Translating Natural Product Methods to Multiply Coupled Columns to Achieve Ultra-Resolution Separations and the Potential Impact of Pressure Induced Changes in Selectivity

ACE® UHPLC and HPLC Columns

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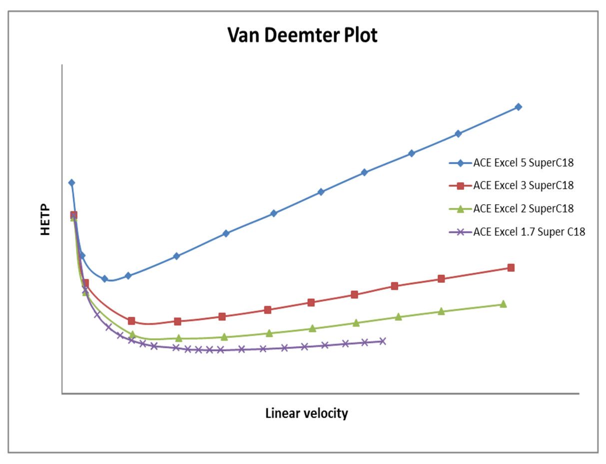
1. INTRODUCTION

- Complex samples require high efficiency separations to provide maximum resolving power for the high number of analytes encountered
- Columns packed with small fully porous or solid core particles can deliver very high efficiency and peak capacity values
- However, column length may be restricted by LC system pressure limits
- UHPLC systems with pressure limits >1,000 bar present possibilities for extending the ***effective*** column length by coupling columns to generate very large column efficiency and peak capacity values
- This work uses method translation principles to migrate methods to longer effective column lengths to achieve ultra-resolution separations.
- Key aspects and potential problems of this approach are considered

2. COLUMN COUPLING

- Column length can be maximised by selecting chromatographic conditions that generate favourable column backpressures
- Acetonitrile was selected over MeOH due to lower viscosity
- Elevated column temperature of 80C was used
- Note that the use of elevated temperature is dependant on sample stability
- To obtain maximum performance from small particles, it is possible to increase the flow rate to obtain increased efficiency



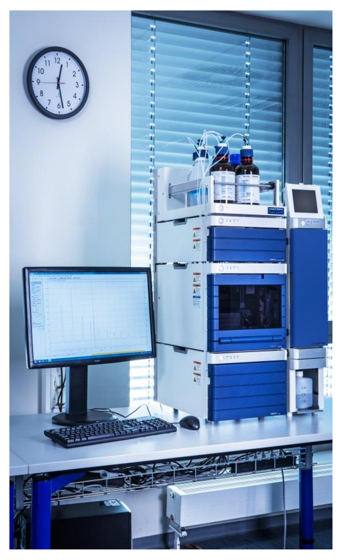
3. EXPERIMENTAL

Samples

100 mg of finely ground Green Tea and St John's Wort tablets extracted with 10.0 mL MeCN:water 1:1 v/v for 15 minutes with ultrasonication. 100 µL supernatant diluted with 300 µL water and filtered using a Whatman Mini-Uniprep syringeless filter (0.45µm polypropylene filter media, VWR P/N 83009-820).

For echinacea, 1.0 mL of preparation was diluted with 9.0 mL MeCN:water 1:1 v/v and filtered.

Chromatographic parameters



System: Hitachi / VWR ChromasterUltra Rs and Agilent 1290

Columns: 3 x ACE Excel 1.7 SuperC18 100 x 3.0 mm
3 x ACE UltraCore 2.5 SuperC18 100 x 3.0 mm

Eluent: A: 0.1% formic acid (aq)
B: MeCN + 0.1% formic acid v/v

Gradient: see next slide

Flow rate: see next slide

Temp.: 80C

Detection: UV, various wavelengths

4. TRANSLATING METHODS TO LONGER COLUMNS

1. Scale gradient volume to new column volume (V_M)* by altering gradient time (t_G) or flow rate (F)

$$t_{G2} = \frac{t_{G1}F_1V_{M2}}{V_{M1}F_2} \qquad F_2 = \frac{t_{G1}F_1V_{M2}}{V_{M1}t_{G2}}$$

2. Scale injection volume (V_{inj}) to new column volume

$$V_{inj2} = V_{inj1} \times \left(\frac{V_{M2}}{V_{M1}}\right)$$

3. To maintain selectivity, correct for change in system dwell (V_D) and V_M.

Determine whether a pre-gradient isocratic hold of x minutes is required or if the injection should be delayed by x minutes*:

$$x = \left[\left(\frac{V_{D1}}{V_{M1}}\right) - \left(\frac{V_{D2}}{V_{M2}}\right)\right] \times \frac{V_{M2}}{F_2}$$

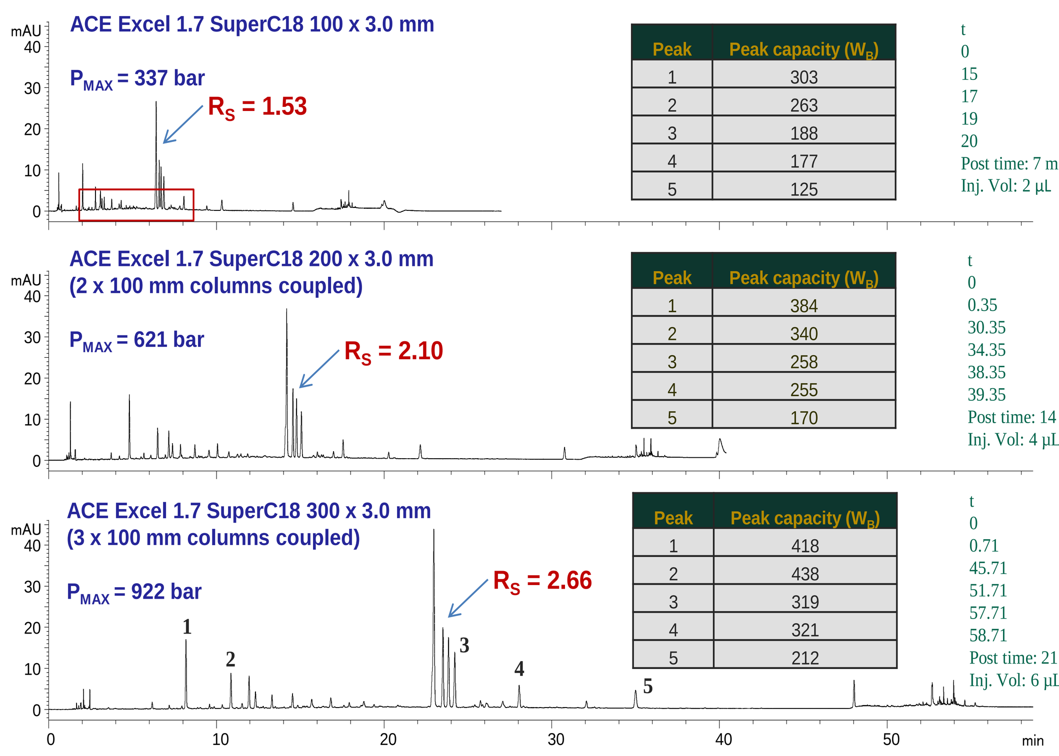
Pre-gradient hold = positive value, delayed injection = negative

*Note – it is recommended to determine V_M experimentally via measurement of a non-retained dead volume marker

* Petersson et al., LCGC Europe 28 (2015) 310-320

5. EXAMPLE 1: ST JOHN'S WORT

- Ultra high efficiency ACE columns packed with 1.7 µm particles
- Method translation: maintain constant linear velocity and scale t_G



Peak	Peak capacity (W_B)
1	303
2	263
3	188
4	177
5	125

t	%B
0	5
15	20
17	95
19	95
20	5

Post time: 7 mins
Inj. Vol: 2 µL

Peak	Peak capacity (W_B)
1	384
2	340
3	258
4	255
5	170

t	%B
0	5
0.35	5
30.35	20
34.35	95
38.35	95
39.35	5

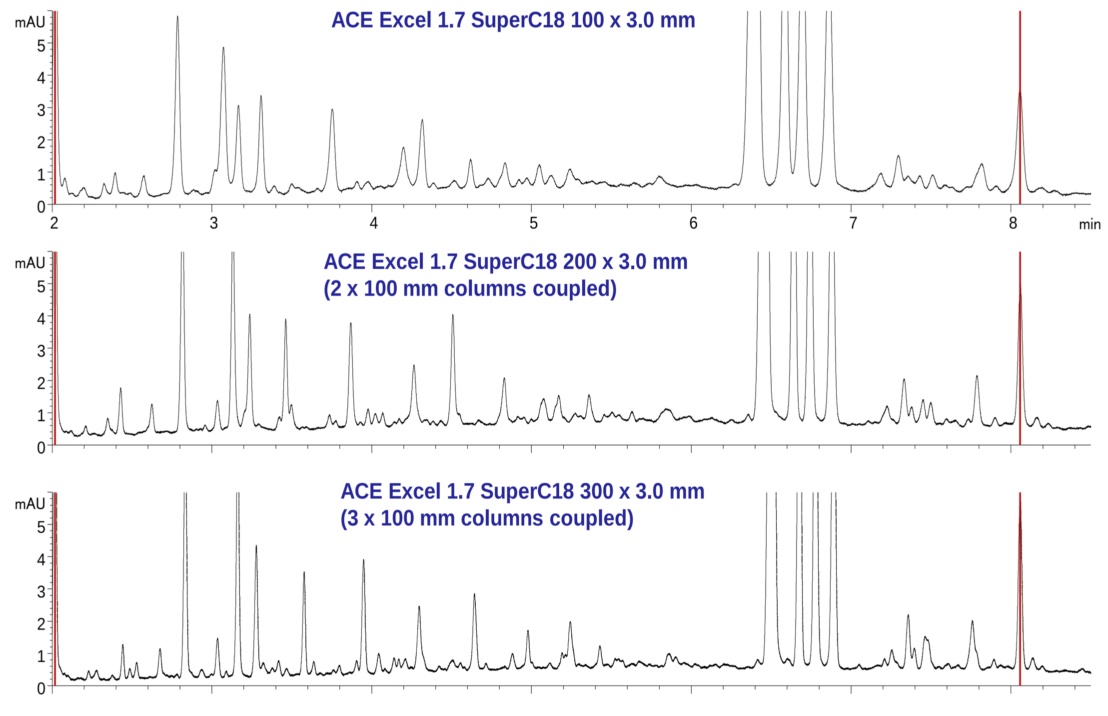
Post time: 14 mins
Inj. Vol: 4 µL

Peak	Peak capacity (W_B)
1	418
2	438
3	319
4	321
5	212

t	%B
0	5
0.71	5
45.71	20
51.71	95
57.71	95
58.71	5

Post time: 21 mins
Inj. Vol: 6 µL

6. EXAMPLE 1: ST JOHN'S WORT...ZOOMED DETAIL



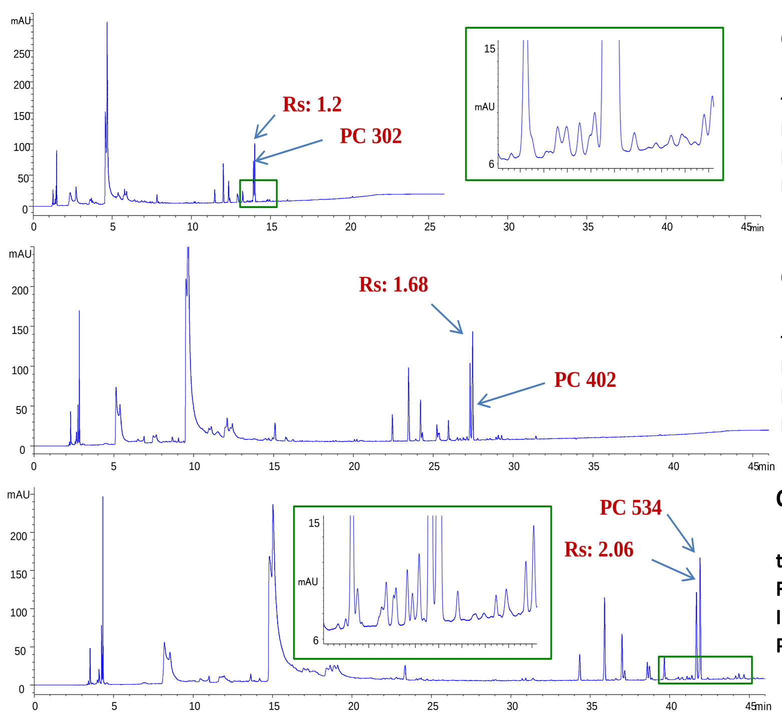
- Increased efficiency and resolution
- Excellent transfer of selectivity between *effective* column lengths

7. EXAMPLE II: ECHINACEA WITH ACE ULTRACORE

- Low backpressure of solid core particles provides additional options

Option 1: translation via changing gradient time

$$t_{G2} = \frac{t_{G1}F_1V_{M2}}{V_{M1}F_2}$$



Column: ACE UltraCore 2.5 SuperC18 150 x 3.0 mm
t_G: 20 min
Flow: 0.43 mL/min
Inj. Vol.: 5 µL
P_{MAX}: 130 bar

Column: ACE UltraCore 2.5 SuperC18 300 x 3.0 mm (2 x 150 x 3.0 mm)
t_G: 40 min
Flow: 0.43 mL/min
Inj. Vol.: 10 µL
P_{MAX}: 223 bar

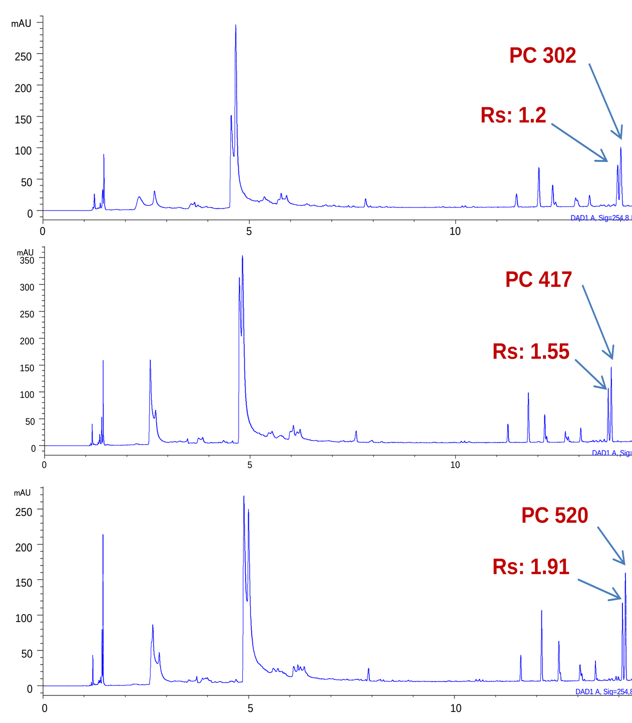
Column: ACE UltraCore 2.5 SuperC18 450 x 3.0 mm (3 x 150 x 3.0 mm)
t_G: 60 min
Flow: 0.43 mL/min
Inj. Vol.: 15 µL
P_{MAX}: 338 bar

8. EXAMPLE II: ECHINACEA WITH ACE ULTRACORE

- Low backpressure of solid core particles provides additional options

Option 2: translation via changing flow rate

$$F_2 = \frac{t_{G1}F_1V_{M2}}{V_{M1}t_{G2}}$$



Column: ACE UltraCore 2.5 SuperC18 150 x 3.0 mm
t_G: 20 min
Flow: 0.43 mL/min
Inj. Vol.: 5 µL
P_{MAX}: 130 bar

Column: ACE UltraCore 2.5 SuperC18 300 x 3.0 mm (2 x 150 x 3.0 mm)
t_G: 20 min
Flow: 0.85 mL/min
Inj. Vol.: 10 µL
P_{MAX}: 431 bar

Column: ACE UltraCore 2.5 SuperC18 450 x 3.0 mm (3 x 150 x 3.0 mm)
t_G: 20 min
Flow: 1.29 mL/min
Inj. Vol.: 15 µL
P_{MAX}: 958 bar

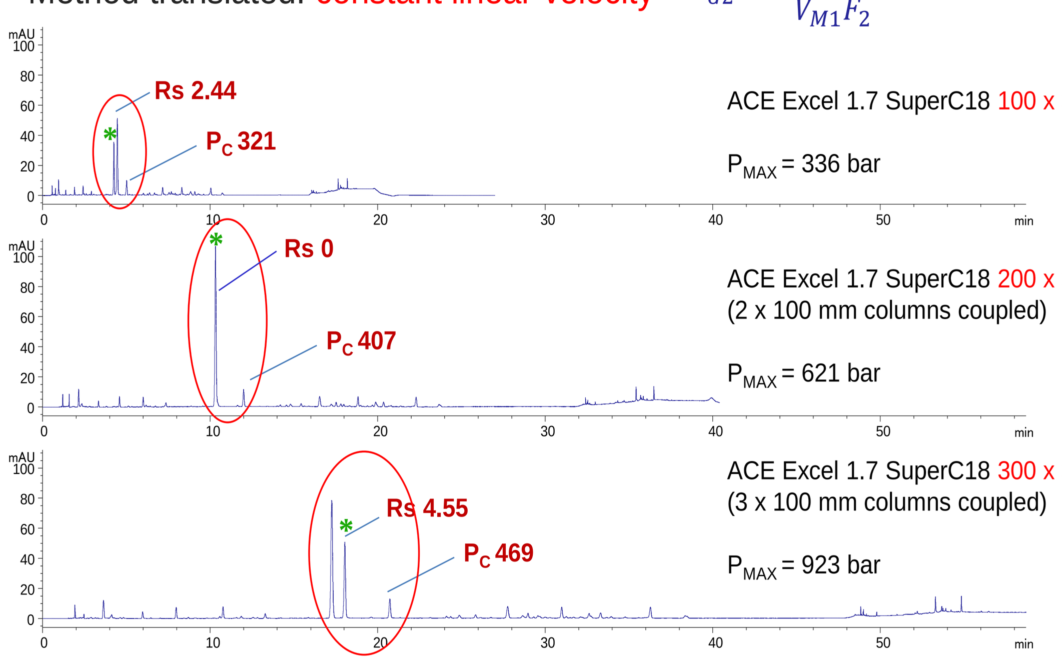
9. POSSIBLE PITFALL: ELEVATED PRESSURE EFFECTS

- High pressure can affect the retention characteristics of analytes
- Studies have shown this can be significant*
- The degree of pressure induced change in retention is a complex phenomenon and can be affected by a multitude of factors including:
  - Physico-chemical properties of the analyte (e.g. polarisability, degree of ionisation, molecular weight, solvation etc.)
  - Mobile phase properties (% organic, pH)
  - Type of stationary phase
- When translating any method to higher pressure separations for increased speed or increased resolution the selectivity of the translated method must be assessed as suitable

* Fallas et al., J. Chromatogr. A 1209 (2008) 195–205

10. EXAMPLE III: GREEN TEA / PRESSURE EFFECTS I

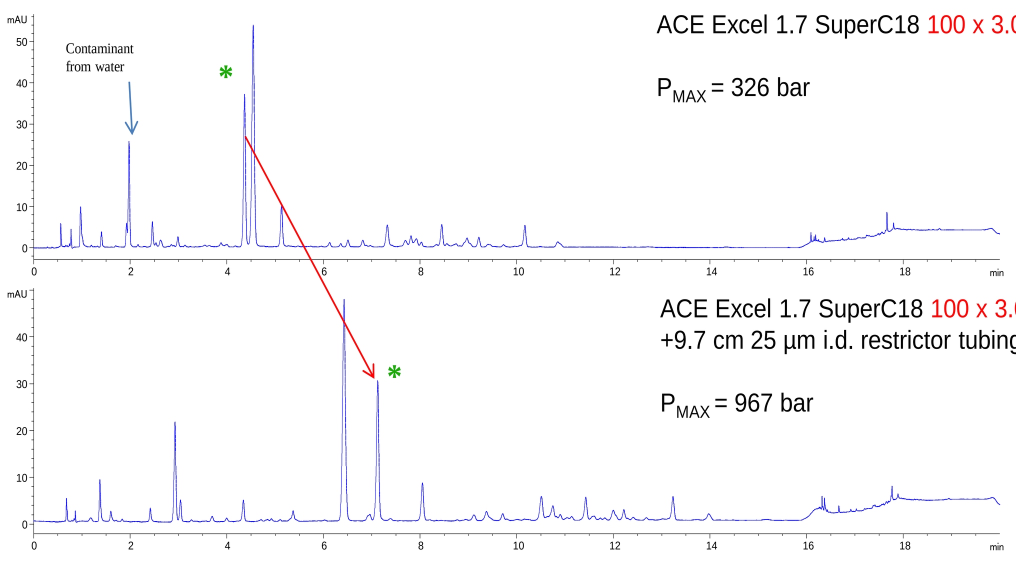
- Method translated: constant linear velocity $t_{G2} = \frac{t_{G1}V_{M2}}{V_{M1}F_2}$



- Peak capacity is increased with increasing column length
- A significant change in selectivity is observed for the two principal peaks

11. EXAMPLE III: GREEN TEA / PRESSURE EFFECTS II

- Separation was re-run on the ACE Excel 1.7 Super C18 100 x 3.0 mm
  - A 9.7 cm length of 25 µm i.d. SS restrictor tubing was inserted post-column to artificially elevate the backpressure



- The observed change in selectivity was confirmed and attributed to the increased column backpressure generated when coupling columns.

12. SUMMARY AND CONCLUSIONS

- A UHPLC column coupling approach to increase effective column lengths to generate very high resolution separations has been shown
- By carefully selecting chromatographic conditions, it is possible to couple up to 3 x ACE Excel 1.7µm SuperC18 100 x 3.0 mm columns
- When translating methods to long column formats, it is important to obey volumetric scaling principles
- Columns packed with solid core particles offer extra flexibility for ultra resolution separations due to reduced backpressure.
- This approach is suitable for the ultra resolution, high sensitivity analysis of complex natural products.
- Whilst column coupling and the resulting higher back pressures are useful, unexpected changes in selectivity (application dependent) may occur.

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