Selectivity: A Potent Ally in RPLC Method Development

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Outline

- Review factors most important for selectivity changes
- Description of complementary alkylphenyl and alkylpentafluorophenyl phases
- Method development strategy
  - which parameters to consider
  - specific approach for flavonoid/phenolic sample example
  - column phase and organic modifier screening
  - optimization using DryLab® 4 ($t_G \times T$)
  - scale separation to HPLC geometry
- Summary

Which Factors[1] Most Affect Selectivity?

MOST Influence

Isocratic Separations

- Column Stationary Phase
- Organic modifier
- Mobile phase pH
  (for ionised analytes only)
- % Organic modifier
- Column temperature
- Buffer choice
- Buffer concentration
- Additive concentration

LEAST Influence

Gradient Separations

- All parameters for isocratic
- Gradient steepness
- k* (that is t_G, F, $\Delta\Phi$, V_m, MW)

$$k^* = \frac{85 \times t_G \times F}{\Delta\Phi \times V_m \times S}$$

- Delay volume
- Column dimensions

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

Power of Changing Multiple Parameters to Change Selectivity and Resolution

$$R_s = \left(\frac{1}{4}\right)\sqrt{N}(\alpha - 1)\left(\frac{k}{1+k}\right)$$

For ΔR_s = 1.5, N = 10,000 and k ≥ 1

(α – 1) = 0.12 and α = 1.12

log α = 0.05 and Snyder proposed $|\delta \log \alpha|_{avg} \geq 0.10$

For a change in both column phase and organic modifier, the expected change is magnified

$|\delta \log \alpha|_{avg} = [(0.20)^2 + (0.19)^2]^{0.5} = 0.28$

Use of different column phases, organic modifiers, pHs, and temperatures can be powerful in changing α and R_s

Relative Impact of Different Changes in RPLC Parameters on Selectivity[1]

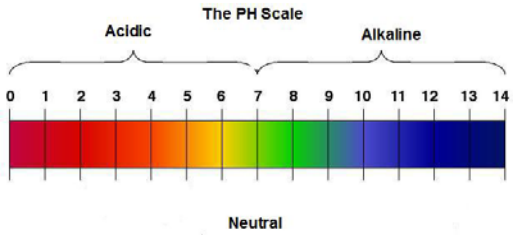
Parameter	Change in Parameter	Maximum $\lvert\delta \log \alpha\rvert_{avg}$
pH	5 pH units	0.70
Organic Modifier	ACN ↔ MeOH	0.20
Gradient Time (t_G)	10-fold	0.20
Orthogonal Column	ΔF_s ~65	0.19
% Organic	10% (v/v)	0.08
Temperature	20°C	0.07
Buffer Concentration	2-fold	0.02

Note:

- **pH and buffer concentration effective for ionizable analytes only**
- **Temperature most effective for ionizable analytes**

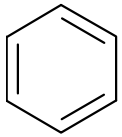
[1] Adapted from Snyder et al., "Orthogonal" separations for reversed-phase liquid chromatography, Journal of Chromatography A, 1101 (2006) 122–135

Considerations for RPLC Method Development



Column Stationary Phase	Organic Modifier Choice	Mobile Phase pH	Additive Choice
Orthogonal phases	Acetonitrile (ACN)	• pH 2	**Low pH**
Complementary interactions	Methanol (MeOH)	• pH 2.8	• TFA
• hydrophobic	ACN/MeOH 1:1 blend	• pH 3.8	• formic acid
• π-π	Ethanol	• pH 4.8	• acetic acid
• dipole-dipole	2-Propanol (IPA)	• pH 6.5–7.0	• ammonium formate
• hydrogen bonding		• pH 7.8	• phosphate/H_3PO_4
• steric resistance (shape)		• pH 9–10.5	• $HClO_4$
			Mid pH
			• ammonium formate
			• ammonium acetate
			• citrate
			• phosphate
			High pH
			• ammonium bicarbonate
			• ammonium hydroxide

The Power of π...Scientific-Led Stationary Phase Design

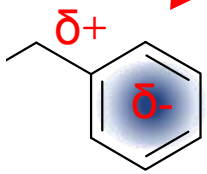


C_6H_6

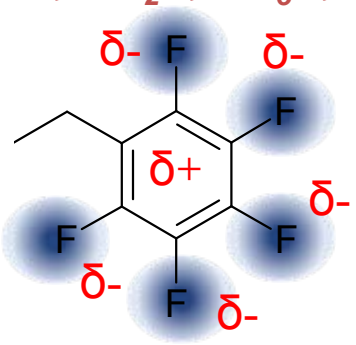
Electron Donating Groups
e.g. NH_2, NR_2, alkyl, OCH_3
OR, CH_3, Ar etc.

Electron Withdrawing Groups
e.g. NO_2, halides, NR_3^+, CO_2H,
CN, CO_2R, SO_3H, COH etc.

e.g.

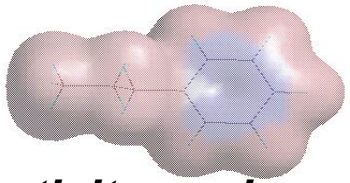


e.g.



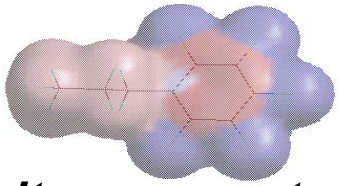
Classic π-π interaction

Electron Rich Ring



Activity: π-donor (π-base)

Electron Deficient Ring



Activity: π-acceptor (π-acid)

How do we exploit these properties for new stationary phases?

C18+Phenyl = ACE® C18-AR

C18+PFP = ACE® C18-PFP

ACE® Phase Orthogonality
Neue Selectivity Factor "S": for 102 acids, bases, neutrals

ACN

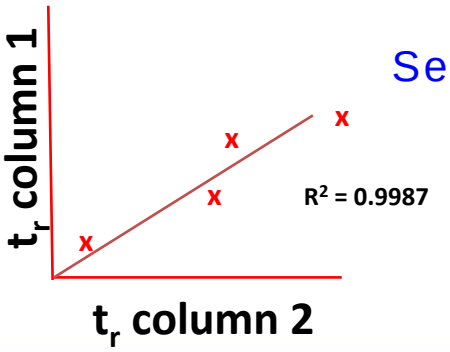
Column 1	Column 2	Selectivity 'S'
C18	C18-AR	8
C18-AR	C18-PFP	8
C18	C18-PFP	7

MeOH

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

MeOH	ACN	Selectivity Value
C18-PFP	C18	19
C18-AR	C18	18
C18-AR	C18-PFP	18
C18-PFP	C18-AR	18
C18-PFP	C18-PFP	18
C18	C18-AR	17
C18	C18-PFP	17
C18	C18	15
C18-AR	C18-AR	15

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



$$\text{Selectivity} = 100 \times \sqrt{(1 - R^2)}$$
$$= 100 \times \sqrt{(1 - 0.9887)}$$
$$= 10.6$$

Objective: Develop a gradient RPLC separation for a complex analyte mixture

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Journal of Chromatography A, 1149 (2007) 73–87

JOURNAL OF CHROMATOGRAPHY A
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Comprehensive two-dimensional liquid chromatography with parallel gradients for separation of phenolic and flavone antioxidants

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Available online 5 February 2007

- J. Chrom. A, 2007
- Considered 31 analytes: phenolic, acidic, flavonoids
- Compared PEG, C18, and phenyl phases both in series (1-D) and in 2-D arrangements
- Previous work (2006) compared C18 and PFP phases in series and in 2-D
- Selected analyte category for method development example

Reasonably Simple Method Development Strategy

- **Column Phase Screening**
  - C18
  - C18-AR
  - C18-PFP
- **Mobile phase**
  - Organic modifier
    - ACN
    - MeOH
  - Aqueous component pH
    - pH 2.8 (0.1% HCOOH)
- **Temperature**
  - 30°C

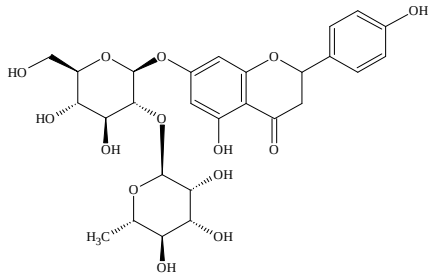
Sample:

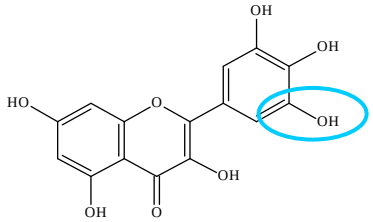
- 19 phenolics, acids and flavonoids used in 2-D method publication
- Preliminary sample: flavonoids on hand
- Columns: 2.1 × 50 mm, 2 μm ACE *Excel*, 1000 bar max. (N ~ 10,000)
- Screen C18, C18-AR, C18-PFP phases using ACN/water (0.1% HCOOH) at 30°C
- Various gradient times: 5, 10, and 15 min from 5 to 95% organic

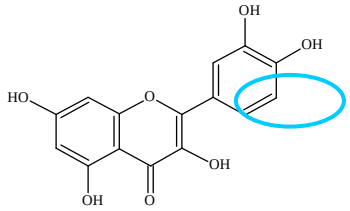
Test available flavonoids on ACE *Excel* Phases

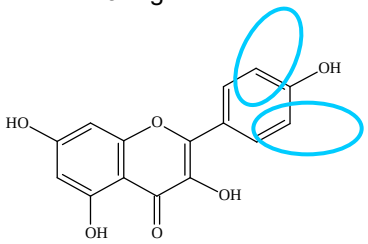
Preliminary Sample

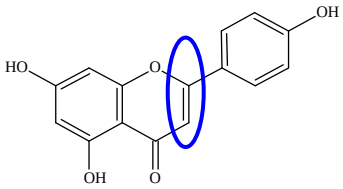
- naringin
- myricetin
- quercetin
- kaempferol
- apigenin
- naringenin
- hesperetin
- biochanin
- flavone

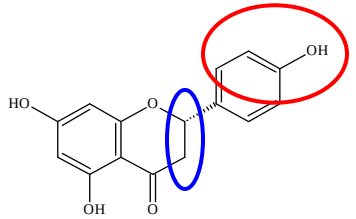

Naringin

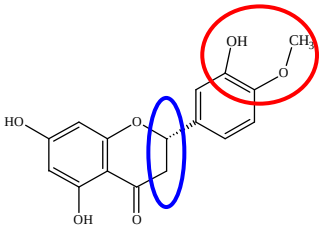

Myricetin

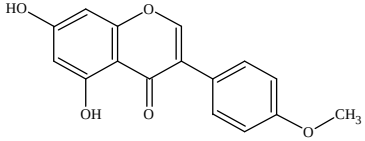

Quercetin

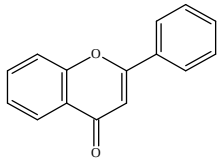

Kaempferol


Apigenin


Naringenin

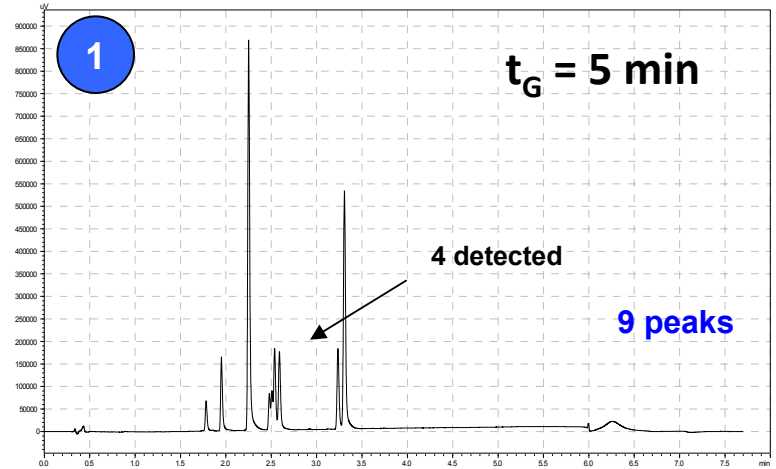

Hesperetin

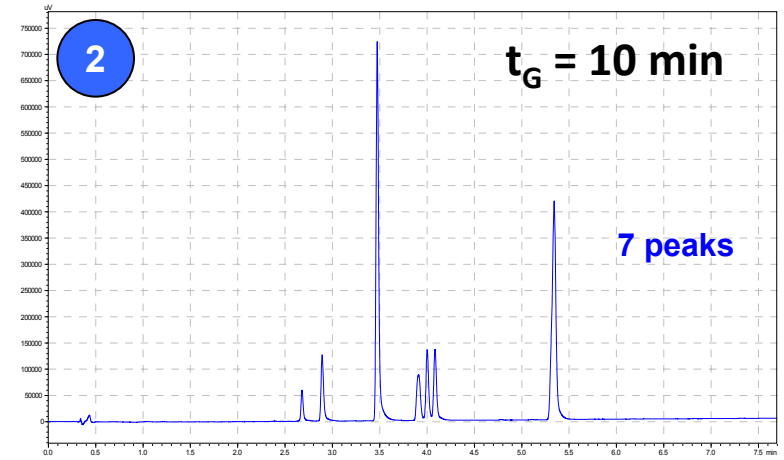

Biochanin A

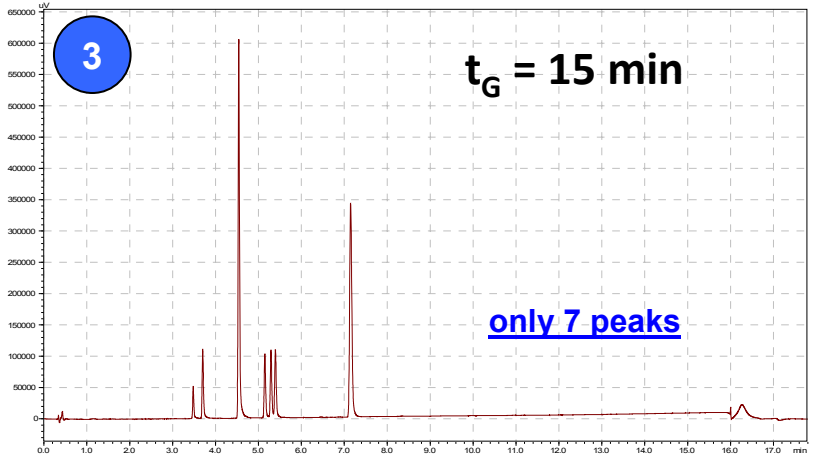

Flavone

ACE C18 Column Screening: 30°C, 3 gradient times

9 Flavonoids





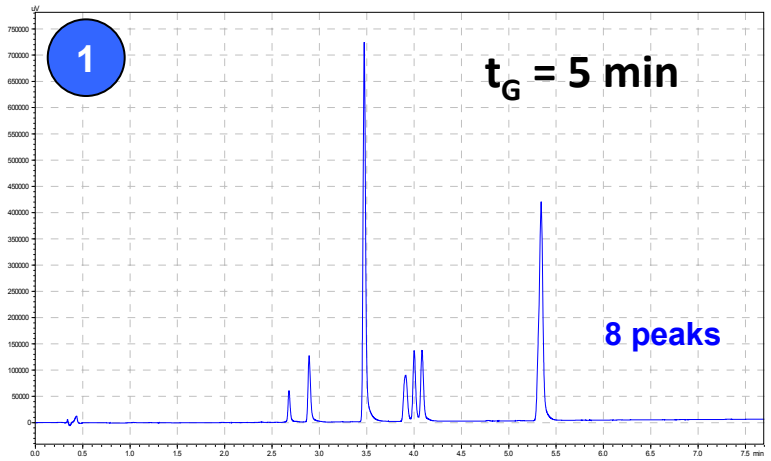


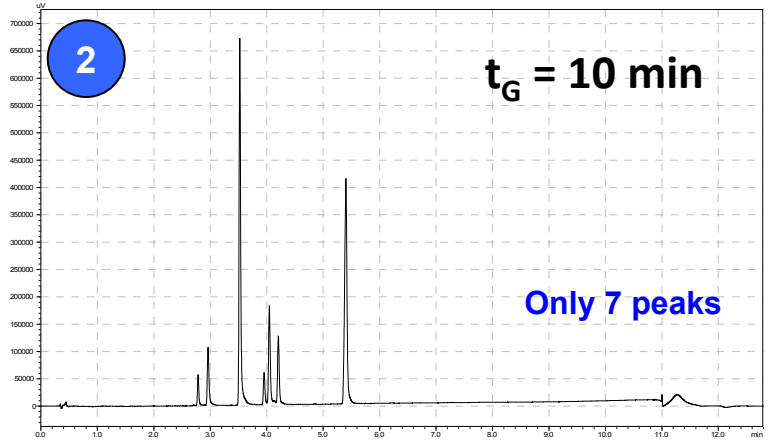
Column:	**ACE Excel 2 C18, 2.1 x 50 mm**
Inst.:	**Shimadzu Nexera**
Flow rate:	**0.5 mL/min**
Temp.:	**30°C**
Detection:	**254 nm, 50 Hz**
Mobile Phase A:	**H2O (0.1% HCOOH)**
Mobile Phase B:	**ACN (0.1% HCOOH)**
Gradient:	**5–95%, times as shown**

Longer gradient times do not always produce better resolution!

ACE C18-AR Column Screening: 30°C, 2 gradient times

9 Flavonoids

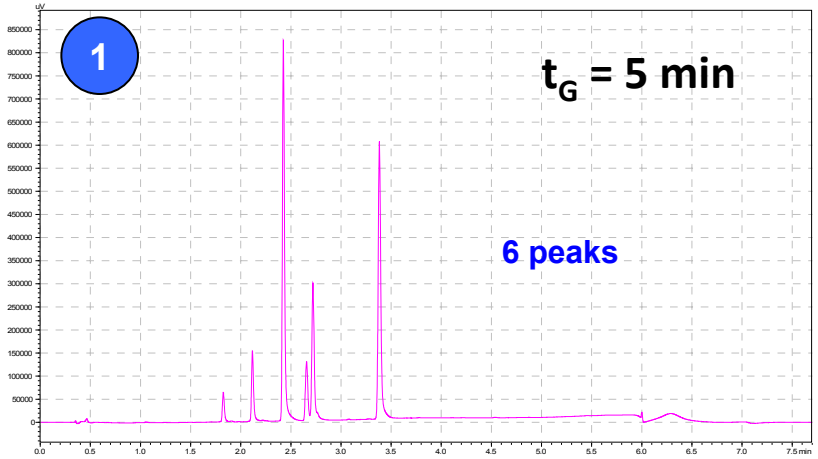


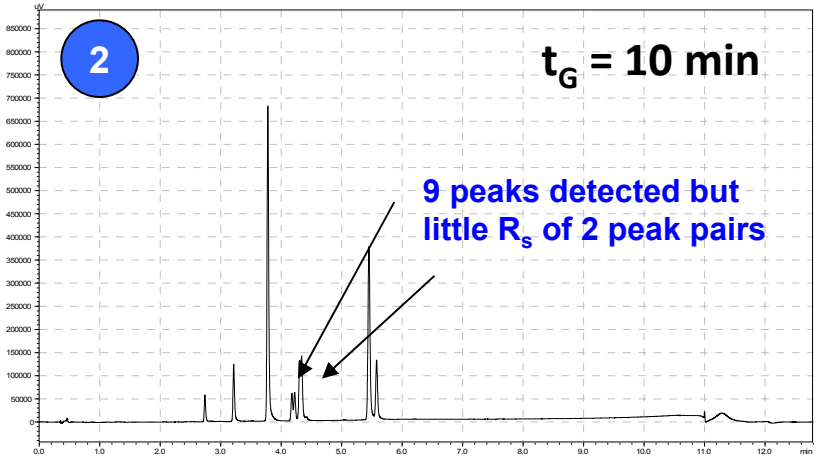


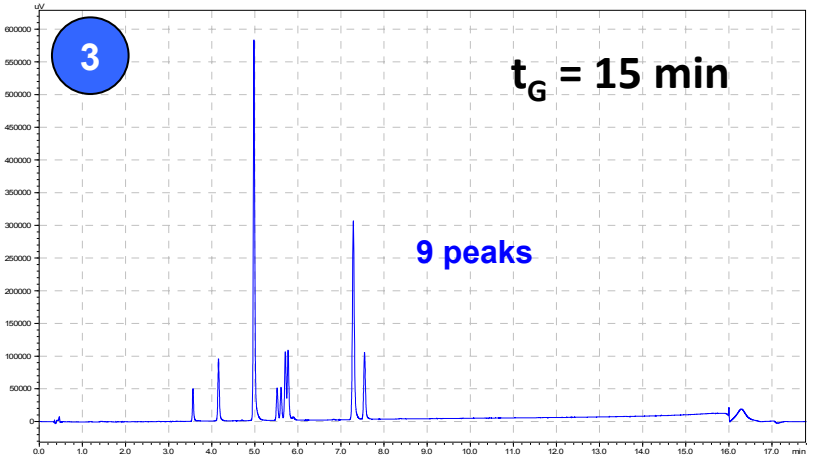
Column:	**ACE Excel 2 C18-AR, 2.1 x 50 mm**
Inst.:	**Shimadzu Nexera**
Flow rate:	**0.5 mL/min**
Temp.:	**30°C**
Detection:	**254 nm, 50 Hz**
Mobile Phase A:	**H2O (0.1% HCOOH)**
Mobile Phase B:	**ACN (0.1% HCOOH)**
Gradient:	**5–95%, times as shown**

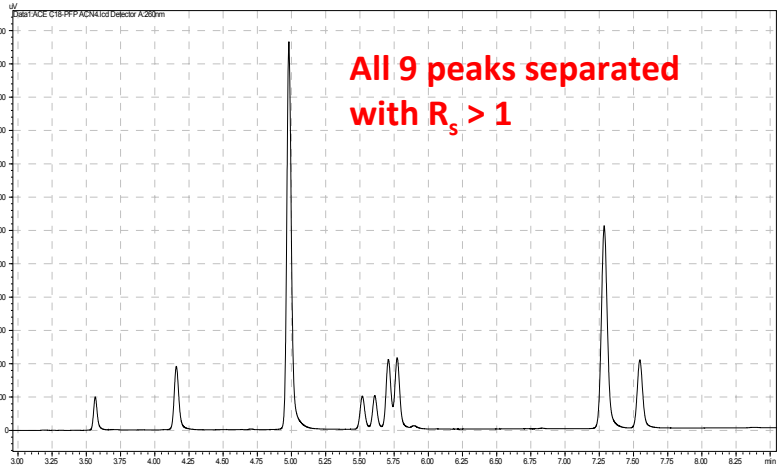
ACE C18-PFP Column Screening: 30°C, 3 gradient times

9 Flavonoids







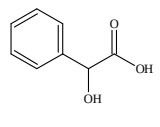


Same instrument and conditions as previous

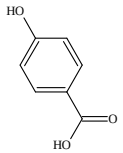
Expanded View of 15-min gradient run

Results of Column Screening, Add more analytes

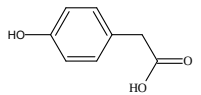
- Improvement in R_s with longer t_G's seen only with ACE C18-PFP
- Add additional analytes to mixture
  - mandelic acid
  - p-hydroxybenzoic acid
  - p-hydroxyphenylacetic acid
  - 6,7-dihydroxycoumarin
  - syringic acid
  - p-coumaric acid
  - ferulic acid
  - morin
  - resveratrol
  - hesperidin
- Use C18-PFP phase for DryLab® 4 optimization (t_G × T)
- Also compare C18 and C18-AR phases using ACN and MeOH and two temperatures (30 and 50°C)
- All subsequent results generated using binary Agilent 1200SL (V_D ~120 µL, 600 bar max.)



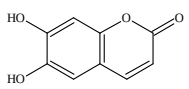
(±)-Mandelic Acid



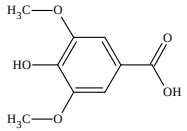
p-Hydroxybenzoic acid



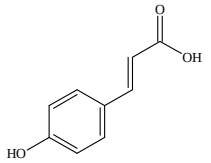
(4-hydroxyphenyl)acetic acid



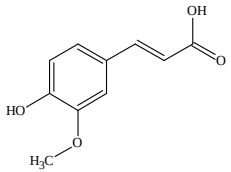
6,7-Dihydroxycoumarin



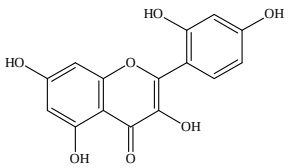
Syringic Acid



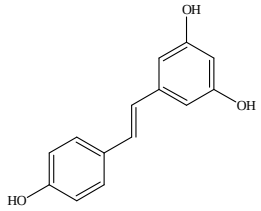
p-Coumaric Acid



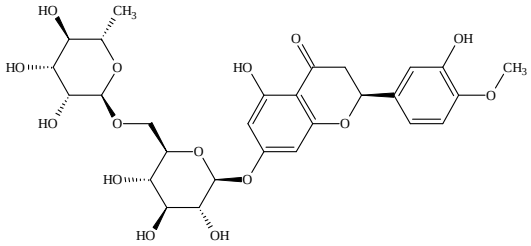
Ferulic Acid



Morin

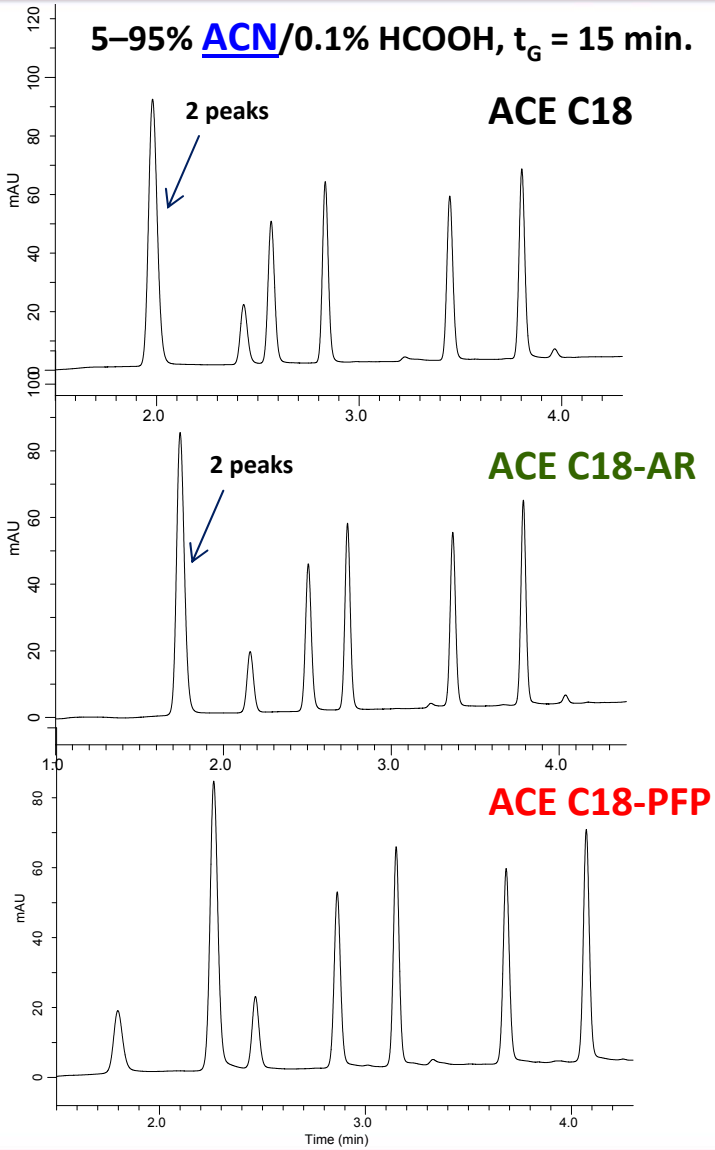


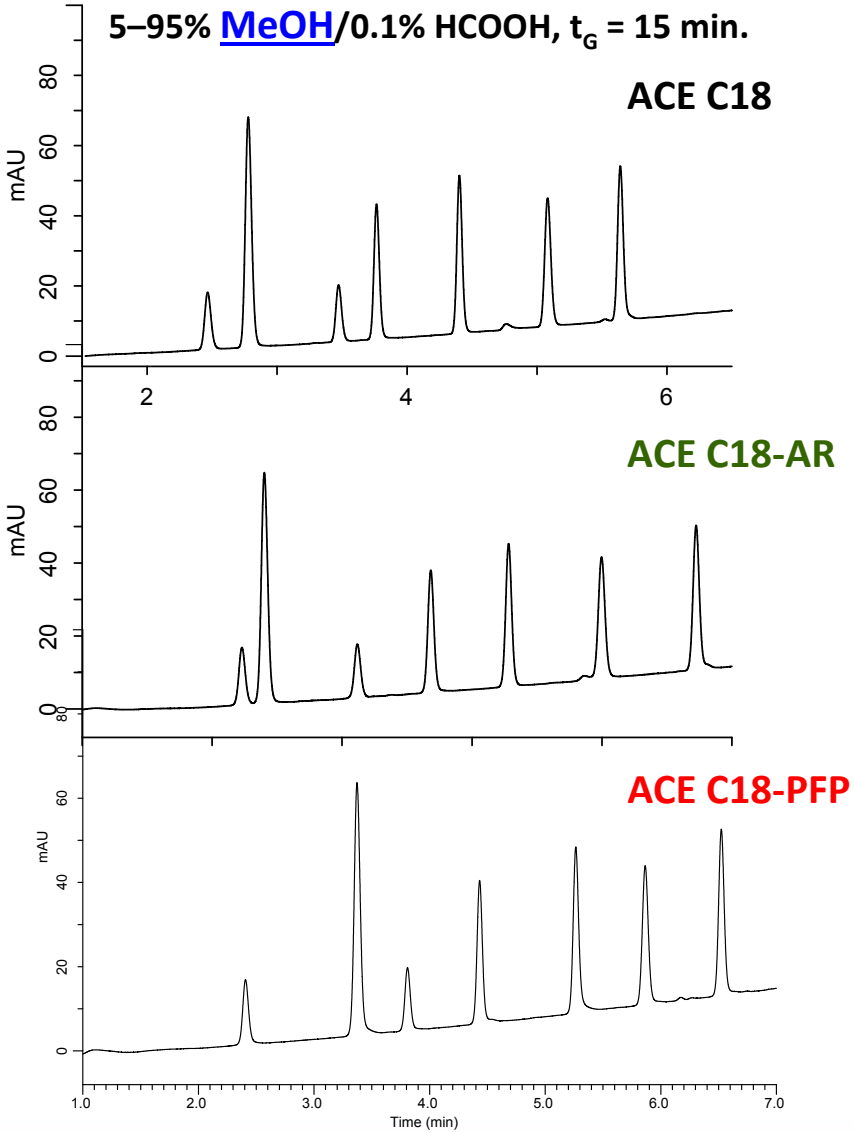
Resveratrol



Hesperidin

Minor selectivity differences using ACN and MeOH with 0.1% HCOOH for 15-min gradients: early eluters

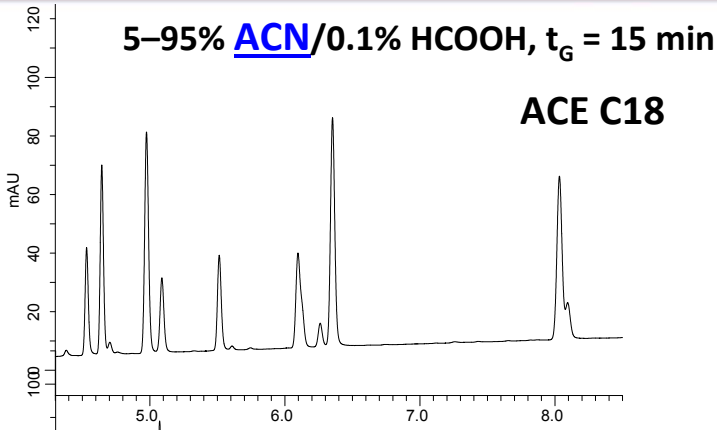




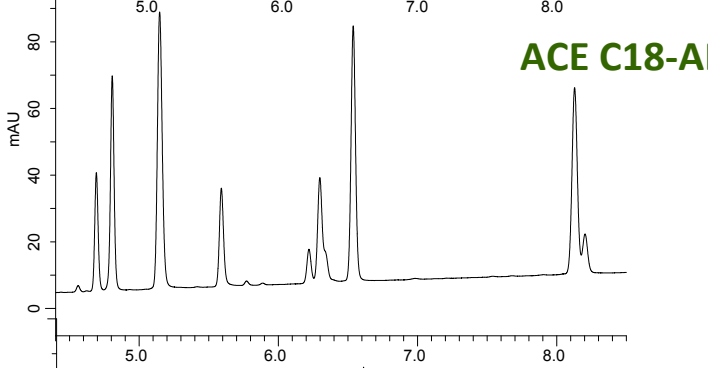
Significant differences using ACN and MeOH with 0.1% HCOOH for 15-min gradients: Later Eluters

5–95% ACN/0.1% HCOOH, t_G = 15 min.

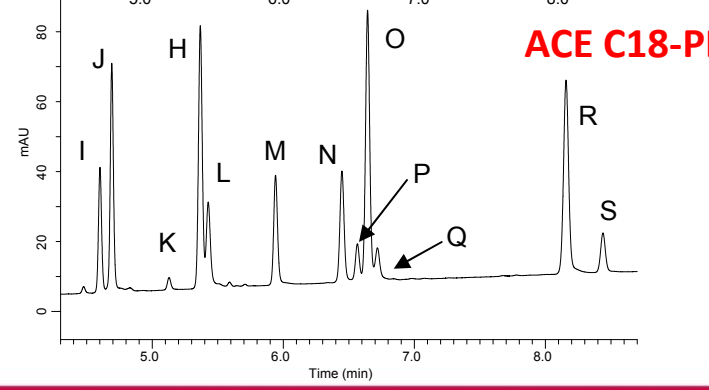
ACE C18



ACE C18-AR

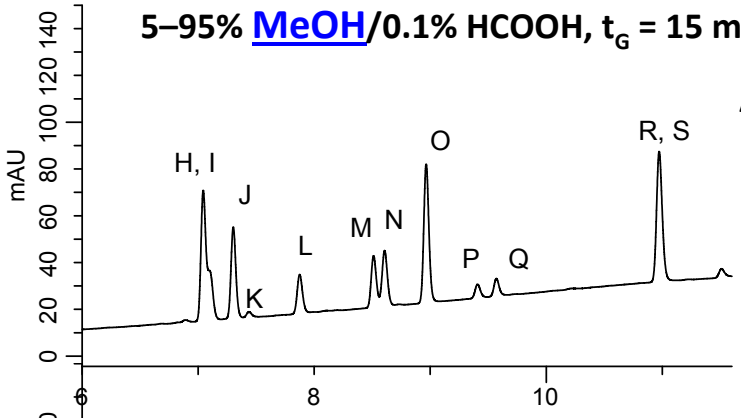


ACE C18-PFP

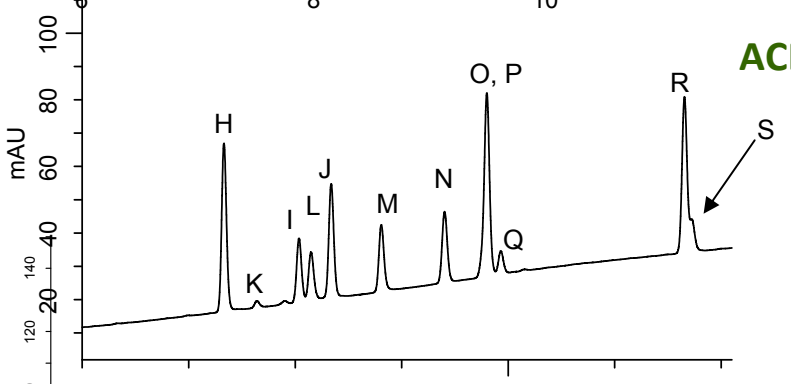


5–95% MeOH/0.1% HCOOH, t_G = 15 min.

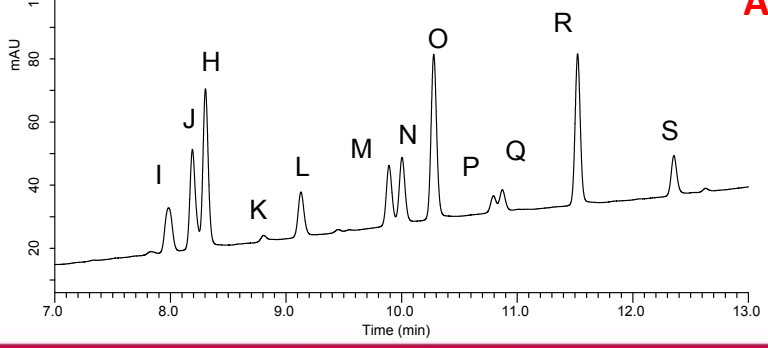
ACE C18



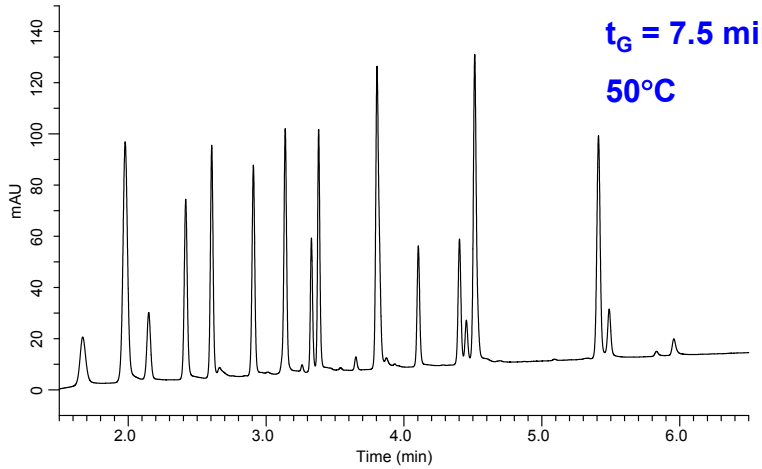
ACE C18-AR



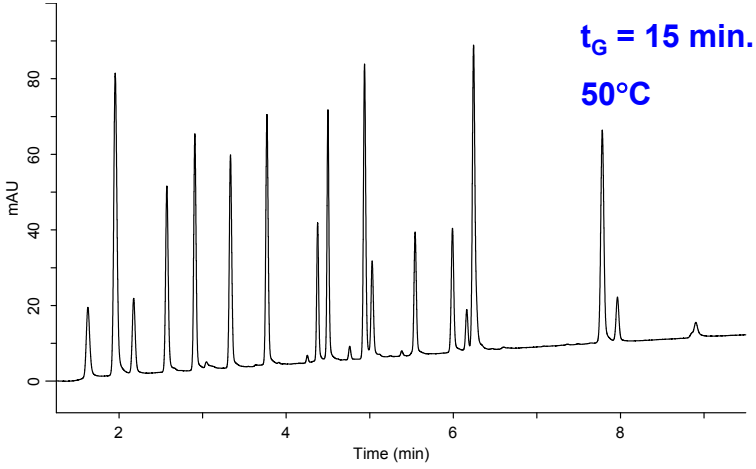
ACE C18-PFP



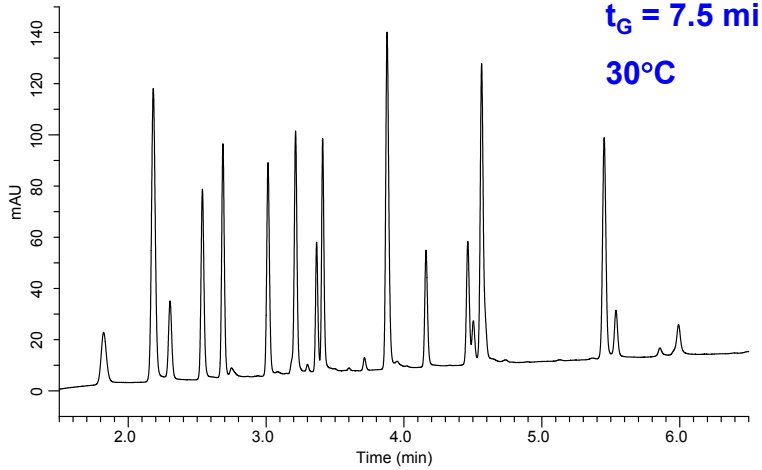
Gradient runs using 2 gradient times and 2 temperatures for DryLab® 4 input: C18-PFP Column



File Name: 50C_7PT5MIN_ACNC18PFP.cdf
Sample Name: 19 compd mix Operator: WAEGHE

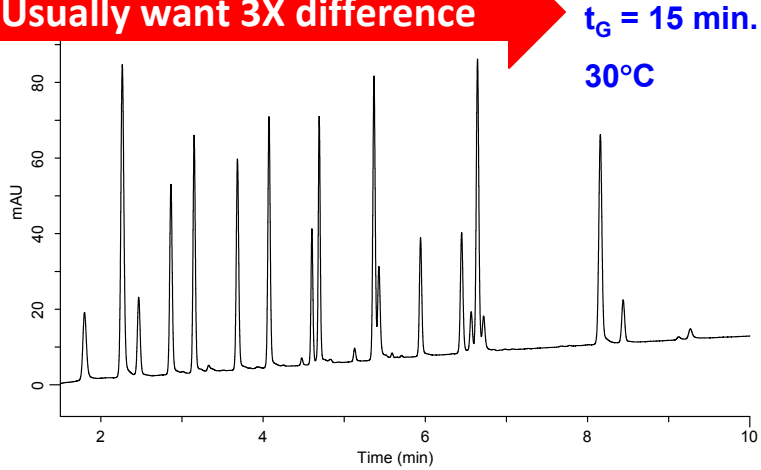


File Name: 50C_15MIN_ACNC18PFP.cdf
Sample Name: 19 compd mix Operator: WAEGHE



File Name: 30C_7PT5MIN_ACNC18PFP.CDF
Sample Name: 19 compd mix Operator: WAEGHE

Usually want 3X difference

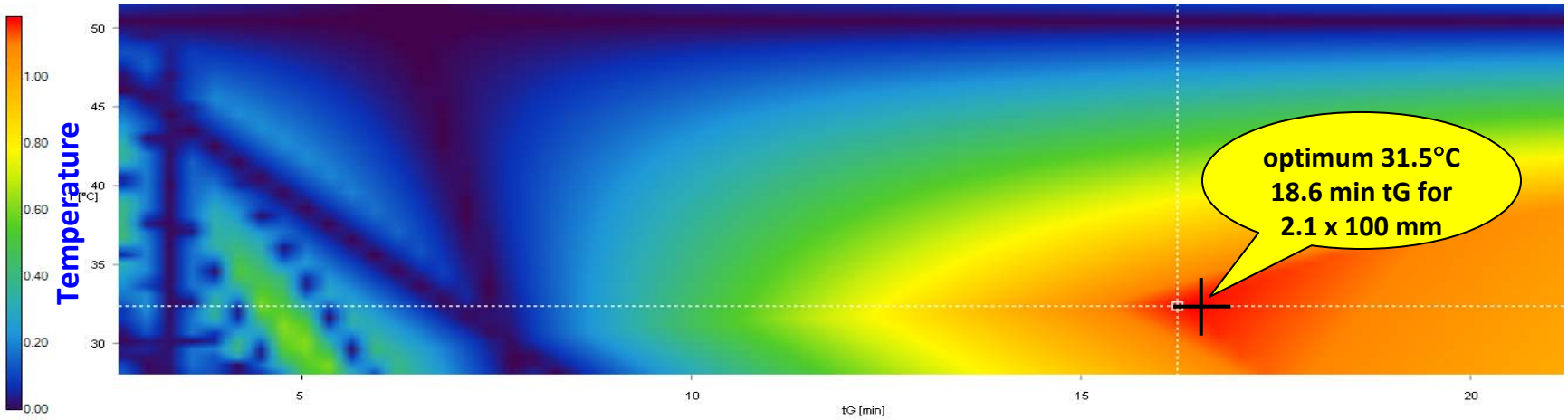


File Name: 30C_15MIN_ACNC18PFP.cdf
Sample Name: 19 compd mix Operator: WAEGHE

5–95% ACN/water with 0.1% HCOOH in each

DryLab® 4: 2-D Resolution Map and Conditions for Optimum Linear Gradient

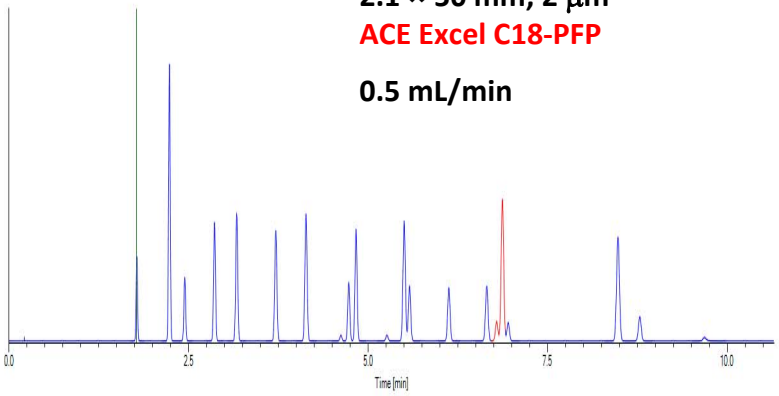
DryLab® 4 courtesy of the Molnar Institute



Gradient time (tG)

2.1 × 50 mm, 2 µm
ACE Excel C18-PFP

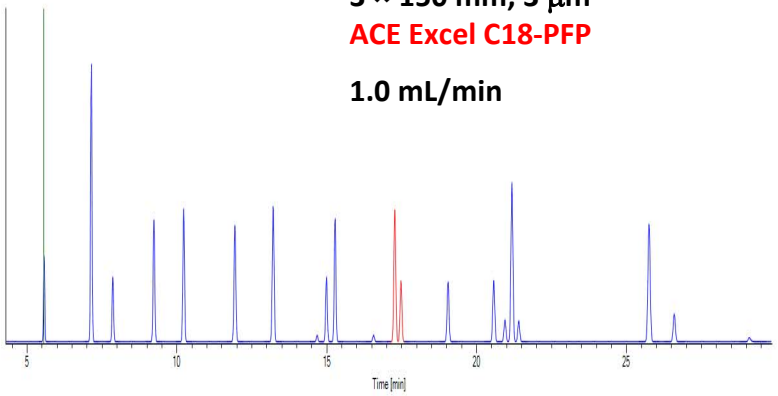
0.5 mL/min



Predicted for optimum linear gradient at 31.5°C
1-58.5% ACN/water (0.1% HCOOH) in 9.3 min

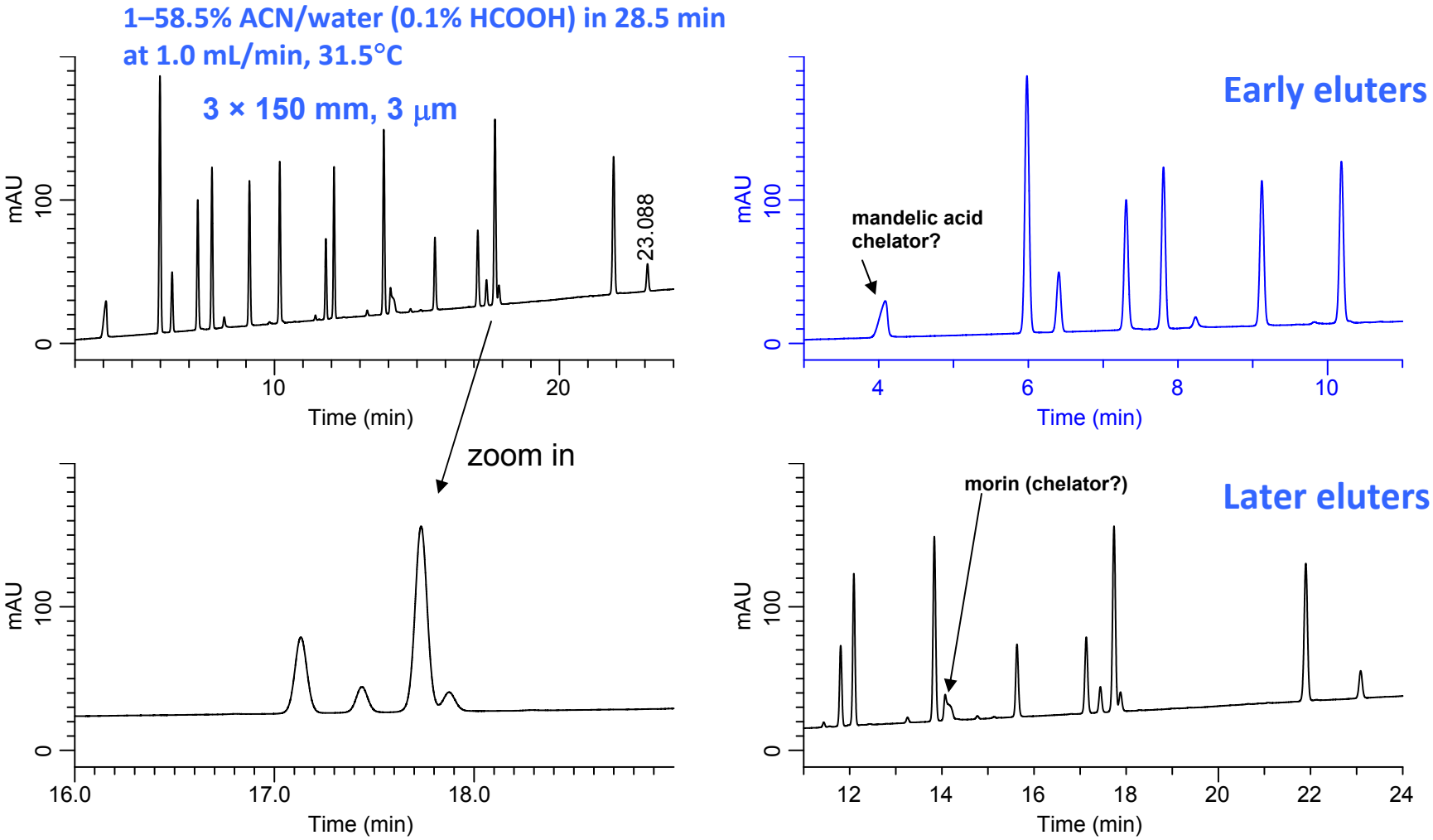
3 × 150 mm, 3 µm
ACE Excel C18-PFP

1.0 mL/min



Predicted for optimum linear gradient at 31.5°C
1-58.5% ACN/water (0.1% HCOOH) in 28.5 min

Transfer Optimum Linear Gradient Separation to ACE *Excel* C18-PFP 3 µm Column for HPLC



Actual chromatograms embedded using **ChromMerge** software

Screening and Optimization
Doesn't Really Take That Long...

Example Scenario 1

Column/Modifier/Temperature

- 2.1 x 50 mm, 2 μm columns
- 3 Column Phases
- 2 Temperatures
- Single pH
- 2 Organic Modifiers
- 3 Gradient Times (5, 10, 15 min.)
- Duplicate injections all conditions

Required Time

- 1.5 hrs. for Temp 1
- 0.5 hr. temp. equilibrium
- 1.5 hrs. for Temp 2
- 3.5 hrs. elapsed time per phase
- ~10.5 hrs. total elapsed time

Example Scenario 2

Column/pH/Temperature

- 2.1 x 50 mm, 2 μm columns
- 3 Column Phases
- 1 Temperatures
- 3 pHs
- 2 Organic Modifiers
- 2 Gradient Times (5, 15 min.)
- Duplicate injections all conditions

Required Time

- 1 hr. for pH 1
- 0.5 hr. pH equilibrium
- 1 hr. for pH 2
- 0.5 hr. pH equilibrium
- 1 hr. for pH 3
- 4 hrs. elapsed time per phase
- ~12 hrs. total elapsed time

Summary and Conclusions

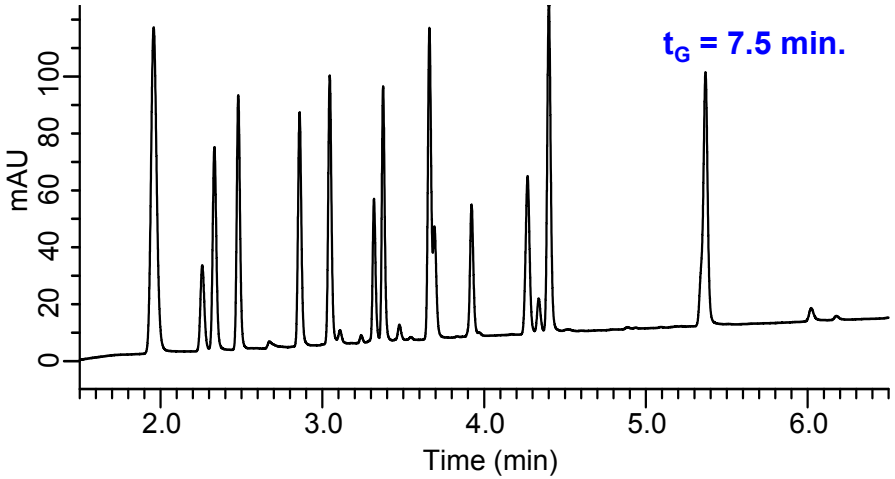
- **A systematic method development approach that incorporates stationary phase and organic modifier screening can be efficient and effective.**
- **ACE Excel 2 μm UHPLC columns with novel, unique, stationary phases and 1000-bar pressure max.**
  - **alkylaryl and alkylpentafluorophenyl groups are useful for polar analytes, structural isomers and analogs.**
  - **Larger particle size and larger frits allow faster flow rates (+30%) at the same pressure as most sub-2-μm columns**

Questions?

- **Acknowledgements**
  - Bob Albrecht, ChromMerge Software
  - Dr. Imre Molnar and Norbert Klapczynski, Molnar-Institute

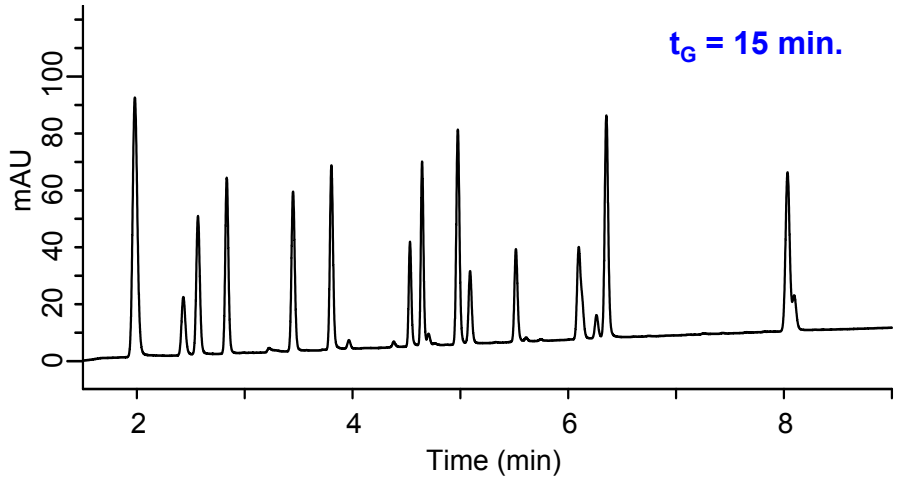
Additional Data (not shown in presentation)

ACE C18: Gradients ACN/water vs. MeOH/water, 30°C only



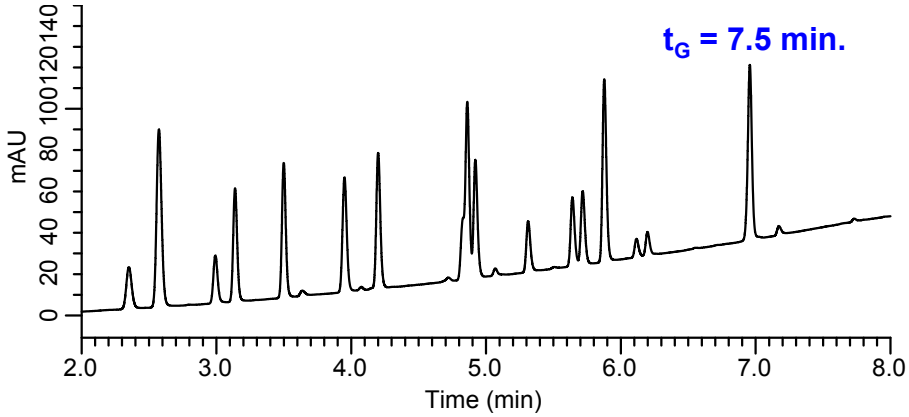
File Name: 30C_7PT5MIN_ACNC18254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

ACN



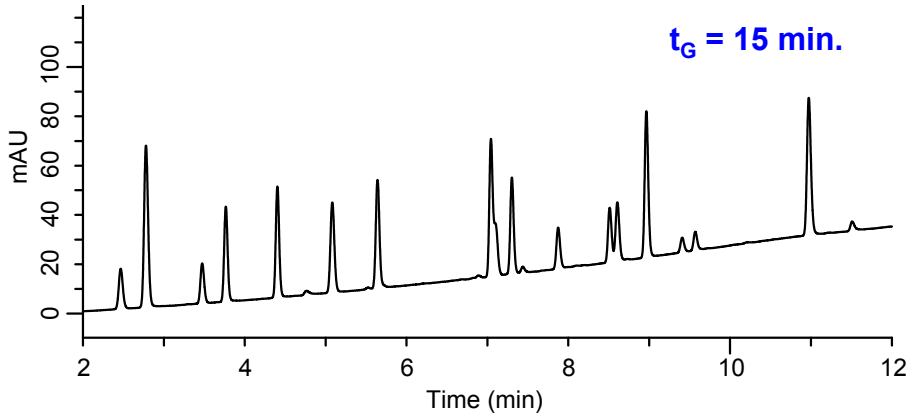
File Name: 30C_15MIN_ACNC18254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

ACN



File Name: 30C_7PT5MIN_MEOHC182ND254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

MeOH

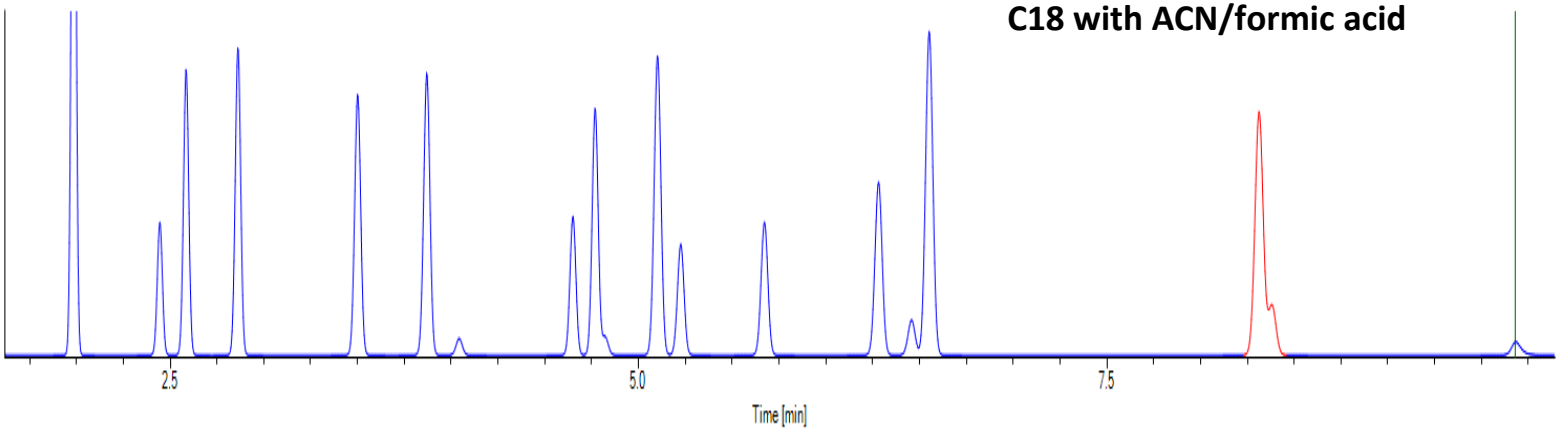


File Name: 30C_15MIN_MEOHC18254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

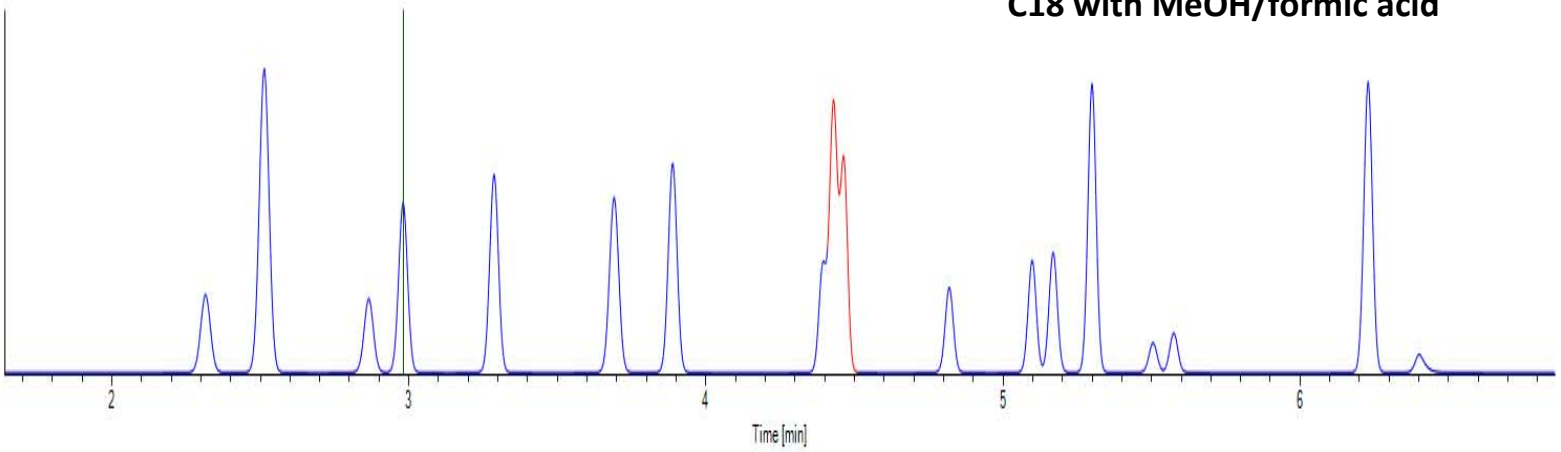
MeOH

ACE C18: Best predicted results with ACN and MeOH gradients at 30℃ using 2.1 x 50 mm ACE *Excel* 2

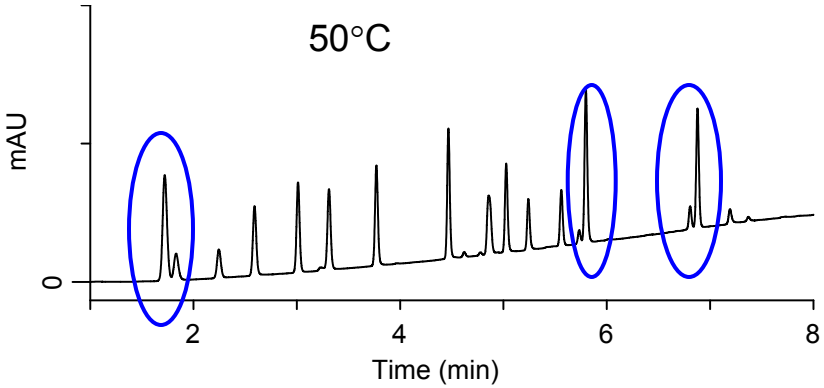
C18 with ACN/formic acid



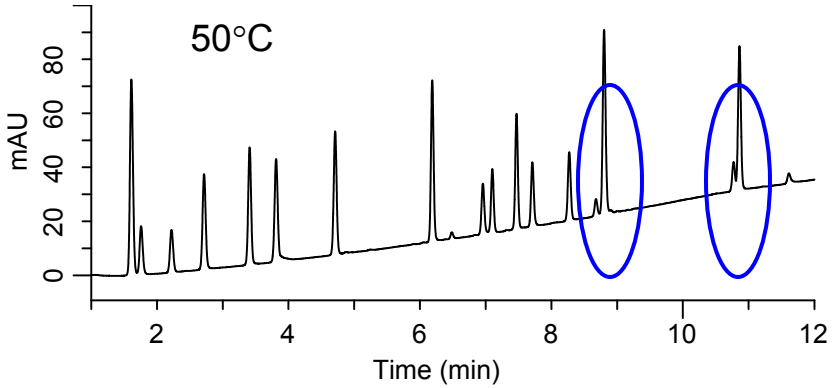
C18 with MeOH/formic acid



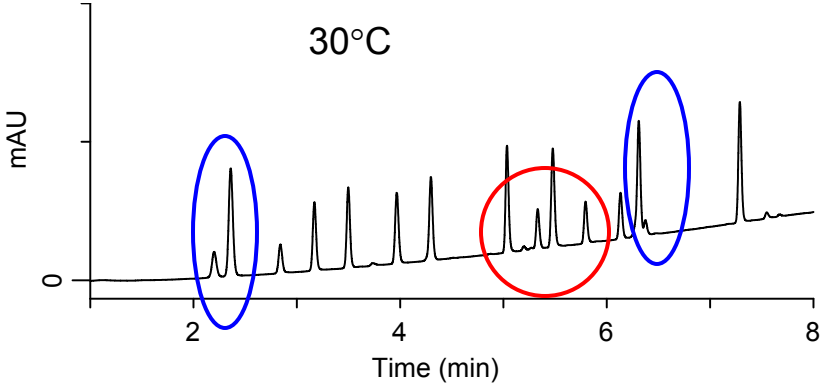
ACE C18-AR with MeOH/water with 0.1% HCOOH gradients at 30 and 50°C



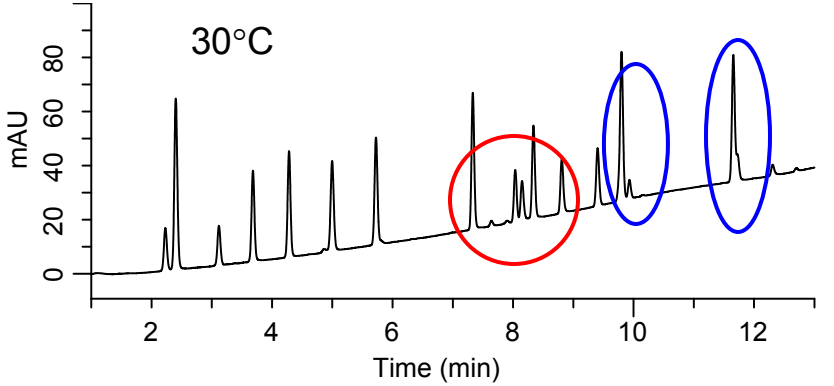
File Name: 50C_7PT5MIN_MEOHC18AR254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE



File Name: 50C_15MIN_MEOHC18AR254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

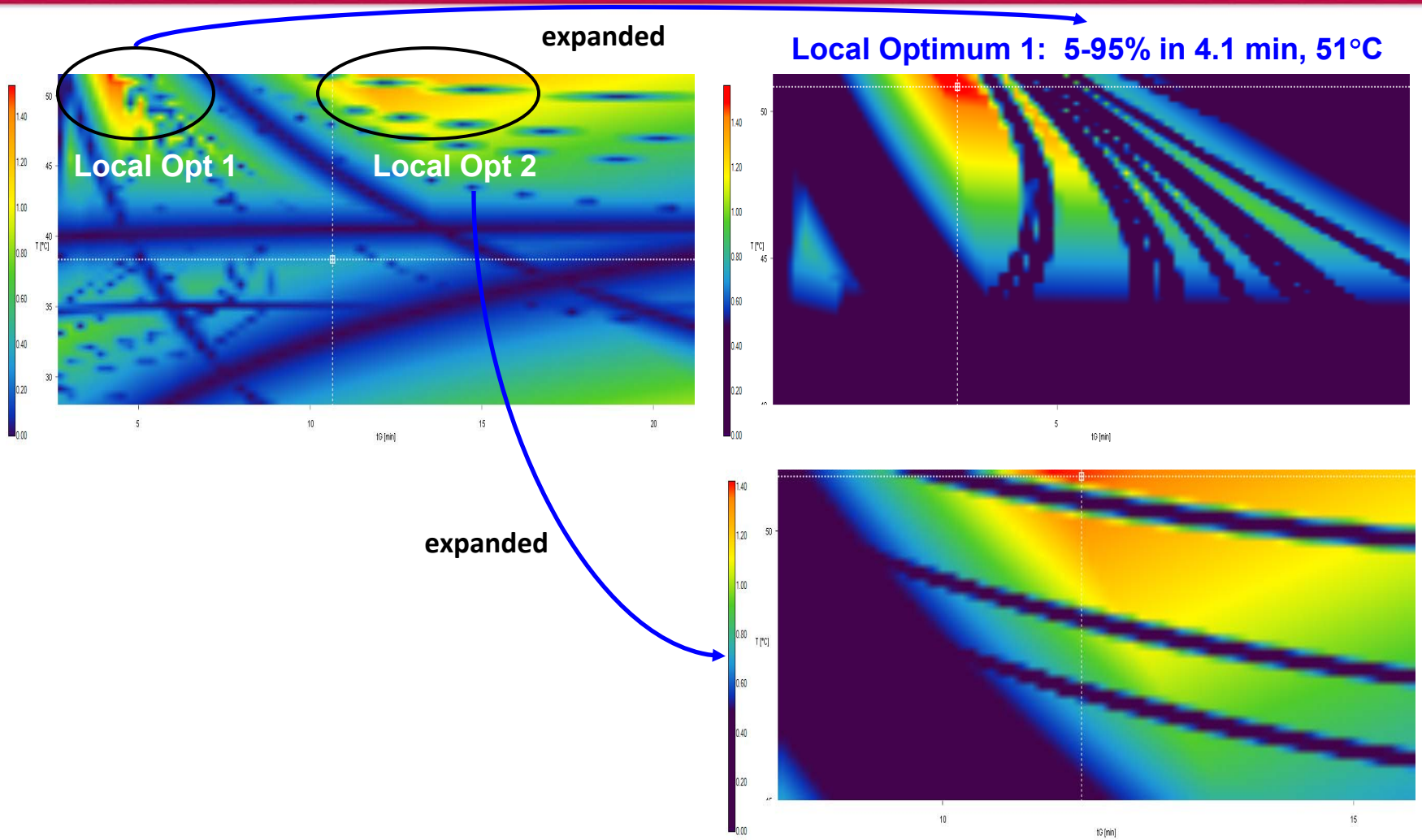


File Name: 30C_7PT5MIN_MEOHC18AR2NDINJ254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE



File Name: 30C_15MIN_MEOHC18AR254100.cdf
Sample Name: 19 compd mix Operator: WAEGHE

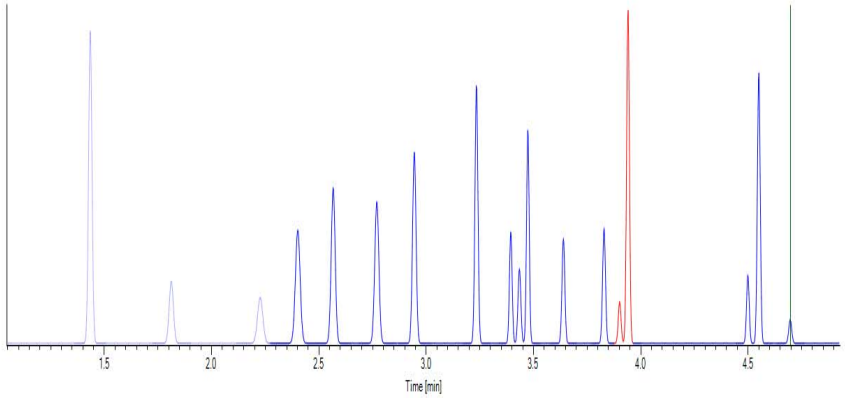
ACE C18-AR: DryLab® 4 2-D Resolution Maps Using MeOH/water (0.1% HCOOH)



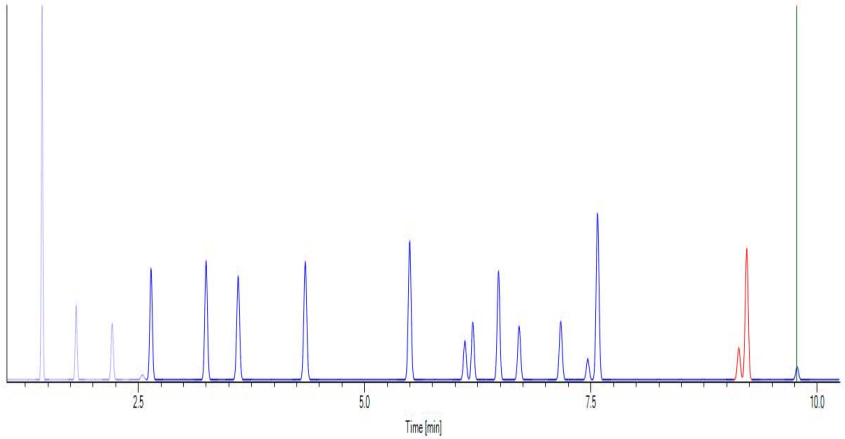
ACE C18-AR with MeOH/water (0.1% HCOOH)

Predicted vs. Actual Chromatograms at Local Optima

Predicted Optima



Local Optimum 1: 5–95% in 4.1 min, 51°C



Local Optimum 2: 5–95% in 11.7 min, 51°C

Actual Runs