Streamlined Reversed-Phase Method Development using a Combined Column Screening and Software Modelling Approach

Alan P McKeown
amckeown@ace-hplc.com

Business Development Director

Advanced Chromatography Technologies Ltd

Overview

- **Method Development and Strategies.**
- **Maximising Selectivity: a Systematic Screening Approach.**
- **Use of ChromSword software for method optimisation.**

Reversed-Phase Method Development

- Method development is typically an iterative process which can take time depending upon separation and sample complexity.

- Several approaches are commonly used for method development:
  - Trial and error
  - Stepwise, one factor at a time (OFAT)
  - Quality by Design (QbD)
  - Systematic Screening

- Logical, well structured method development processes can help to efficiently develop separations and robust LC methods.

Systematic Screening

- A systematic screening approach applies a common screening protocol to explore specific chromatographic parameters which maximise selectivity.
- Can be semi-automated with a suitable LC instrument eg VWR-Hitachi ChroMaster LC instrument.
- The screening process may identify a combination of these parameters that provide a separation.

Resolution, Selectivity, Efficiency & Retention

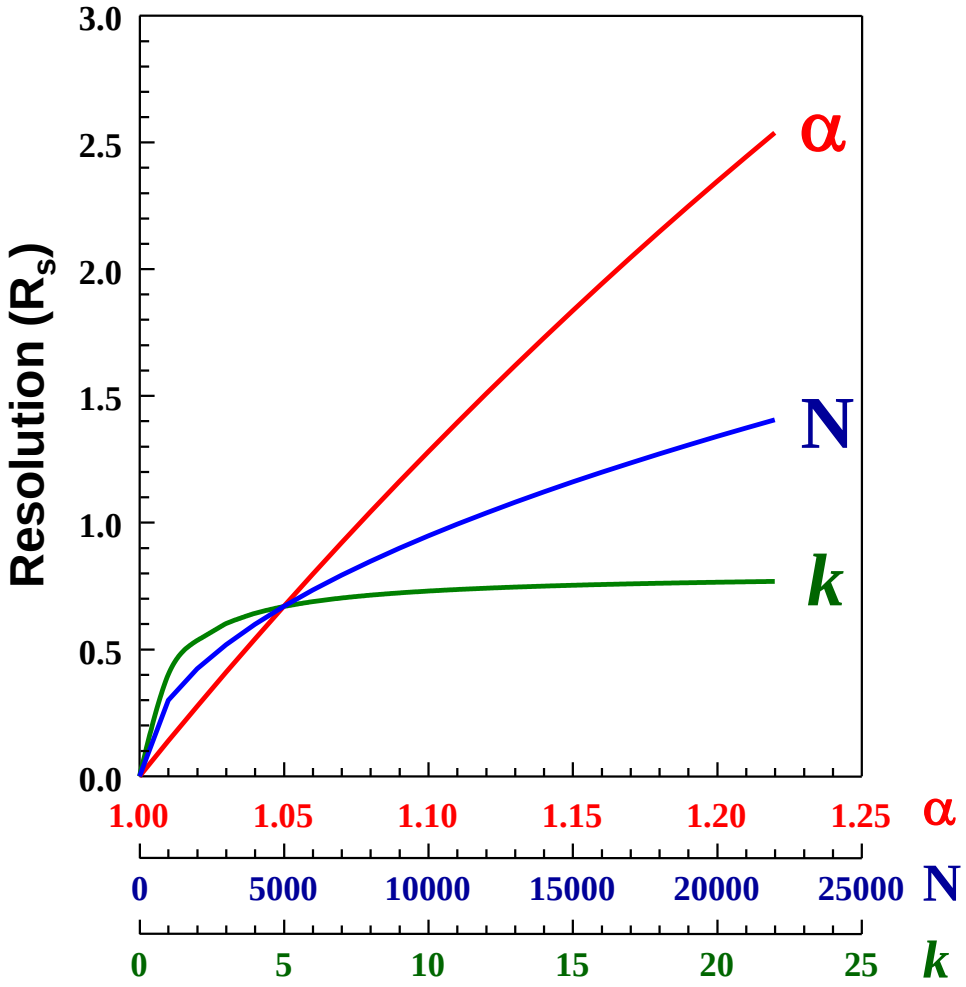
Particle size, column length, dispersion etc

Phase design, eluent etc

Efficiency **Selectivity** **Retention**

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

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



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

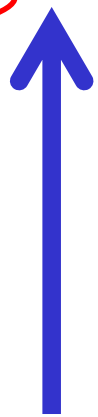
Which Factors[1] Affect Selectivity?

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

Isocratic Separations

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

MOST Influence



LEAST Influence

Gradient Separations

All parameters for isocratic **PLUS**

Gradient steepness

k^* (t_G, F, V_m, ΔΦ, M)

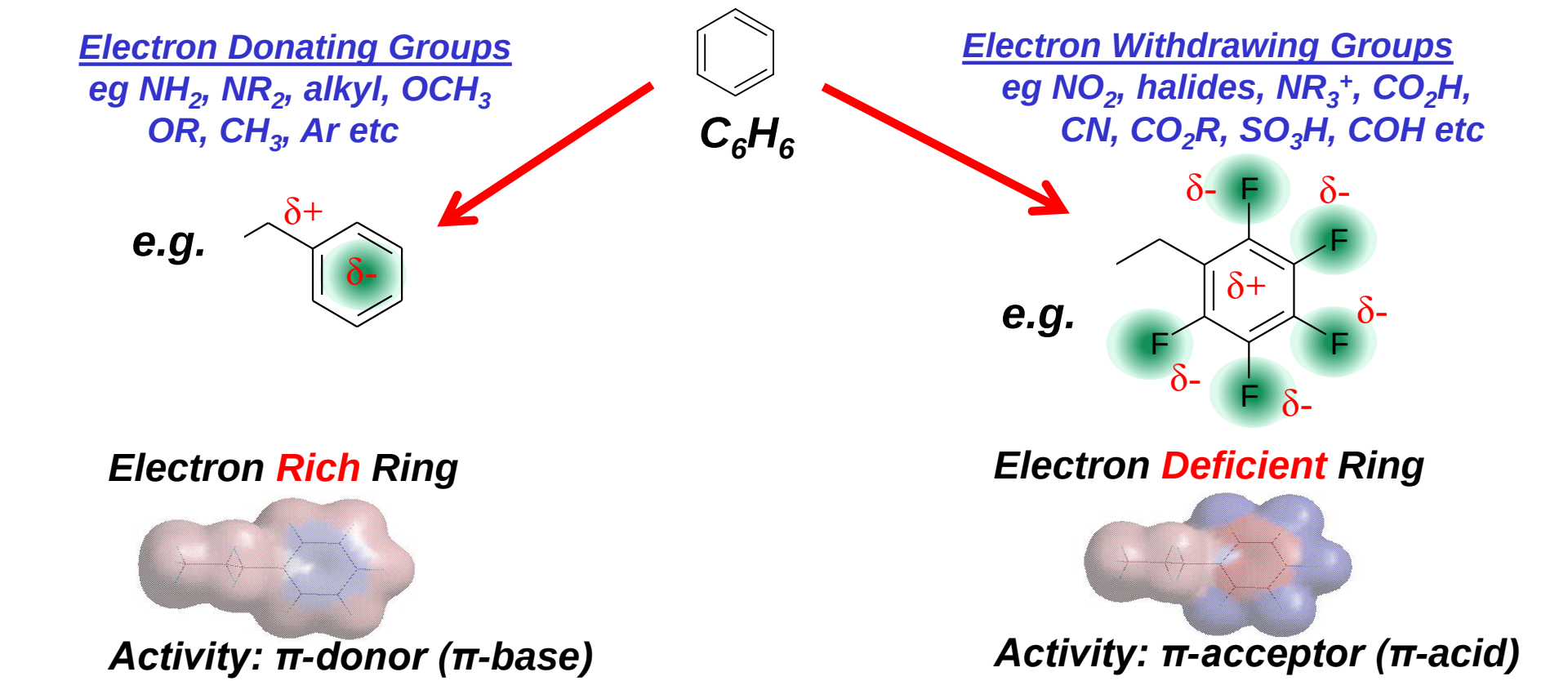
$$k^* = \frac{t_G F}{\Delta \Phi V_m M}$$

Dwell volume

Column dimensions

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

Scientific Led Stationary Phase Design: Aromatic Phases



How do we exploit these properties for new stationary phases?

C18+Phenyl = ACE® C18-AR

C18+PFP = ACE® C18-PFP

Both phases have multiple mechanisms of interaction, low bleed and are 100% wettable: i.e. maximize selectivity

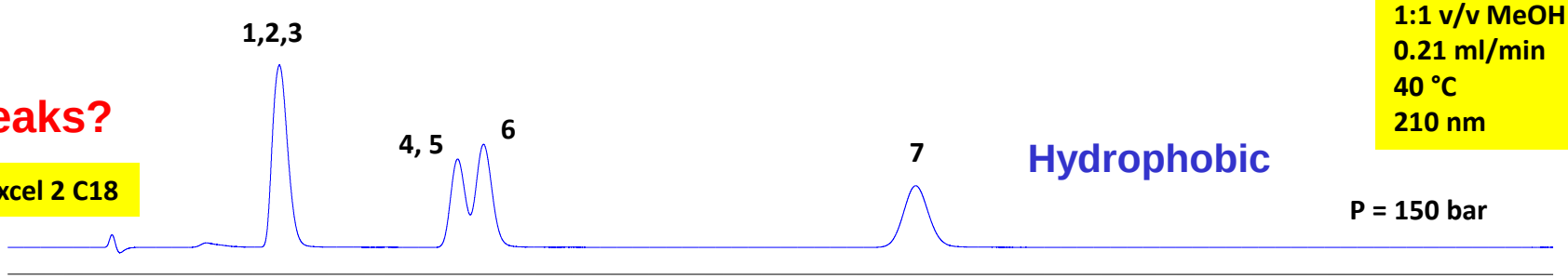
Stationary Phase Selectivity: Regioisomers

Isocratic analysis

1:1 v/v MeOH:H2O
0.21 ml/min
40 °C
210 nm

4 peaks?

ACE Excel 2 C18

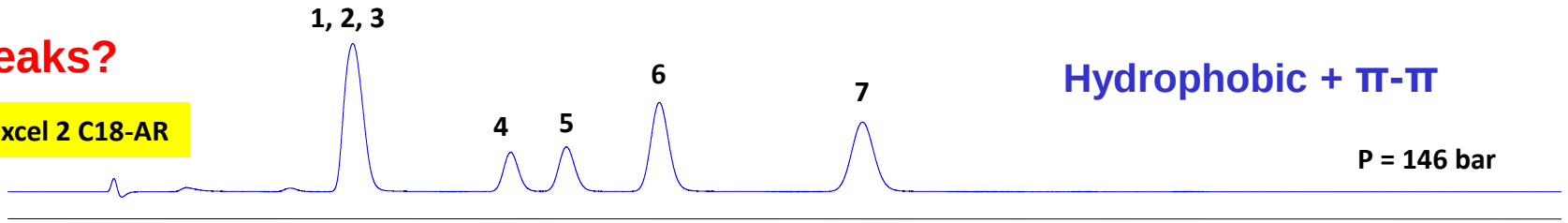


Hydrophobic

P = 150 bar

5 peaks?

ACE Excel 2 C18-AR

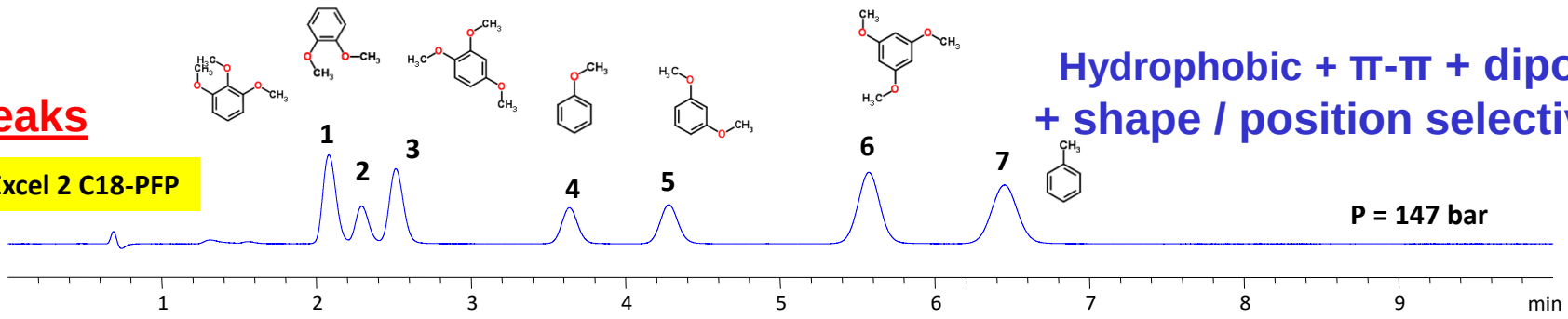


Hydrophobic + π-π

P = 146 bar

7 peaks

ACE Excel 2 C18-PFP



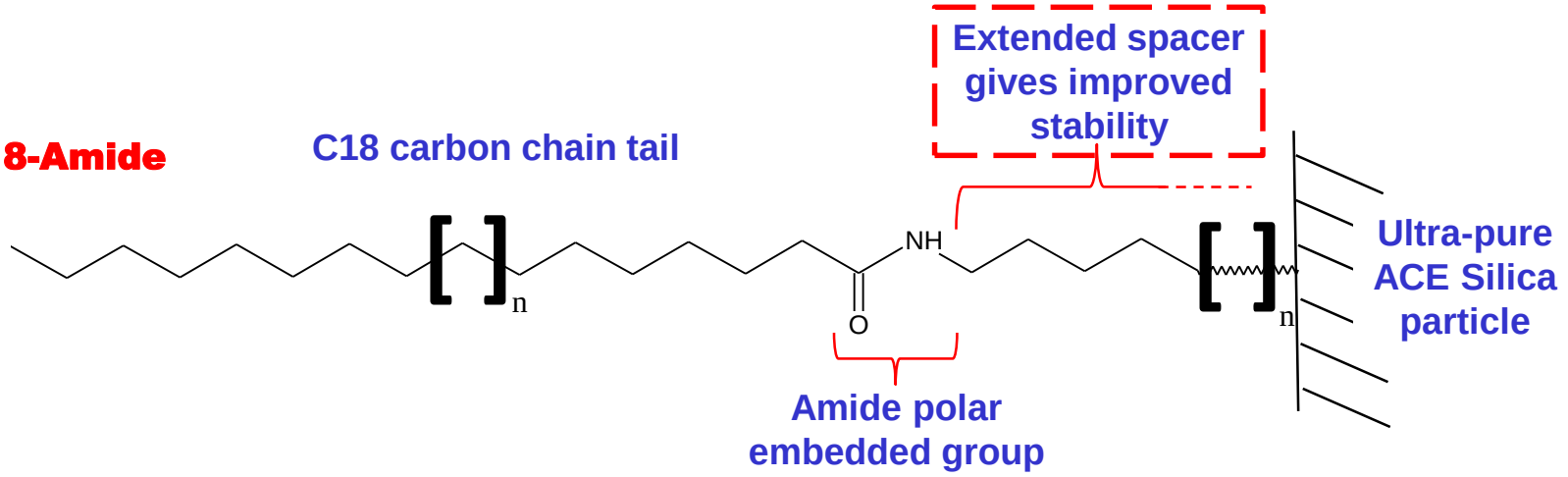
Hydrophobic + π-π + dipole + shape / position selectivity

P = 147 bar

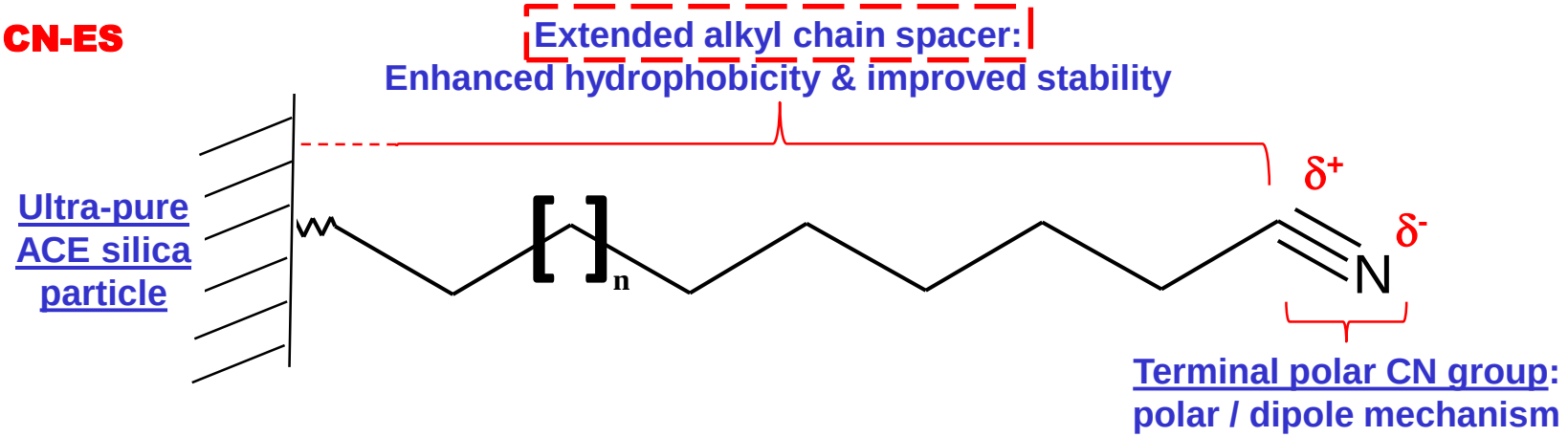
Stationary Phase Selectivity Is Powerful

Scientific Led Stationary Phase Design: Other Phases

ACE C18-Amide



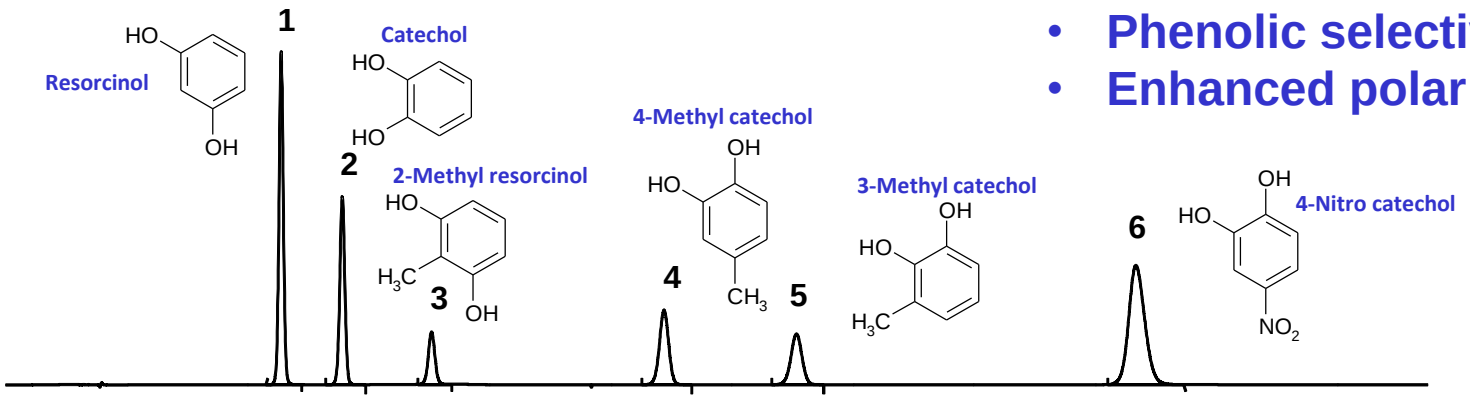
ACE CN-ES



Multiple mechanisms of interaction, low bleed and are 100% wettable: i.e. maximize selectivity

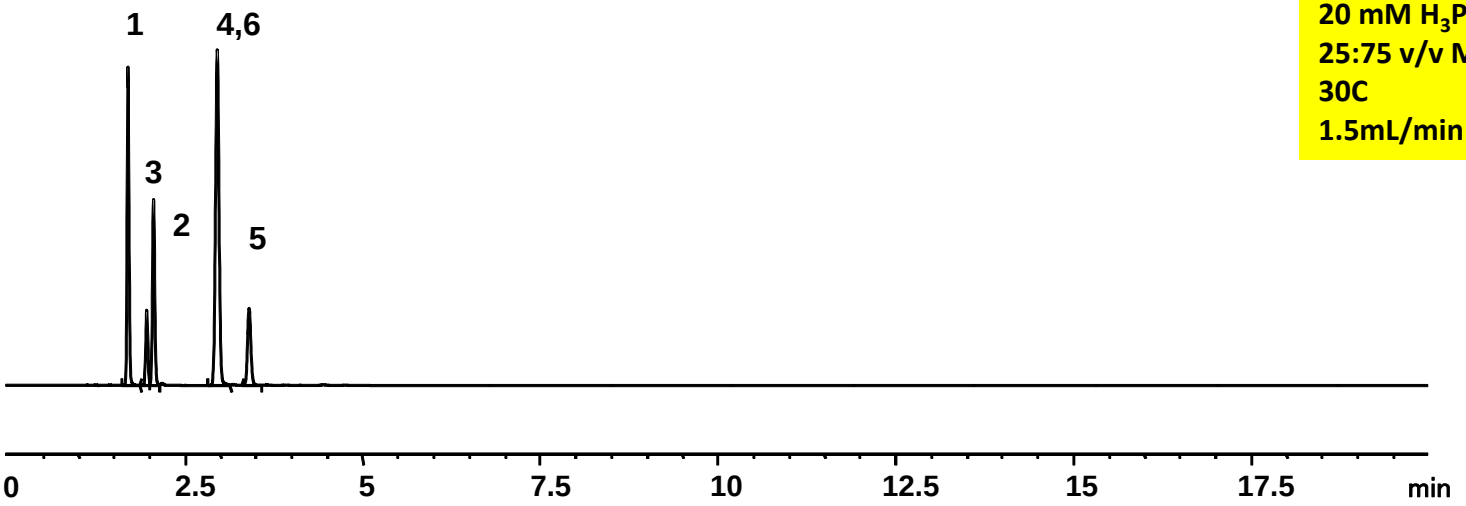
Catechols and Resorcinols Separations

ACE C18-Amide



- **Phenolic selectivity**
- **Enhanced polar retention**

ACE C18



150 x 4.6mm, 3µm
Isocratic analysis

20 mM H_3PO_4 in
25:75 v/v MeCN/H_2O
30C
1.5mL/min

Stationary Phase Key Mechanisms of Interactions

- **Tanaka[1] characterisation can help understand mechanisms and weightings with different column chemistries**

Bonded Phase	Separation Mechanism and Relative Strength[1]				
	Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
ACE C18	****	-	-	*	**
ACE C18-AR	****	*** (donor)	*	**	***
ACE C18-PFP	****	*** (acceptor)	****	***	****
ACE SuperC18	****	-	-	-	**
ACE C18-Amide	****	-	**	****	**/***
ACE CN-ES	***	*	***	**	*

Approximate value – determined by semi-quantitative mechanism weightings and/or by reference to other ACE phases using >100 characterising analytes.

[1] K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Arki, N. Tanaka, *J. Chromatogr. Sci.* 27 (1989) 721.

Stationary Phase Key Mechanisms of Interactions

- **Specifically designed phases to maximize selectivity**

Bonded Phase	Separation Mechanism and Relative Strength[1]				
	Hydrophobic Binding	π-π Interaction	Dipole-Dipole	Hydrogen Bonding	Shape Selectivity
ACE C18	****	-	-	*	**
ACE C18-AR	****	*** (donor)	*	**	***
ACE C18-PFP	****	*** (acceptor)	****	***	****
ACE SuperC18	****	-	-	-	**
ACE C18-Amide	****	-	**	****	**/***
ACE CN-ES	***	*	***	**	*

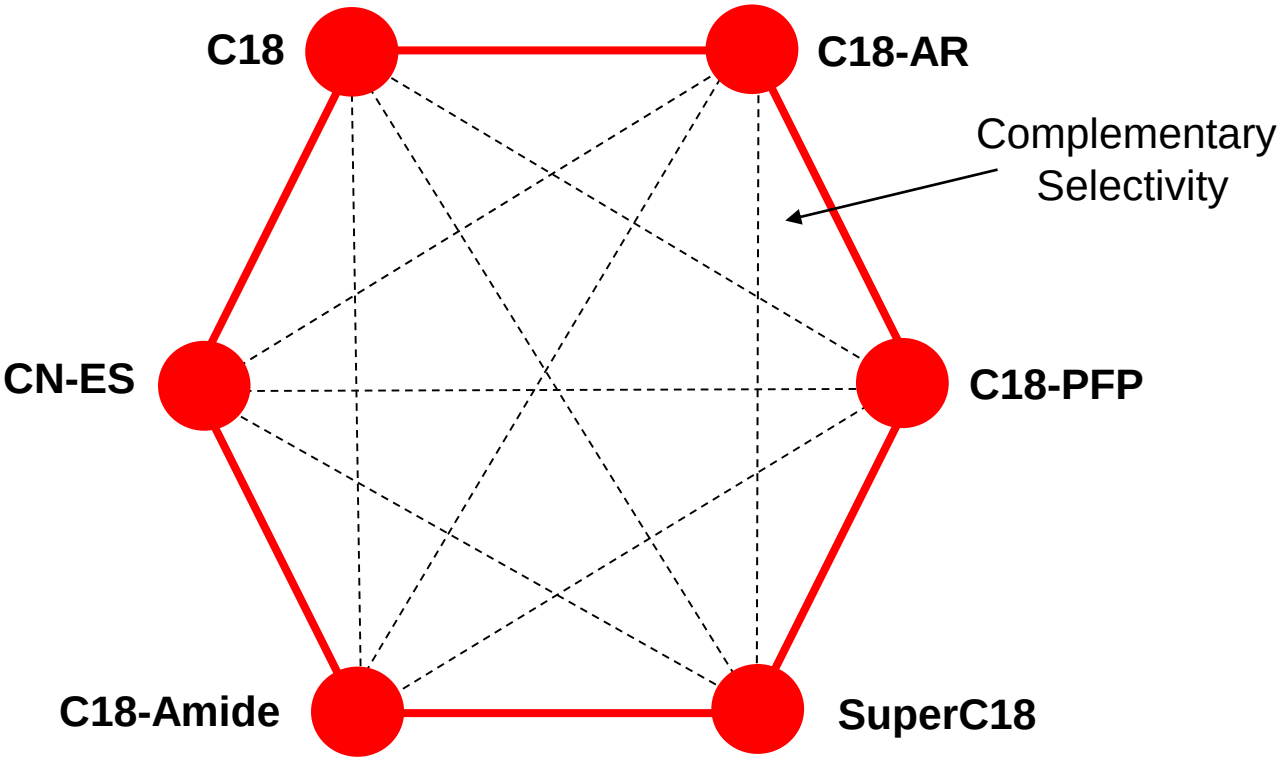
Approximate value – determined by semi-quantitative mechanism weightings and/or by reference to other ACE phases using >100 characterising analytes.

- **Multiple mechanisms of interaction: ideal for method development**

Selectivity, Method Development: 6 Column Switcher

- 6 stationary phases, 1 solvent, low pH

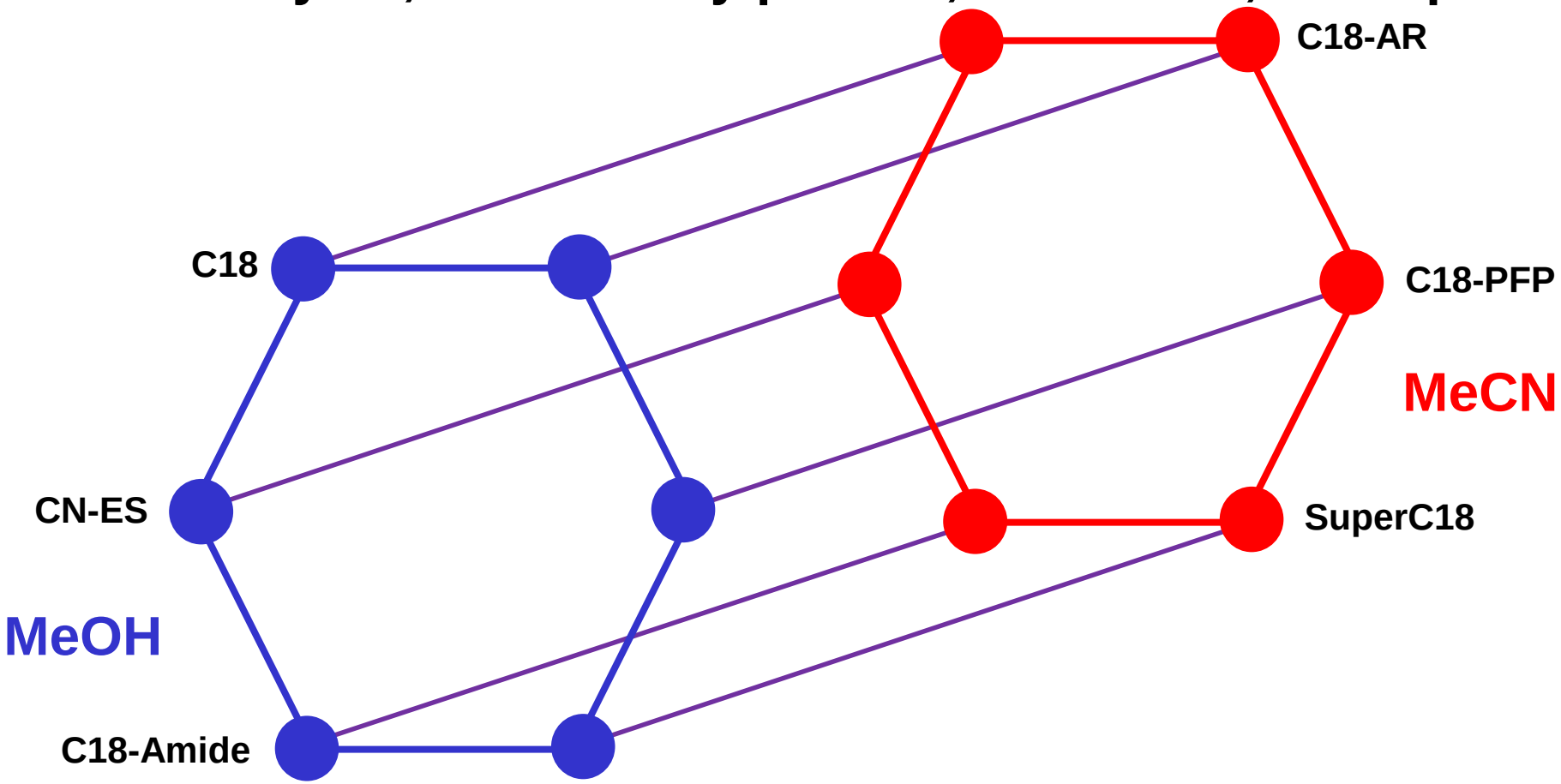
MeCN



6 Column Method Development Platform
Based Upon The Power of Phase Chemistry / Selectivity

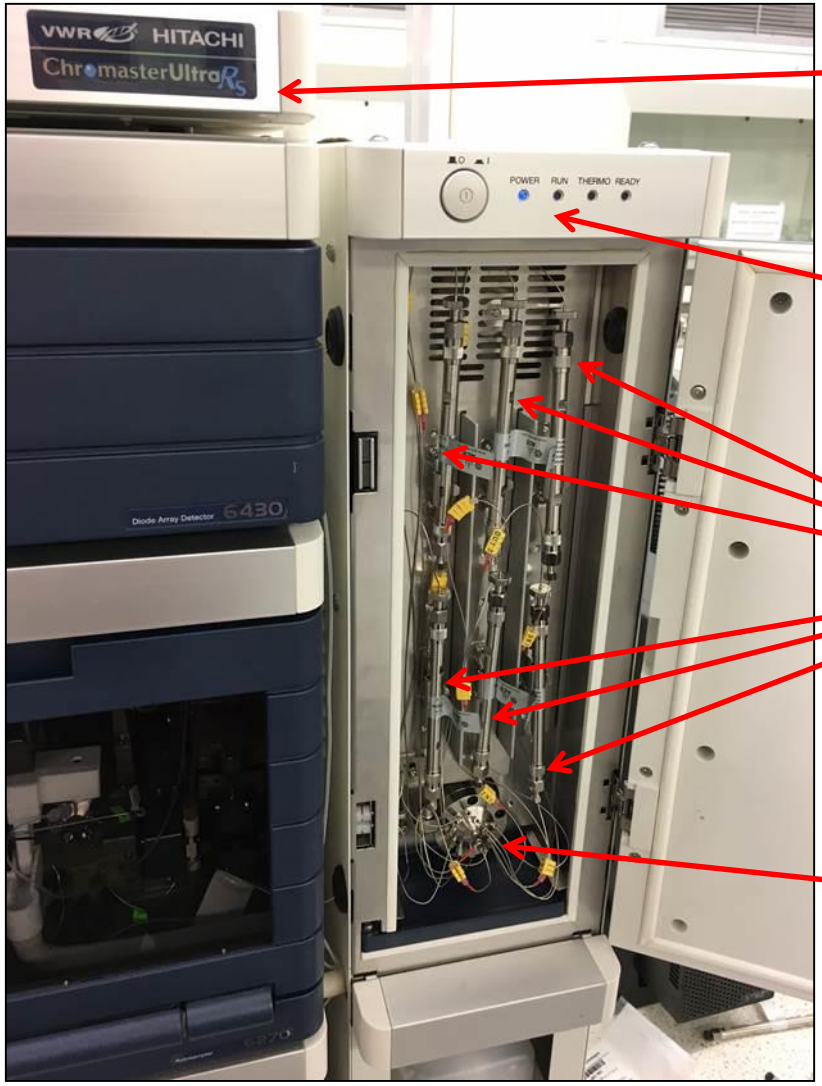
Explore Selectivity for Method Development

- 45 analytes, 6 stationary phases, 2 solvents, 1 low pH



6 Column, 2 Solvent Method Development Platform Based Upon The Power of Selectivity

Total Selectivity, Method Development: Screening Platform



UHPLC Instrument

Thermostatted oven

6 x ACE Column
(100 x 3mm, 2µm)

Switching valve

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 100 x 3mm column:

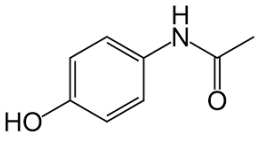
t_G	= 5 minutes
F	= 1.2 mL/min
$\Delta\phi$	= 0.95
V_m	= 0.459 mL
M	= 5

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

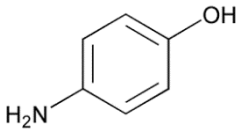
- Ideally retention (or *k** in gradient elution) should be >2 and <20 for initial method development

Paracetamol Plus Some Impurities For Method Development

1.	Acetaminophen	API (a.k.a. Paracetamol)
2.	4-aminophenol	Synthesis/degradation product
3.	Hydroquinone	Deg product of 4-aminophenol
4.	2-aminophenol	Included as would be a synthesis impurity of (8) if not fully removed
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

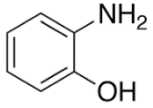


Acetaminophen (Paracetamol)
pKa = 9.86
LogP = 0.34
LogD 5.5 = 0.4
LogD 7.4 = 0.4

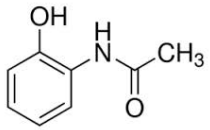


4-aminophenol
pKa = 5.28, 10.17
LogP = -0.29
LogD 5.5 = -0.04
LogD 7.4 = 0.16

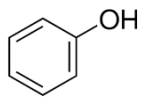
Hydroquinone
pKa = 10.33
LogP = 0.64
LogD 5.5 = 0.53
LogD 7.4 = 0.53



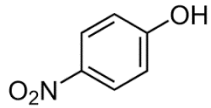
2-aminophenol
pKa = 4.84, 10.01
LogP = 0.44
LogD 5.5 = 0.58
LogD 7.4 = 0.64



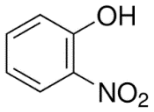
2-acetamidophenol
pKa = 8.79
LogP = 0.72
LogD 5.5 = 0.79
LogD 7.4 = 0.79



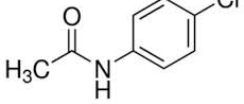
Phenol
pKa = 9.95
LogP = 1.48
LogD 5.5 = 1.63
LogD 7.4 = 1.63



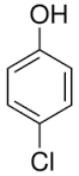
4-nitrophenol
pKa = 7.23
LogP = 1.57
LogD 5.5 = 1.7
LogD 7.4 = 1.31



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



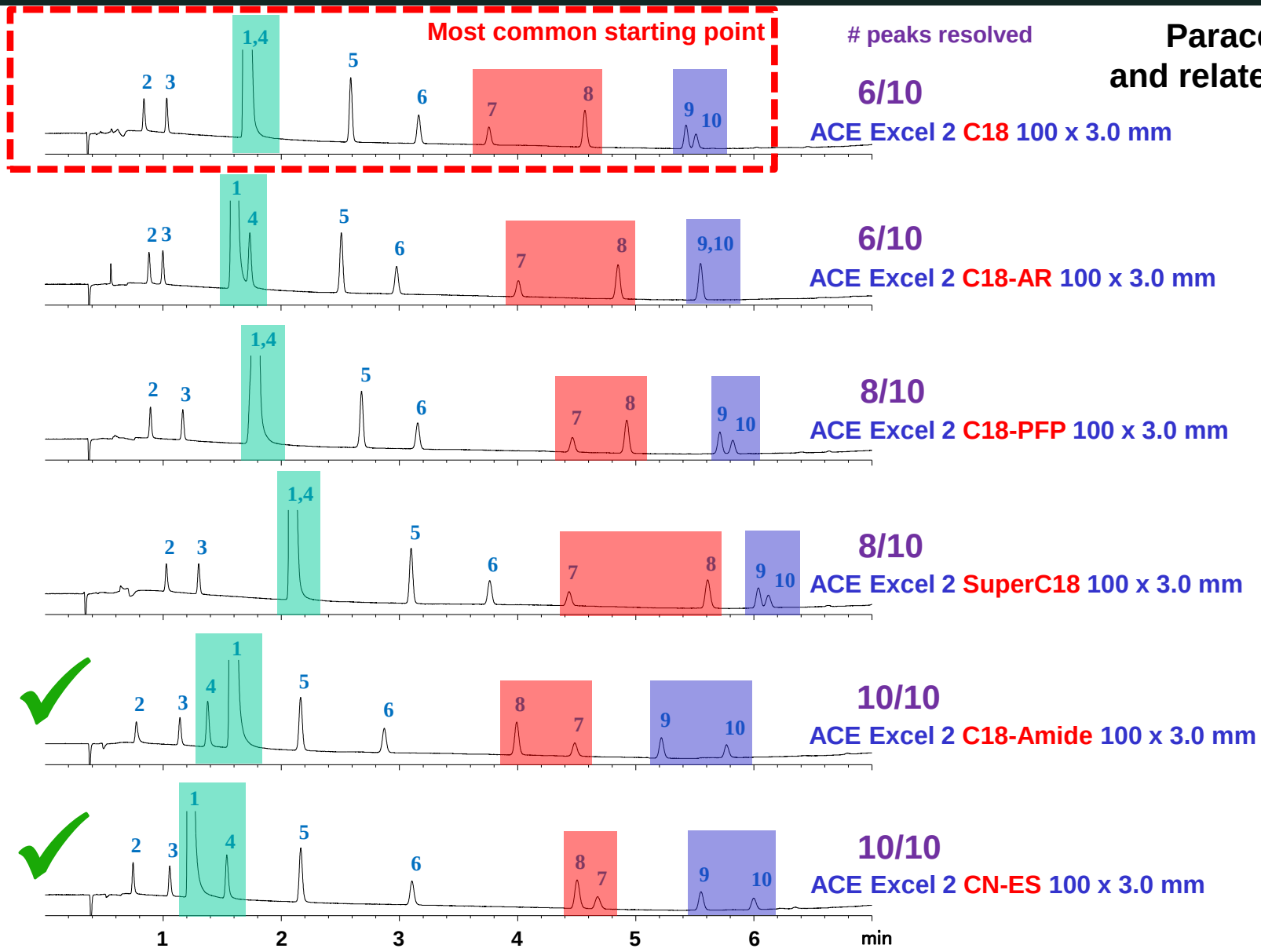
4-chloroacetanilide
pKa = -1.97/14.25
LogP = 2.05
LogD 5.5 = 2.14
LogD 7.4 = 2.14



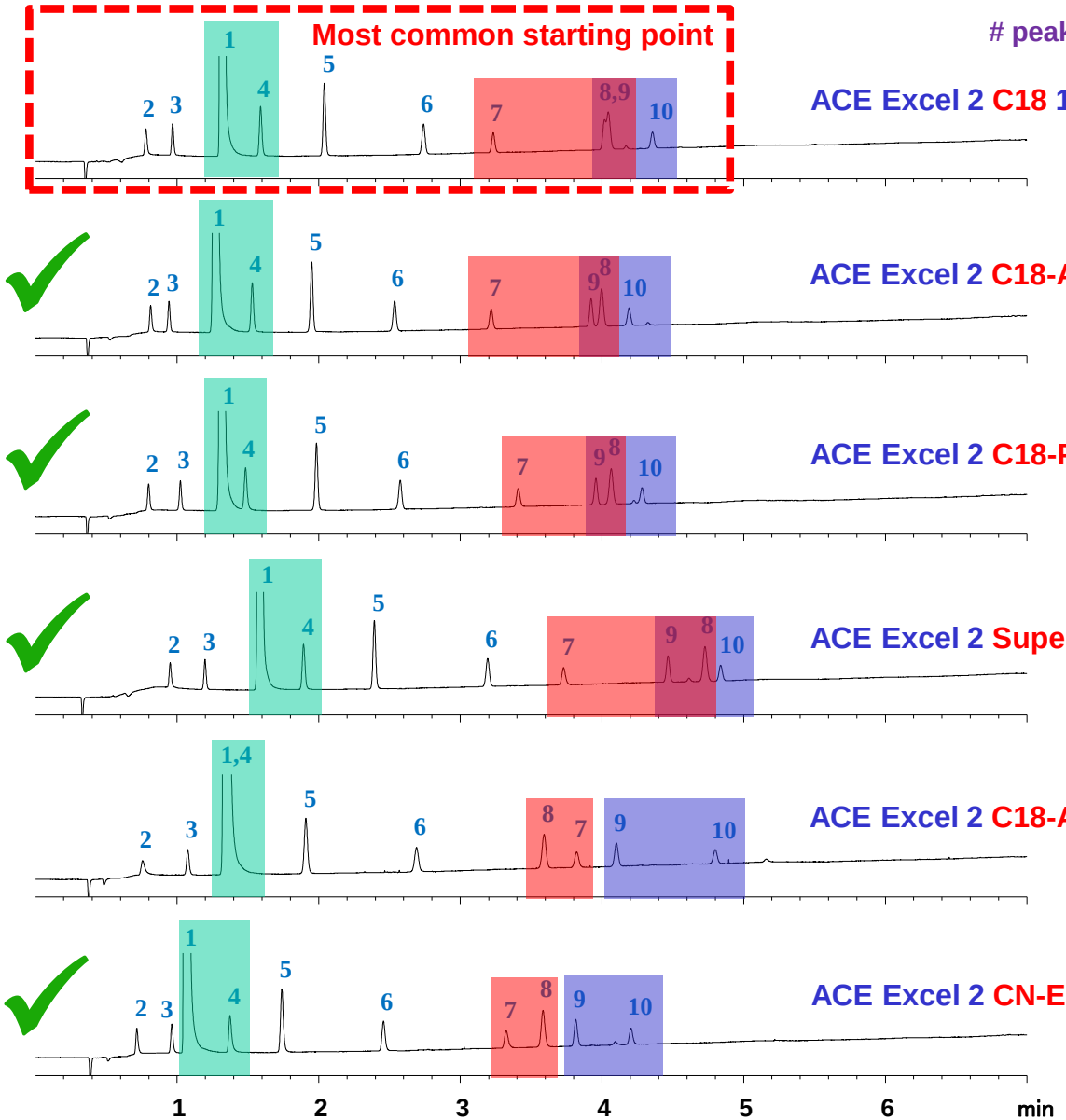
4-chlorophenol
pka = 9.3
LogP = 2.43
LogD 5.5 = 2.43
LogD 7.4 = 2.42

General scouting conditions:
100 x 3.0 mm columns
A: 20 mM Ammonium acetate pH 6.0
B: 20 mM Ammonium acetate pH 6.0 in MeOH or MeCN:H_2O (9:1 v/v)
Gradient: 5 to 95%B in 10 mins
Temp: 40 °C, 2 µL injection, Flow rate: 1.2 mL/min
Sample: Acetaminophen with rel subs at 0.5% w/w

Total Selectivity, Method Development: Screening Platform



Total Selectivity, Method Development: Screening Platform

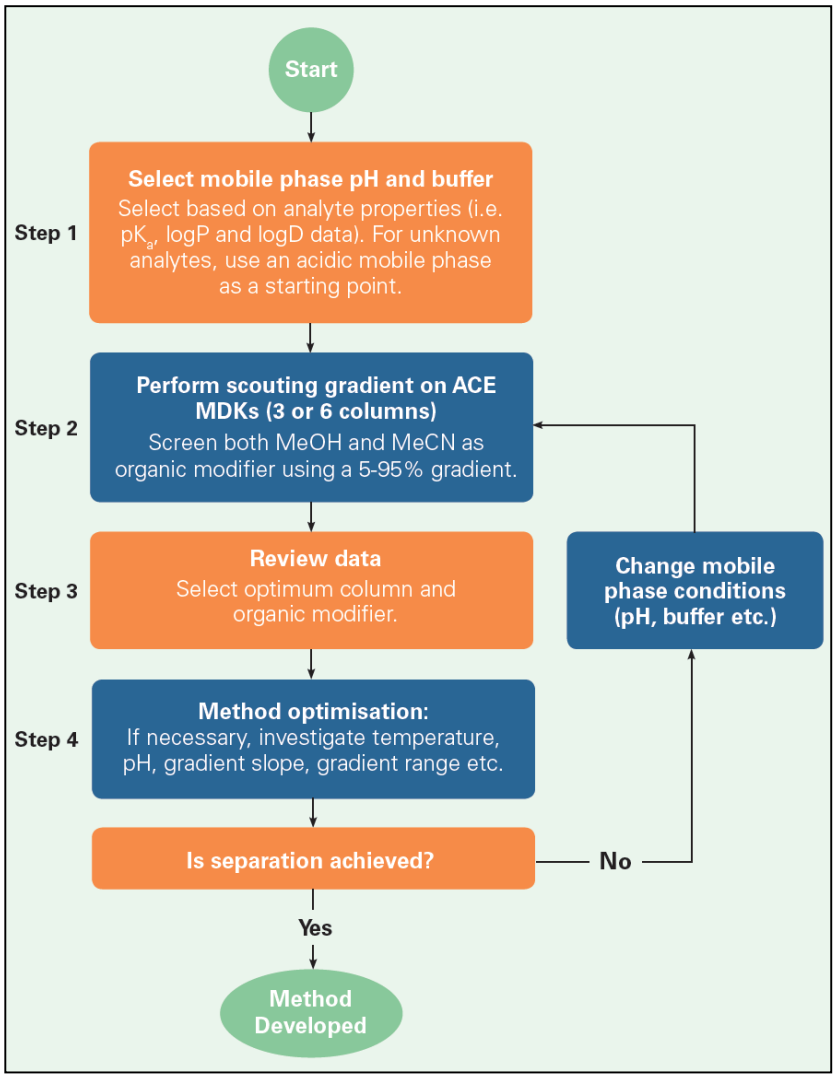


Acetaminophen and related analytes

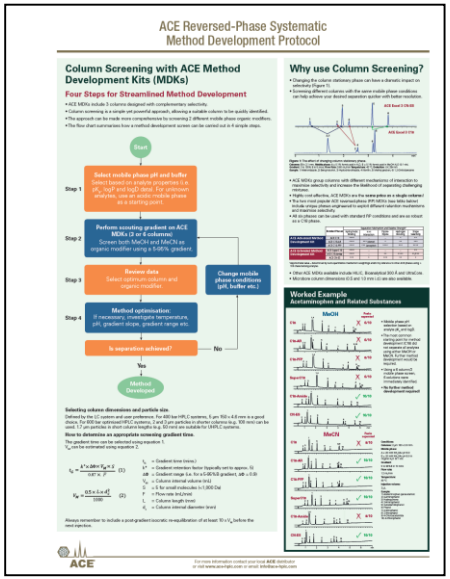
Six options direct from the screening data.

No further development required.

The Complete Method Development Screening Solution



- The **ACE Reversed-Phase Method Development Flow Chart** provides a **simple to follow** tool for **efficient** and streamlined **method development** using the **ACE novel phases**.
- Available as a **free A2 Poster**.



Optimising the separation

- The screening data is used to select a column/organic modifier combination for method optimisation.
- Method optimisation may require investigation of other parameters:
  - Gradient time and %B range
  - Temperature
  - pH
- Method development software can be used to facilitate the optimisation.

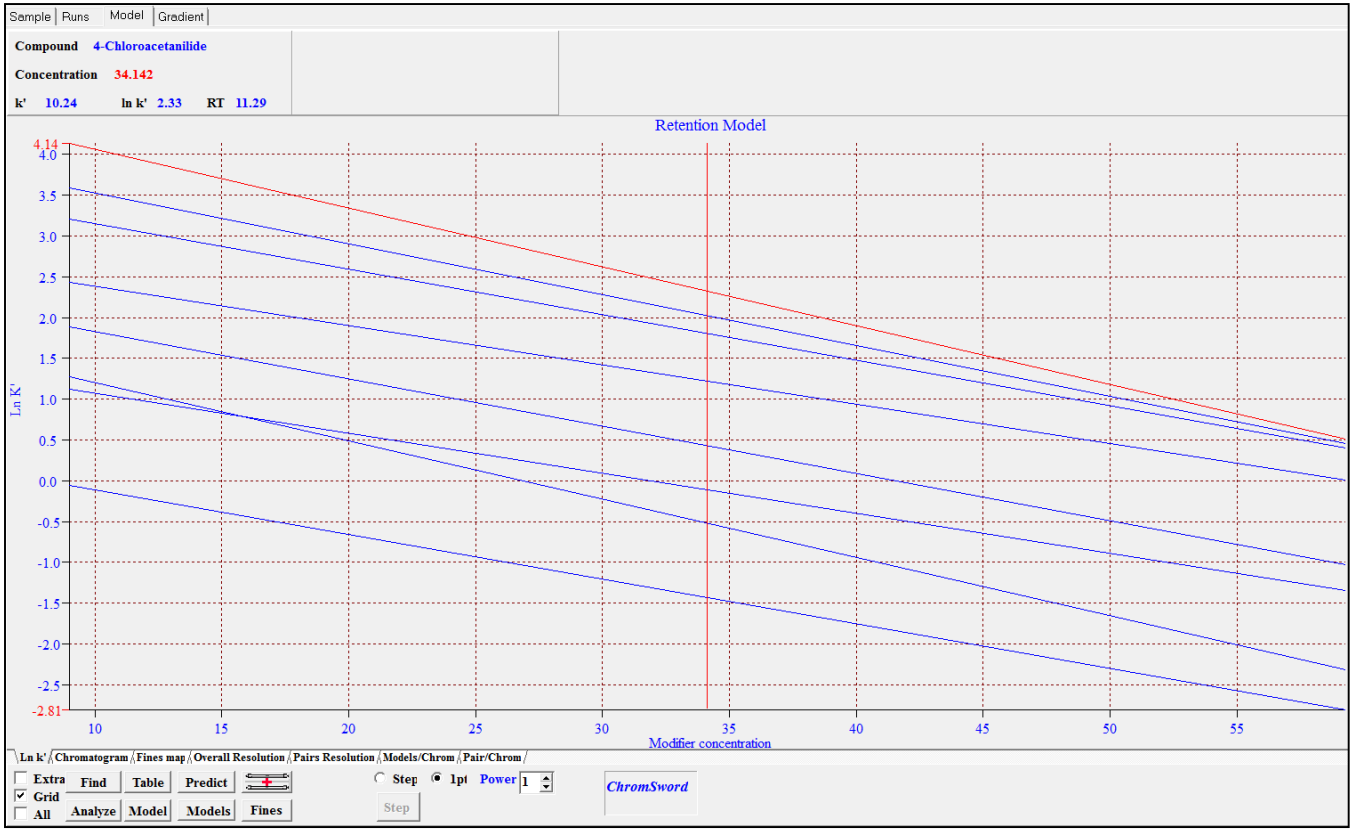
ChromSword 2

ChromSword

- **Method development software can be used to model, predict and optimise separations in:**
  - **Reversed Phase Chromatography**
    - % Organic
    - Gradient time
    - pH
    - Temperature
  - **Also Compatible with Normal Phase and Ion Exchange Chromatography**

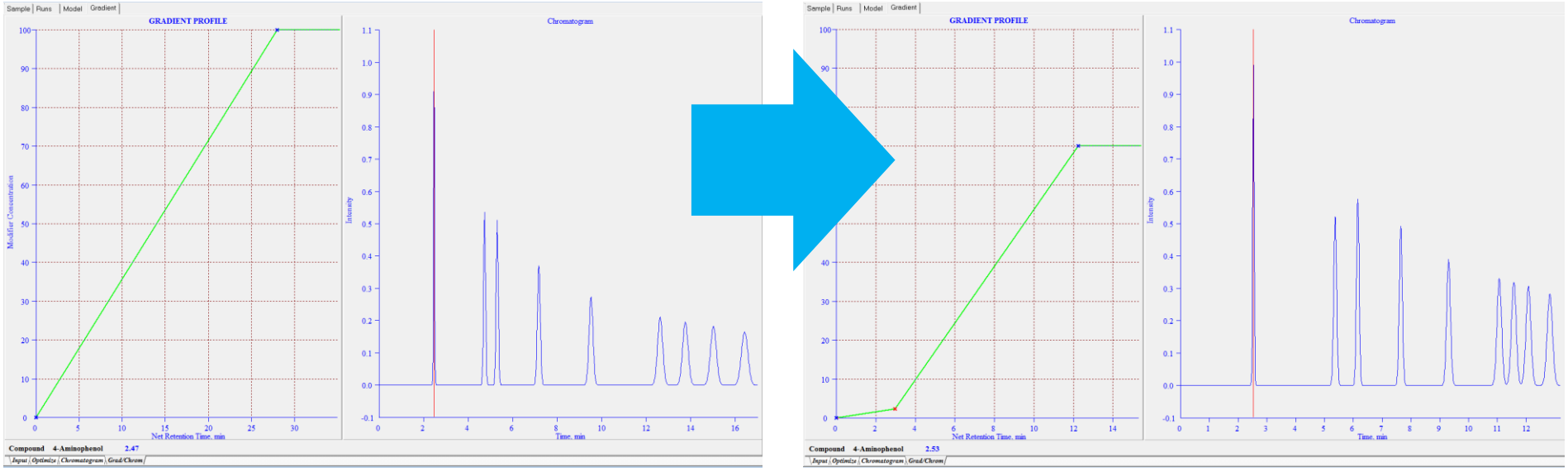
Method Optimisation with ChromSword 2

- Input from as few as two initial runs into the software to build a tG retention model.
- Additional runs can also be included to improve and validate the accuracy of the model.



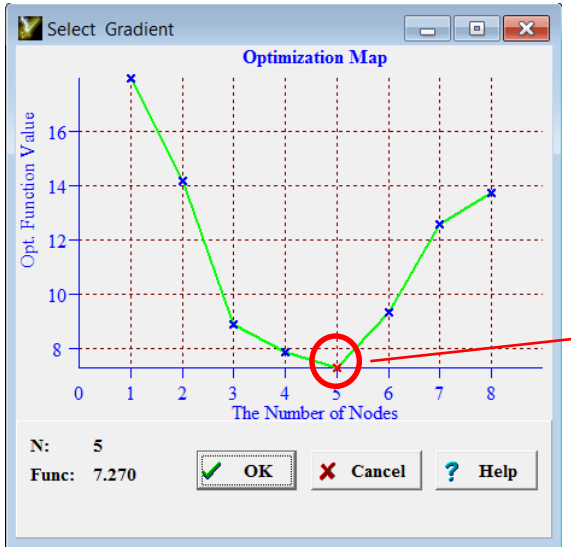
Method Optimisation with ChromSword 2

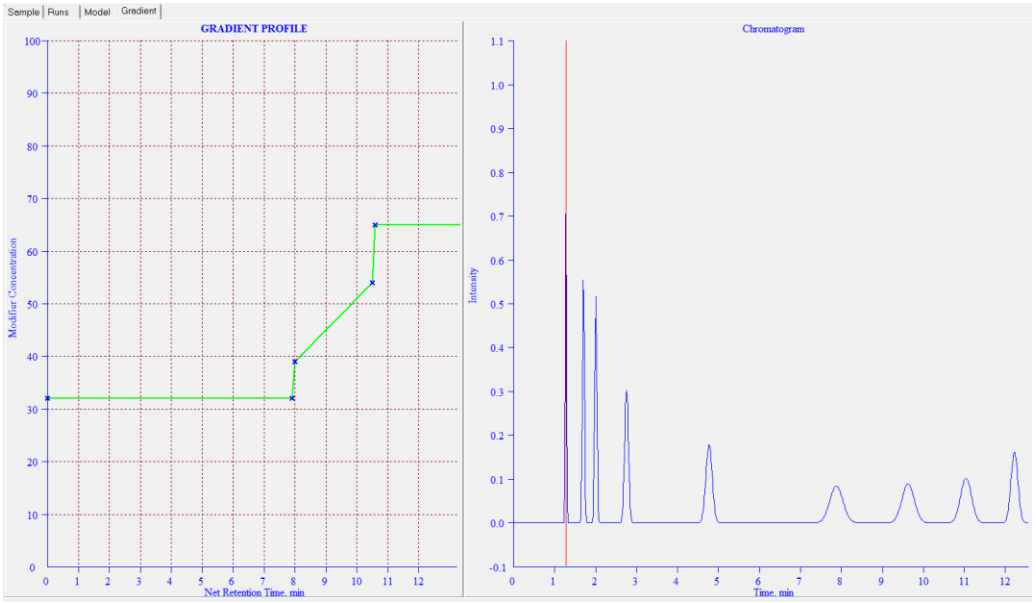
- Input from as few as two initial runs entered into the software to build a retention model.
- Additional runs can also be included to improve and validate the accuracy of the model.
- **Can now simulate the separation and adjust manually.**



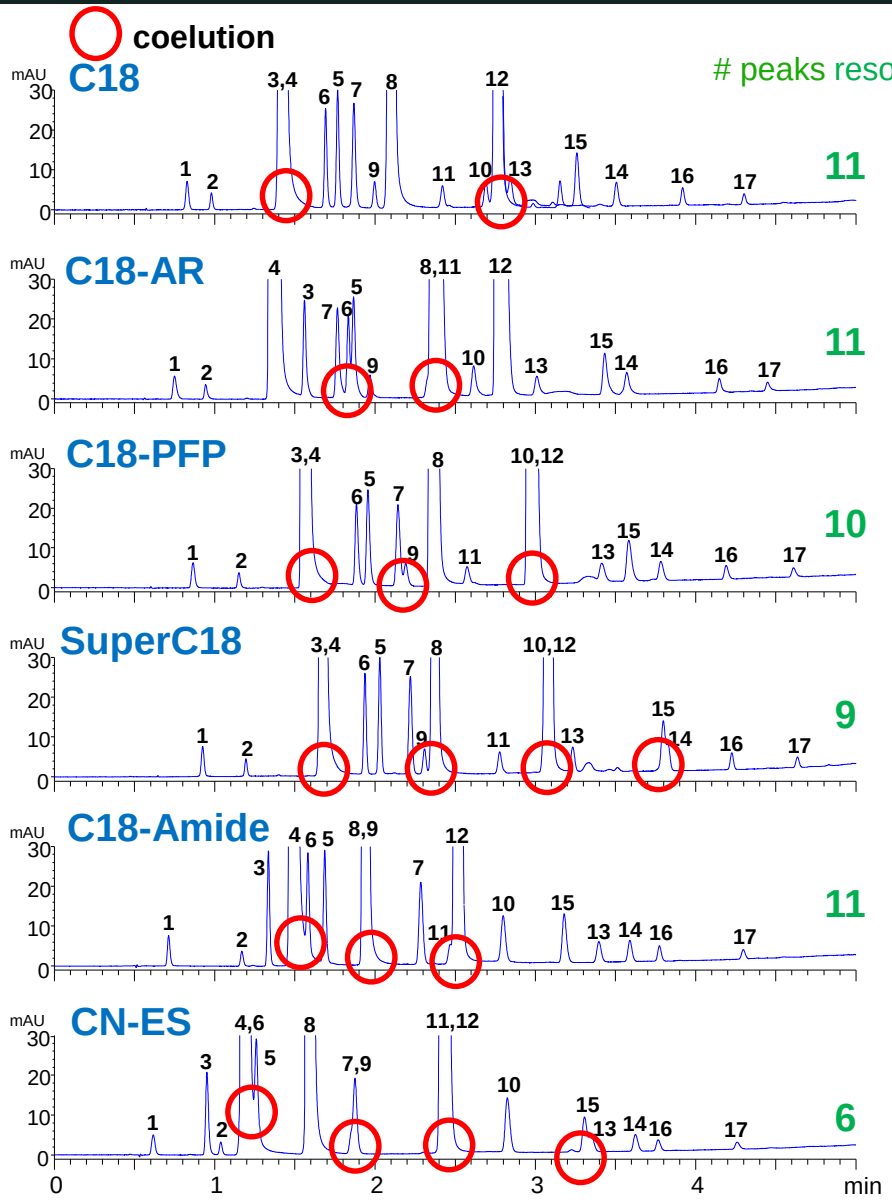
Method Optimisation with ChromSword 2

- Input from as few as two initial runs entered into the software to build a retention model.
- Additional runs can also be included to improve and validate the accuracy of the model.
- Can now simulate the separation and adjust manually.
- **ChromSword can also automatically optimise the separation.**





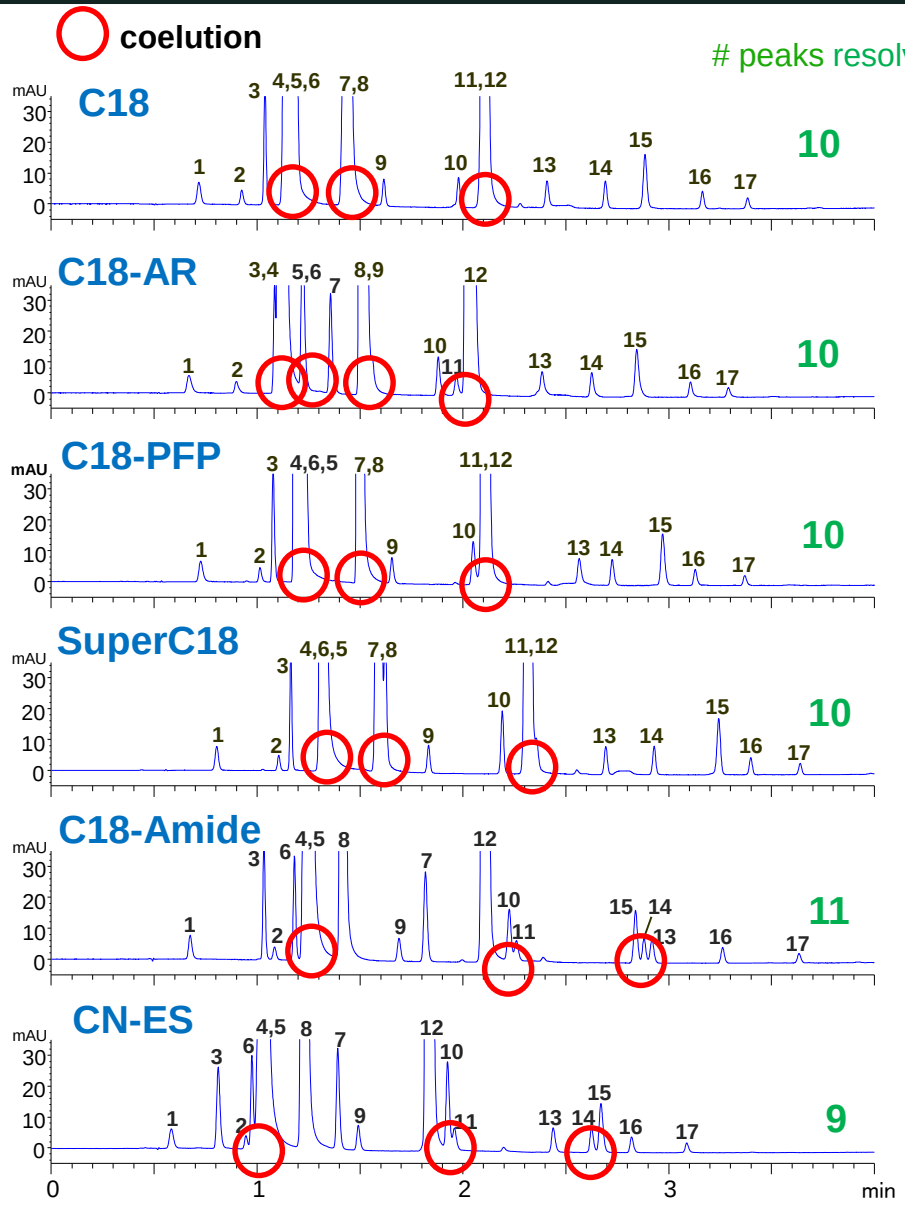
6 Column Screen of Triple API sample + Impurities (17 peaks)



- Methanol provides better retention and selectivity for the more hydrophilic analytes.
- The screening approach provides multiple options to pursue for obtaining a full separation.
- The ACE C18, C18-AR and C18-Amide all resolve 11 of the 14 impurity peaks.

1. 2-Aminophenol, 2. Hydroquinone, 3. Theobromine, 4. Paracetamol, 5. Theophylline, 6. Paraxanthine, 7. 4-Hydroxybenzoic acid, 8. Caffeine, 9. 2-Acetamidophenol, 10. 2-Hydroxybenzoic acid, 11. Phenol, 12. Aspirin, 13. 4-Nitrophenol, 14. 4-Chloroacetanilide, 15. 2-Nitrophenol, 16. Acetylsalicylsalicylic acid, 17. Salsalate

6 Column Screen of Triple API sample + Impurities (17 peaks)



- Columns: 6 x ACE Excel 2, 100 x 3.0 mm
- System: ChromasterUltra Rs

- A: 10 mM ammonium formate pH 3.0
- B1: 10 mM ammonium formate pH 3.0 in MeCN:H_2O 9:1 v/v
- B2: 10 mM ammonium formate pH 3.0 in MeOH:H_2O 9:1 v/v

- Gradient: 5-95% B in 5 minutes
- Temp: 40 °C
- Flow rate: 1.2 mL/min
- Detection: UV, 270 nm

- Sample:
  - Aspirin: 5 mg/mL
  - Paracetamol: 3.3 mg/mL
  - Caffeine: 0.75 mg/mL
  - Impurities spiked at 0.1% (wrt aspirin)

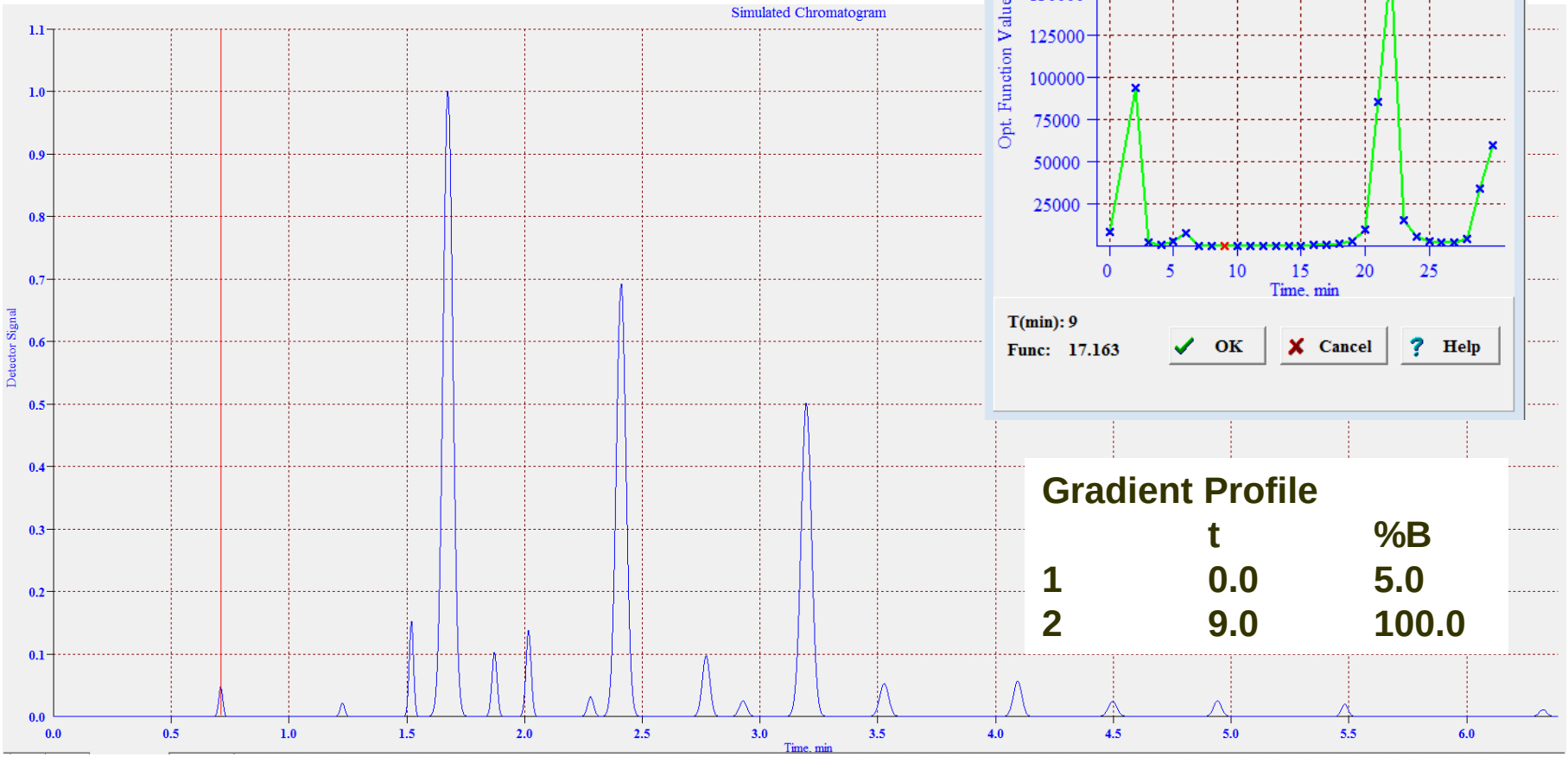
1. 2-Aminophenol, 2. Hydroquinone, 3. Theobromine, 4. Paracetamol, 5. Theophylline, 6. Paraxanthine, 7. 4-Hydroxybenzoic acid, 8. Caffeine, 9. 2-Acetamidophenol, 10. 2-Hydroxybenzoic acid, 11. Phenol, 12. Aspirin, 13. 4-Nitrophenol, 14. 4-Chloroacetanilide, 15. 2-Nitrophenol, 16. Acetylsalicylsalicylic acid, 17. Salsalate

Optimising the separation with ChromSword 2

- **Selected column: ACE Excel 2 C18-Amide 100 x 3.0 mm**
- **A: 10 mM ammonium formate pH 3.0**
- **B: 10 mM ammonium formate pH 3.0 in MeOH:H_2O 9:1 (v/v)**
- **The 5 minute screening gradient run was input into ChromSword.**
- **10 and 15 minute gradients were also run and input to generate the tG model.**
- **The separation was optimised and the optimum linear gradient was predicted.**
- **(Further optimisation was achieved: prediction of multi-step gradients).**

Optimising the separation with ChromSword 2

- **Optimum predicted linear gradient**



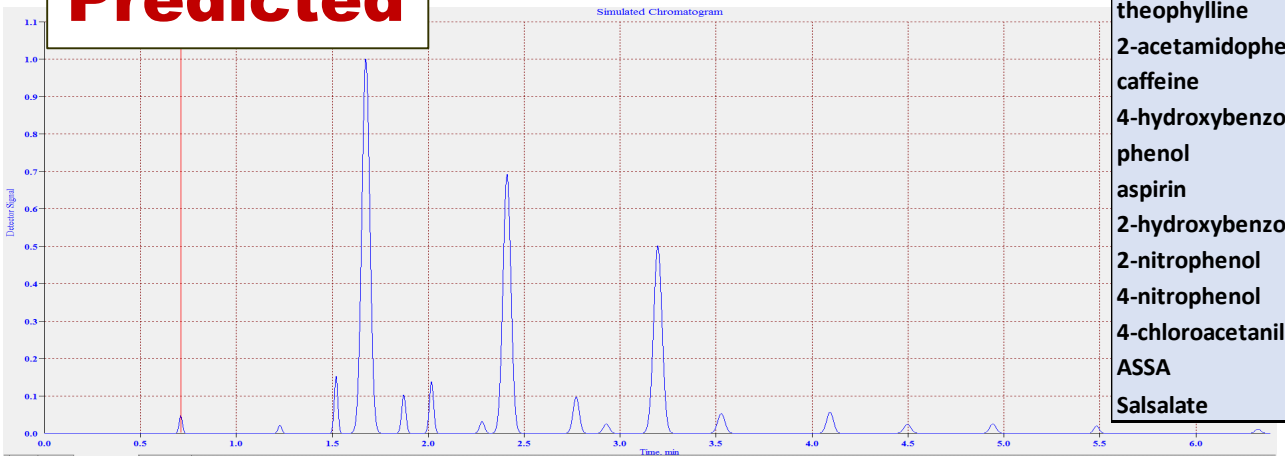
Gradient Profile

	t	%B
1	0.0	5.0
2	9.0	100.0

Optimising the separation with ChromSword 2

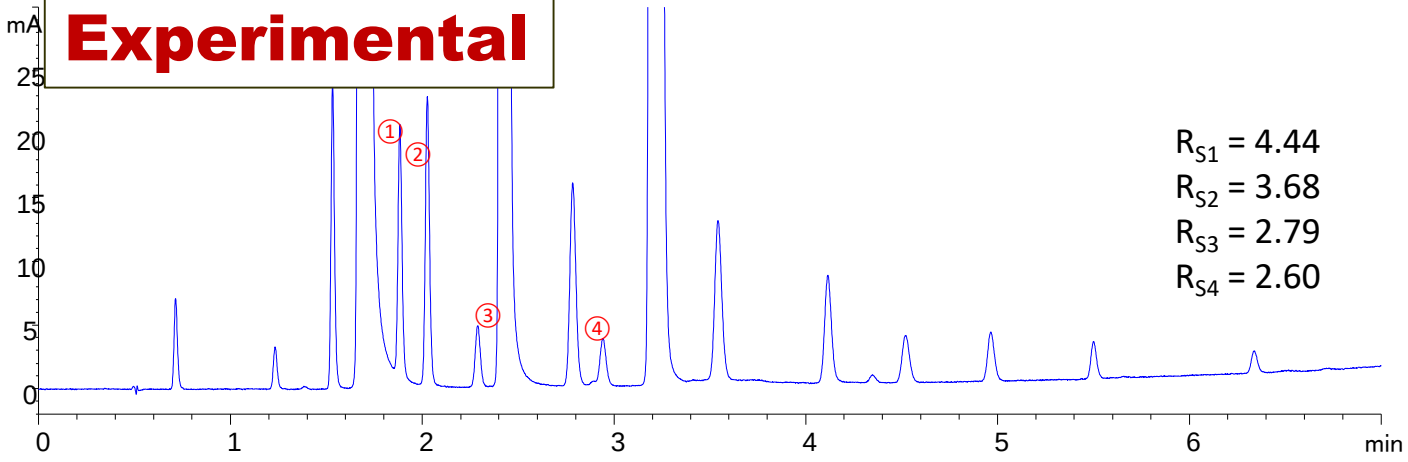
- Optimum predicted linear gradient

Predicted



Compound	Predicted t_R	Experimental t_R	% error
2-aminophenol	0.71	0.714	-0.6
hydroquinone	1.23	1.233	-0.2
theobromine	1.52	1.533	-0.9
paracetamol	1.67	1.684	-0.8
paraxanthine	1.87	1.882	-0.6
theophylline	2.02	2.025	-0.2
2-acetamidophenol	2.28	2.289	-0.4
caffeine	2.41	2.421	-0.5
4-hydroxybenzoic acid	2.77	2.785	-0.5
phenol	2.93	2.943	-0.4
aspirin	3.20	3.209	-0.3
2-hydroxybenzoic acid	3.53	3.542	-0.3
2-nitrophenol	4.09	4.115	-0.6
4-nitrophenol	4.50	4.521	-0.5
4-chloroacetanilide	4.94	4.965	-0.5
ASSA	5.48	5.5	-0.4
Salsalate	6.32	6.338	-0.3

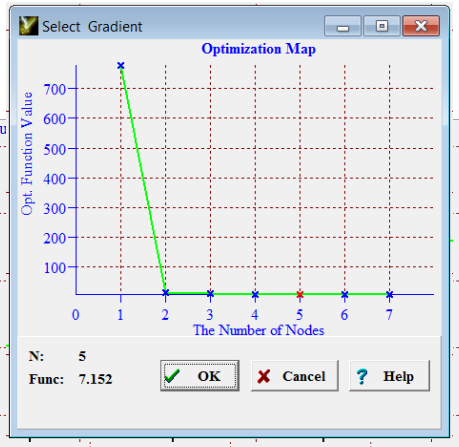
Experimental



Optimising the Separation with ChromSword 2

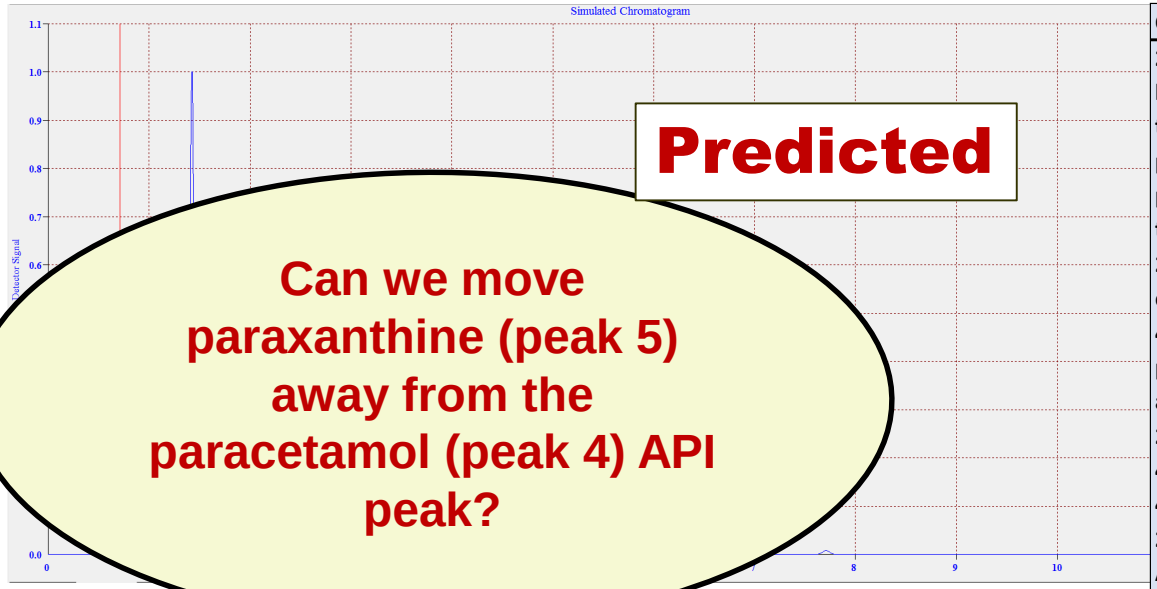
- **Step Gradient**: Target run time 10 minutes
- A 5 node gradient is predicted to give the optimum separation

Gradient Profile	
t	%B
0.0	12.0
1.7	18.0
1.9	31.0
4.4	31.0
5.1	44.0



Optimising the separation with ChromSword 2

- **Step Gradients: Target run time 10 minutes**

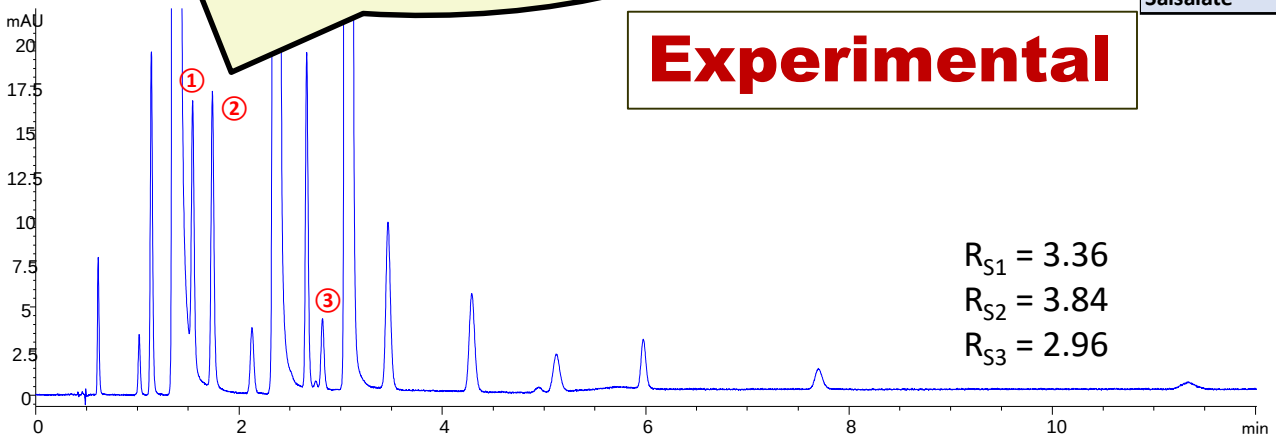


Predicted

Can we move paraxanthine (peak 5) away from the paracetamol (peak 4) API peak?

Compound	Predicted t_R	Experimental t_R	% error
2-aminophenol	0.71	0.612	13.8
hydroquinone	1.05	1.015	3.3
theobromine	1.18	1.135	3.8
paracetamol	1.42	1.368	3.7
paraxanthine	1.62	1.543	4.8
theophylline	1.83	1.738	5.0
2-acetamidophenol	2.20	2.128	3.3
caffeine	2.40	2.358	1.7
4-hydroxybenzoic acid	2.66	2.665	-0.2
phenol	2.82	2.820	0.0
aspirin	3.07	3.062	0.3
2-hydroxybenzoic acid	3.47	3.463	0.2
4-nitrophenol	5.12	5.122	0.0
4-chloroacetanilide	5.97	5.975	-0.1
2-nitrophenol	4.30	4.289	0.3
ASSA	7.71	7.699	0.1
Salsalate	11.18	11.338	-1.4

Experimental



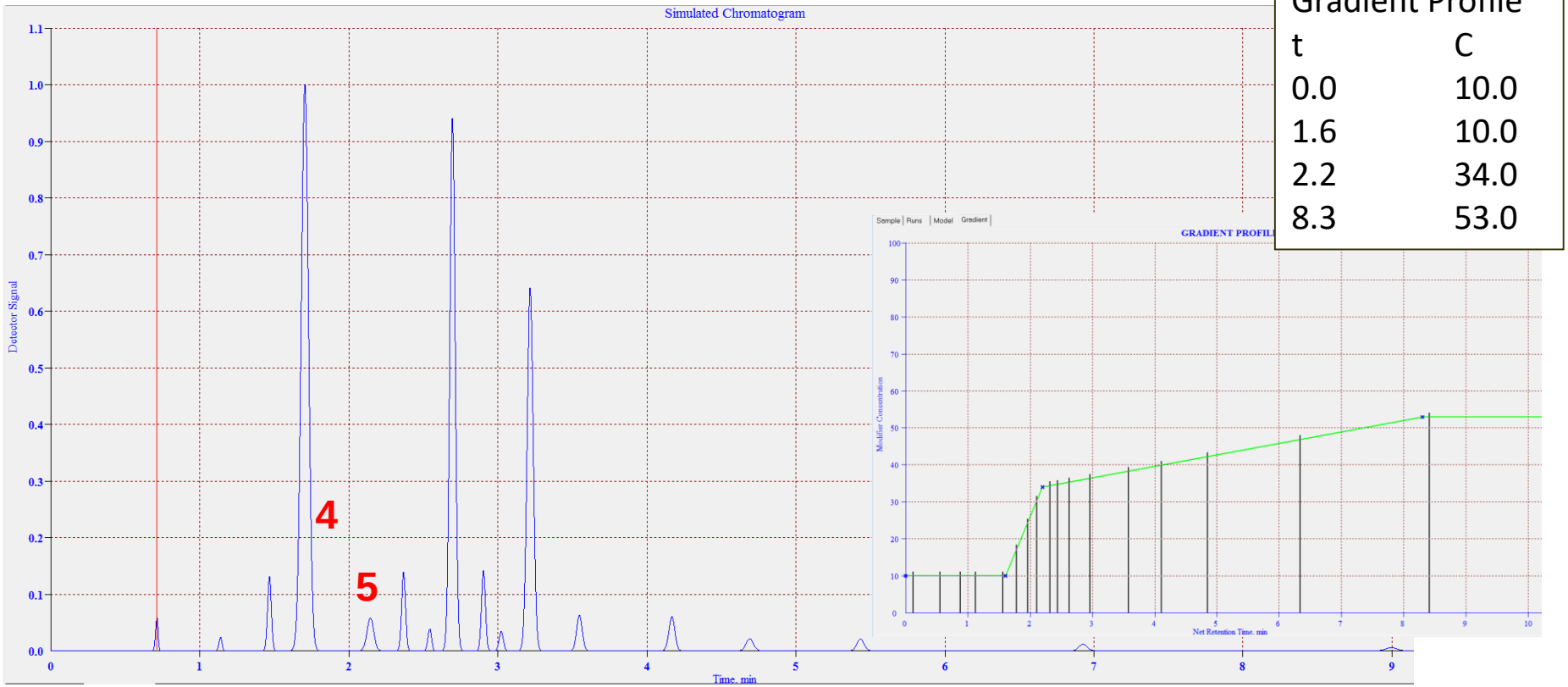
R_{S1} = 3.36
R_{S2} = 3.84
R_{S3} = 2.96

Gradient Profile	
t	%B
0.0	12.0
1.7	18.0
1.9	31.0
4.4	31.0
5.1	44.0

Optimising the separation with ChromSword 2

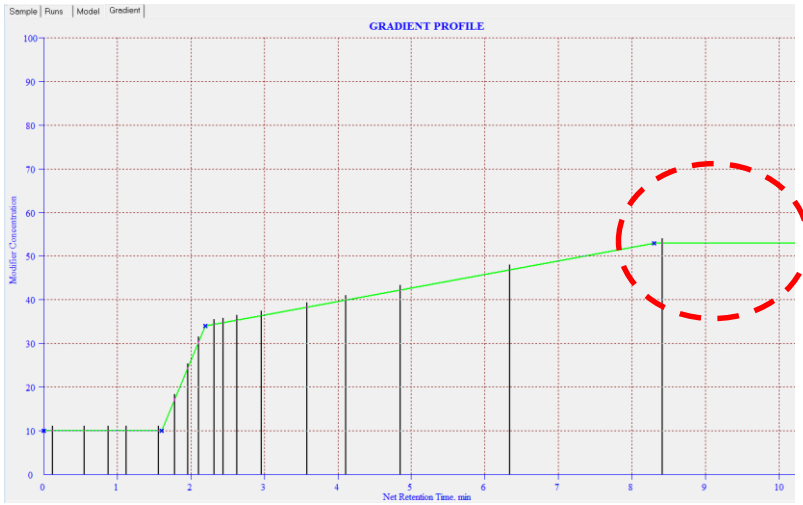
- Increase weighting (i.e. importance) for the separation of peaks 4 and 5.
- A 4 node gradient is predicted to give the optimum separation.

Gradient Profile	
t	C
0.0	10.0
1.6	10.0
2.2	34.0
8.3	53.0

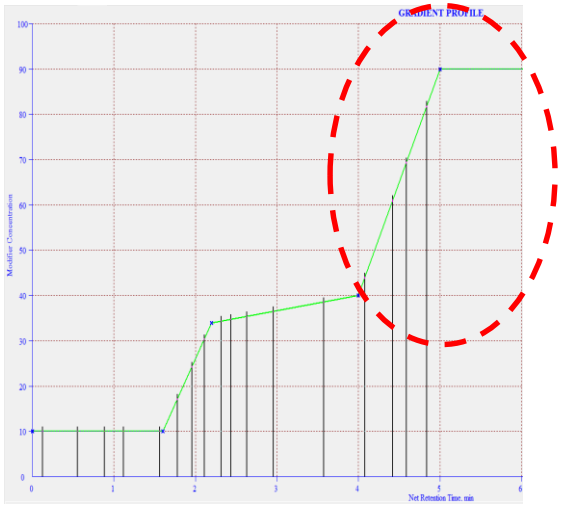


Optimising the separation with ChromSword 2

- **Profile manual editing also possible to ‘tweak’ if necessary**
- **The final gradient step was manually edited to reduce run time.**



t	%B
0.0	10.0
1.6	10.0
2.2	34.0
8.3	53.0

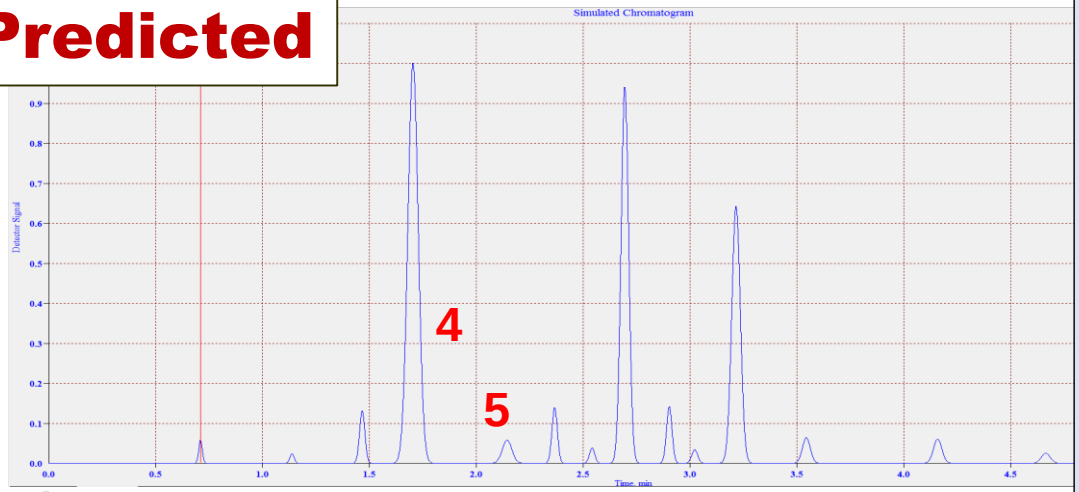


t	%B
0.0	10.0
1.6	10.0
2.2	34.0
4.0	40.0
5.0	90.0

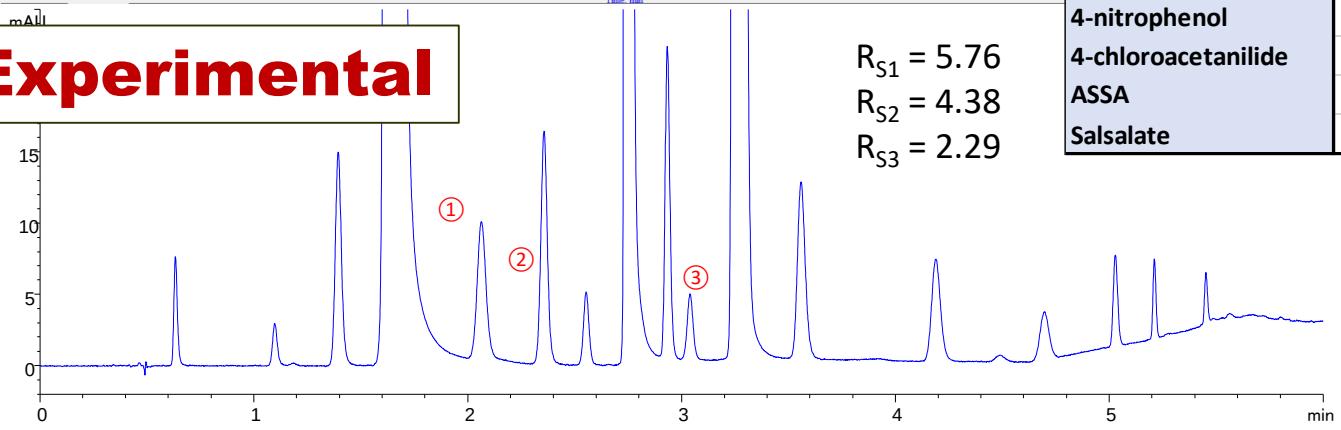
Optimising the separation with ChromSword 2

- **Final separation.**

Predicted



Experimental

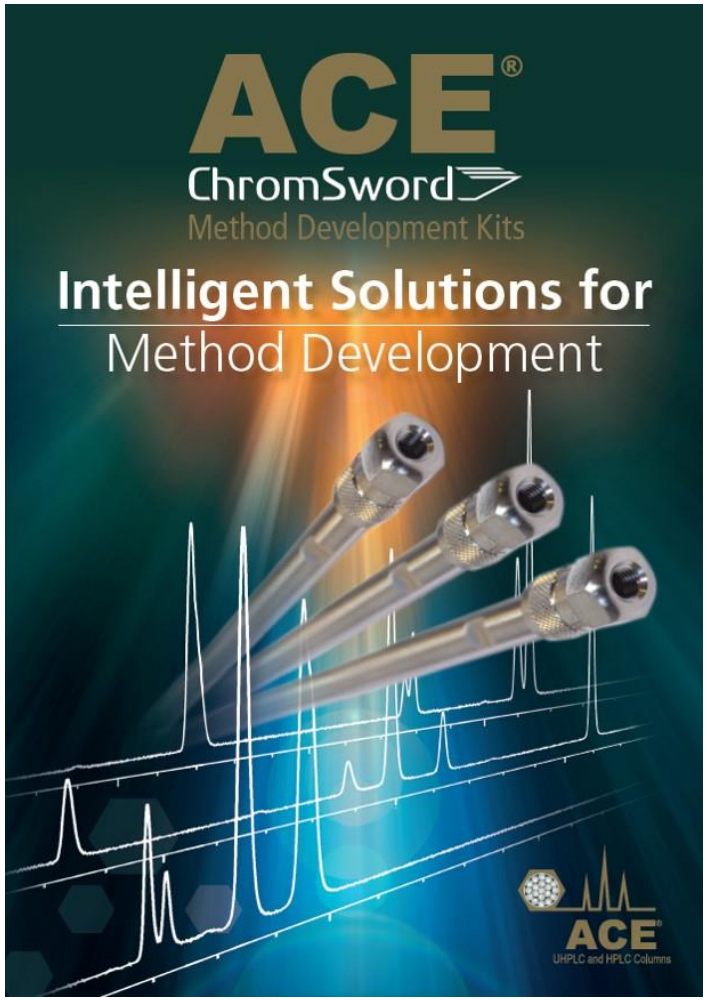


Compound	Predicted t_R	Experimental t_R	% error
2-aminophenol	0.71	0.633	**10.8**
hydroquinone	1.14	1.098	**3.7**
theobromine	1.47	1.395	**5.1**
paracetamol	1.70	1.633	**3.9**
paraxanthine	2.14	2.064	**3.6**
theophylline	2.37	2.357	**0.5**
2-acetamidophenol	2.54	2.554	**-0.6**
caffeine	2.69	2.747	**-2.1**
4-hydroxybenzoic acid	2.90	2.933	**-1.1**
phenol	3.02	3.039	**-0.6**
aspirin	3.21	3.26	**-1.6**
2-hydroxybenzoic acid	3.54	3.559	**-0.5**
2-nitrophenol	4.16	4.189	**-0.7**
4-nitrophenol	4.66	4.697	**-0.8**
4-chloroacetanilide	5.01	5.027	**-0.3**
ASSA	5.17	5.21	**-0.8**
Salsalate	5.42	5.452	**-0.6**

Summary and Conclusions

- **Systematic screening is a logical and productive approach to LC method development.**
- **Stationary phase and organic modifier are two of the most powerful parameters for exploring the selectivity of a separation.**
- **The 6-column, 2-solvent platform described is designed to quickly identify the most promising column/organic solvent combination for a separation.**
- **ChromSword 2 provides an efficient tool for method optimisation once the column/mobile phase have been selected.**

ACE-CS2.0 Method Development Solution



Thank You For Your Attention

www.mac-mod.com

info@mac-mod.com