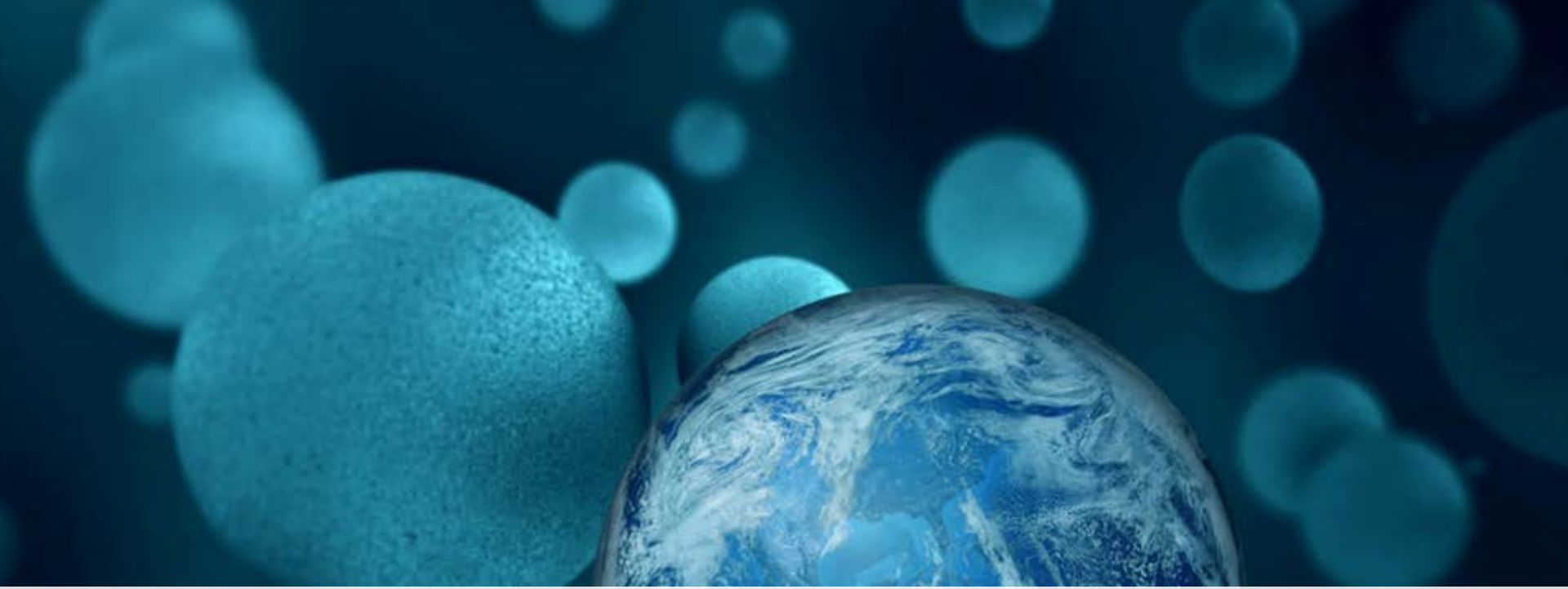

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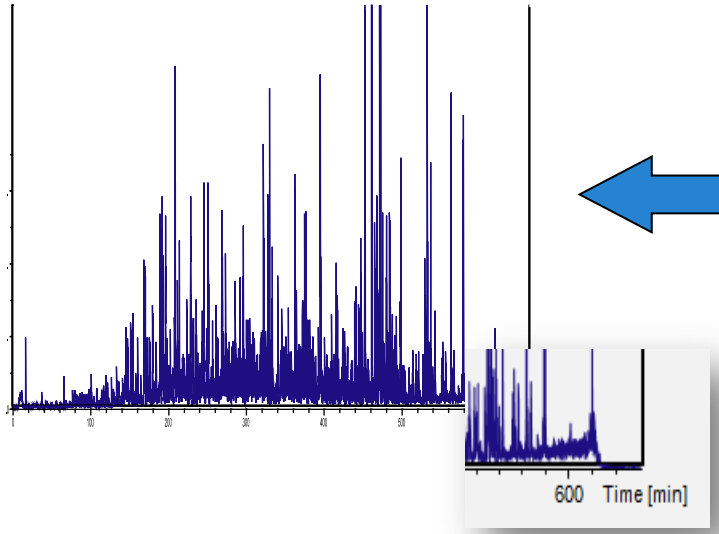
From HPLC to UHPLC: How fast can I be, and does fastest always mean best?

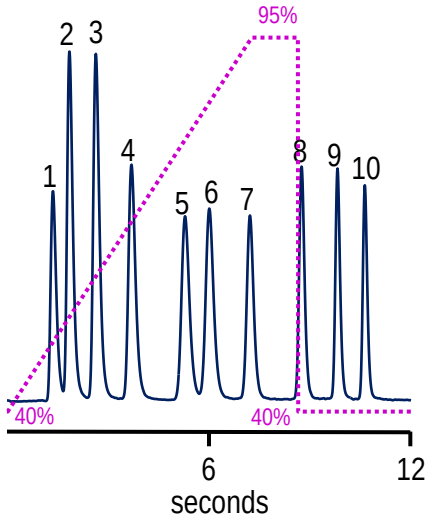
Dr. Markus M. Martin
Thermo Fisher Scientific, Germering/Germany

UHPLC potential



Up to 1000 peaks in a single run

Thermo Scientific™
Vanquish™ UHPLC system



10 peaks in 10 seconds

Focus on high resolution:
Peak capacity

Focus on high speed:
Sample throughput

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Accelerating by UHPLC – How fast can it be?

- Good UHPLC methods are at least 10x faster than HPLC methods.

- Yes, but wait…:
  - The speedup potential based on the pure pressure capabilities of the two technologies accounts for a maximum of factor 4 only. All improvements beyond that mostly come in line with a reduced resolution in UHPLC.

How do efficiency, resolution, and analysis time interact with particle size?

- What the van Deemter theory on band broadening tells us:

$$H = A + \frac{B}{u} + C \cdot u$$

  - Minimal plate height (HETP) H ~ dp, and plate count N ~ 1/dp, resp.
  - Half as large particles produce thus twice as many plate counts (efficiency)

  - And besides:
    - Speed of analysis (= linear velocity u_{min}) ~ 1/dp

- Speed-up potential:

  Resolution remains the same if L/dp = constant

How do efficiency, resolution, and analysis time interact with particle size?

- What the van Deemter theory on band broadening tells us:

$$H = A + \frac{B}{u} + C \cdot u$$

  - Minimal plate height (HETP) H ~ dp, and plate count N ~ 1/dp, resp.
  - Half as large particles produce thus twice as many plate counts (efficiency)
  - Speed-up potential: Resolution remains the same if L/dp = constant

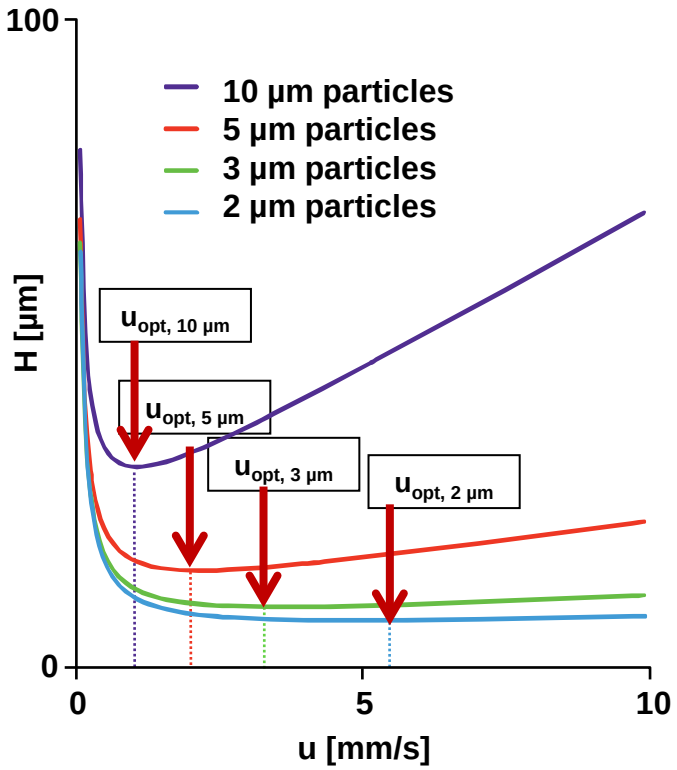
- Looking at the Purnell equation we learn:

$$\textbf{Resolution } R_S = \frac{1}{4}\sqrt{N} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2}{1 + k_2}$$

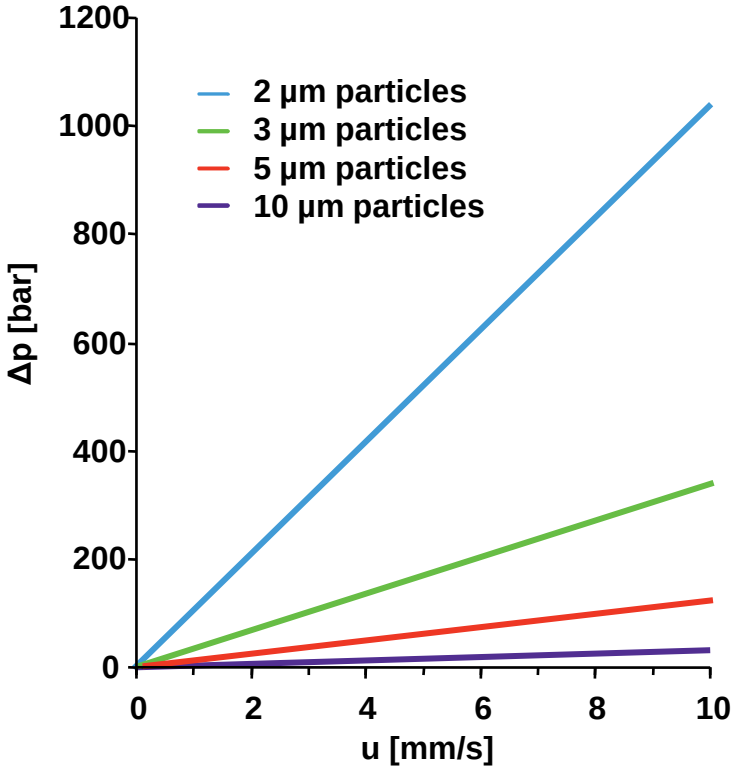
  - R ~ $\sqrt{N}$, and thus R ~ $\sqrt{1/dp}$
  - Double the resolution needs four times smaller particles
  - …or a four times longer column with the same packing material

Particle size and Pressure Drop, or: There‘s no free Lunch

a) Separation performance



b) Column pressure



- The column pressure rises with the inverse square of the particle size:

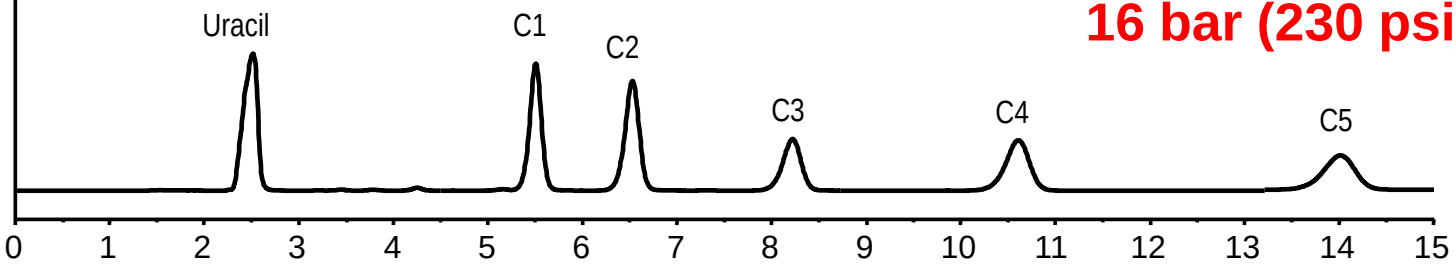
$$\Delta p \sim 1/dp^2$$

Smaller Particles while cutting down the LC Column Length

250 x 4.6 mm, *dp*=10 µm, F= 1 mL/min

Isocratic separation

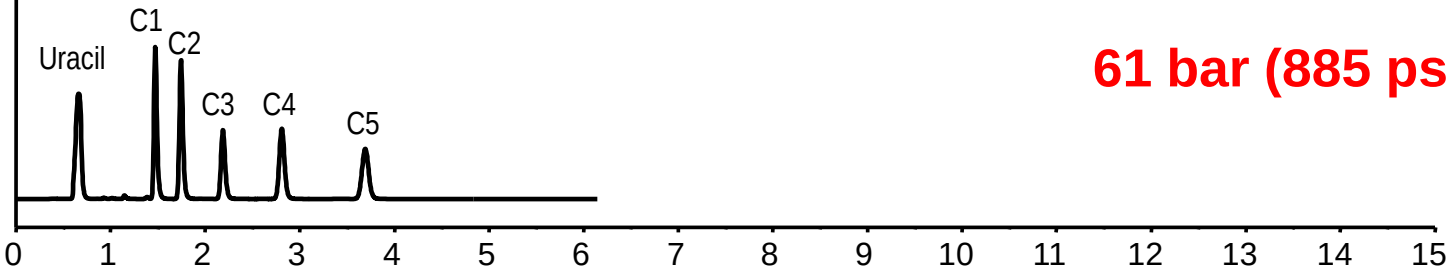
16 bar (230 psi)



L/dp = const.
F · dp = const.

125 x 4.6 mm, *dp*=5 µm, F= 2 mL/min ➔ 4-fold acceleration

61 bar (885 psi)



Sample: phenylalkanes C1 – C5, Uracil
Stationary phase: ProntoSIL 120 C18 AQ (BISCHOFF Analysentechnik u. –geräte GmbH);
Mobile phase: 20/80 (v/v) H_2O/ACN; Temperature: 20 °C

rules: L/dp = const. and dp·F = const.

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Smaller Particles while cutting down the LC Column Length

250 x 4.6 mm, *dp*=10 µm, F= 1 mL/min

Isocratic separation

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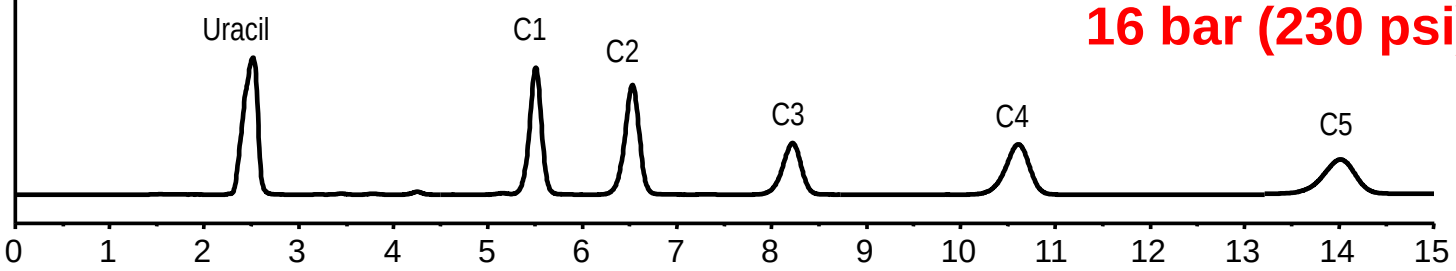

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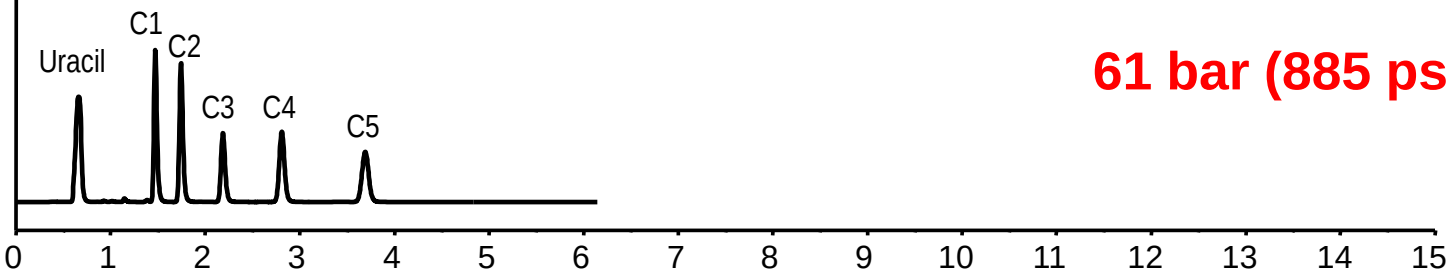
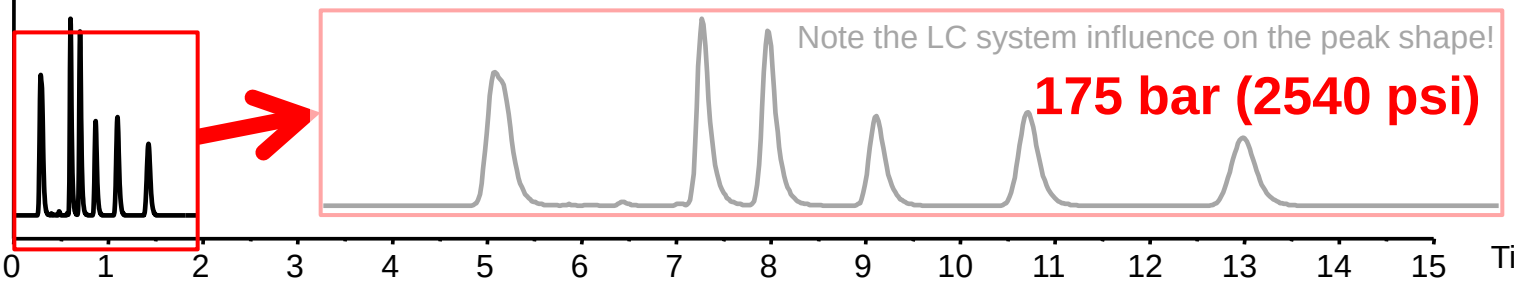
125 x 4.6 mm, *dp*=5 µm, F= 2 mL/min ➔ 4-fold acceleration

61 bar (885 psi)



L/dp = const.
F · dp = const.

75 x 4.6 mm, *dp*=3 µm, F= 3.3 mL/min ➔ 11-fold acceleration



Sample: phenylalkanes C1 – C5, Uracil
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Mobile phase: 20/80 (v/v) H_2O/ACN; Temperature: 20 °C

rules: L/dp = const. and dp·F = const.

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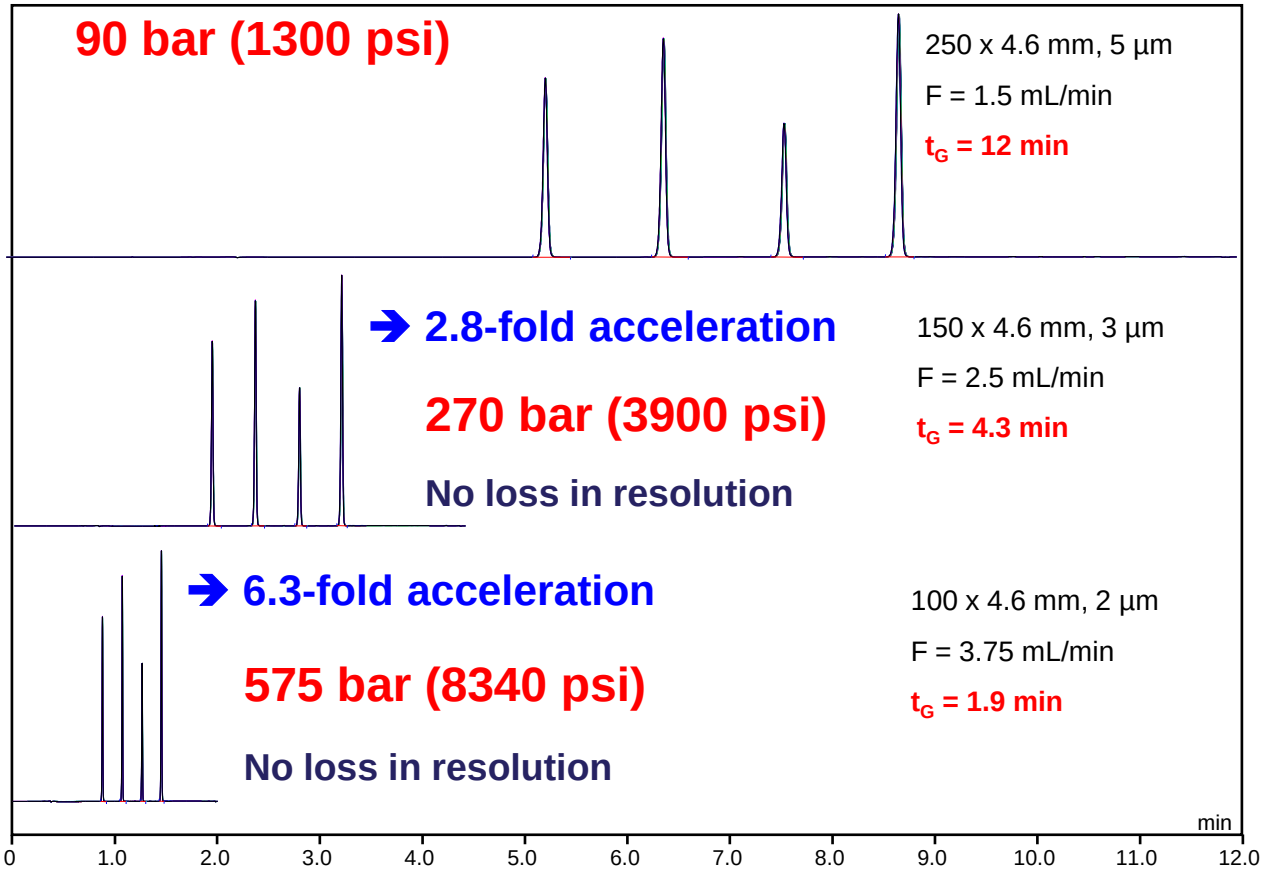
Does this also work with Gradient Methods?

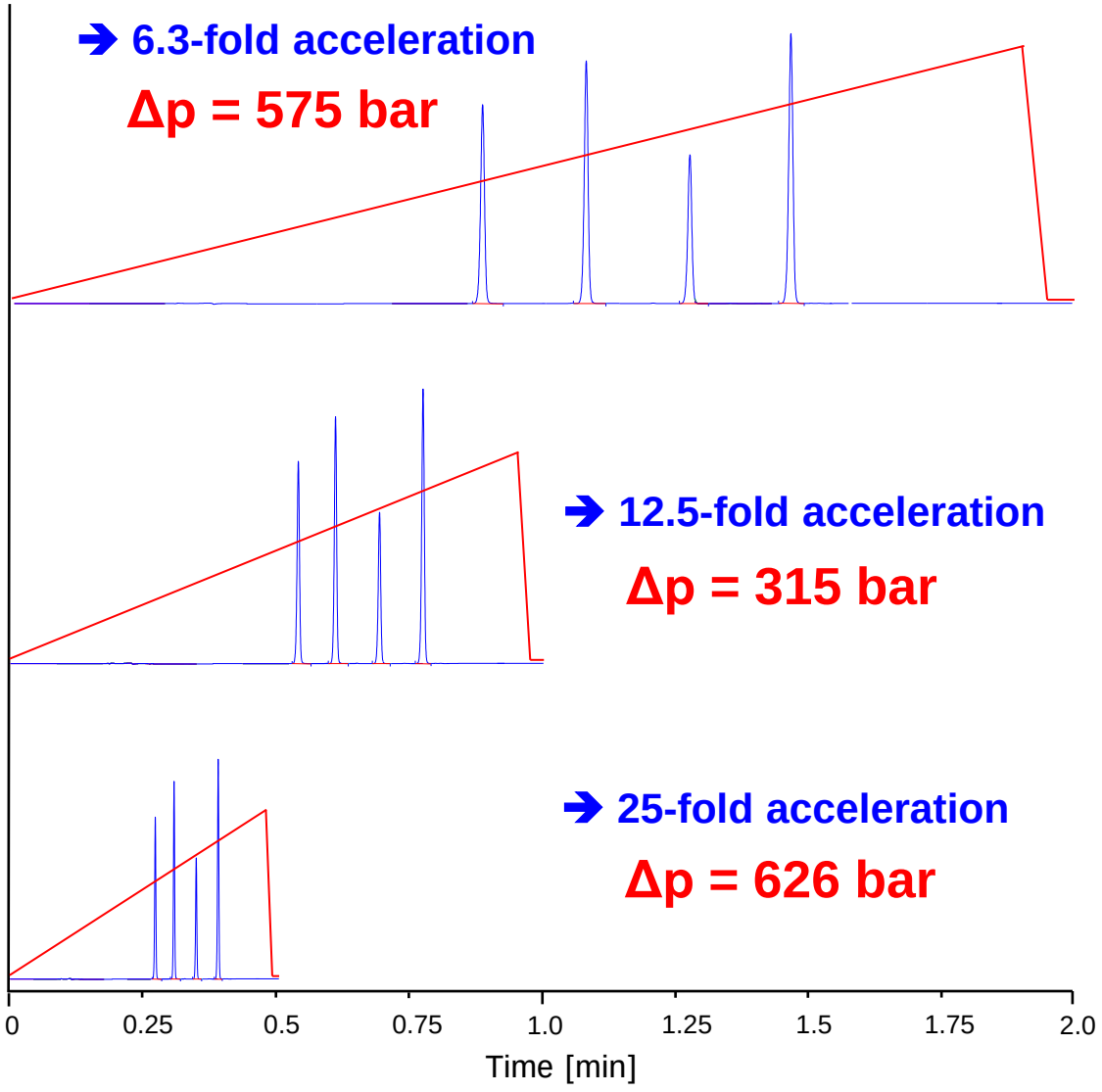


L/dp = const.
F · dp = const.
t_G·F/L = const.

L/dp = const.
F · dp = const.
t_G·F/L = const.

- Additional rule: t_G·F/L = const.
- If we follow all the mentioned scaling laws, then…
- …pressure increases linearly with the speed of analysis
- **But: Do we always need a UHPLC system for separations on (sub-)2 µm particles?**

Supercharge by Half the Column Length ☺



100 x 2.1 mm, 2.2 µm

F = 0.75 mL/min

t_G = 1.9 min

V_{inj} = 2 µL

50 x 2.1 mm, 2.2 µm

F = 0,75 mL/min

t_G = 0.95 min

V_{inj} = 1 µL

50 x 2.1 mm, 2.2 µm

F = 1.50 mL/min

t_G = 0.48 min

V_{inj} = 1 µL

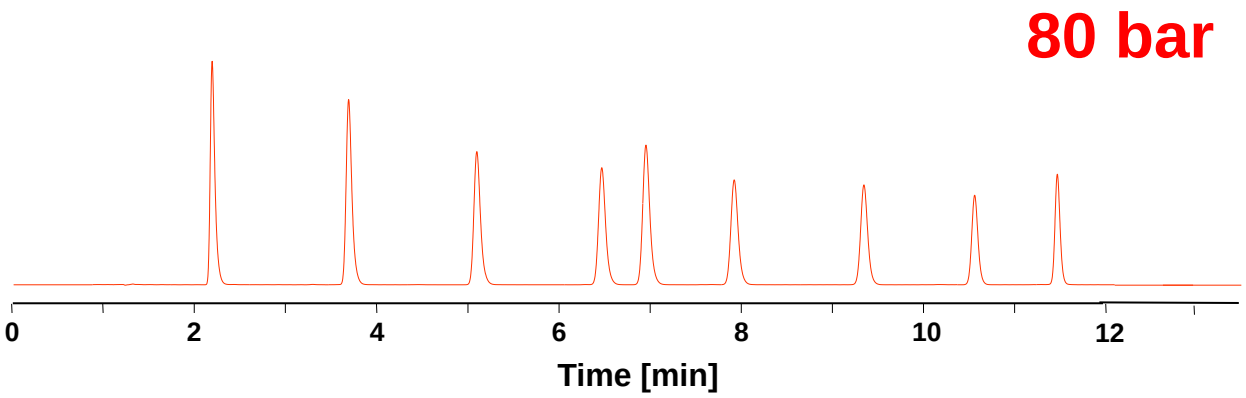
Would there even be a Chance for a 60-fold Speed-up?

150 x 4.6 mm, 5 µm

F = 1.5 mL/min

t_G = 12 min

T = 30 °C

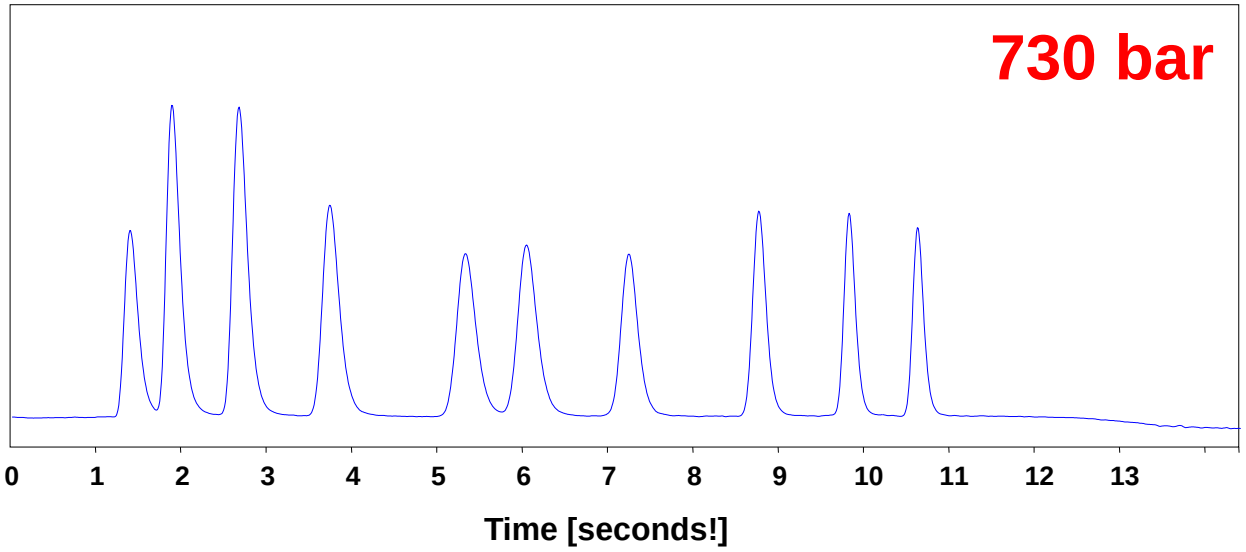


30 x 2.1 mm, 1.8 µm

F = 3.7 mL/min

t_G = 12 s

T = 100 °C



- Only 9x the pressure, but 60x the speed – What‘s wrong here?

Would there even be a Chance for a 60-fold Speed-up?

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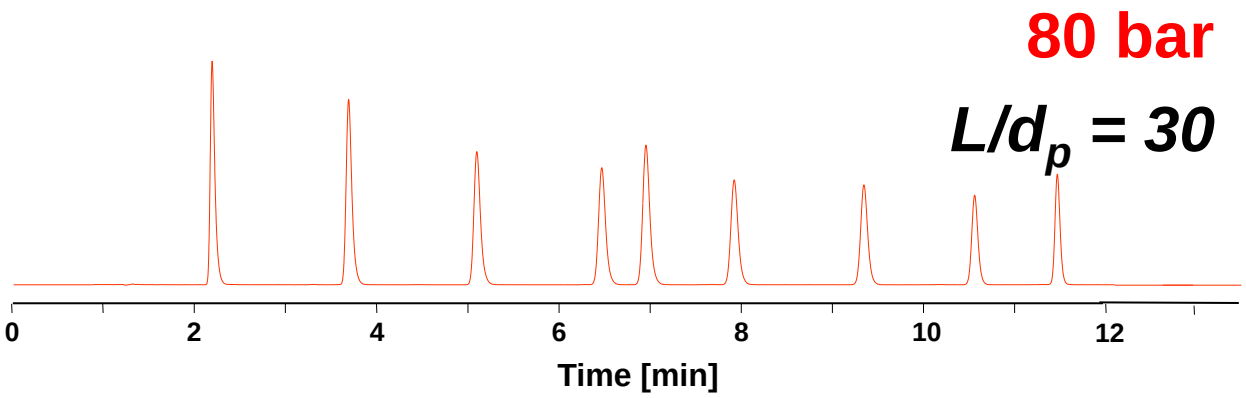
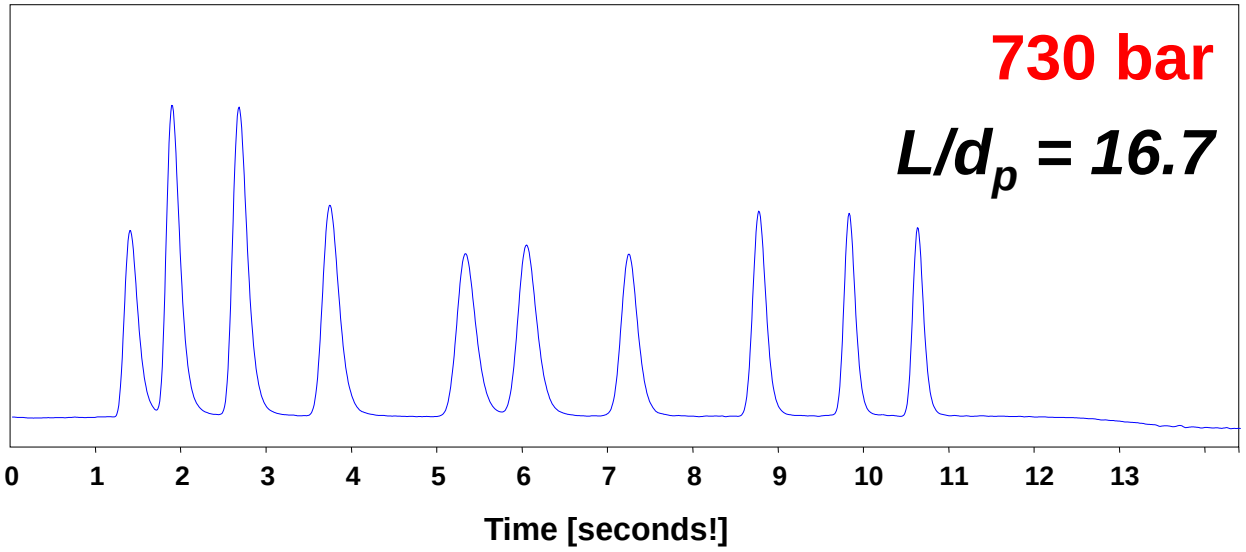


30 x 2.1 mm, 1.8 µm

F = 3.7 mL/min

t_G = 12 s

T = 100 °C



- Only 9x the pressure, but 60x the speed – What‘s wrong here?
- L/dp not constant / smaller resolution – A compromise in quality

Take-Home Message 1: Concept for Speed Optimization

- Analysis speed:
  - $t_{analysis}$ ~ 1/p (At constant resolution R)

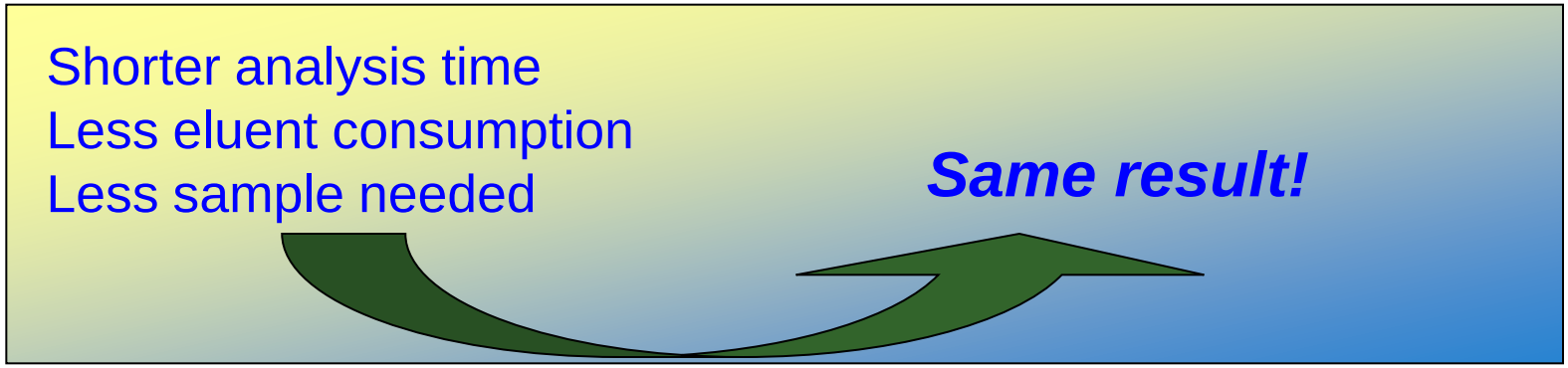
- A 1000 bar UHPLC system gives you ‘only’ 2.5 times faster separations than a 400 bar HPLC system.

Take-Home Message 1: Concept for Speed Optimization

Use small particles in short columns

Leads to same efficiency in shorter time

Enables faster separations with less peak broadening



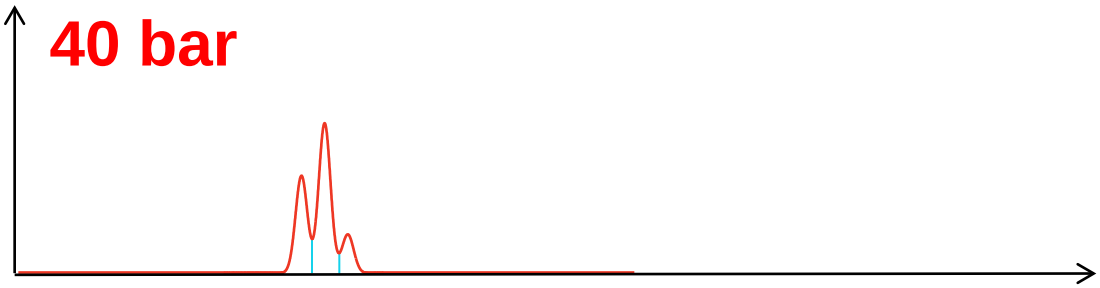
Limitations of the Speed-up Potential

- Does a smaller particle size always result in a better peak resolution?

- Yes, but wait…:
  - This only applies if the column length and the linear velocity are adjusted accordingly. And it always comes at the price of a substantial rise in pressure.

Which Options do I have for Optimization?

a)



100 x 4.6 mm, 5 µm

F = 1.5 mL/min

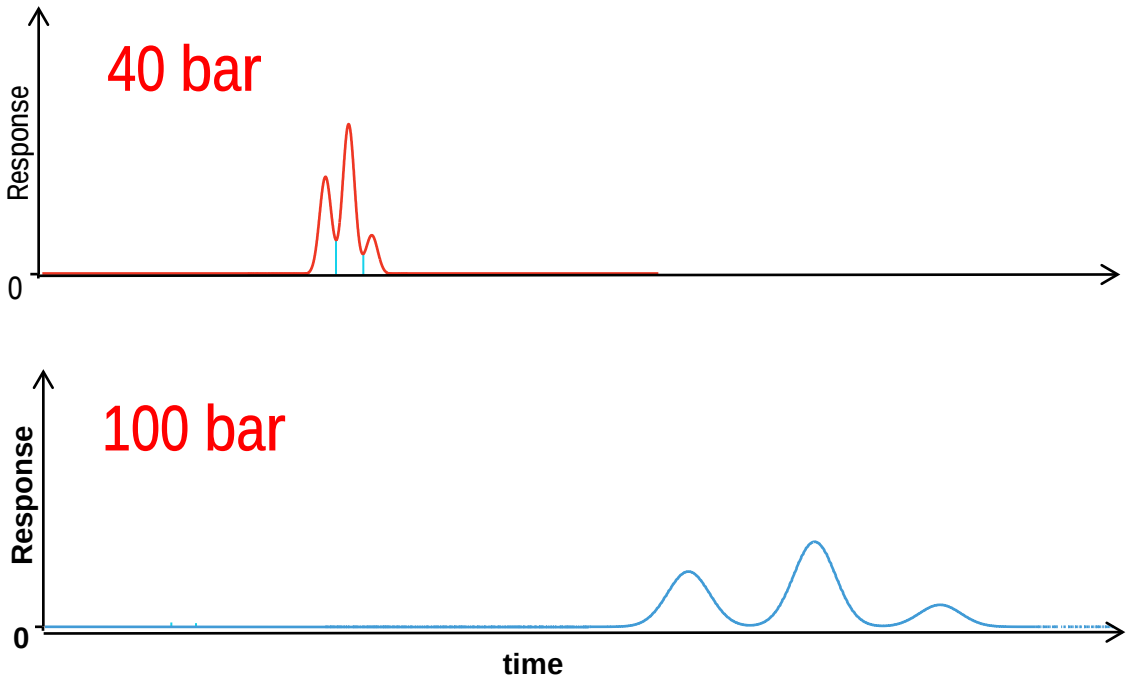
Rs = 0.94

L/dp = 20

- b) More plates on the same phase material → Make the column longer
- c) More plates at the same column dimensions → Smaller particles
- d) A combination of both?

Better Resolution at *lower* Speed

b) More plates at the same phase material by a longer column



100 x 4.6 mm, 5 µm

F = 1.5 mL/min

Rs = 0.9

L/dp = 20

250 x 4.6 mm, 5 µm

F = 1.50 mL/min

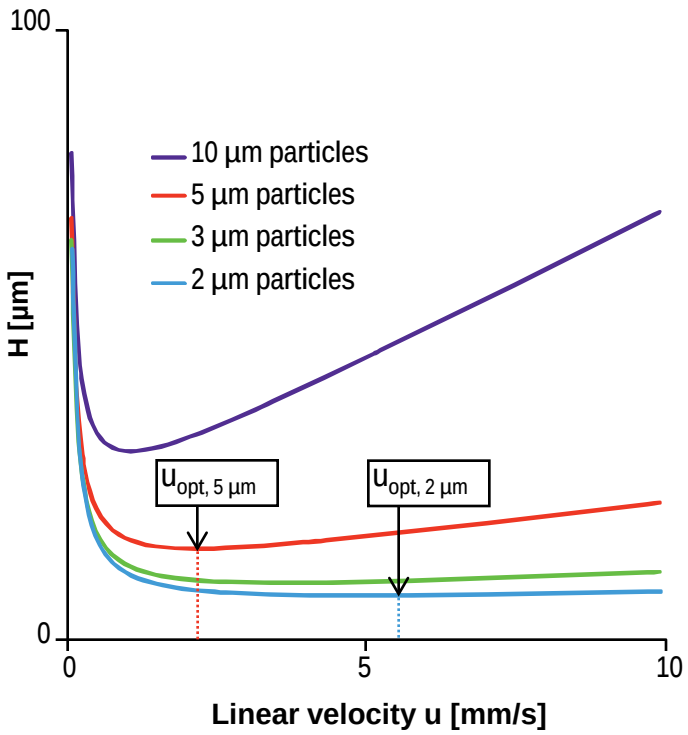
Rs = 1.5

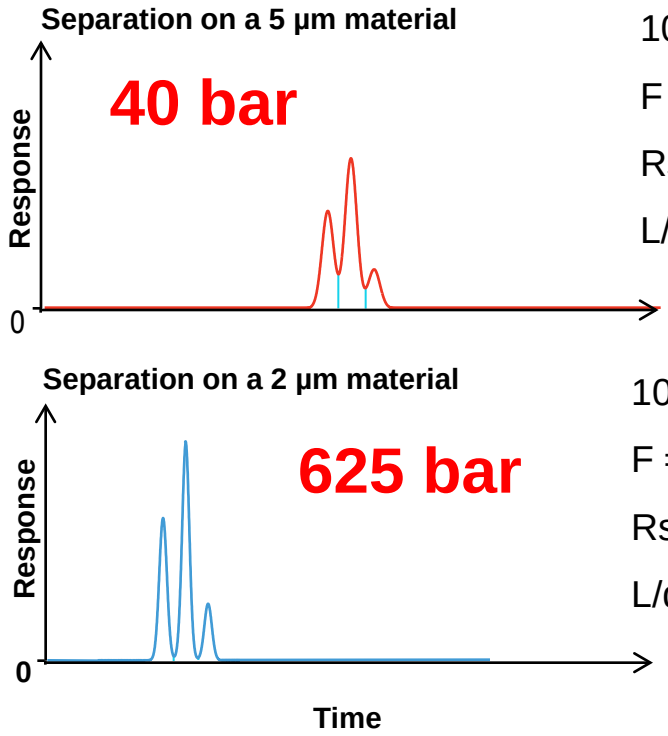
L/dp = 50

- Leads to 1.6x higher resolution at 2.5x higher pressure
  – And 2.5x longer analysis time
- Useful approach if analysis time does not matter (too much) and the chromatogram does not need to be a beauty

Better Resolution at *higher* Speed

c) More plates at the same column dimensions using smaller particles





100 x 4.6 mm, 5 µm

F = 1.50 mL/min

Rs = 0.9

L/dp = 20

100 x 4.6 mm, 2 µm

F = 3.75 mL/min

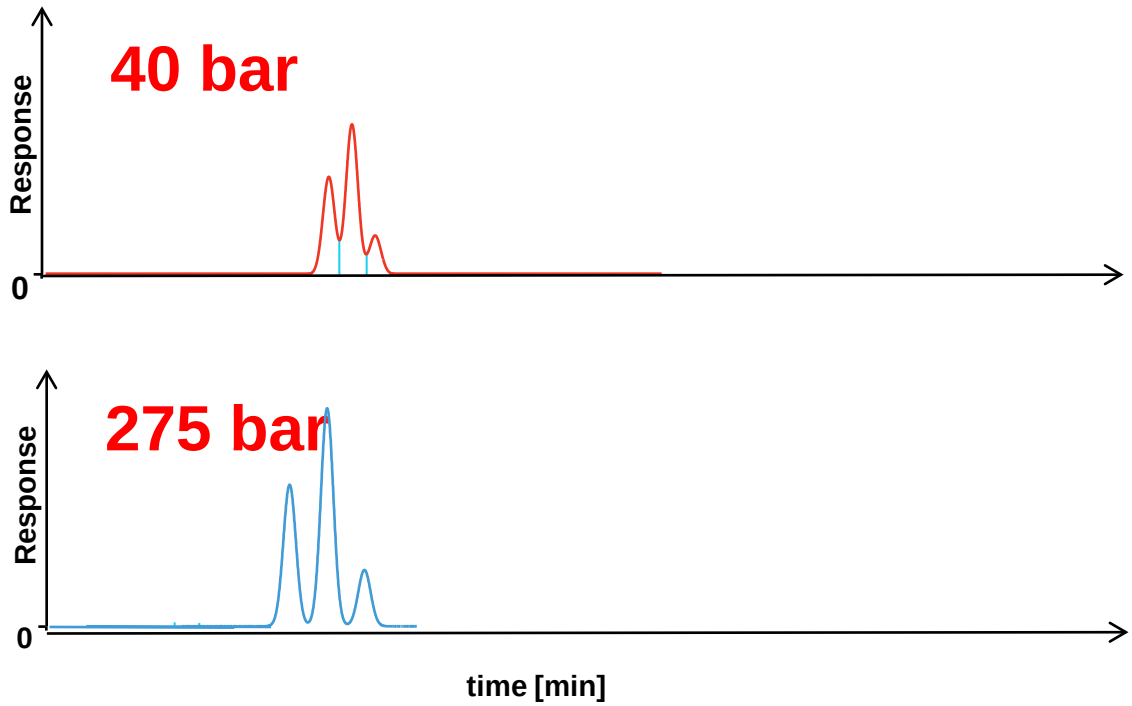
Rs = 1.5

L/dp = 50

- 16-fold pressure for only 1.6x higher resolution – Was it worth it?
- Analysis speeds up by factor 2.5 – But is this a requirement?

Better Resolution at *the same* Speed

d) Smartly combine L and dp in a way that enables (approx.) the same speed of analysis:



100 x 4.6 mm, 5 µm

F = 1.5 mL/min

Rs = 0.9

L/dp = 20

150 x 4.6 mm, 3 µm

F = 2.5 mL/min

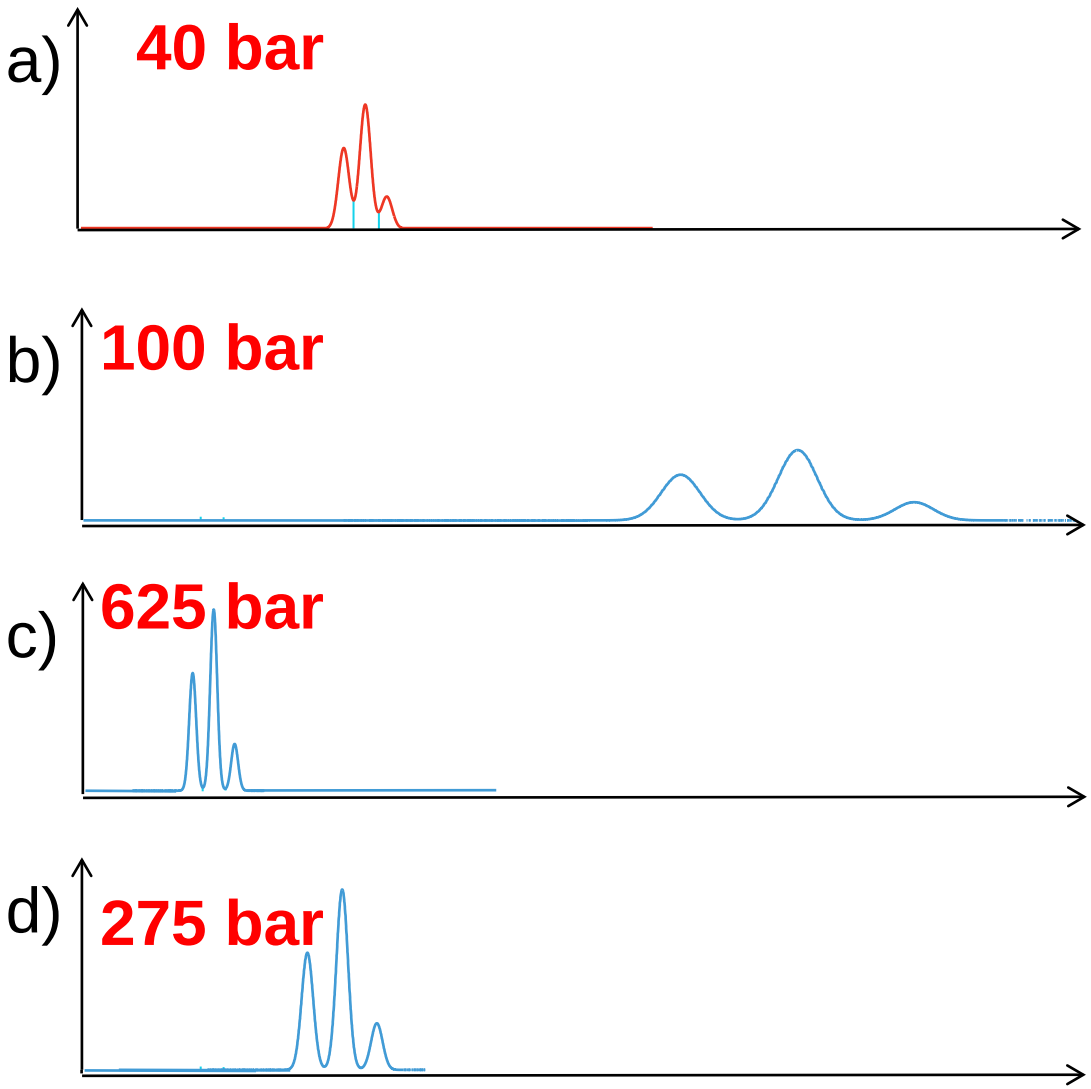
Rs = 1.5

L/dp = 50

- 1.6x higher resolution at 6.9x higher pressure – And 1.1x the analysis time

Be aware that $R \sim \Delta p^4$

Summarizing the different Approaches



100 x 4.6 mm, 5 µm

F = 1.5 mL/min

Rs = 0.94

L/dp = 20

250 x 4.6 mm, 5 µm

F = 1.50 mL/min

Rs = 1.5

L/dp = 50

100 x 4.6 mm, 2 µm

F = 3.75 mL/min

Rs = 1.5

L/dp = 50

150 x 4.6 mm, 3 µm

F = 2.5 mL/min

Rs = 1.5

L/dp = 50

Take-Home Message 2: Avoid kinetic Resolution Tuning…

...or do it systematically right to ideally exploit the pressure capabilities:

- Peak resolution
  - $R \sim p^4$ (At constant analysis time)
- Better resolution by longer columns with same phase material:
  - Costs analysis time
- Better resolution by smaller particles in the same column hardware (And adjusted flow rate):
  - Speeds up the separation, at **substantially** higher pressure
- Better resolution by moderate adjustment of column length and particle size → **Best variant**:
  - Keeps analysis time the same at only moderate pressure increase

Speed-up of Gradient Methods

- If a gradient method is transferred to a shorter column, the slope of the gradient always has to be increased by the same factor as the column is shortened in order to obtain a good result.

Do the Speed-up Rules also apply to Gradient Methods?

- The *Gradient Volume Concept* (GVC):

Gradient volume V_G:

$$V_G = F \cdot t_G$$

F	:	Flow rate [mL/min]
t_G	:	Length of gradient segment
V_C	:	Column volume
L	:	Column length
d_c	:	Column inner diameter

- Keep V_G constant if a flow rate modulation is used to speed-up a gradient method.

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L	:	Column length
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- Keep V_G constant if a flow rate modulation is used to speed-up a gradient method.
- Keep the ratio V_G/V_C constant to port gradient separations to a column with different dimensions.

$$\frac{F \cdot t_G}{V_C} \equiv const.$$

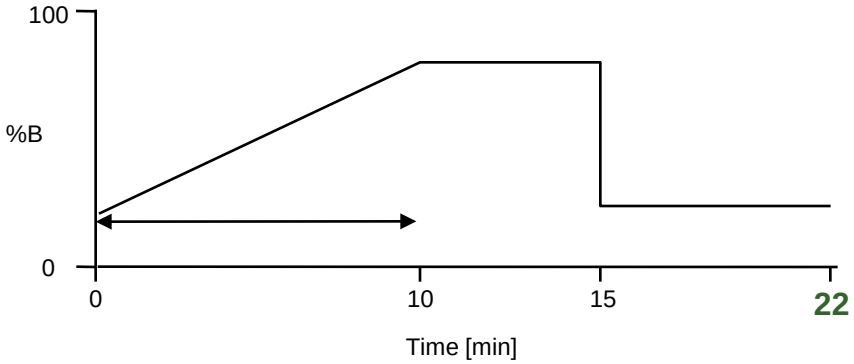
$$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{L_{new} \cdot d_{c,new}^{\,2}}{L_{old} \cdot d_{c,old}^{\,2}}$$

- Then peaks do elute always at the same eluent composition.
- Chromatographic separation only changes in terms of analysis time.

How do I practically translate a Gradient Program?

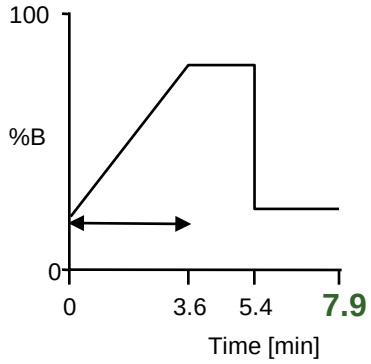
4.6 x 250 mm column, 5 µm
1.5 mL/min (u = 2.2 mm/s)

Column volume (V_C) = 4.15 mL



2.1 x 150 mm column, 3 µm
0.52 mL/min (increase u = 3.7 mm/s)

Column volume (V_C) = 0.52 mL



First step of original gradient 0-10 min

t_G = 10 min

F = 1.5 mL/min

V_C = 4.15 mL

$$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{V_{C,new}}{V_{C,old}}$$

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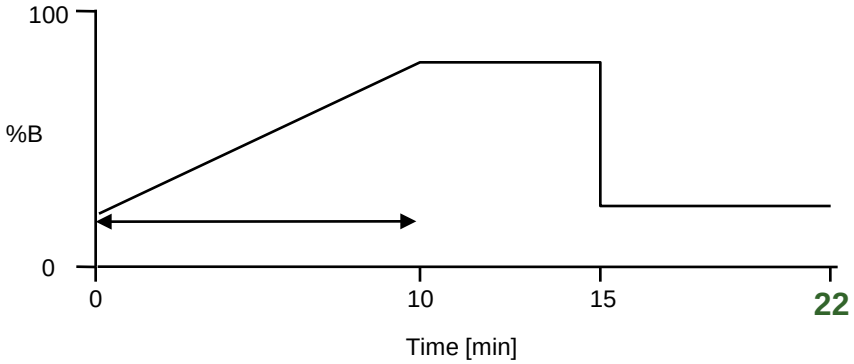

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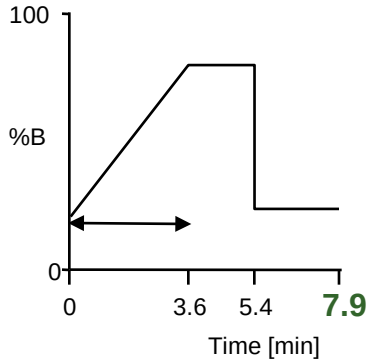

First step of original gradient 0-10 min

t_G = 10 min

F = 1.5 mL/min

V_C = 4.15 mL

$$t_{G,new} = t_{G,old} \cdot \frac{F_{old}}{F_{new}} \cdot \frac{V_{C,new}}{V_{C,old}}$$

New first step of gradient 0-3.6 min

$$t_{G,new} = 10 \cdot \frac{1.5}{0.52} \cdot \frac{0.52}{4.15}$$

= 3.6 min

HPLC Method Development Calculator: http://www.separatedbyexperience.com/products/GradientMethod.aspx

Rules for the Gradient Volume/Column Volume Ratio

$V_G = 5\ V_M$

- Recommended for trace analysis of known compounds
- Small gradient volumes lead to small peak volumes
- Results in less peak dilution and thus better limits of detection (LOD)

$$V_M = \varepsilon_t \cdot r^2 \cdot \pi \cdot L$$

L : Column length [mm]
r : Column radius [mm]
ε_t : Total porosity (ca. 0.7)

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$V_G = 10 - 15\ V_M$

- For optimal resolution at moderate run times
- Recommended if not enough selectivity achievable

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L : Column length [mm]
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Rules for the Gradient Volume/Column Volume Ratio

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$V_G = 10 - 15\ V_M$

- For optimal resolution at moderate run times
- Recommended if not enough selectivity achievable

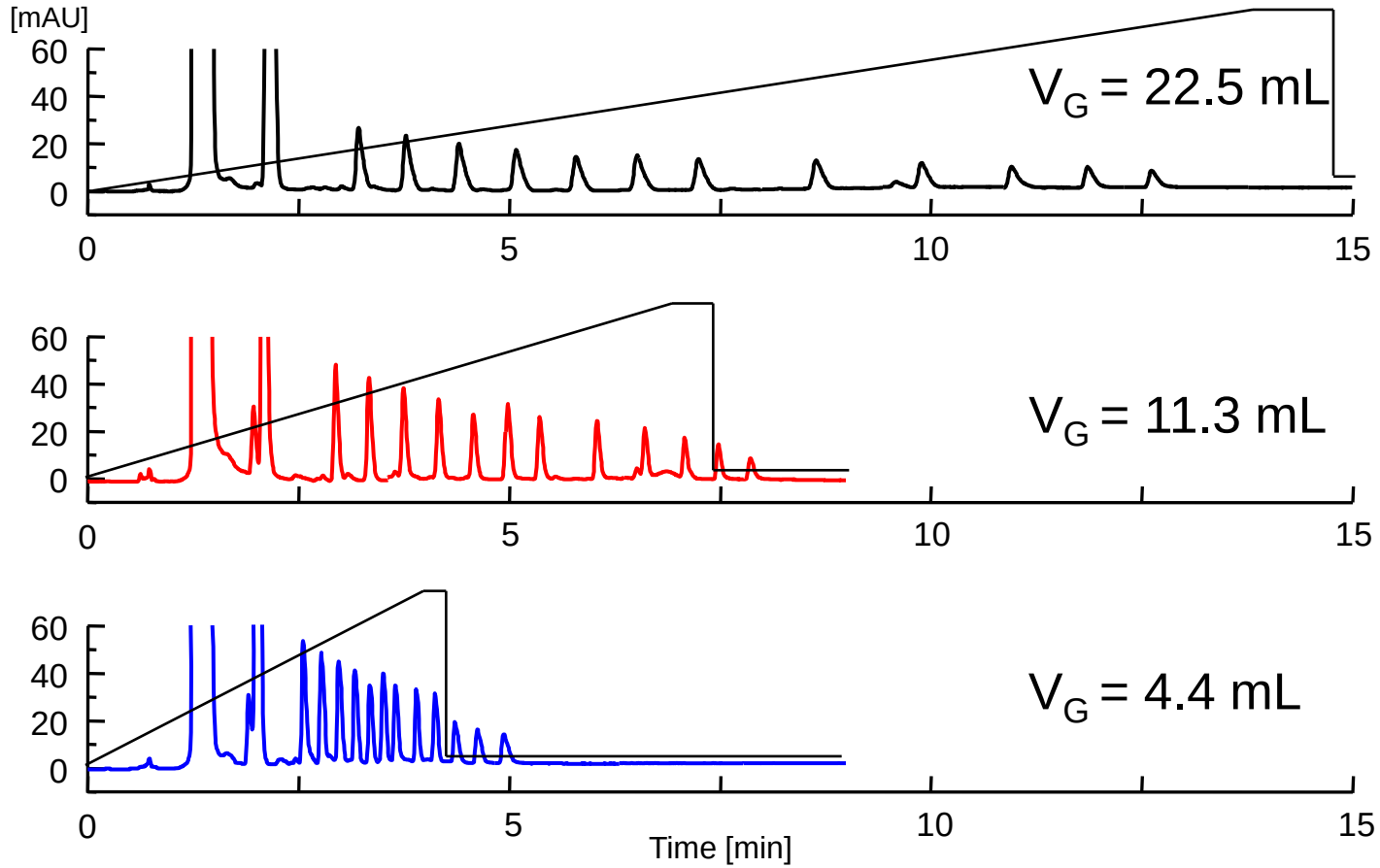
$V_G > 15\ V_M$

- For complex multi-component analyses
- Recommended for highest peak capacity

$$V_M = \varepsilon_t \cdot r^2 \cdot \pi \cdot L$$

L : Column length [mm]
r : Column radius [mm]
ε_t : Total porosity (ca. 0.7)

Increase the Gradient Slope – Reduce the Gradient Volume



- With decreasing gradient volume…
- …the peak height increases.
- …the selectivity/relative retention reduces.
- …the elution order can revert due to selectivity changes.

Take-Home Message 3: The Gradient Volume Concept

- If the gradient volume of a gradient separation in relation to the column void volume is kept constant,
  - Then peaks elute always at the same eluent composition
  - And a constant gradient volume enables an acceleration of the gradient separation by an increased flow rate, without significantly changing the relative retention of the analytes.

- If the gradient volume is not reduced in proportion to the column volume, but remains relatively too large,
  - Then there is almost no gain in analysis time,
  - Then relative retention times may change,
  - Then usually the resolution increases, but the peak heights (S / N) decrease.

Summary

- The speed of analysis increases linearly with the column pressure.
- To *double the resolution* of a given method with a known column material due to better efficiency, you need

EITHER

  - Packing particles having a diameter of *a quarter* of the starting material,
  - What translates into *16 times* the back pressure
  - At the same analysis time

Summary

- The speed of analysis increases linearly with the column pressure.
- To *double the resolution* of a given method with a known column material due to better efficiency, you need

EITHER

  - Packing particles having a diameter of *a quarter* of the starting material,
  - What translates into *16 times* the back pressure
  - At the same analysis time

OR

  - A *four times longer* column packed with the same particles,
  - Which generates only a *fourfold* higher back pressure,
  - But at the same time the analysis time also *quadruples*.

Summary

- The speed of analysis increases linearly with the column pressure.
- To *double the resolution* of a given method with a known column material due to better efficiency, you need

EITHER

  - Packing particles having a diameter of *a quarter* of the starting material,
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  - At the same analysis time

OR

  - A *four times longer* column packed with the same particles,
  - Which generates only a *fourfold* higher back pressure,
  - But at the same time the analysis time also *quadruples*.

- *The price for time AND quality is pressure*
  - Less is sometimes more, depending on the analytical goal.
- UHPLC gives us a greater freedom to exploit the principles of chromatography

Any questions?

Do you have additional questions or do you want to talk to an expert from Thermo Fisher Scientific?

Please send an E-Mail to analyze.eu@thermofisher.com and we will get back to you.