UHPLC Method Development and HPLC Method Upgrade: Importance of Selectivity and Efficiency

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Abstract

Liquid chromatographic methods are now often developed in research laboratories using modern UHPLC and UPLC instrumentation. However, when it's time to transfer those UHPLC methods to conventional HPLC equipment in quality control or manufacturing laboratories, it is usually necessary to increase particle size, column ID, and occasionally, column length to accommodate the lower pressure limits and higher dispersion of HPLC systems.

With the introduction of 2-µm ACE® *Excel*™ UHPLC columns, the excellent performance that chromatographers have come to expect from conventional ACE® columns is now available for UHPLC/UPLC in 1000-bar hardware. These UHPLC columns are also manufactured with 2-micron inlet frits, which make the columns more rugged for long life compared to sub-2-µm columns.

Examples will be presented which demonstrate the use of these columns when developing and transferring new UHPLC separations, and for improving legacy HPLC methods. The efficiency and unique selectivities of the C18, alkylphenyl, and alkylpentafluorophenyl phases make it easy to screen analysis conditions when developing new methods. In addition, separations developed on these UHPLC columns can be easily transferred to HPLC systems in the same column hardware with 3- and 5-µm particles. Finally, the advantages of improving the speed and/or resolution of legacy HPLC methods will also be shown.

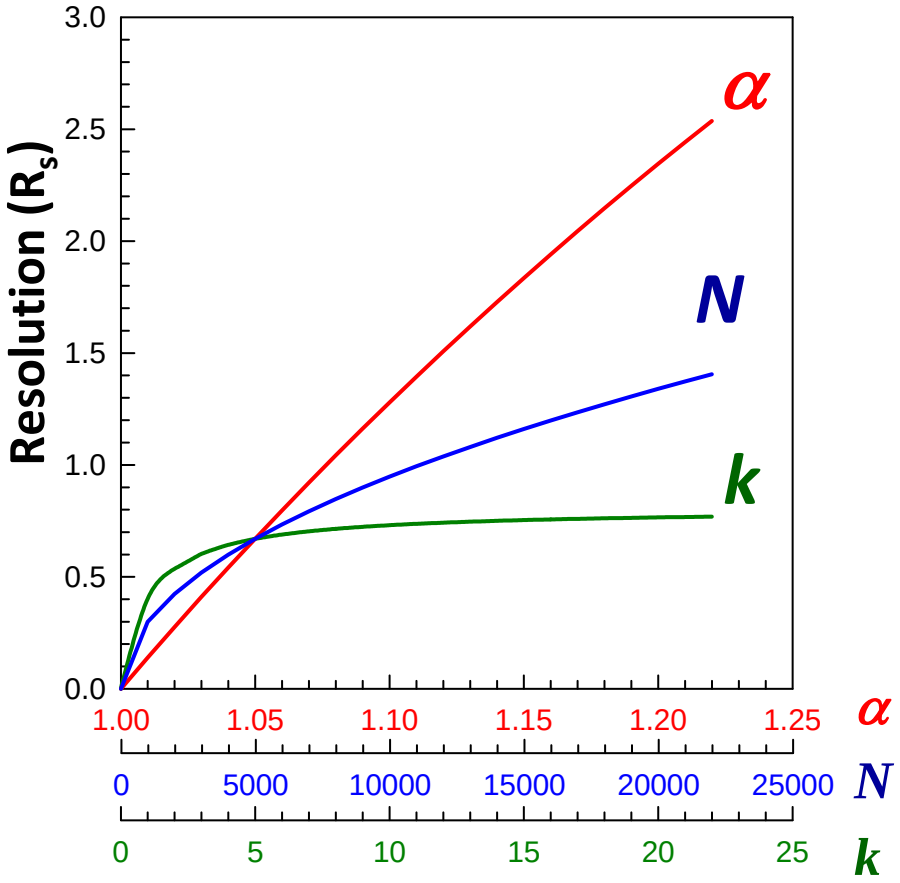
Modern Trends and Practices for Method Development and Validation

- **High productivity of people and instrumentation**
  - Develop fast methods that are more productive = higher throughput
  - Develop faster methods more quickly = less time to final method
    - Allows more time for thorough evaluation of separation parameters that affect selectivity and resolution = more robust and rugged methods
- **High quality methods and results**
  - Follow Quality by Design principles and practices
    - Lower likelihood of method and sample rework
    - Fewer “surprises” from potentially “hidden” impurities
  - More adaptable methods if/when processes or formulations change
  - Easier method transfer and implementation in manufacturing labs
  - Easier method acceptance by regulatory agencies and reviewers

Keys to Successful Transfer and Implementation for UHPLC Methods

- **Robust and rugged methods**
  - key parameters evaluated and "optimized" per performance goals
  - excellent peak shape for bases, acids, and neutrals
- **Reproducible retention and selectivity**
  - among different particles sizes
    - UHPLC to HPLC, analytical to semi-prep and preparative scale
  - excellent batch-to-batch reproducibility
- **Reliable and rugged column for acceptably long use**
  - excellent column bed stability at pressures up to 1000-bar
  - bonded phase stability over pH range needed
  - column frit porosity acceptable for use with biologically-derived samples and using typical sample and instrument hygiene

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) change resolution (R_s) much faster than *N* or *k*.**

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

Combine high efficiency with unique selectivity to speed method development and enable faster analyses.

[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.

ACT: Engineering New Stationary Phases with Aromatic Functionality for ACE® Columns

- **New phases with alternative selectivity, but with the robust properties of the C18 ligand**
  - **Reproducible (column-to-column & batch-to-batch)**
  - **Excellent column lifetime and low bleed**
  - **Superb efficiency provided by ultra-inert, ultra-pure silica particle**
- **Phases with aromatic functionality include phenyl and pentafluorophenyl (PFP) based ligands**
  - **Aromatic functionality potentially offer unique interactions with analytes (vs. C18) giving alternative selectivity**
  - **C18-phenyl (C18-AR[1]) and C18-PFP offer complementary selectivities, but comparable overall retention**
  - **Provide enhanced retention of polar compounds**
  - **Many aromatic functionality-based phases can be used in 100% aqueous eluents**

[1]AR = "aryl", derived from an aromatic ring

ACE and ACE *Excel* ™ Bonded Phase Characteristics

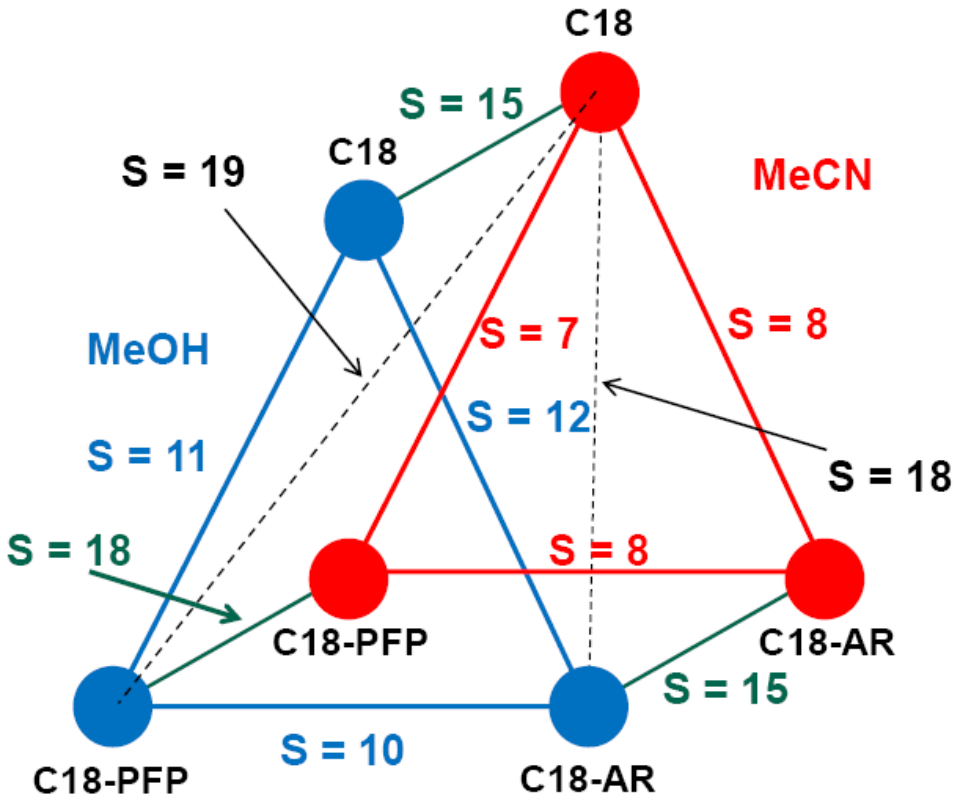
ACE Phase	Separation Mechanism	Target Analytes	Recommended Applications
C18, C8, C4	Hydrophobic interactions	• Analytes differing in hydrophobicity; • Polar, moderately polar, and nonpolar analytes; • Uncharged acids and bases (ion suppression); • Ionized acids or bases using ion-pairing	Homologous compounds differing by $-CH_2$, alkyl, aryl groups Non-aqueous RPLC (NARP)
C18-AR	Hydrophobic and π–π interactions	• Analytes differing in hydrophobicity; • Electron-poor analytes with electron delocalization and electron-withdrawing groups (halogens, nitro groups, ketones, esters, acids	Basic analytes, heterocycles, proton donors and acceptors, halogenated aromatics Highly aqueous mobile phases
C18-PFP	Hydrophobic, π–π, hydrogen bonding, dipole-dipole, and steric interactions	• Analytes differing in hydrophobicity • Electron-rich analytes with electron-delocalization and electron-donating groups (phenols, aromatic ethers, amines), • Analytes with proton-donating groups • Analytes with differing dipole moments	Stereoisomers, steroids, taxanes, substituted aromatics, nucleosides Highly aqueous mobile phases
Phenyl	Hydrophobic and π–π interactions	• Analytes differing in hydrophobicity; • Electron-poor analytes with electron delocalization and electron-withdrawing groups (halogens, nitro groups, ketones, esters, acids	Basic analytes, heterocycles, proton donors and acceptors, halogenated aromatics Highly aqueous mobile phases

The contribution of each separation mechanism to retention depends on each analyte's physicochemical properties, its structure, and the chromatographic conditions used (organic modifier, pH, etc.).

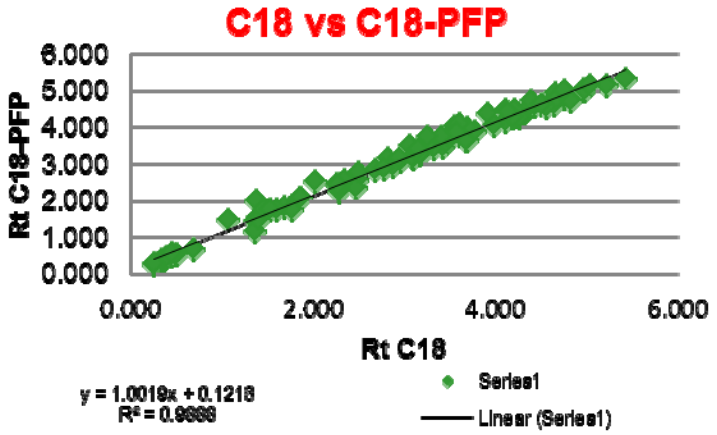
Phase Orthogonality:
Complementary yet similar in retention (~10 < S < ~20)

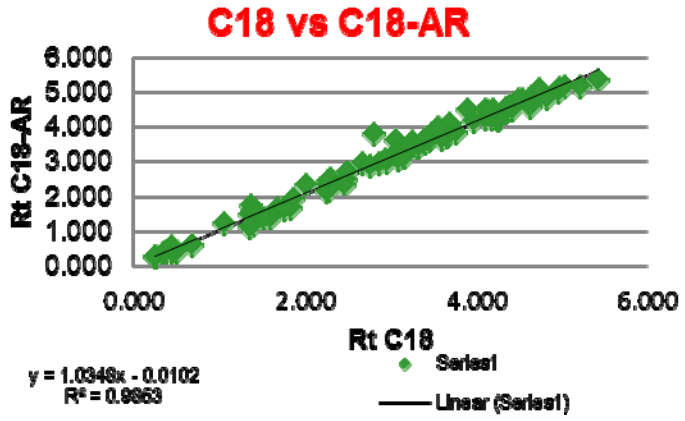
Neue Selectivity Descriptor*

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



Data Generated using >100 Acidic, Basic and Neutral Analytes





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

Ranking ACE® Phase Relative Orthogonality with MeOH and MeCN

MeOH

Column 1	Column 2	Selectivity "S"
C18	C18-AR	12
C18	C18-PFP	11
C18-AR	C18-PFP	10

MeCN

Column 1	Column 2	Selectivity "S"
C18	C18-AR	8
C18-AR	C18-PFP	8
C18	C18-PFP	7

Between MeOH and MeCN

MeOH	MeCN	Selectivity "S"
C18-PFP	C18	19
C18-AR	C18	18
C18-AR	C18-PFP	18
C18-PFP	C18-AR	18
C18	C18-AR	17
C18	C18-PFP	17

Demonstrates value of using 3 phases with 2 organic modifiers for screening during method development

Introducing ACE *Excel* UHPLC Columns…

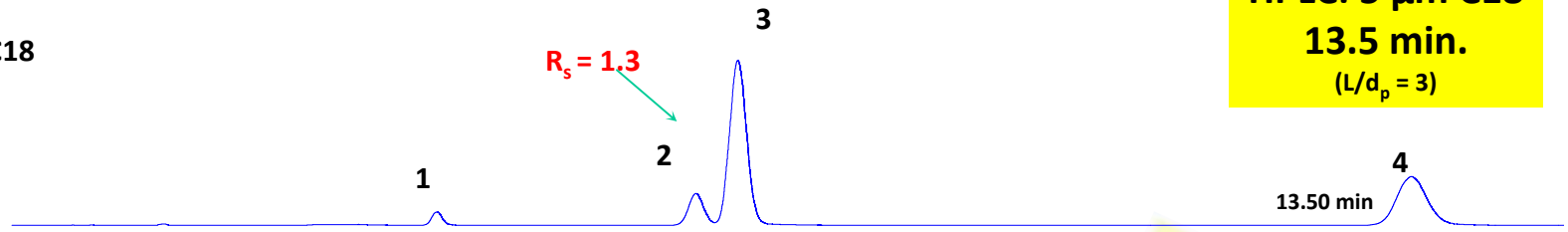
- When
  - Introduced in Sept-Oct, 2011
- What
  - 2 µm particles with 200,000 N/m efficiency
  - Same selectivity as conventional ACE column phases with excellent peak shape and lot-to-lot reproducibility
  - Available in C18, C18-AR, C18-PFP as well as CN, C4, C8, Phenyl, and AQ in 100 Ångstrom pore size
  - All phases available in same 1000-bar hardware with 3- and 5-µm particles
- Benefits
  - ~20% Lower back pressure than sub-2-µm UHPLC columns
    - can use higher flow rates for shorter run times
  - Rugged 1000-bar hardware with specially designed 2-µm frits
    - less plugging than sub-2-µm columns
  - Optimum linear velocity range: 1–3.5 mm/sec

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

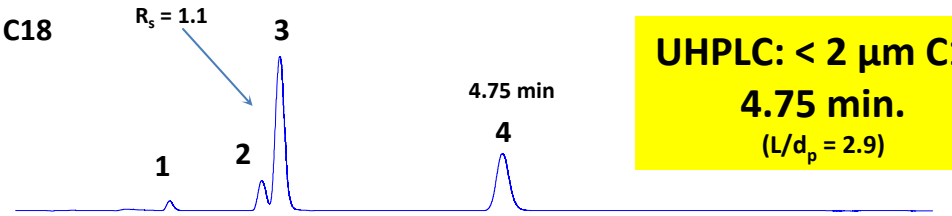
Aim – Obtain R_s > 1.7 in shortest possible time

HPLC: 5 µm C18
13.5 min.
(L/d_p = 3)

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



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



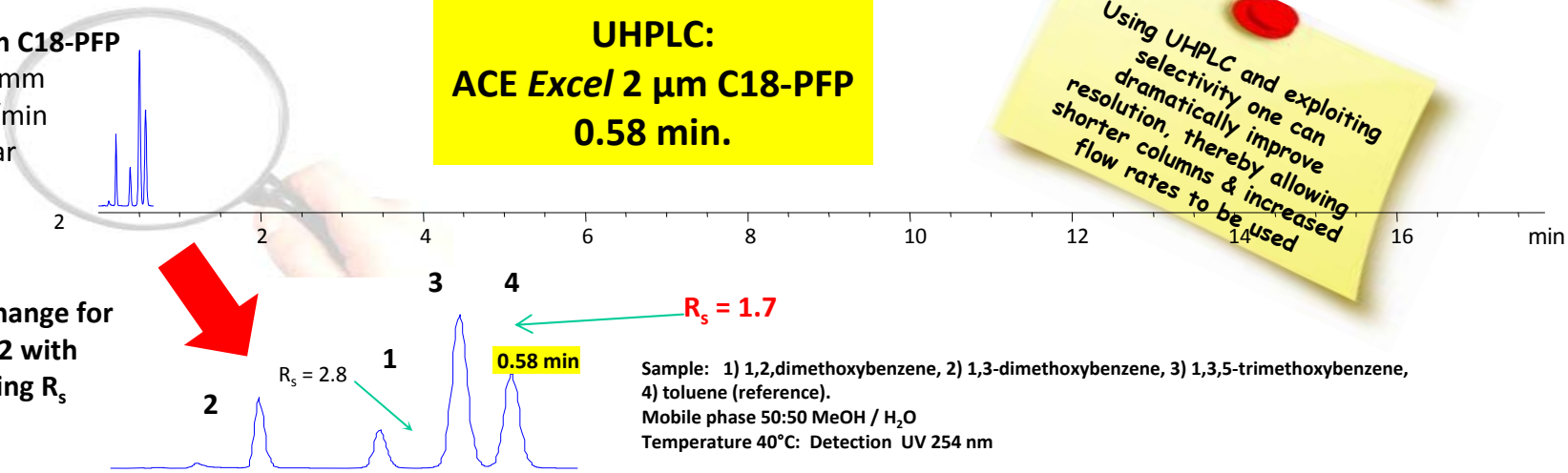
UHPLC: < 2 µm C18
4.75 min.
(L/d_p = 2.9)

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* 2 µm C18-PFP
0.58 min.

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



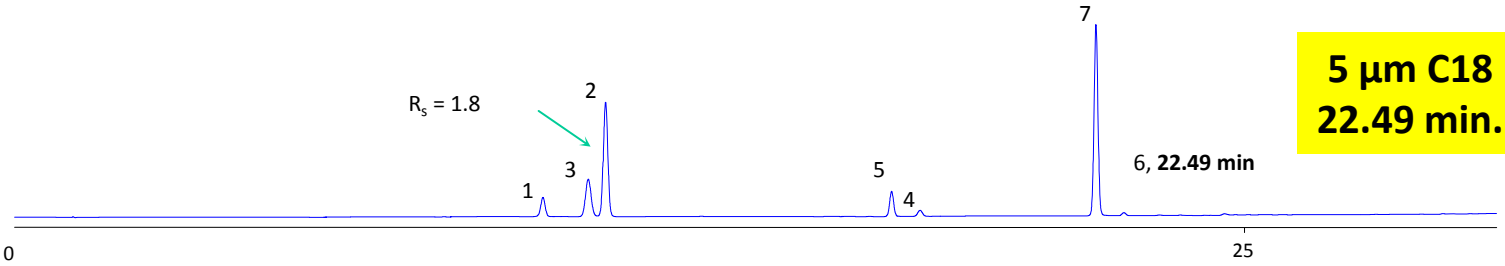
elution order change for peaks 1 and 2 with better limiting R_s

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

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

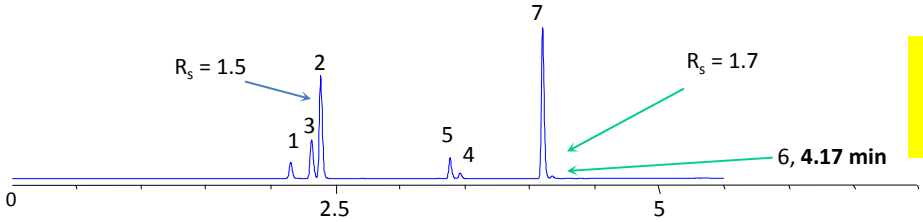
Sample: 1, aspirin; 2, phenacetin; 3, 1,3-dinitrobenzene ; 4, ethyl benzoate; 5, nimesulide; 6, ibuprofen; 7, indomethacin.

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



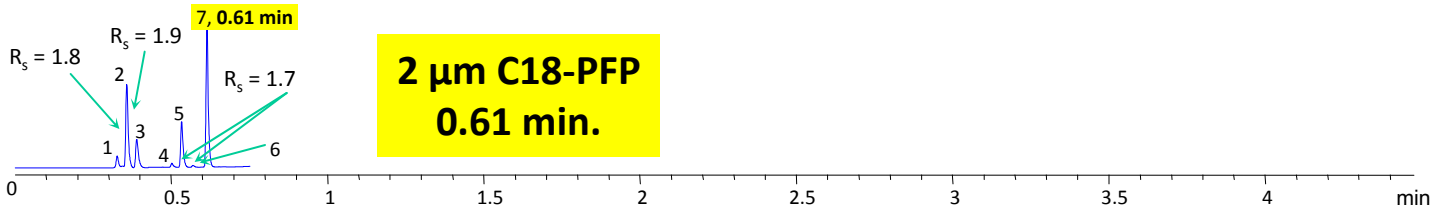
5 µm C18
22.49 min.

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

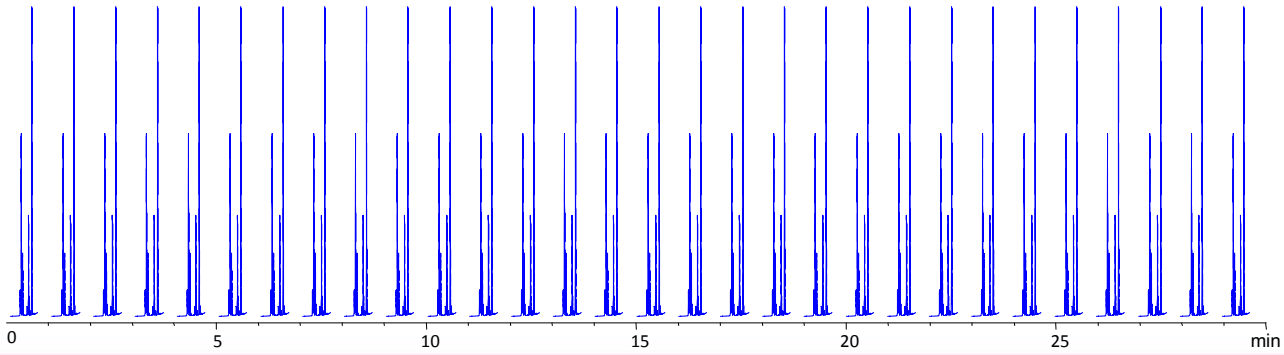


< 2 µm C18
4.17 min.

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



2 µm C18-PFP
0.61 min.

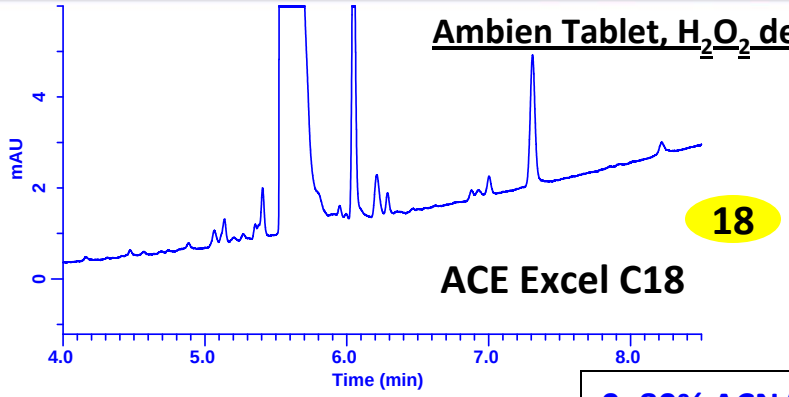


$R_s \geq 1.7$

Gradient RPLC Column Phase Screening:

Optimum selectivity + UHPLC efficiency

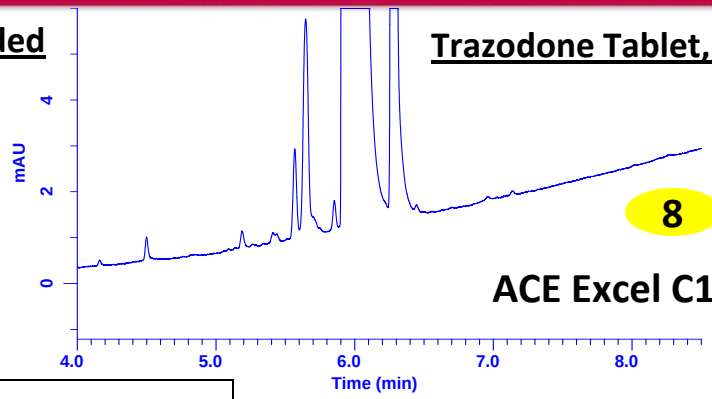
Ambien Tablet, H_2O_2 degraded



18

ACE Excel C18

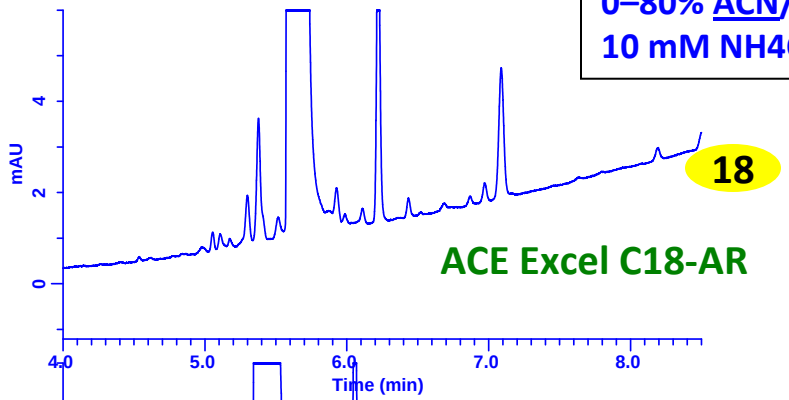
Trazodone Tablet, H_2O_2 degraded



8 # Peaks

ACE Excel C18

0–80% ACN/0.1% HCOOH,
10 mM NH4COO, 12-minutes



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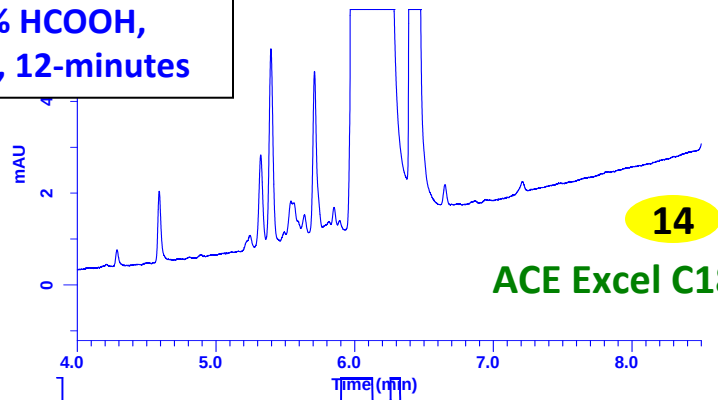
ACE Excel C18-AR



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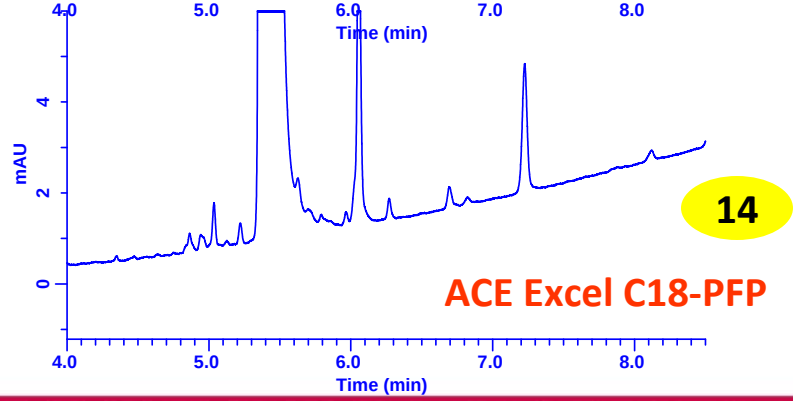
ACE Excel C18-AR



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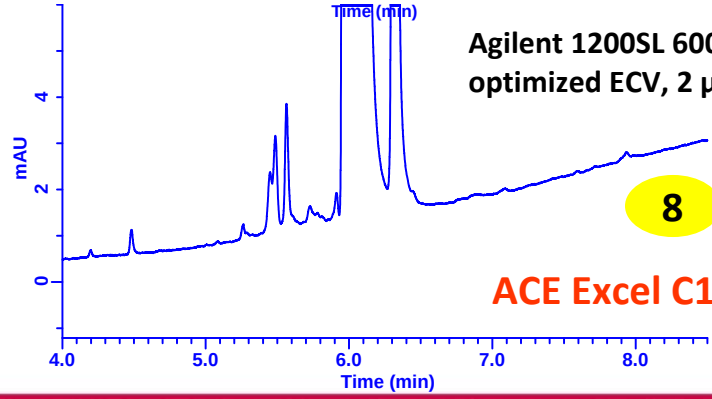
ACE Excel C18-PFP

Agilent 1200SL 600 bar system
optimized ECV, 2 µL flow cell



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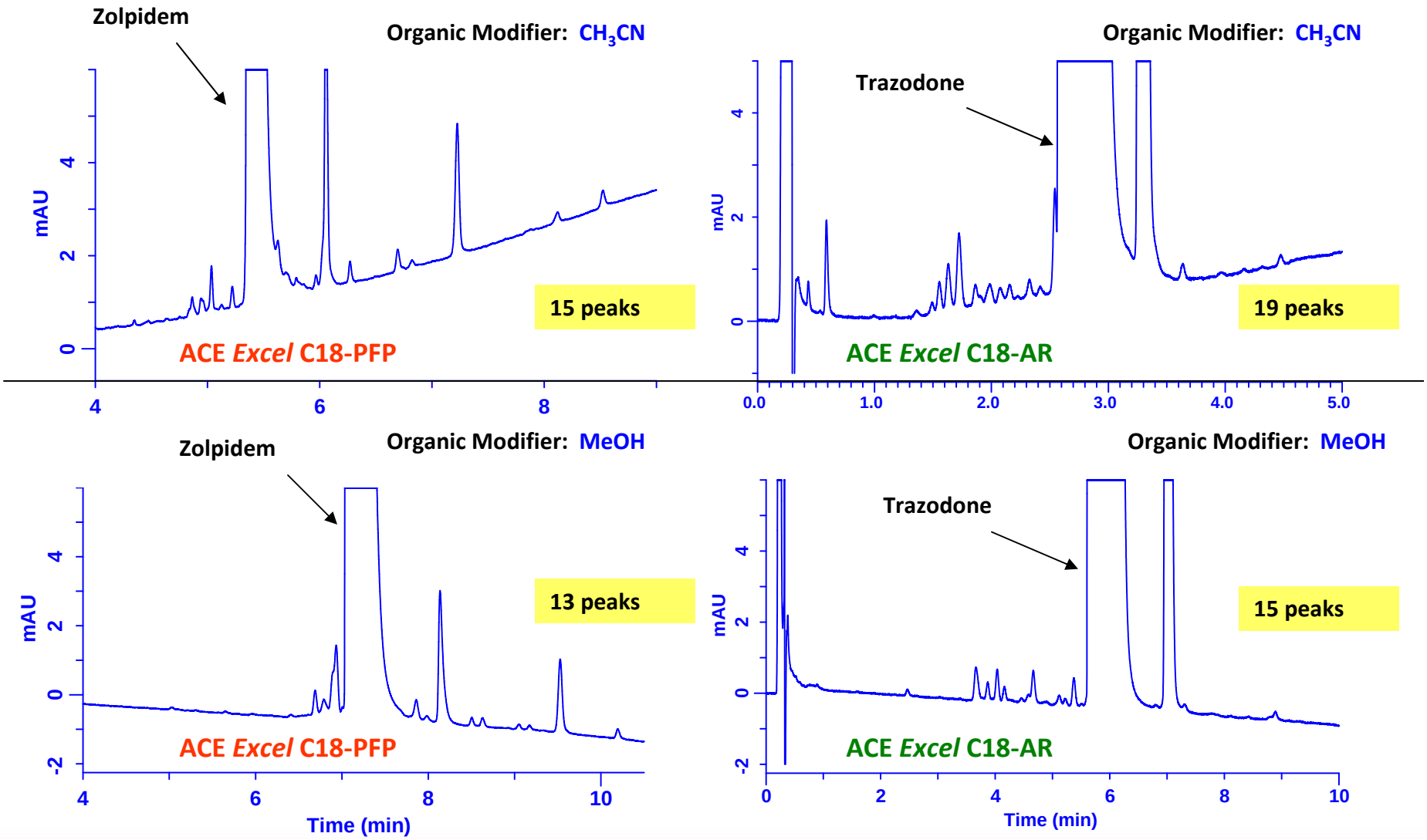
ACE Excel C18-PFP

Zolpidem and Trazodone Peroxide-Degraded Tablets

Column and Condition Screening: Impurity Method Development/Screening Phases

Columns: 2.1 x 50 mm, 2 µm ACE *Excel,* Gradient: 0 to 80% Organic in 12 min., 30°C, 0.5 mL/min, 254 nm



ACE Excel C18-AR and C18-PFP Columns provide unique selectivity for impurity profiling with or without changing organic modifiers too.

Example Fast RPLC Method Development Strategy

1. Screen short, high efficiency orthogonal column phases with 5-10 minute linear gradients at a temperature of 20-40°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 100 or 150 mm, 5 µm column often recommended.
3. Use different organic modifiers: ACN, MeOH and 1:1 v/v ACN/MeOH, if appropriate.
4. Use different LC/MS-ready aqueous components at two or more pHs
   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) for optimization.
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.

Fast Method Screening with 2.1 × 50 mm ACE *Excel*:
2 Organic Modifiers, 2 pHs, at Single Temperature

ACE Excel
2.1 x 50 mm, 2 µm

Gradient Delay Volume, V_D	0.090	mL
ID	2.1	mm
Length	50	mm
d_p	2	µm
Column, Volume, V_m	0.113	mL
Flow Rate	0.60	mL
k_e	7.0	
h	2.1	
Plates	9900	

Gradient Program

	Time	%B
	0.0	3
3 V_m	0.6	3
	7.5	95
	9.4	95
	9.5	3
10 Col Vols + 3 Sys Vols Equil.	11.8	3
Total Run time	11.8	min.

Ideal Injection Sequence

Acquisition Method	Vial	# Inj.
ACN_pH2.75_25C	Blank	2
ACN_pH2.75_25C	Sample	2
MeOH_pH2.75_25C	Blank	2
MeOH_pH2.75_25C	Sample	2
ACN_pH5.75_25C	Blank	2
ACN_pH5.75_25C	Sample	2
MeOH_pH5.75_25C	Blank	2
MeOH_pH5.75_25C	Sample	2
	Total # Runs	16

Time can be saved further by reducing # of replicates

- ~12 min total run time for each gradient from 3 to 95% organic at single temperature = 16 × 12 = 192 min.
- Complete screening for one column phase at 2 pHs with ACN and MeOH in ~2–3 hrs
- Screen 2 stationary phases in ~5–6.5 hrs, 3 phases in ~9.5 hrs

Summary

- **Novel C18 phases: C18-AR and C18-PFP + C18**
  - Benefits of C18 bonded phases (retention, stability) combined with the additional separation mechanisms offered by Phenyl and PFP groups
  - Selectivities are complementary among phases with same organic modifier, and more so when organic modifier is changed too
  - Changes in relative retention between phases are significant, but not severe
  - Enables fast screening of stationary phases and conditions to identify optimum selectivity and resolution to speed method development
- **ACE *Excel* 2 µm Columns with 1000-bar Hardware**
  - All ACE *Excel* 100 Ångstrom phases available in UHPLC and HPLC format (including C4, CN, Phenyl, C8, AQ) in 2-, 3- and 5-µm columns in 1000-bar hardware
  - Engineered with lower back pressures compared to other sub-2-µm phases due to 2-µm particle size and frit technology
- **Transferability/Scalability to Analytical, Semi-Prep and Prep HPLC**
  - Fully scalable to ACE® analytical, semi-prep and preparative columns in 3-µm 5-µm and 10-µm particle sizes in all phases
  - Fully compatible with all commercial HPLC and UHPLC instrumentation using appropriate fittings