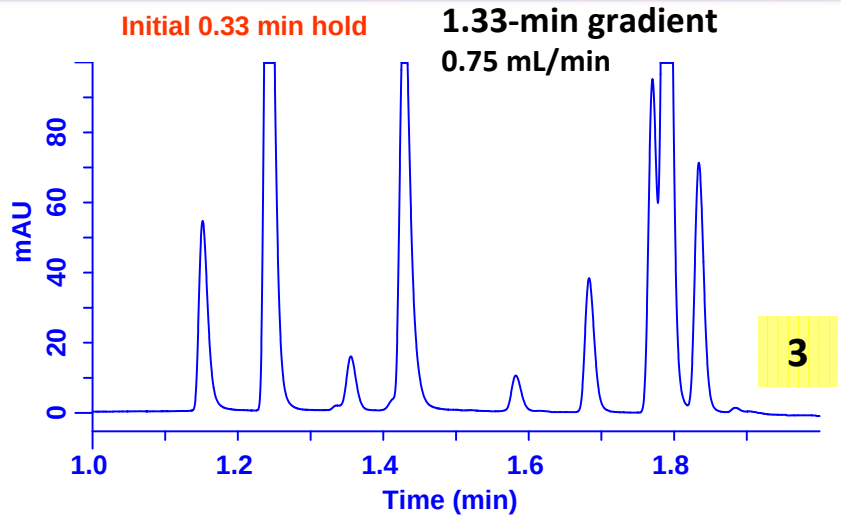
4) phenacetin, 5) p-cresol, 6) acetophenone, 7) quercetin, 8) carvedilol, 9) 1,3-dinitrobenzene, 10) tolmetin 11) 3,4-dichlorobenzoic acid, 12) ethyl benzoate 13) toluene 14) ibuprofen 15) indomethacin, 16) 1,2-dichlorobenzene

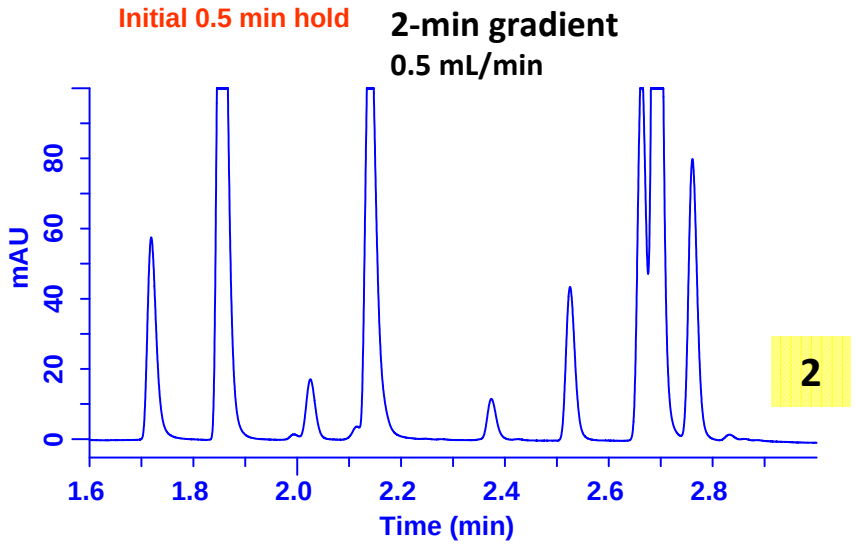


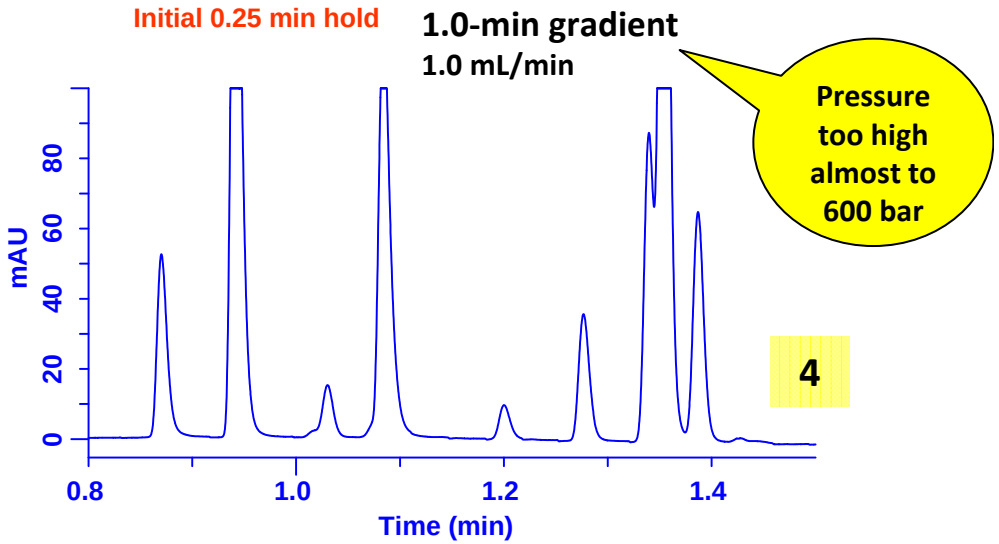


Fast Gradients with ACE Excel C18 2 µm UHPLC Cols
9 Analyte Mixture "thrown together"



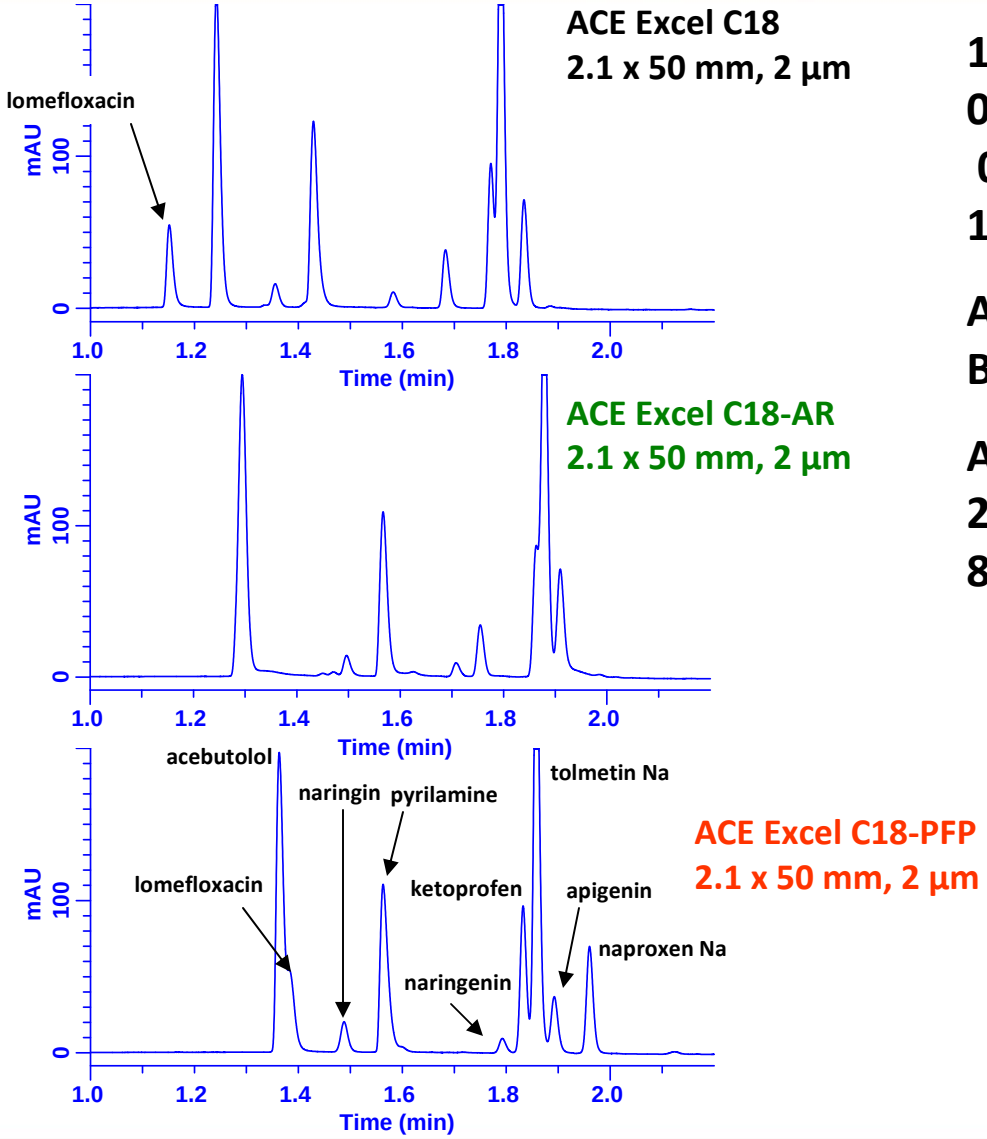






15–80% MeOH/0.1% HCOOH, 10 mM NH_4COO, 40°C, 1 µL injs., 2 µL flow cell, 80 Hz

ACE Excel 2 µm UHPLC Columns (1000 bar)



1.3-minute Screening Gradients
0.33-min hold at start
0.75 mL/min,
15 to 80% MeOH, 40° C, 1 µL injections,

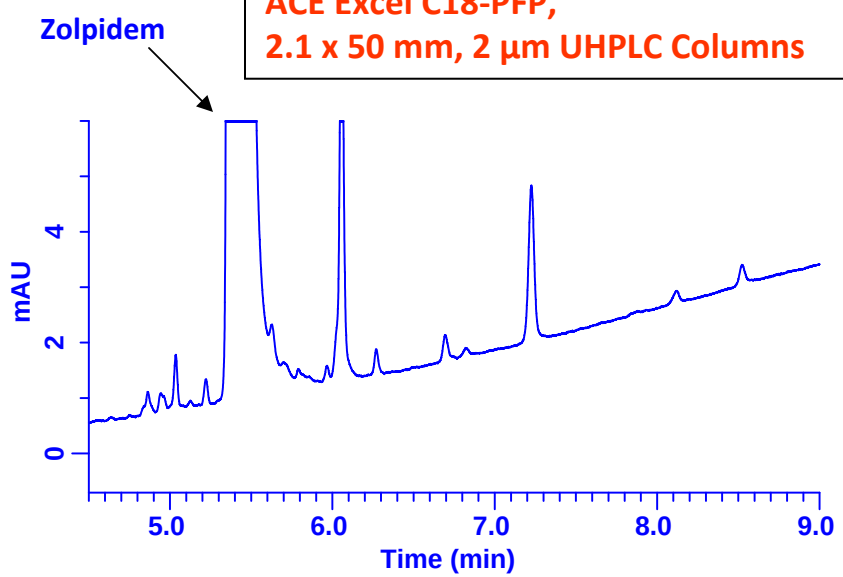
A: 0.1% HCOOH with 10 mM NH4COO
B: 80:20 MeOH/mobile phase A

Agilent 1200SL, V_D ~120 µL
2 µL flow cell
80 Hz data rate

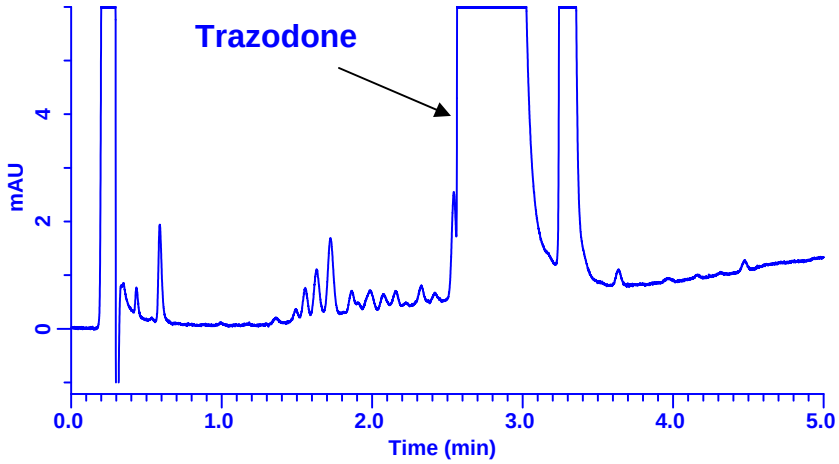
Ambien and Trazodone Tablets: Peroxide Degradants

heated 60°C for several hrs in 50:50 MeOH/3% H2O2

ACE Excel C18-PFP,
2.1 x 50 mm, 2 µm UHPLC Columns

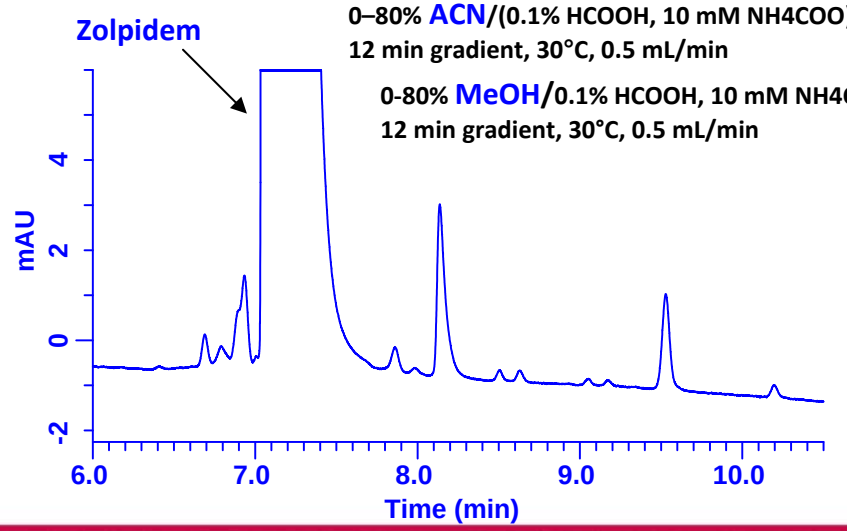


ACE Excel C18-AR, 2.1 x 50 mm, 2 µm

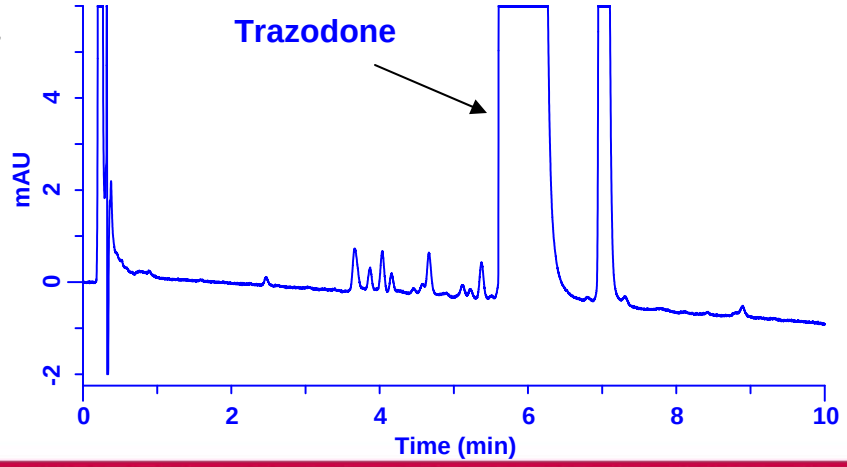


0–80% ACN/(0.1% HCOOH, 10 mM NH4COO)
12 min gradient, 30°C, 0.5 mL/min

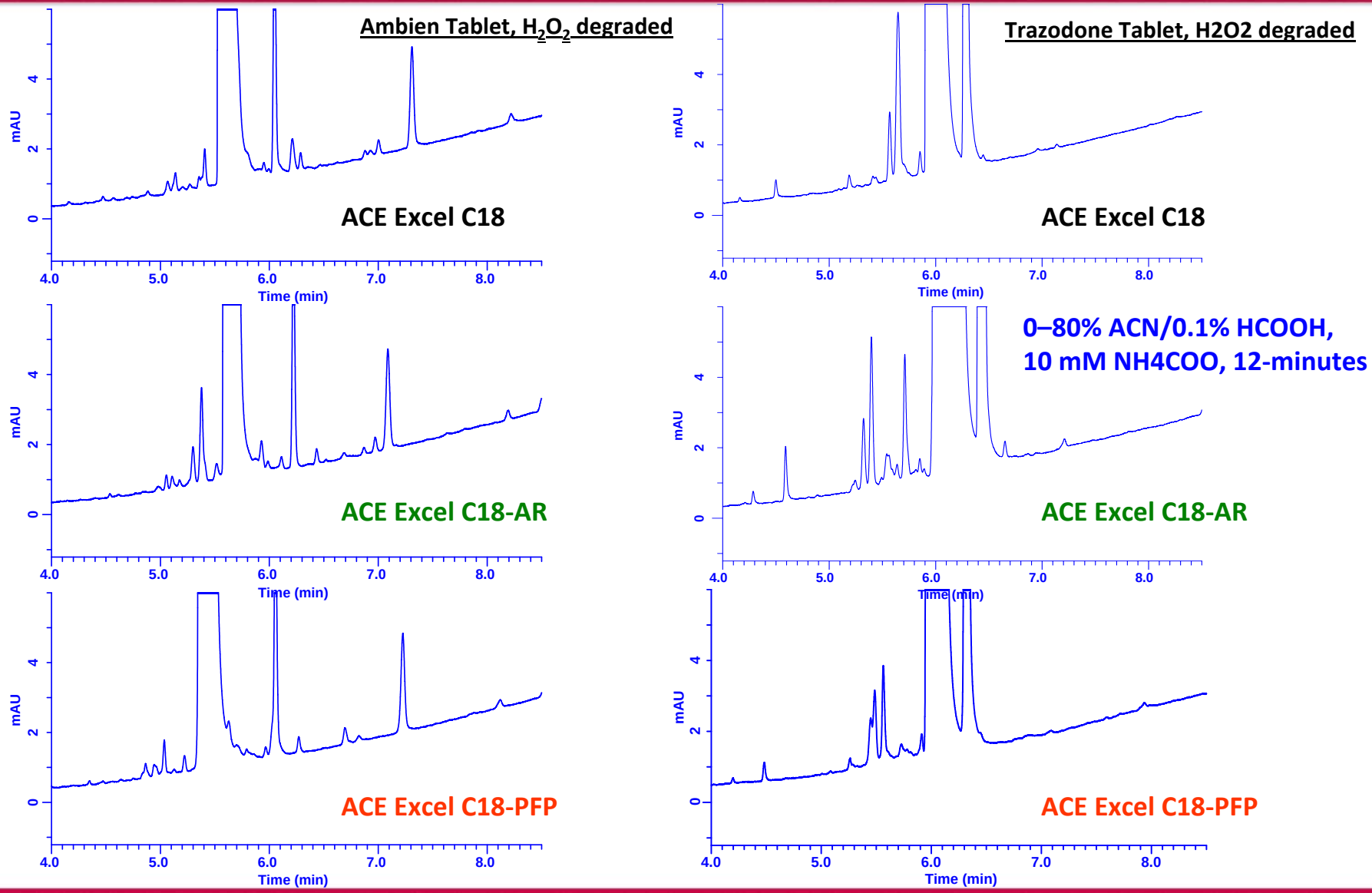
0-80% MeOH/0.1% HCOOH, 10 mM NH4COO,
12 min gradient, 30°C, 0.5 mL/min



ACE Excel C18-PFP, 2.1 x 50 mm, 2 µm



Gradient Screening: Marked Selectivity Differences Among Phases



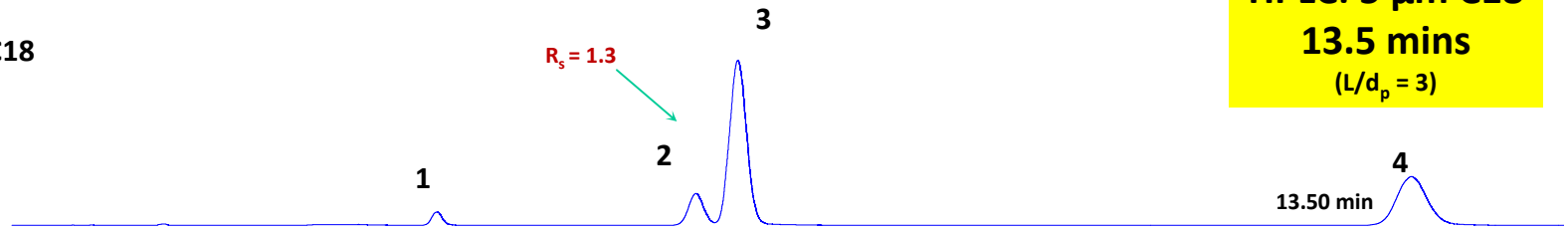
Summary: Fast, Robust RPLC Method Development

- Careful consideration and evaluation of important separation selectivity parameters are effective and critical for final method quality.
- Use of multiple column phase selectivities helps to ensure separations accomplish their objectives.
- Orthogonal, complementary alkyl, alkylphenyl, and alkylpentafluorophenyl phases make it easier to carry out method development.
- Use of short, efficient columns speeds column screening and optimization (3 × 50 mm, 2.7 µm and 3 × 75 mm, 3 µm, e.g.)
- Usually expensive, and time- and resource-intensive
- Development of a robust and rugged separation following QbD (Quality by Design) approach helps to minimize or avoid future problems and builds in quality.

Using ACE *Excel* C18-PFP to increase throughput (Isocratic)

Aim – Obtain R_s > 1.7 in shortest possible time

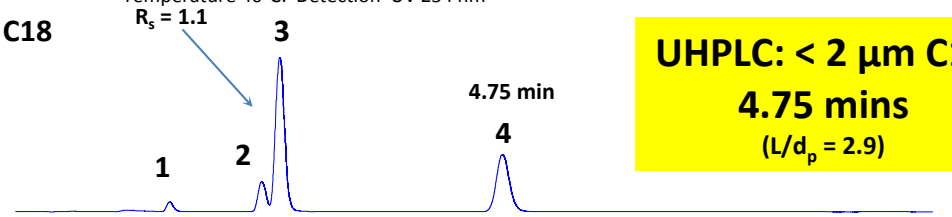
Competitor A 5 µm C18
150 x 4.6 mm
1.00 mL/min
163 bar



HPLC: 5 µm C18
13.5 mins
(L/d_p = 3)

Sample: 1) 1,2,dimethoxybenzene, 2) 1,3-dimethoxybenzene, 3) 1,3,5-trimethoxybenzene, 4) toluene (reference). Mobile phase 50:50 MeOH / H_2O
Temperature 40°C: Detection UV 254 nm

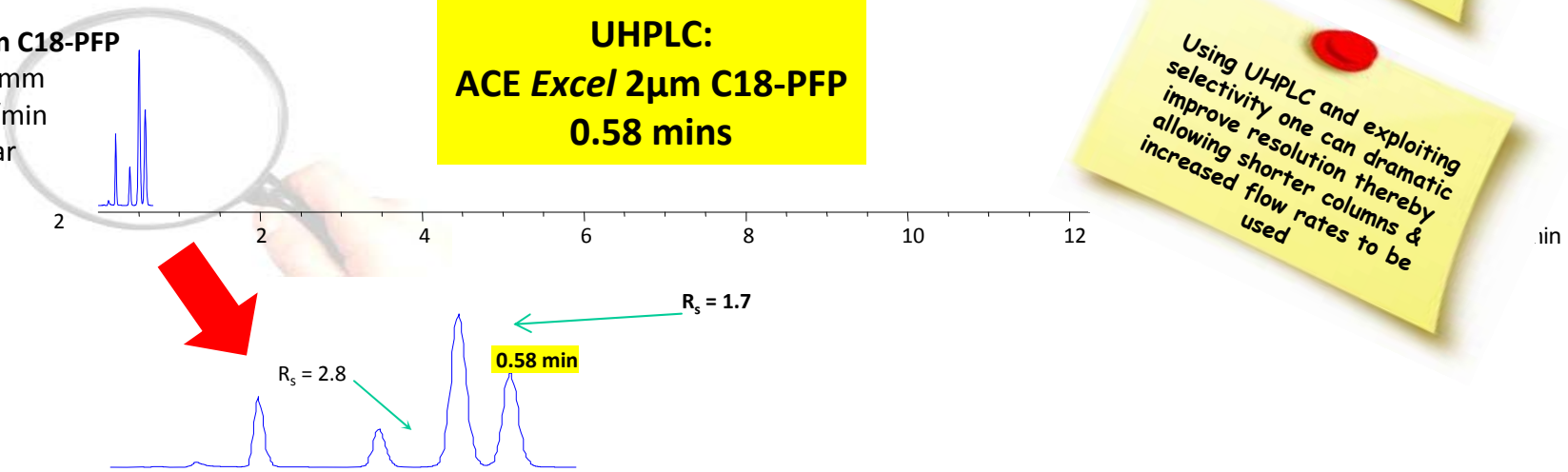
Competitor B < 2 µm C18
50 x 2.1 mm
0.21 mL/min
246 bar



UHPLC: < 2 µm C18
4.75 mins
(L/d_p = 2.9)

Keeping the L/d_p ratio constant one should maintain R_s and decrease the run time

ACE *Excel* 2 µm C18-PFP
30 x 2.1 mm
1.30 mL/min
492 bar

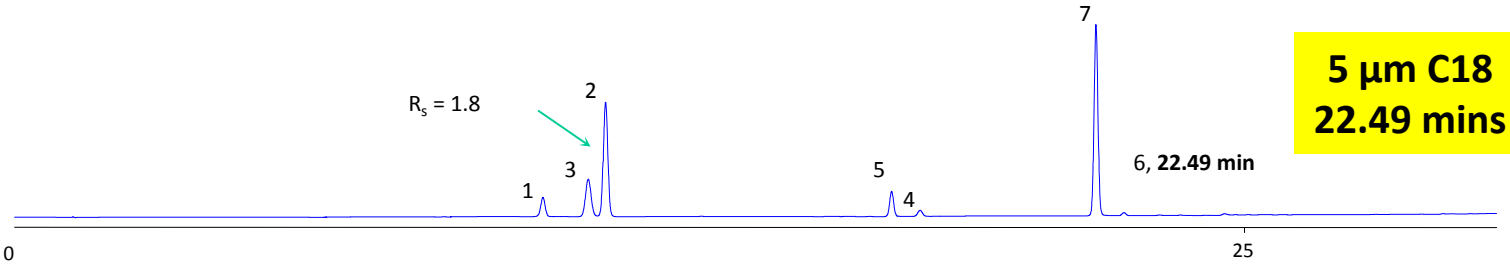


UHPLC:
ACE *Excel* 2µm C18-PFP
0.58 mins

Using UHPLC and exploiting selectivity one can dramatic improve resolution thereby allowing shorter columns & increased flow rates to be used

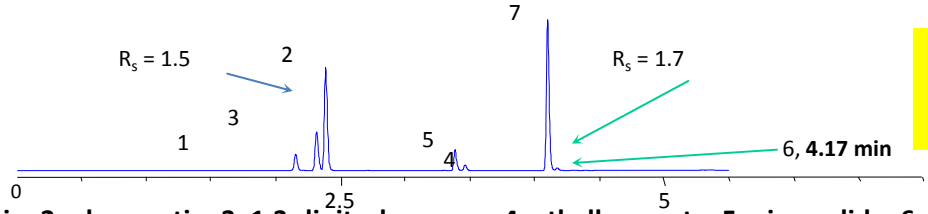
Using ACE *Excel* columns to increase throughput (Gradient)

Competitor A 5 µm C18
100 x 4.6 mm
1 ml/min, t_G = 29 min
max pressure: 92 bar
40 min cycle time



5 µm C18
22.49 mins

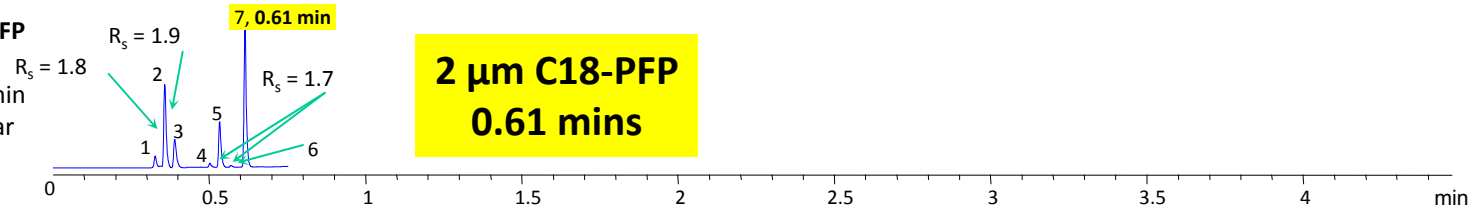
Competitor B < 2 µm C18
50 x 2.1 mm
0.6 ml/min, t_G = 5 min
max pressure: 367 bar
9 min cycle time



< 2 µm C18
4.17 mins

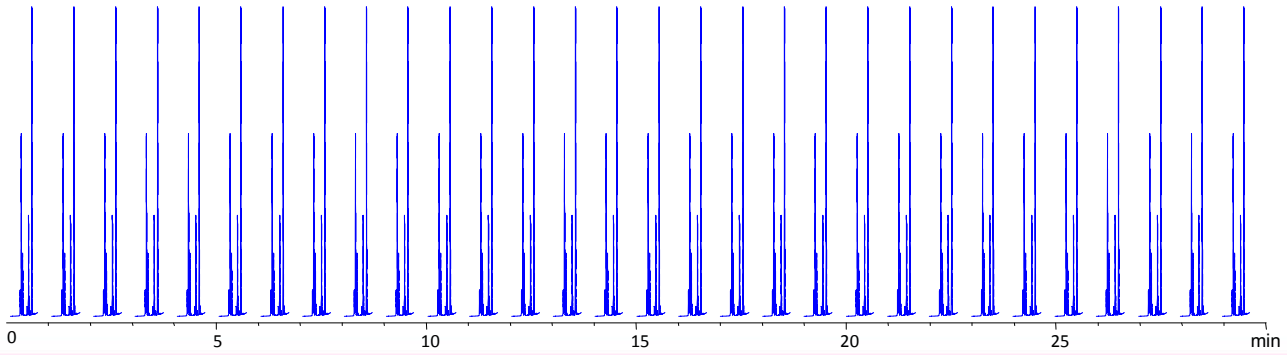
1, aspirin; 2, phenacetin; 3, 1,3-dinitrobenzene ; 4, ethylbenzoate; 5, nimesulide; 6, ibuprofen; 7, indomethacin.

ACE *Excel* 2 µm C18-PFP
30 x 2.1 mm
2.5 ml/min, tG = 0.7 min
max pressure: 914 bar
1 min cycle time

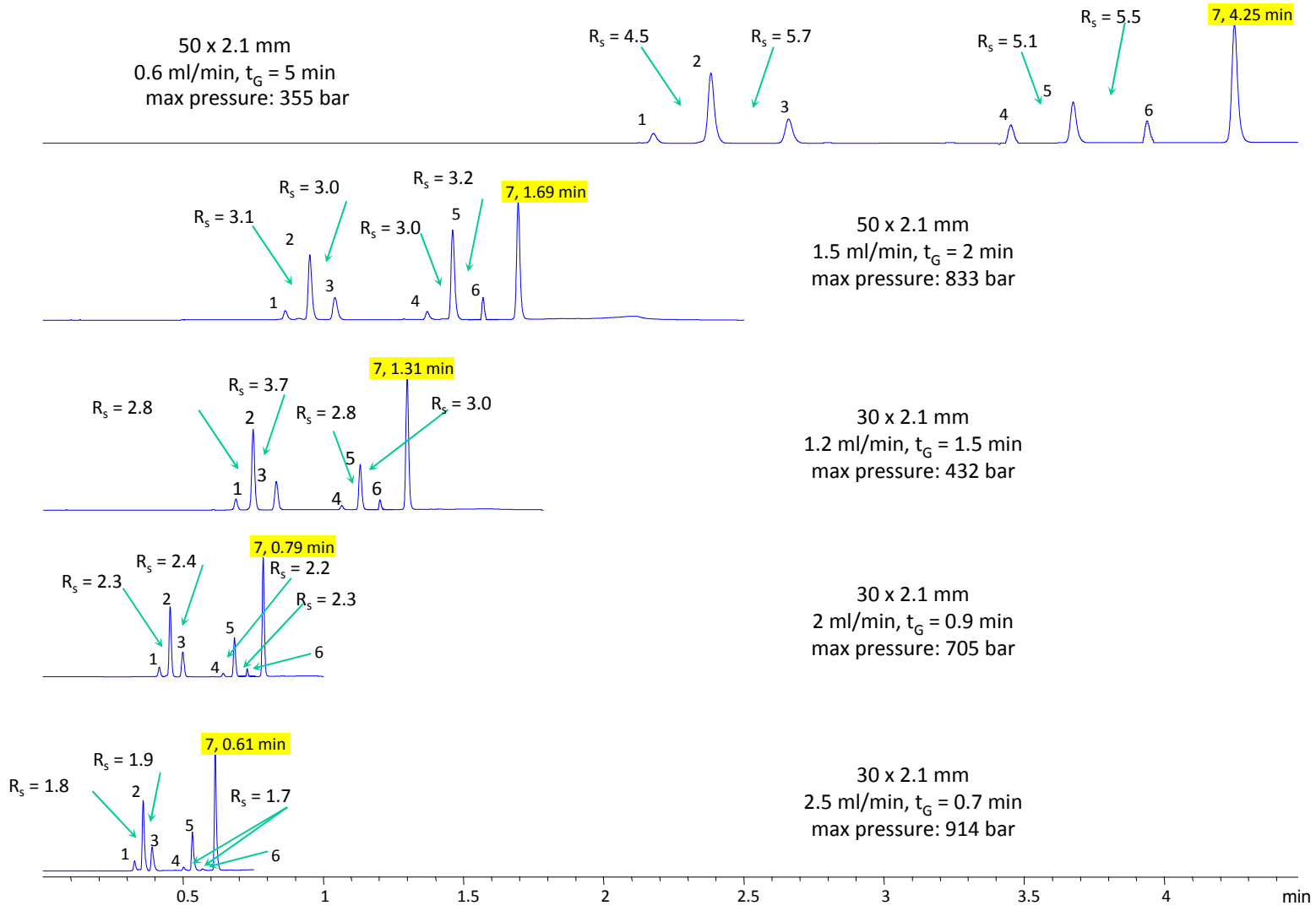


2 µm C18-PFP
0.61 mins

$R_s \geq 1.7$



Using ACE Excel columns to increase throughput (Gradient)



ACE *Excel* 2µm C18-PFP column

1, aspirin; 2, phenacetin; 3, 1,3-dinitrobenzene ; 4, ethylbenzoate; 5, nimesulide; 6, ibuprofen; 7, indomethacin.

Fast RPLC Method Development Strategy

1. **Screen short, high efficiency orthogonal phases with fast gradients at temperatures of 20-30°C.**
2. **Select 3 x 50 mm or 2.1 x 50 mm 2 or 3 µm size—similar in efficiency to 4.6 x 150 mm, 5 µm column often recommended in past by many experts.**
3. **Use different organic modifiers: ACN, MeOH and 1:1 v/v ACN/MeOH, if appropriate.**
4. **Use different LC/MS-ready aqueous components**
   a) **10 mM ammonium formate (pH 3.0), 0.1% HCOOH**
   b) **10 mM ammonium acetate (pH 4.75-5.75), adjusted with HOAc**
   c) **10 mM ammonium acetate (pH 6.8, unbuffered)**
   d) **10 mM ammonium bicarbonate (pH 7.8)**
5. **Identify best 1 or 2 combinations of stationary phase and mobile phase (organic modifier/pH)**
6. **Generate input data for DryLab® 2010 using 2 different gradient times (differing by 3-fold) at 2 temperatures (t_G x T expt.)**
7. **Identify optimum(a) and assess robustness.**
8. **Run optimized conditions to verify performance.**
9. **Compare performance vs. method goals.**