A practical guide to maximizing the Reversed Phase LC (RPLC) separation's peak capacity.

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Outline

1. Background
2. Trends vs. different operational parameters
3. General approach

Maximise peak capacity rapidly

1. Background

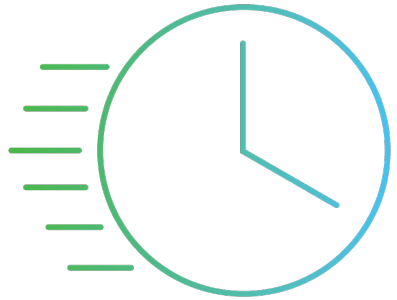
Method development is expensive

Multivariate relationship between practical parameters:

I. Gradient time (t_G)
II. Temperature (T)
III. Flow rate (F)
IV. Final eluent composition (Φ_{final})

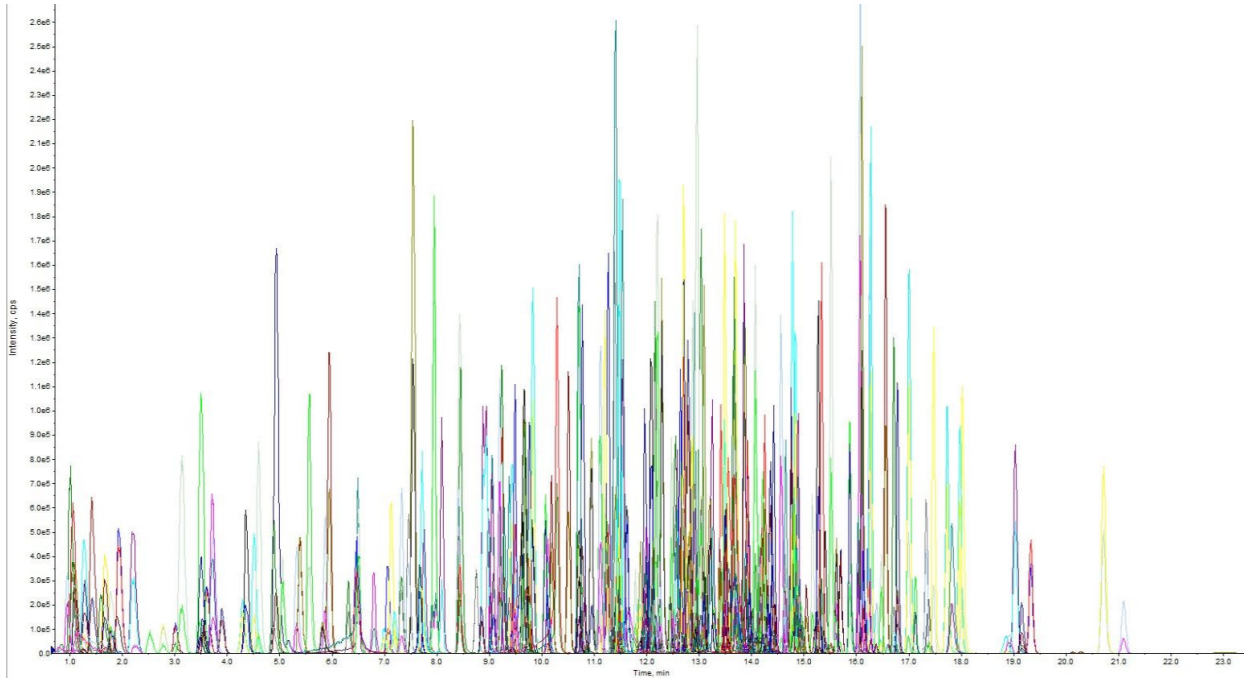
Understanding the trends - can help you develop methods fast

Maximize n_c rapidly



LC-MS/MS Analysis of 300 Pesticides

Maximized n_c rapidly



Column: Avantor® ACE® UltraCore 2.5 SuperC18

Particle Size:2.5 µm

Dimensions: 100 x 2.1 mm

Mobile Phases: A: 5 mM ammonium formate in H_2O/MeOH (9:1 v/v)

B: 5 mM ammonium formate in H_2O/MeOH (1:9 v/v)

Gradient:

Time (mins)	% B
0.0	30
0.5	30
15.0	100
22.0	100
22.1	30
27.0	30

Flow Rate: 0.3 mL/min

Injection: 6 µL

Temperature: 24°C

Detection: AB SCIEX 4000 QTRAP

TurbolonSpray ESI positive mode

Capillary voltage: 5000 V

Heater gas temperature: 450 °C

Sample: Sample prepared using QuEChERs methodology.

Method validated using cucumber matrix spiked at 0.01 mg/kg. 265 analytes successfully validated (analytes in blue).

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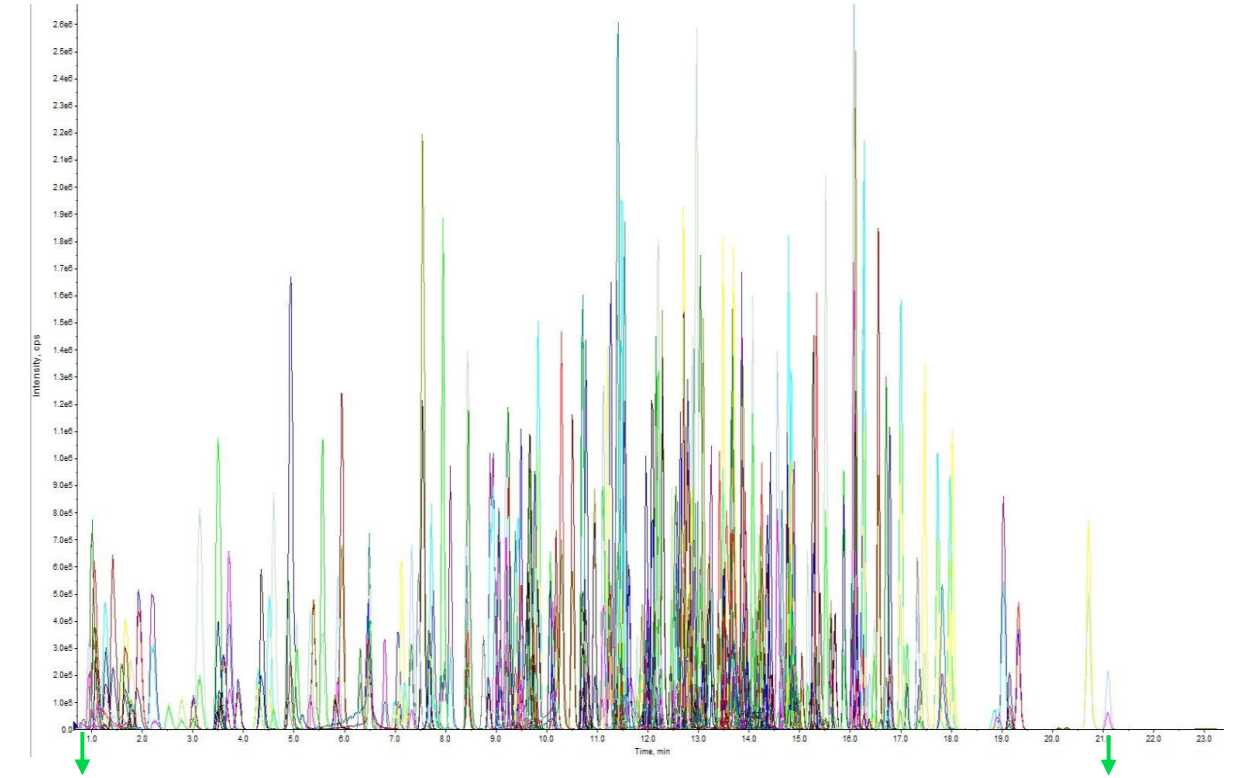
Measuring the LC separation's sample peak capacity

$$n_c = \frac{t_{R,last} - t_{R,first}}{4\sigma_{avg}}$$

$$n_c = \frac{\text{separation window}}{\text{average peak width}}$$



$$n_c = 20.8/0.25 = 83$$

Dolan JW, Snyder LR, Djordjevic NM, Hill DW, Waeghe TJ. J. Chromatogr. A. 857 (1999) 1–20.

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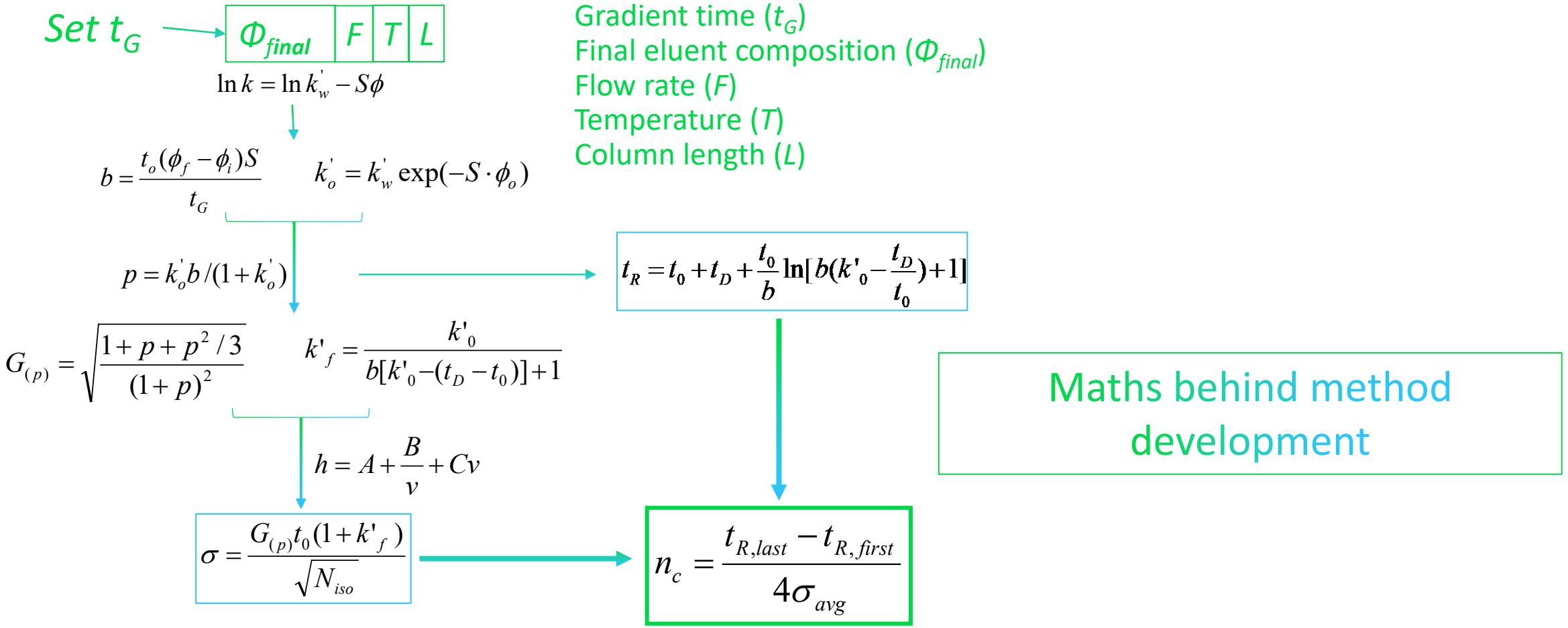
Roadmap for low molecular weight (LMW) samples



Gradient time (t_G)
Final eluent composition (Φ_{final})
Flow rate (F)
Temperature (T)
Column length (L)

Maths behind method development

A. Soliven, I.A. Haidar Ahmad, M.R. Filgueira, P.W. Carr, J. Chromatogr. A 1273 (2013) 57.
L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model, John Wiley & Sons, Inc., 2007.

Key aspects - LMW modelling retention time

- ***LSS model*: dependence at different temperatures *(T)***

  $\ln k'_w = A_k + B_k T + C_k \ln T \qquad S = A_s + B_s T$

- **"Performance" of the column at different *L* & *F***

  $h = a + b/v + cv$

- **Diffusion coefficients (*T* dependent)**

  1. Viscosity → Chen and Horvath approximation
  2. Diffusion coefficient → Wilke–Chang approximation

J. Chromatogr. A 1273 (2013) 57.

✓ Computational approach

t_R experimental vs. t_R predicted

T (°C)	t_g (min)	Av. Exp. t_R (min)	Predicted t_R
40	5	3.2	3.2
	15	4.0	4.0
	45	4.5	4.5
60	5	2.5	2.5
	15	2.9	2.9
	45	3.0	3.0
80	5	1.8	1.9
	15	2.0	2.0
	45	2.1	2.1

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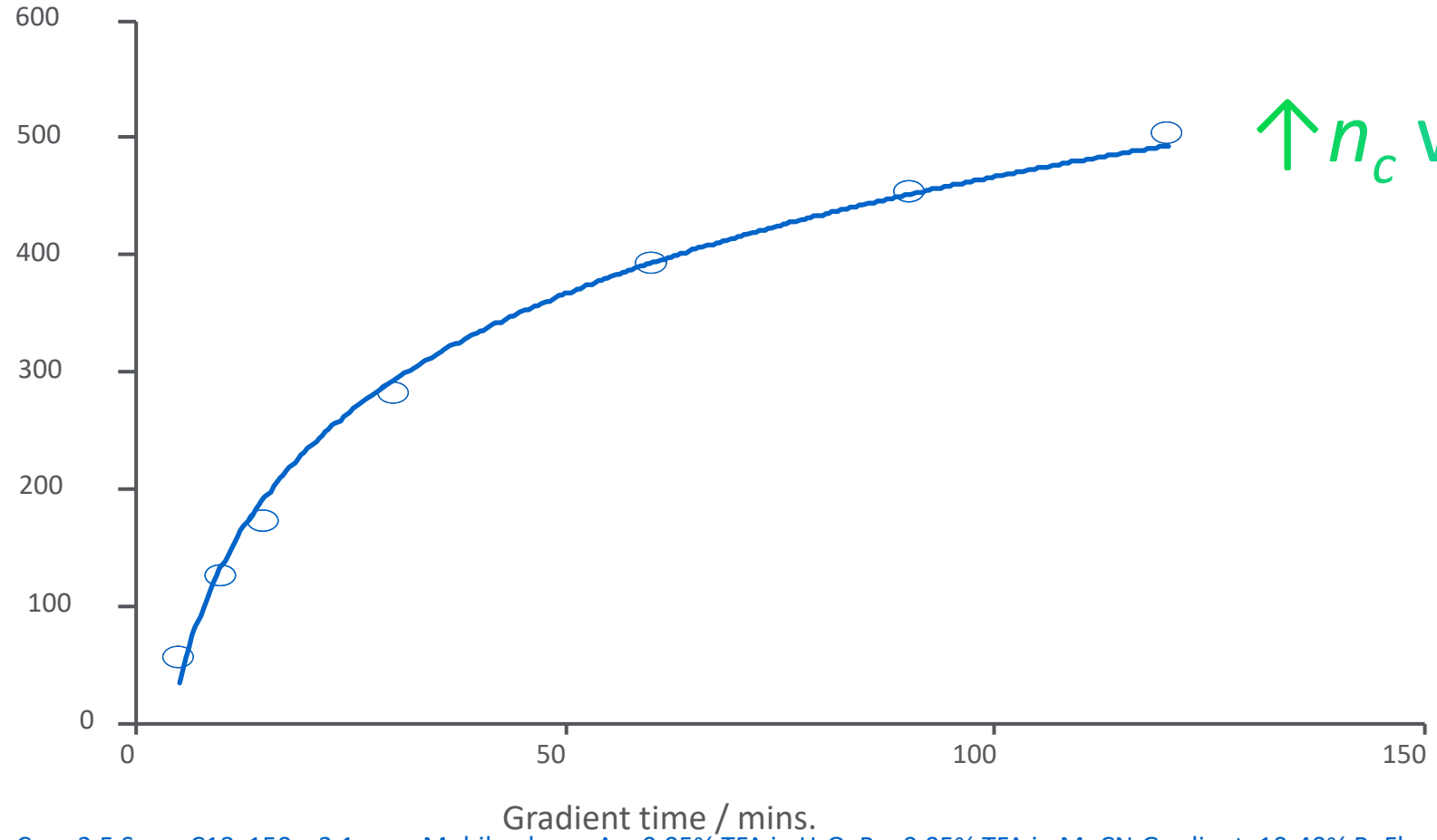
2. Trends in n_c vs. practical parameters

Gradient time (t_G)
Temperature (T)
Flow rate (F)
Final eluent composition (Φ_{final})

Complex relatively larger molecules: fixed L, effect of t_G

X. Wang, W.E. Barber, P.W. Carr, J. Chromatogr. A 1107 (2006) 139-151.



$$P_C = \frac{t_G}{w+1}$$

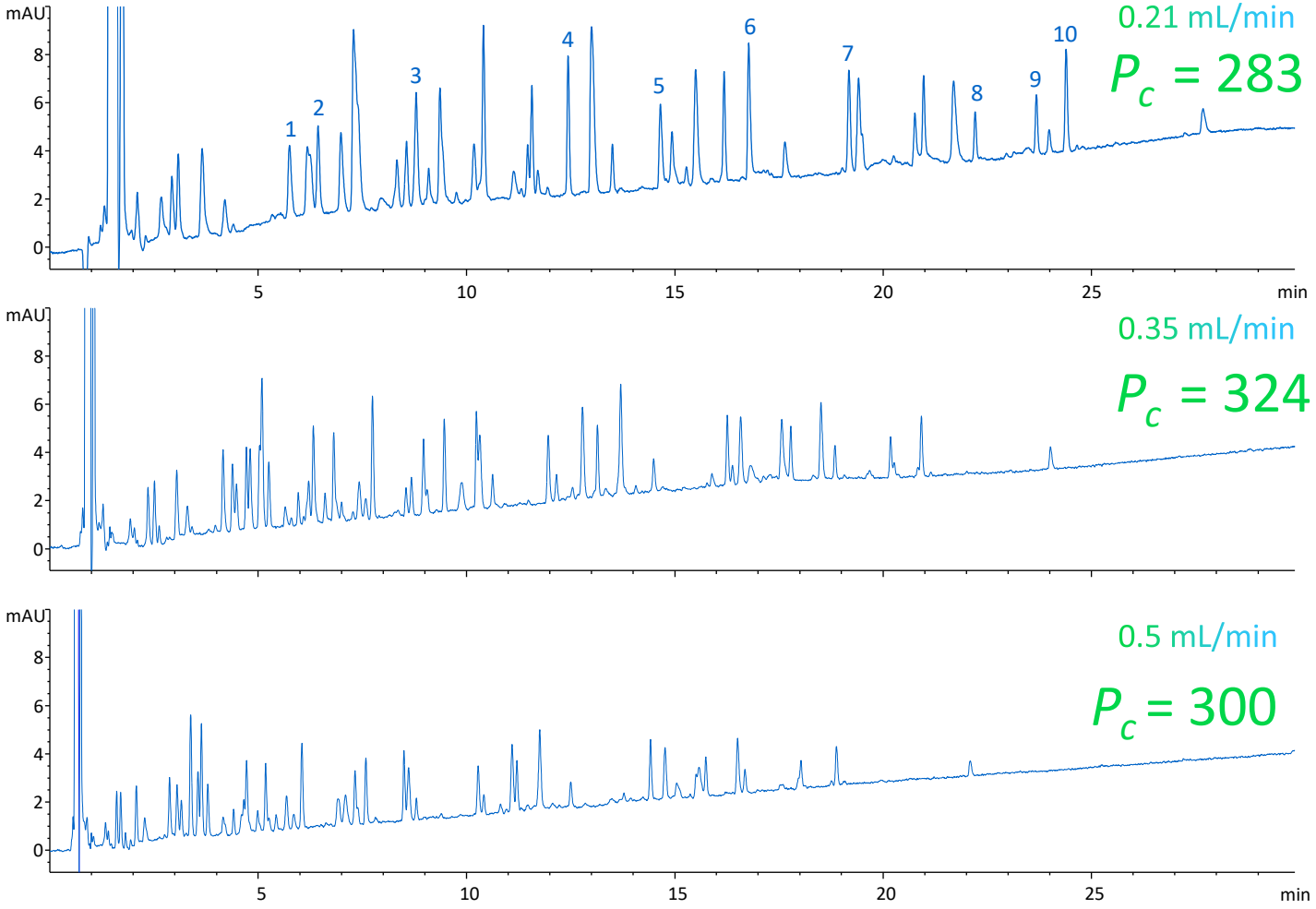
$\uparrow n_c$ with $\uparrow$time

Column: Avantor® ACE® UltraCore 2.5 SuperC18, 150 x 2.1 mm; Mobile phase: A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN;Gradient: 10-40% B; Flow rate: 0.21 mL/min; Temperature: 60°C; Detection: UV, 214 nm; Injection volume: 20 µL; Sample: BSA tryptic digest.

Complex relatively larger molecules: fixed *L*, effect of *F*

J. Chromatogr. A 1107 (2006) 139-151.

$$P_C = \frac{t_G}{w + 1}$$



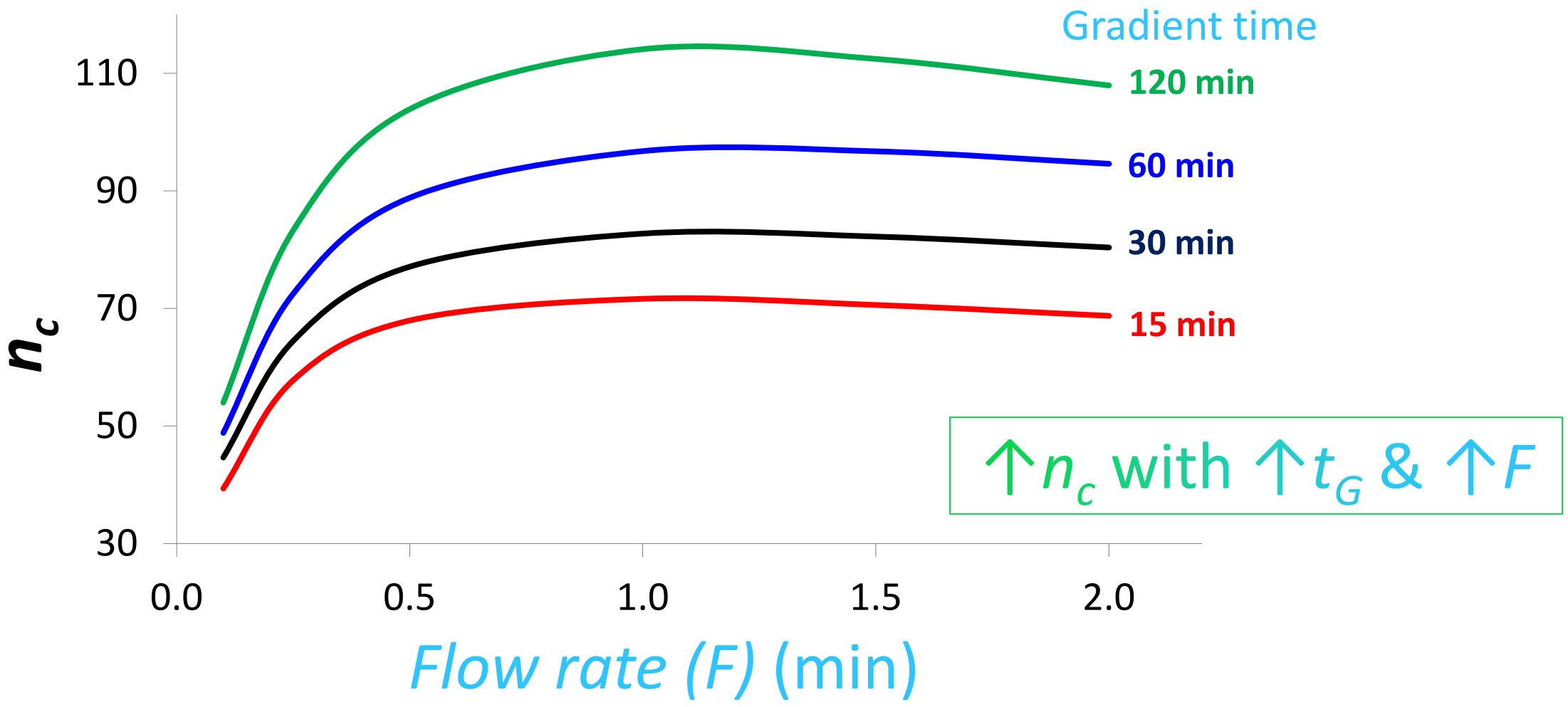
$\uparrow n_c \neq \uparrow F$

F must be optimized

Column: Avantor® ACE® UltraCore 2.5 SuperC18, 150 x 2.1 mm; Mobile phase: A = 0.05% TFA in H_2O, B = 0.05% TFA in MeCN;Gradient: 10-40% B in 30 minutes; Temperature: 60°C; Detection: UV, 214 nm; Injection volume: 20 µL; Sample: BSA tryptic digest.

Proprietary & confidential

Complex small molecules: fixed *L*, effect of *F and* t_G



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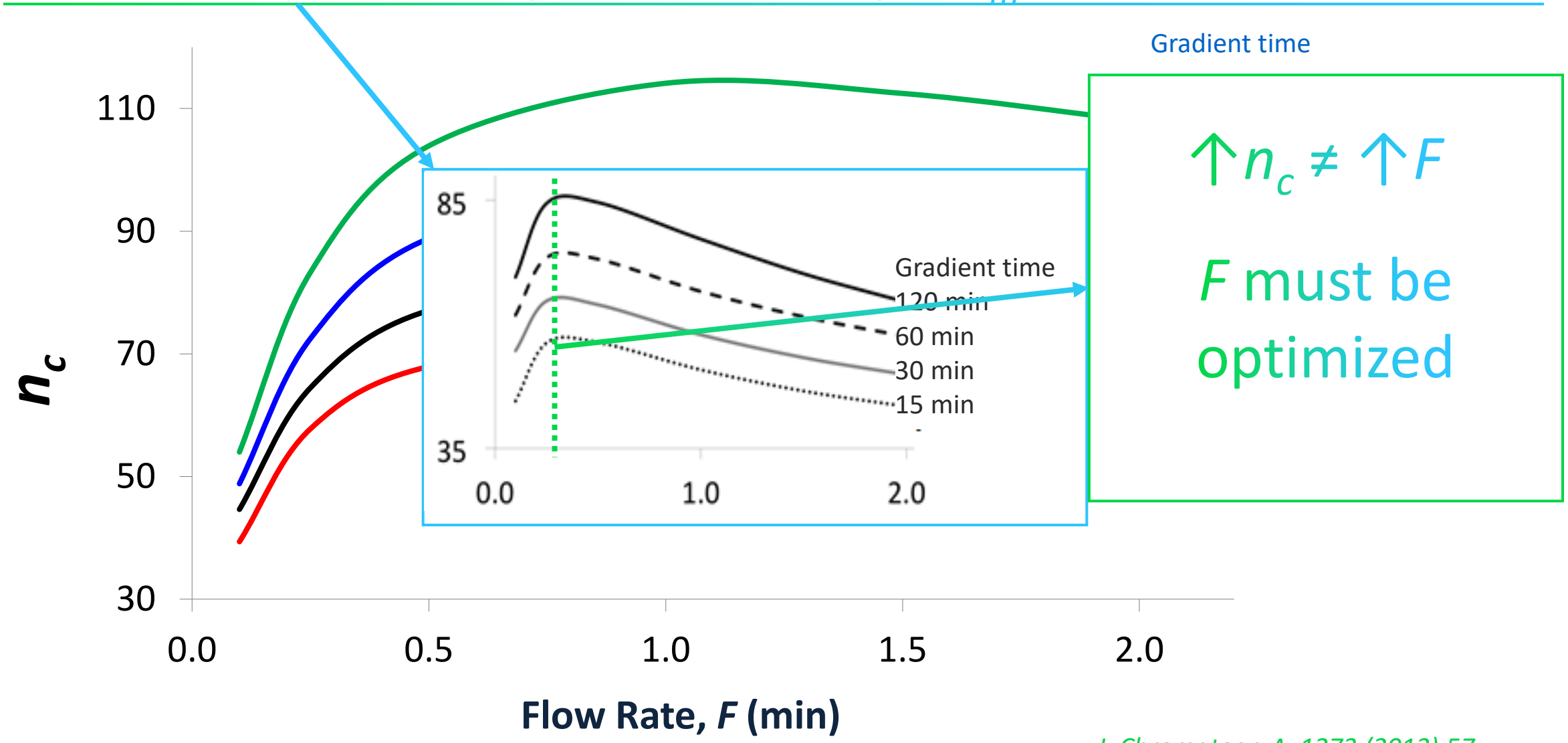
Effect of solute size (replaced with larger D_m)



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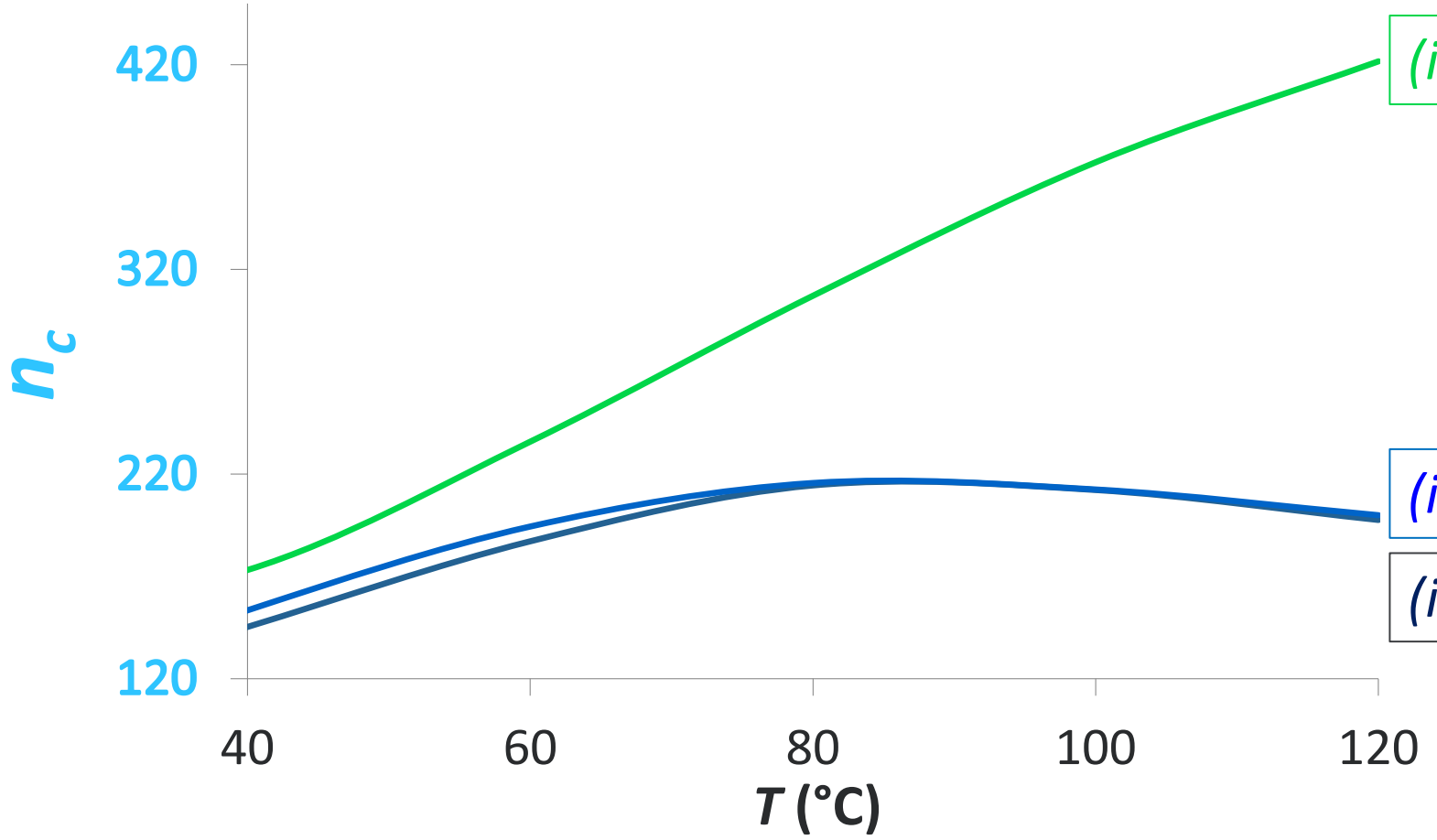
Different optimisation strategies



(i) Φ_{final}, ***F*, and *L***

↑n_c need to ↑T and optimize *F* & *L*

(ii) Φ_{final} and *F*

(iii) Φ_{final} only

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3. General approach for complex samples

Protocol for peptides

Column length is fixed:

1. Select the maximum allowable gradient time
2. Select the highest tolerable temperature
3. Optimize the flow rate
4. Adjust the final mobile phase (ϕ_{final}) so that the last solute elutes at the end of the gradient

X. Wang, W.E. Barber, P.W. Carr, J. Chromatogr. A 1107 (2006) 139-151.

Protocol for small molecules

- Run the Snyder-Dolan (S-D) test 5-100%B screening gradient
  - Using your sample of interest (not a standardised test mixture)
- Use the S–D test as above to determine if it is advisable to separate the mixture by gradient elution.
- To “pass” the S-D test
  - The solutes should occupy more than 25–40% of the gradient window ($T_{R,\,Last} - T_{R,\,First}$)/$t_G \geq 0.25–0.40$
- If use of a gradient is deemed inappropriate the protocol to maximise peak capacity will probably not work.
- Instead one should use an optimized isocratic separation.

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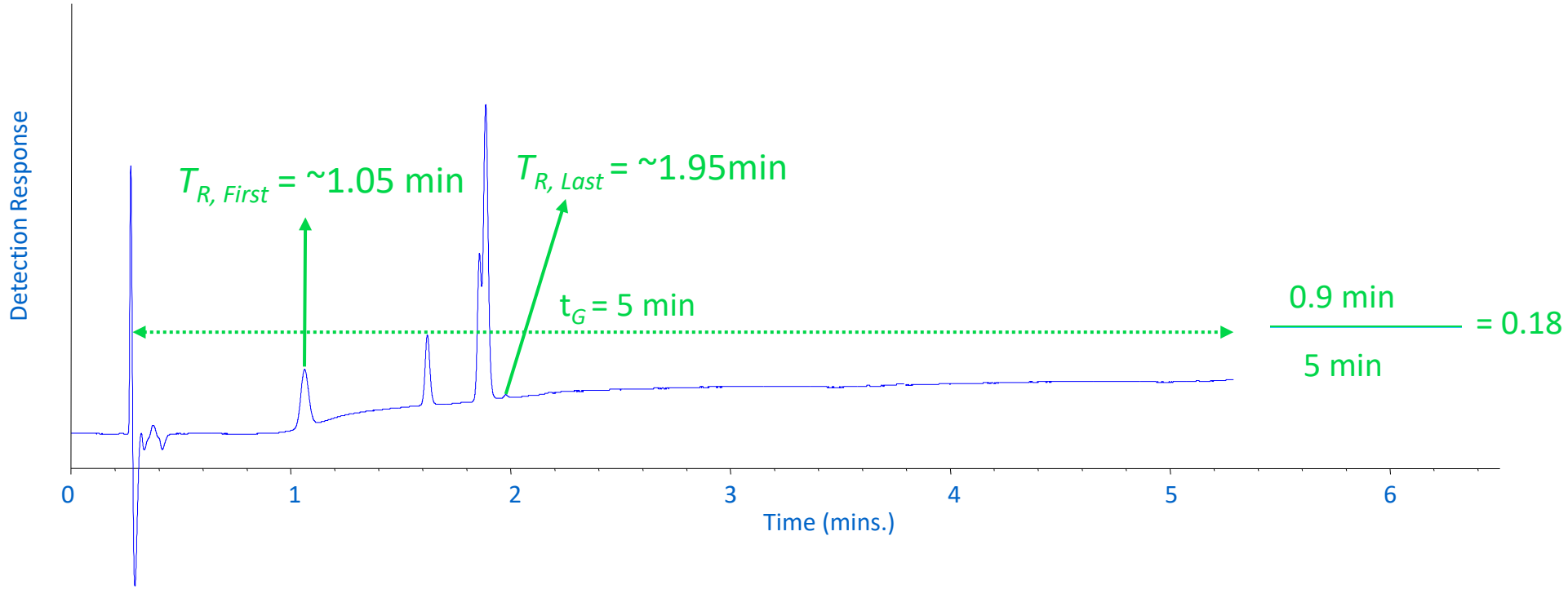
S-D test: 5-100%B screening method

The solutes should occupy more than 25–40% of the gradient window $(T_{R,\,Last} - T_{R,\,First})/t_G \geq 0.25–0.40$

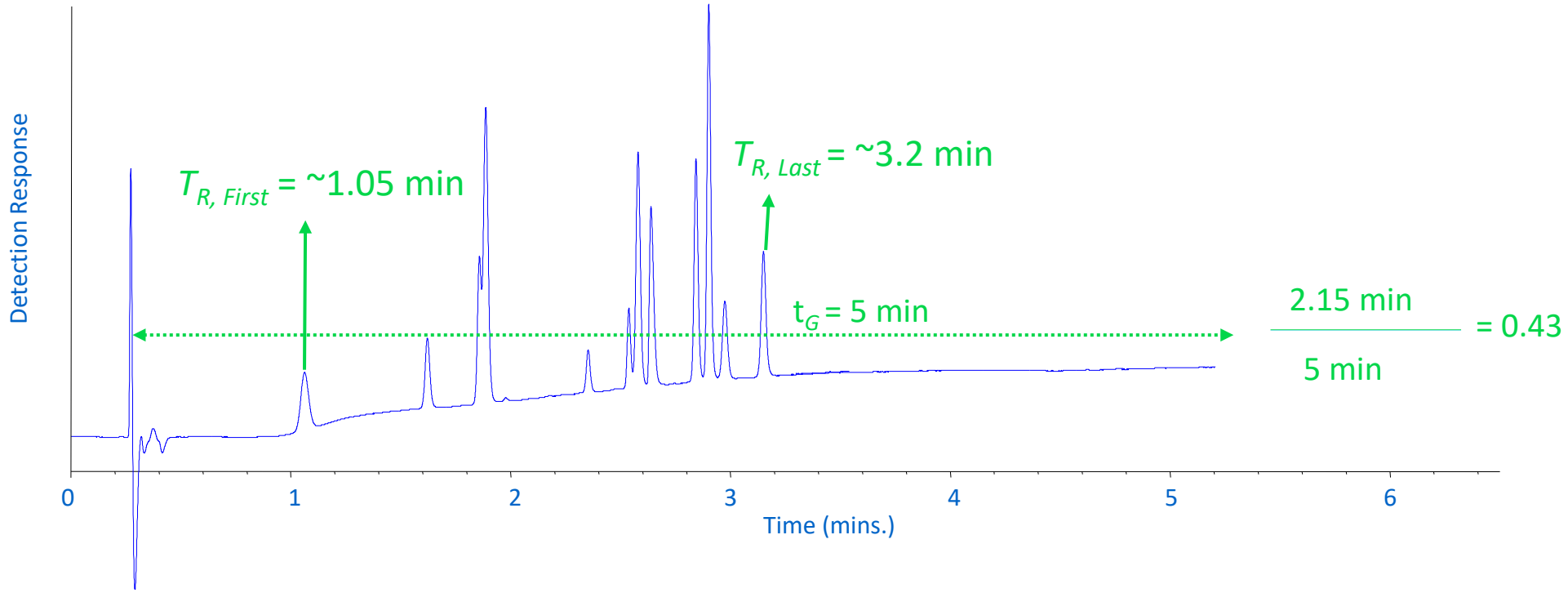


- The solutes occupy 18% of the gradient window
- Do not follow protocol to maximise peak capacity
- Requires isocratic mobile phase conditions

L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model, John Wiley & Sons, Inc., 2007.

Smaller molecules – Snyder-Dolan (S-D) Test

The solutes should occupy more than 25–40% of the gradient window $(T_{R,\,Last} - T_{R,\,First})/t_G \geq 0.25–0.40$

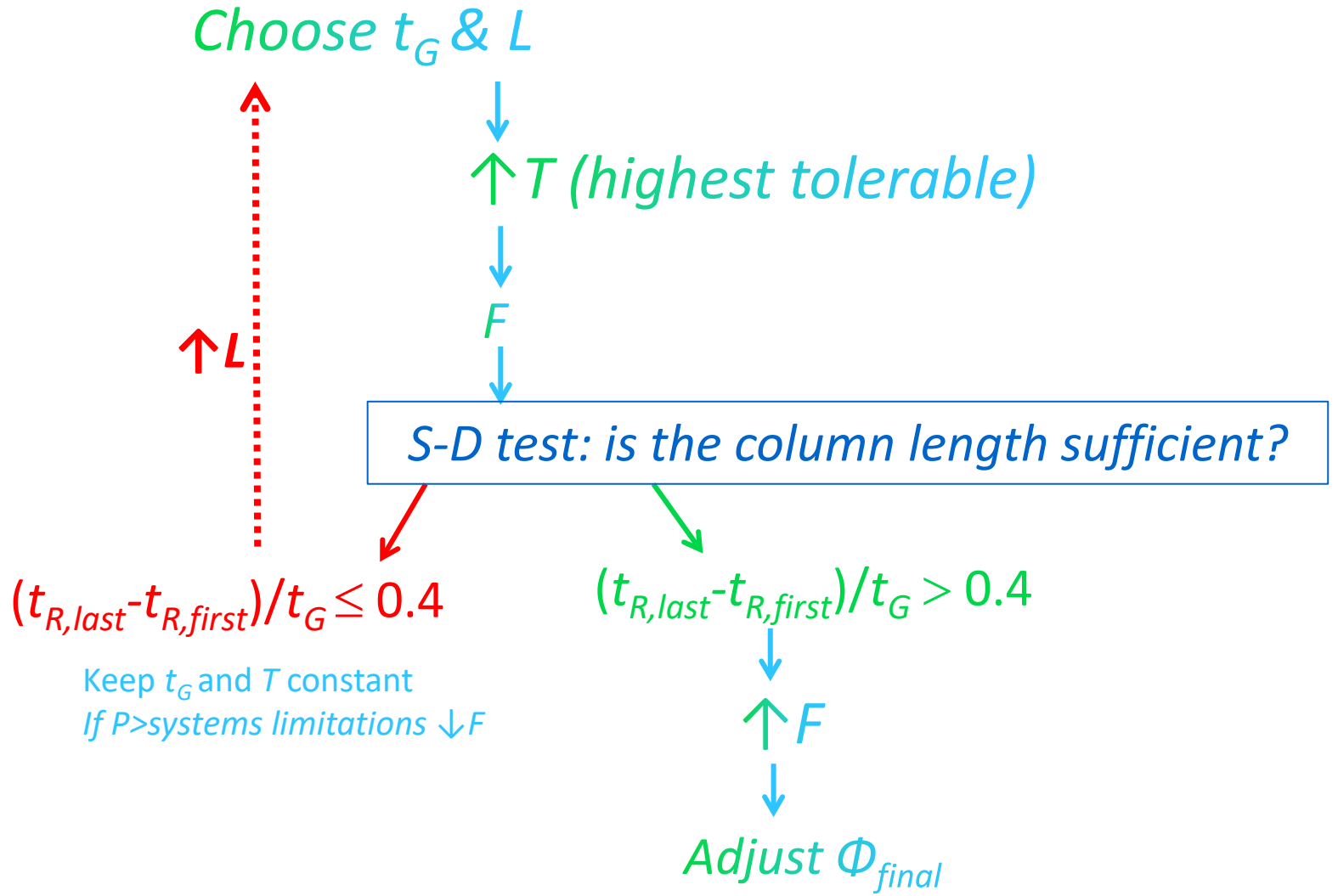


$$\frac{2.15 \text{ min}}{5 \text{ min}} = 0.43$$

- The solutes occupy 43% of the gradient window
- Proceed with the protocol to maximise peak capacity
- Requires gradient mobile phase conditions

L.R. Snyder, J.W. Dolan, High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model, John Wiley & Sons, Inc., 2007.

Maximise n_c for LMW complex samples



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Robustness of the protocol

20 combinations of t_G & L
Protocol optimisation vs. Solver* optimisation

Max. differences: F 0.3 mL/min, Φ_{final} 0.02 & n_c 3 %

L (cm)	t_g (min)	n_c^*	n_c^S	F^* (mL/min)	F^S (mL/min)	ϕ_{final}^*	ϕ_{final}^S	P^* (bar)	P^S (bar)
5	5	72	72	3.5	3.2	0.29	0.3	398	362
	15	–[b]	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–	–
	60	–	–	–	–	–	–	–	–
	120	–	–	–	–	–	–	–	–
10	5	80	81	1.7	1.8	0.44	0.44	382	400
	15	119	119	1.7	1.8	0.30	0.30	382	400
	30	–	–	–	–	–	–	–	–
	60	–	–	–	–	–	–	–	–
	120	–	–	–	–	–	–	–	–
20	5	71	73	0.9	0.9	0.62	0.61	380	400
	15	118	120	0.9	0.9	0.46	0.46	380	400
	30	148	150	0.9	0.9	0.38	0.37	380	400
	60	–	–	–	–	–	–	–	–
	120	–	–	–	–	–	–	–	–
40	5	42	45	0.4	0.4	0.85	0.83	375	400
	15	97	100	0.4	0.4	0.63	0.62	375	400
	30	130	135	0.4	0.4	0.54	0.53	375	400
	60	163	167	0.4	0.4	0.45	0.44	375	400
	120	201	207	0.4	0.4	0.37	0.36	375	400

[a] Fixed variables: $\phi_{initial} = 0.05$ and $V_D = 0.30$ mL, particle size = 3.5 µm, 2.1 mm i.d, $T = 70$ °C. * denotes computed results following the new protocol and S denotes results obtained by Solver.
[b] – denotes the choice of L and t_g did not pass the S–D test.

*Note: *Solver is a free optimisation add-on tool in Microsoft Excel*

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Application of the protocol

Maximised n_c rapidly - fast method development

n_c 170 for the sample matrix (maize seed extract)

Often used for 2DLC separations

Experimental conditions:

t_G = 15 min

L = 10 cm (2.1 mm i.d)

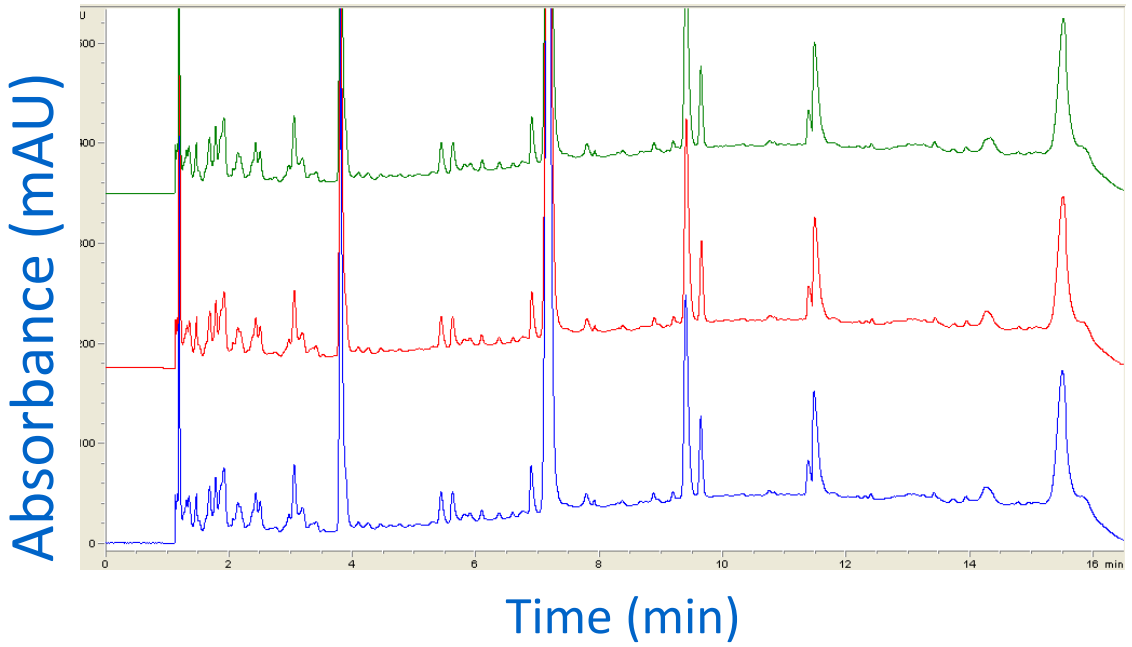
T = 70°C

F = 1.3 mL/min

Φ_{final} = 0.38

Delayed inj. 5 µL of sample

(t_D = 0.87 min)



Median peak width:

Maize = 0.084 min

Standard set of indoles = 0.080 min

Outline

1. Background
2. Trends vs. different operational parameters
3. General approach and alternative strategies

Maximise peak capacity rapidly

Acknowledgements

Avantor Associates
- Reviewing content: Gemma Lo, Tony Edge, and Gemma Howse
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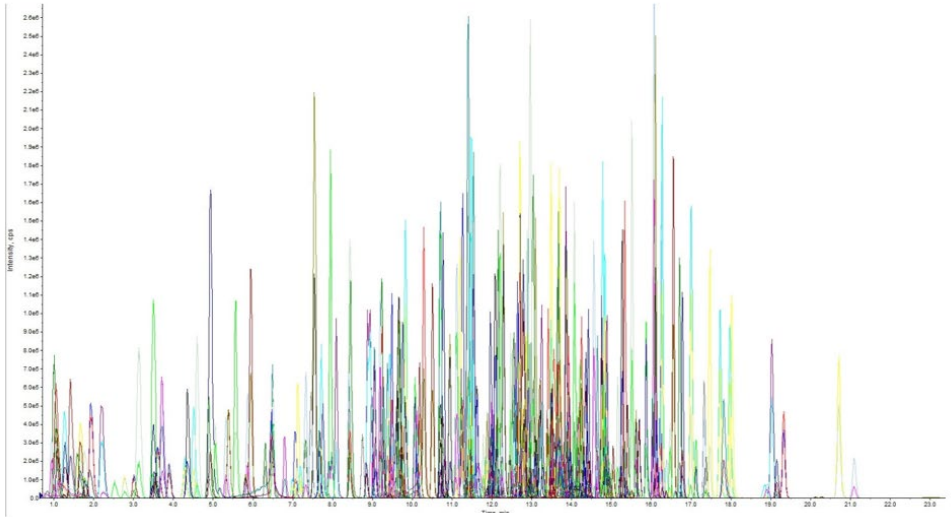
Imad Haidar Ahmad, Marcelo Filgueira, Xiaoli Wang, and Peter Carr

Thank you

Maximized n_c rapidly



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