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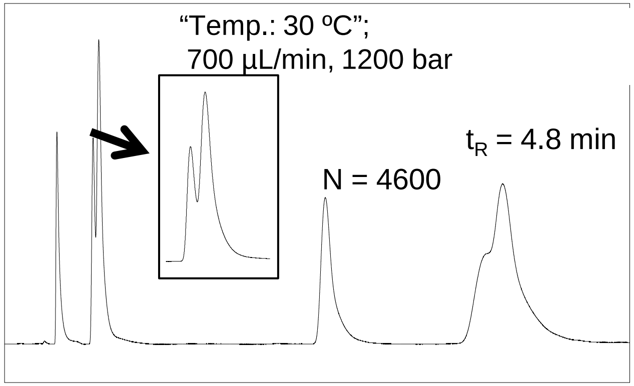
# From HPLC to UHPLC: Method Transfer, What Should I Pay Attention to?

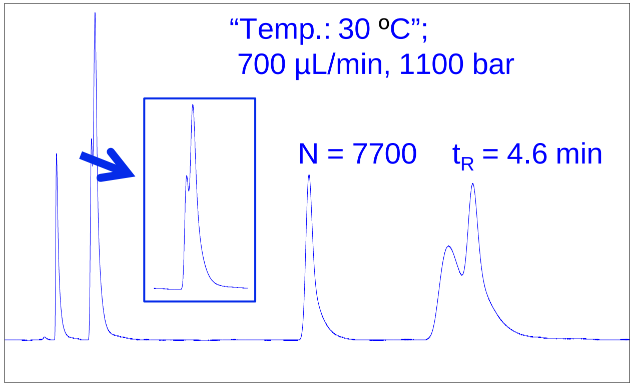
Dr. Frank Steiner
Thermo Fisher Scientific, Germering/Germany

# A Well-Known Situation in the Lab

- Why does a chromatogram recorded on system X…




- …look so different on system Y?



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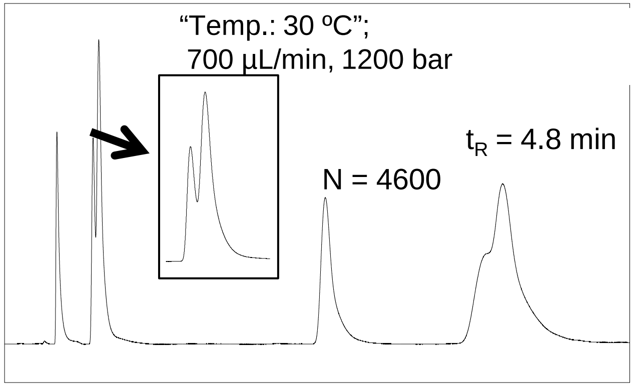


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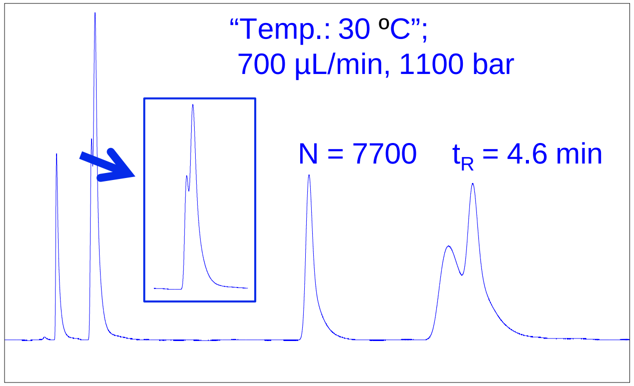


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  - Main reason: Gradient delay volume (GDV)

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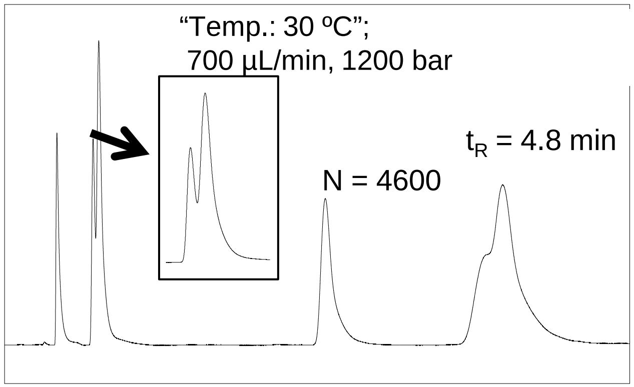


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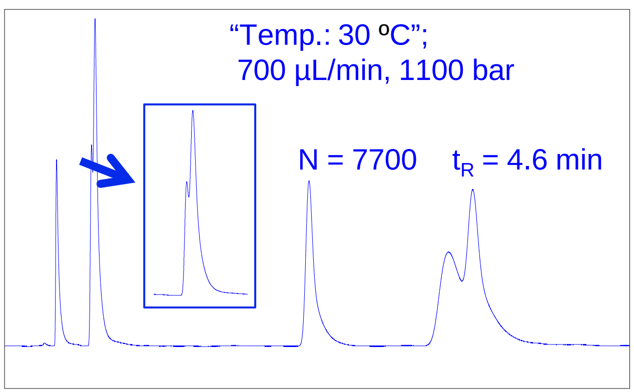
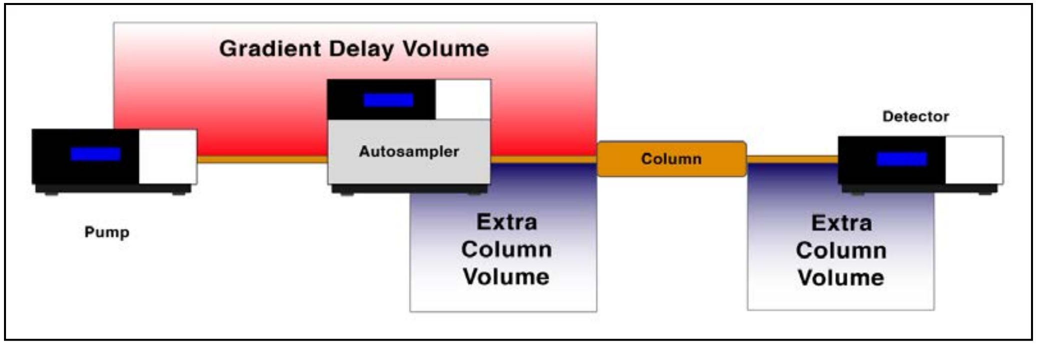



- Reasons are differences in the fluidics…
  - Gradient separations mostly affected
  - Impacts primarily retention times
  - Main reason:
    Gradient delay volume (GDV)
- …and the thermostatting concept
  - Affects both isocratic and gradient separations
  - Impacts retention times and peak shape (efficiency)
  - Reasons: Operational principle of the column thermostat, eluent pre-heating

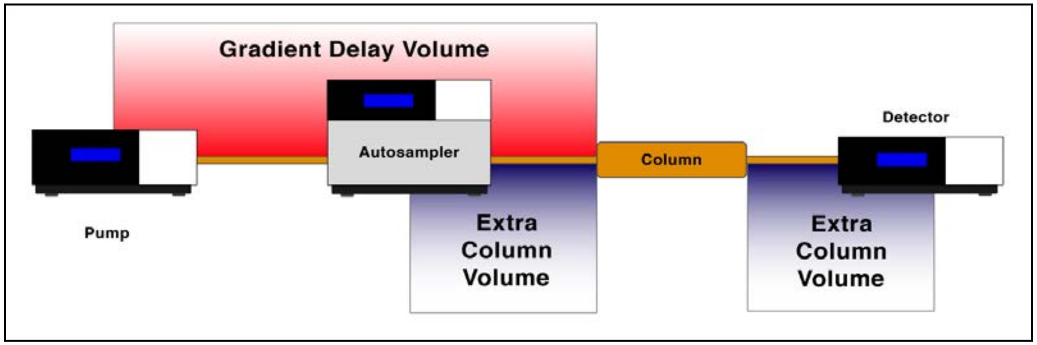
# The Gradient Delay Volume or even a Journey of a Thousand Miles begins with a Single (Isocratic) Step

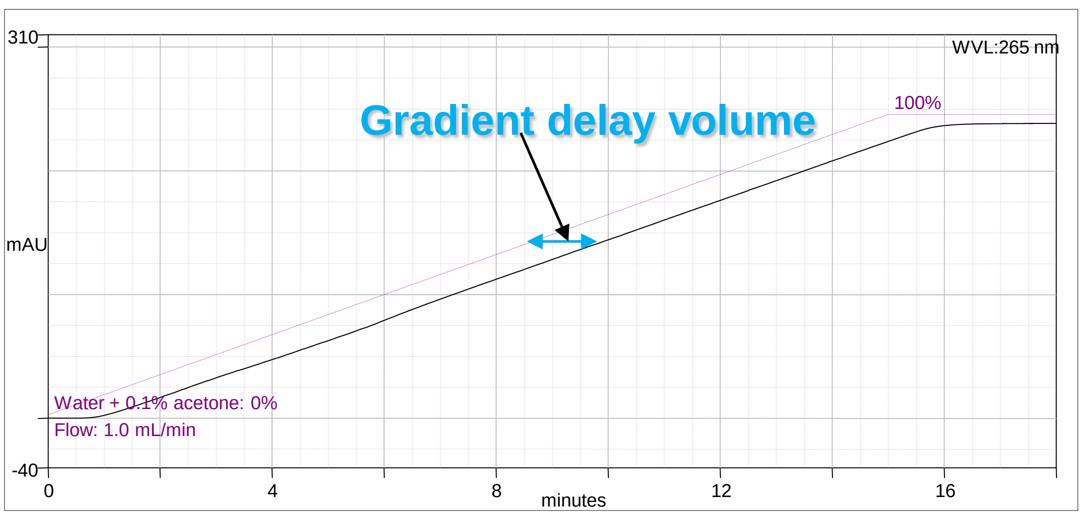
# The Gradient Delay Volume (GDV)




- GDV influences or generates
  - An isocratic step at the very beginning of every gradient separation
  - The gradient sharpness
  - The required equilibration time and thus the total analysis time
- Weakly retained compounds are much more affected by the GDV influence that late-eluting compounds.

# The Gradient Delay Volume (GDV)

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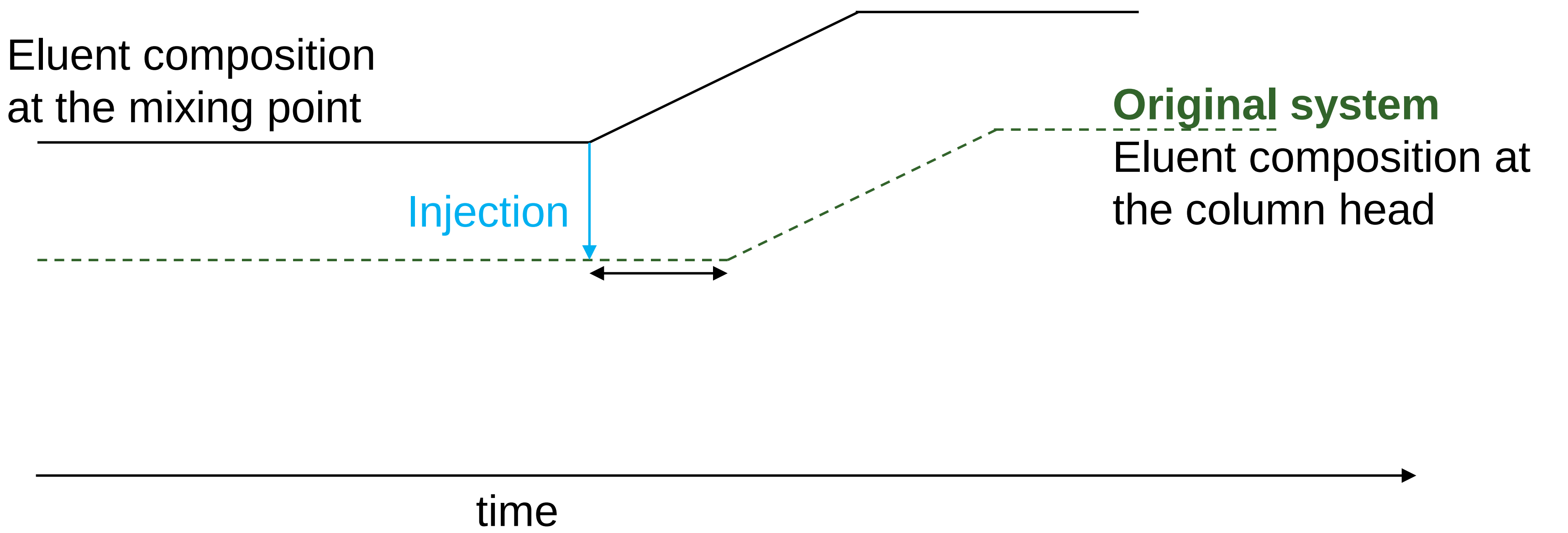
⇨ Needs special attention with steep/ballistic gradients and/or low flow rates

# What Does the GDV Do with My Method Transfer?



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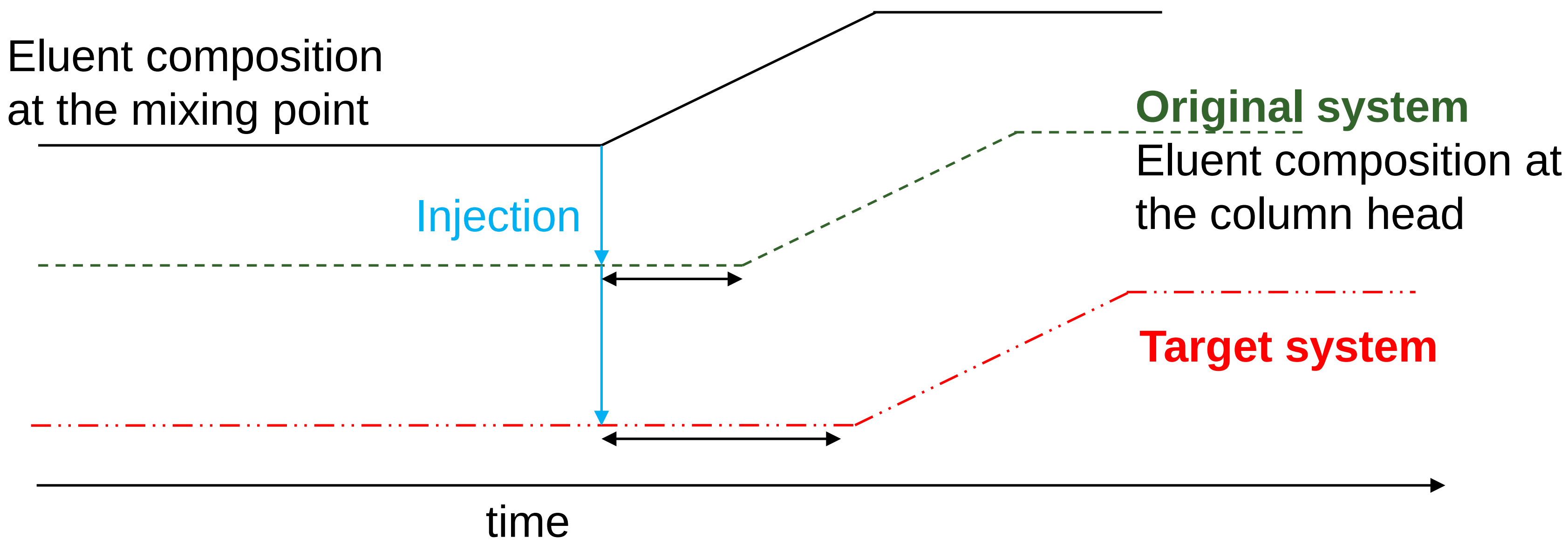


- Compare the GDVs (Represented as GDV per column volume)
- If the target system gives you a smaller isocratic segment:

  → Add an additional hold-up step before the gradient start to ensure identical isocratic start conditions
- If the target system gives you a larger isocratic segment:

  → Minimize the isocratic segment (E.g. with microflow kits, smaller tubing I.D., autosampler bypass)

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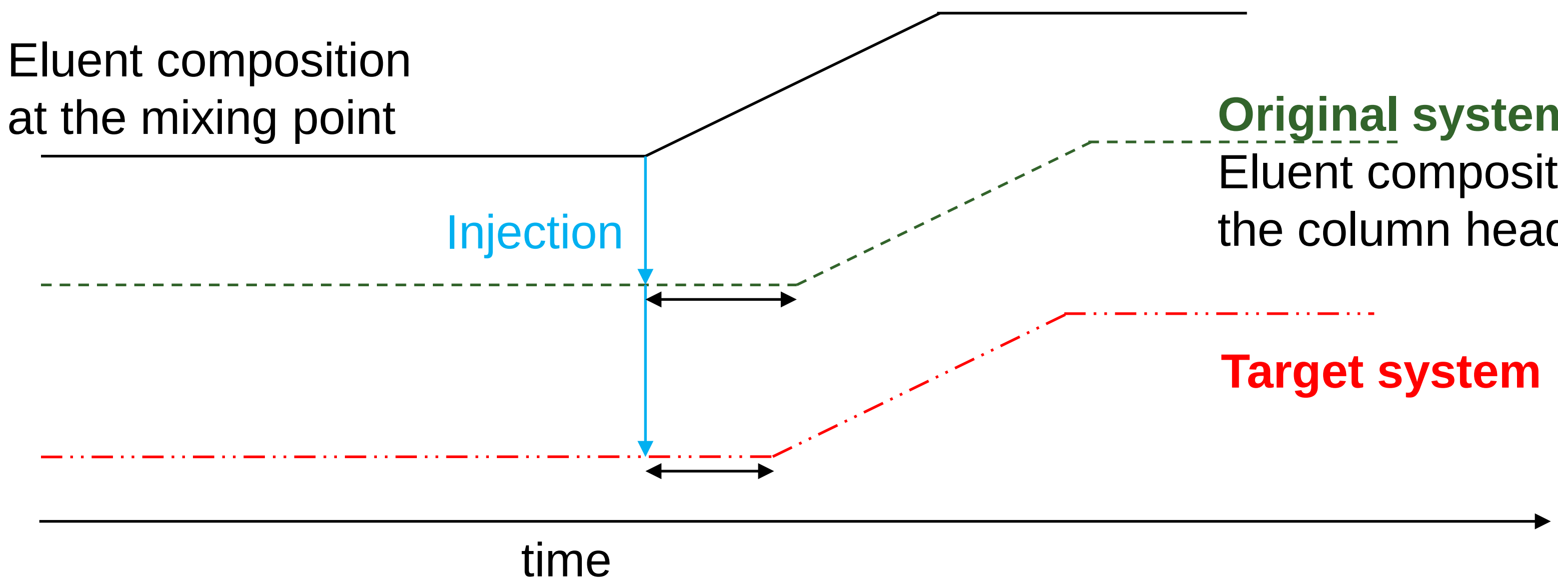


$$GDVper(CV) = \frac{GDV}{CV}$$

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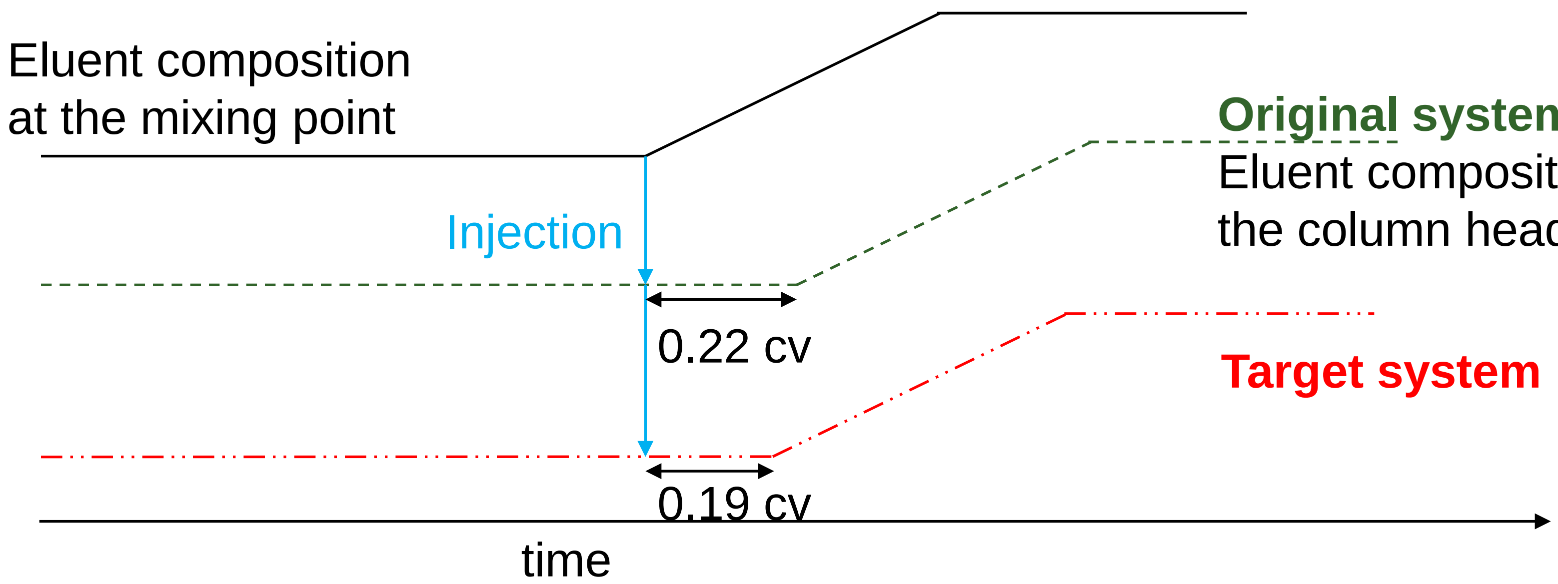


Original system with 0.9 mL GDV and 4.15 mL CV:

0.22 GDV per column volume

Target system with 0.1 mL GDV and 0.52 mL CV:

0.19 GDV per column volume

$$GDVper(CV) = \frac{GDV}{CV}$$

- Change the GDV to column volume ratio as little as possible!



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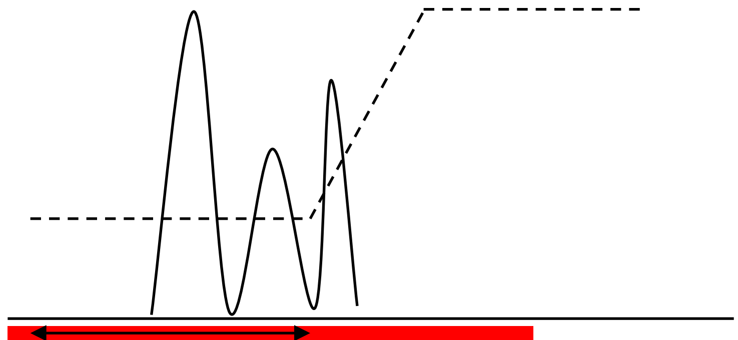
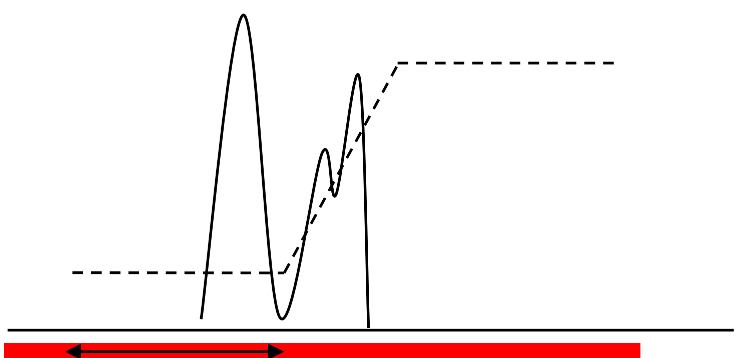
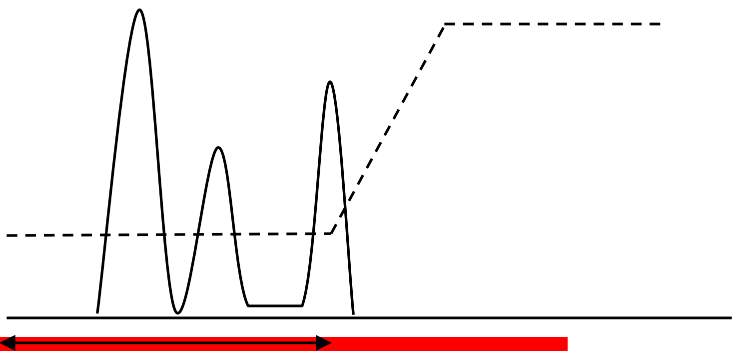
# Different GDVs and their Influence on Early-Eluting Compounds



Original system



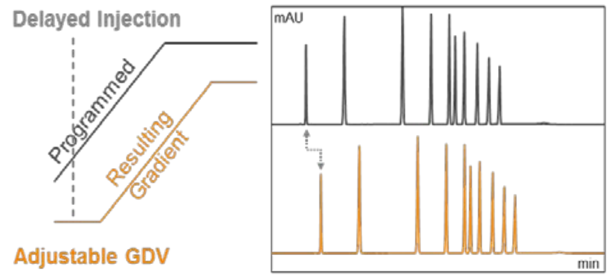
System with a smaller GDV



System with a larger GDV

# How a UHPLC System can Help to Handle GDV Challenges

- **Larger GDV required**
  - Add additional volumes to the flow path:
    - Individually settable metering device in the autosampler to fine-tune GDV
    - Adjust mixer and (Thermo Scientific™ Viper™) capillaries
  - Add isocratic hold-up step at the beginning of the separation






Chromeleon


Pump H and F


Sampler HT and FT

Thermo Scientific™ Chromeleon™ software


Thermo Scientific™ Vanquish™ **Horizon** system


Thermo Scientific™ Vanquish™ **Flex** system


Thermo Scientific™ UltiMate™ 3000 (Bio)RS system

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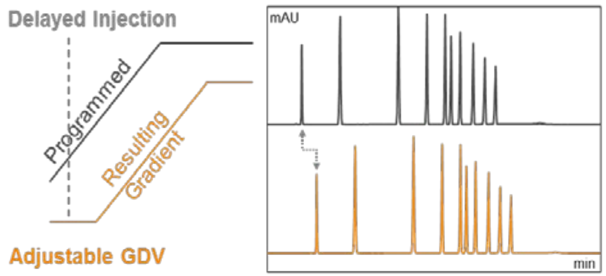
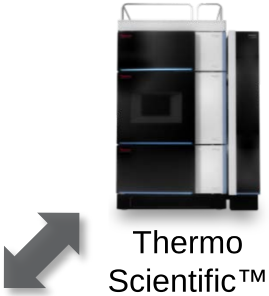



- **Smaller GDV required**
  - Reduce volumes in the flow path:
    - Individually settable Metering Device in the autosampler to fine-tune GDV
    - Adjust mixer and (Viper) capillaries
  - Delayed Injection at the program start


Chromeleon

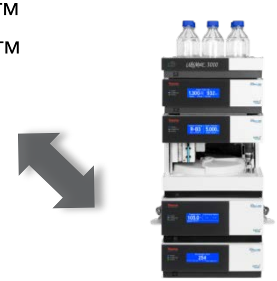
Pump H and F

Sampler HT and FT



Thermo Scientific™ Vanquish™ **Horizon** system

Thermo Scientific™ Vanquish™ **Flex** system



Thermo Scientific™ UltiMate™ 3000 (Bio)RS system

# Thermostatting with UHPLC or

# “Some like it Hot”

# The Role of Temperature in UHPLC Separations

- Temperature is a key factor for selectivity optimization.
- Retention depends inversely on temperature:

$$\ln k = -\frac{\Delta H^0}{R} \cdot \frac{1}{T} + \frac{\Delta S^0}{R} + \ln \beta$$ (Van‘t Hoff equation)

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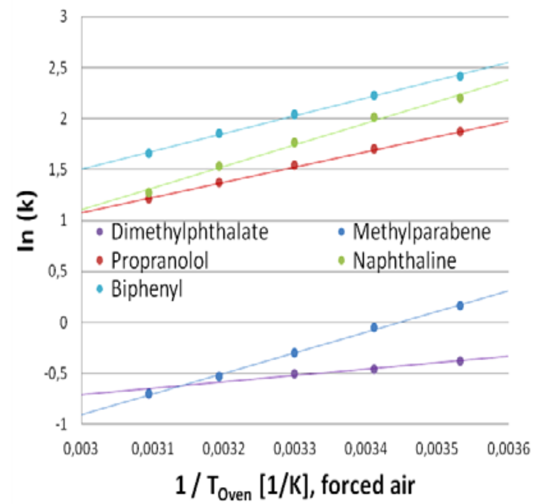


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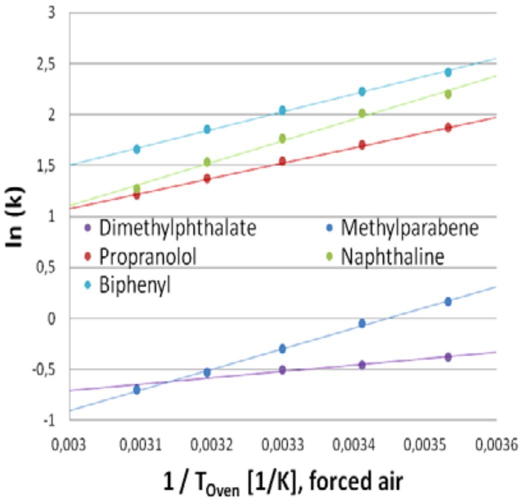


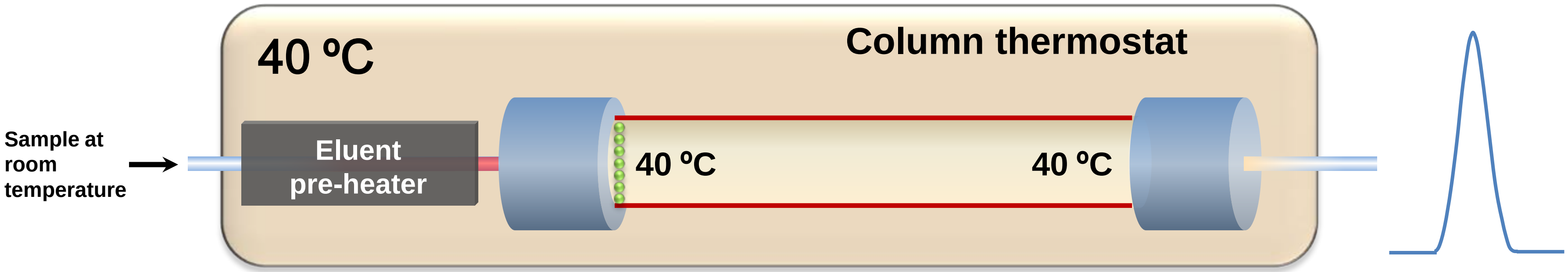

- Even minor temperature variations can lead to changes in the elution order.
- High pressure in UHPLC creates frictional heat inside the column.
- Two design concepts for column compartments:
  - Forced air thermostats – Continuous air circulation inside the column chamber
  - Still air thermostats – No air circulation with the goal to thermally insulate the separation column

- Still air thermostatting always leads to better chromatographic resolution due to higher efficiencies / plate counts.
- Therefore, UHPLC methods which measurably increase the temperature inside the column by viscous friction always benefit from still air thermostatting.

- Yes, but wait…:
  - This *can* be true, but it doesn‘t have to, if the rising internal column temperature results in a lower selectivity of critical peak pairs.

# Temperature Effect with Viscous Friction Inside the Column

HPLC column, run without notable frictional heating:



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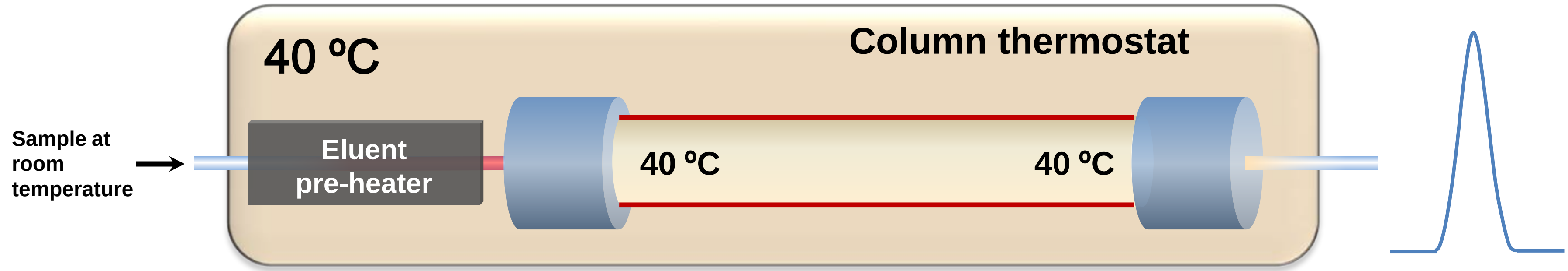
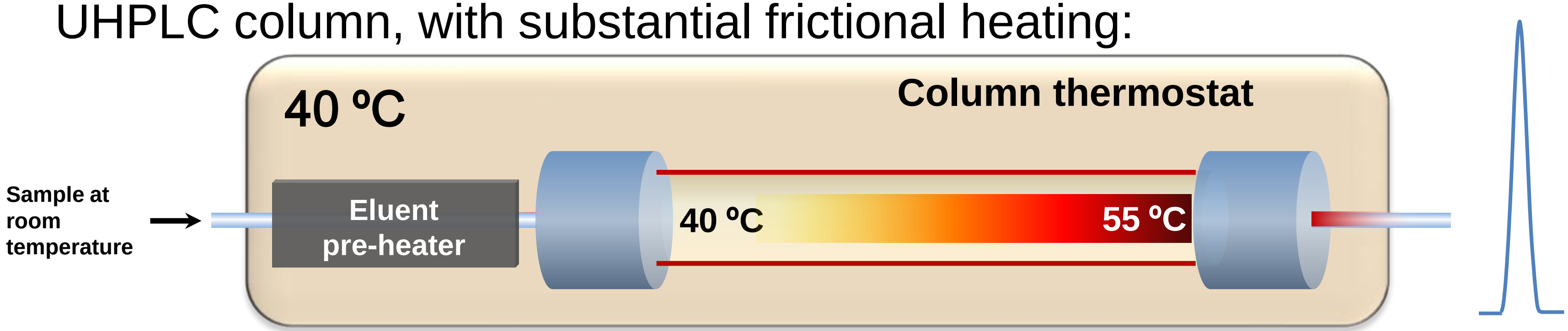



UHPLC column, with substantial frictional heating:



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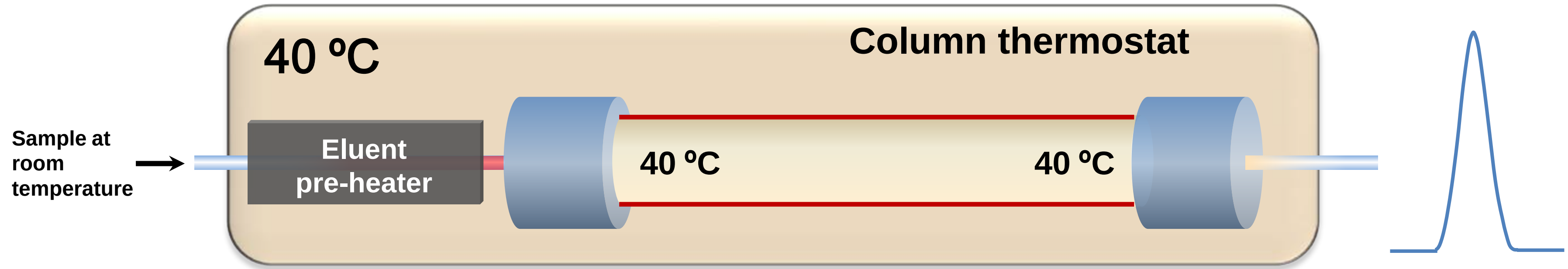


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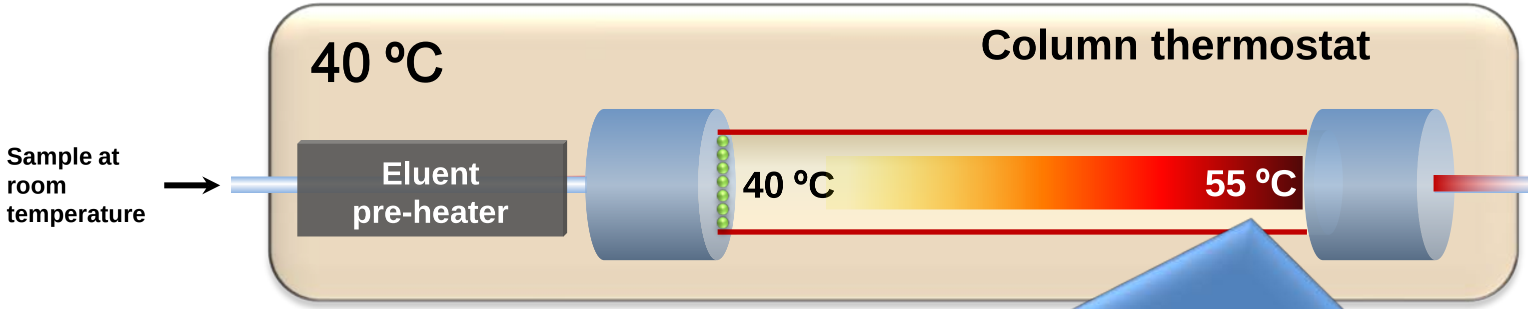


**Mismatch with radial temperature gradients:**

- Column heats up substantially beyond the compartment temperature
  - Lower viscosity leads to higher linear velocity
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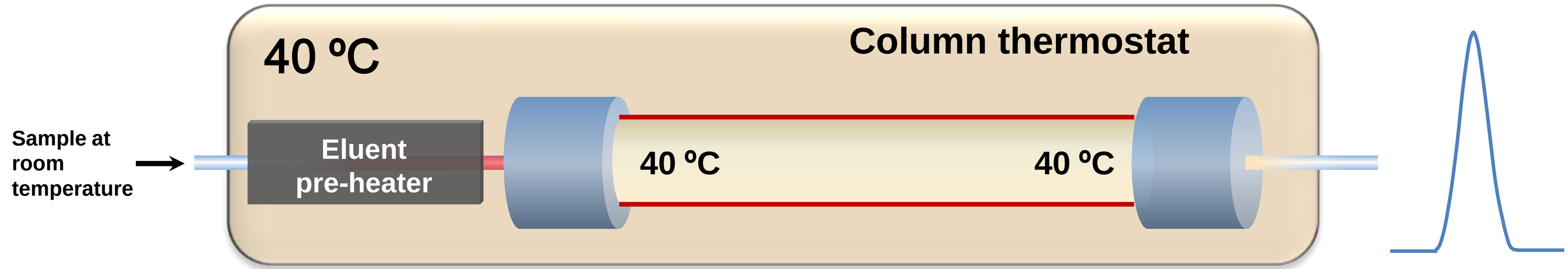


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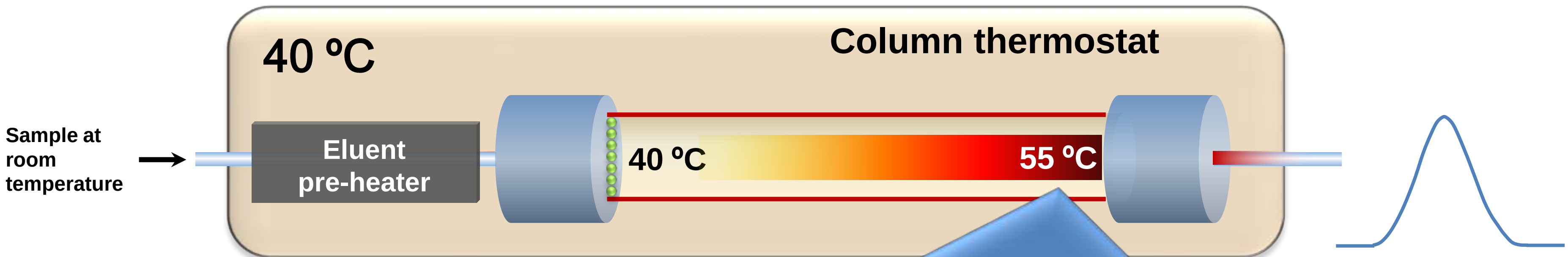


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# Frictional Heat Impacting Efficiency and Retention

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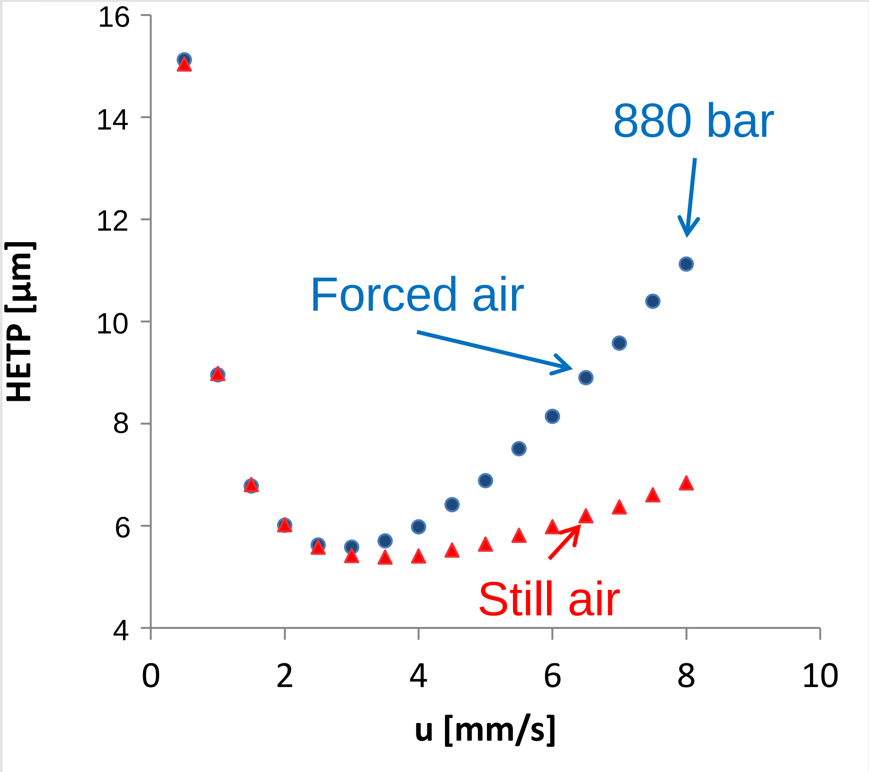


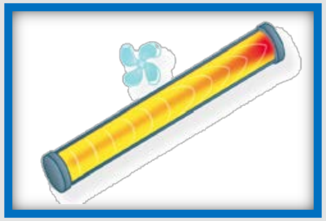

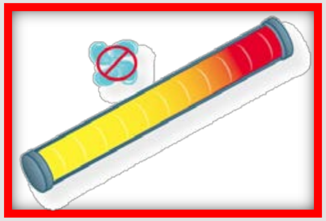

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| Mobile phase: | 50% water/ 50% acetonitrile |
| Sample: | Hexanophenone |
| Temperature: | CC and pre-heater 30 ºC, forced air and still air, resp. |

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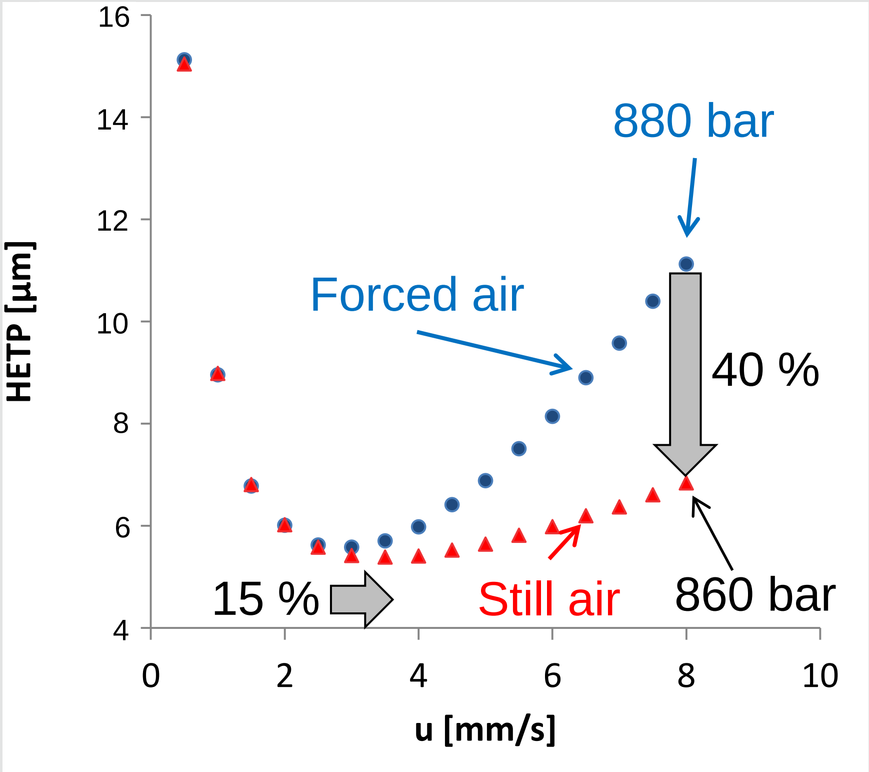


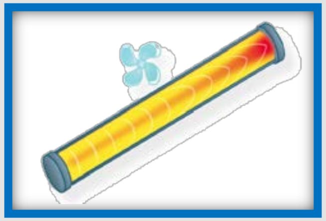

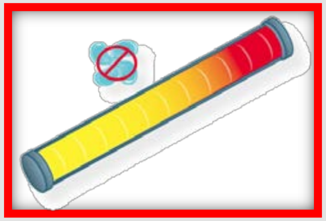

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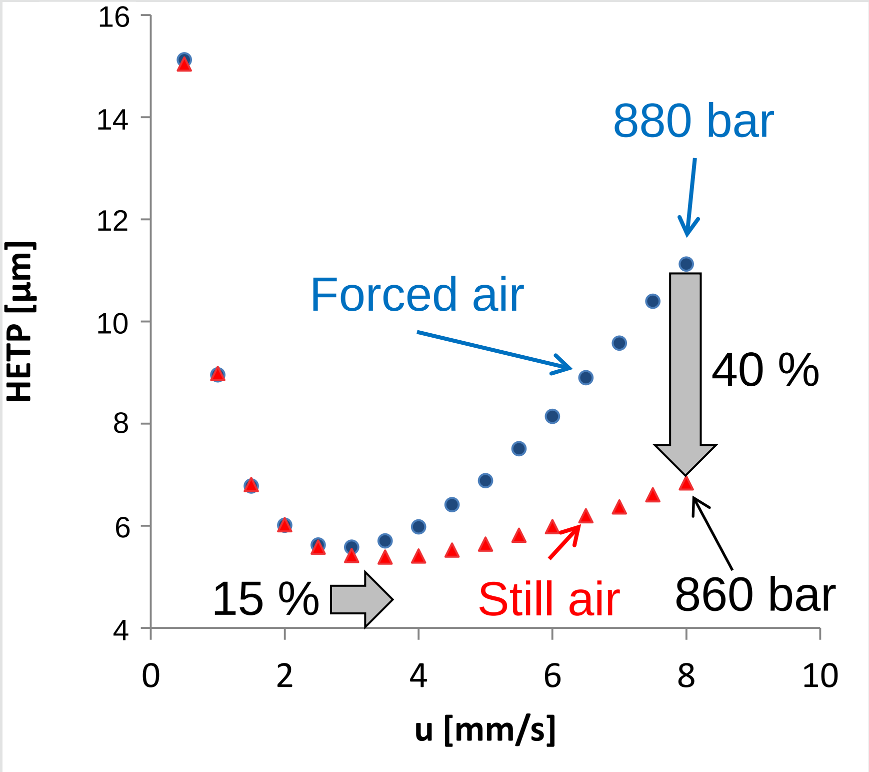
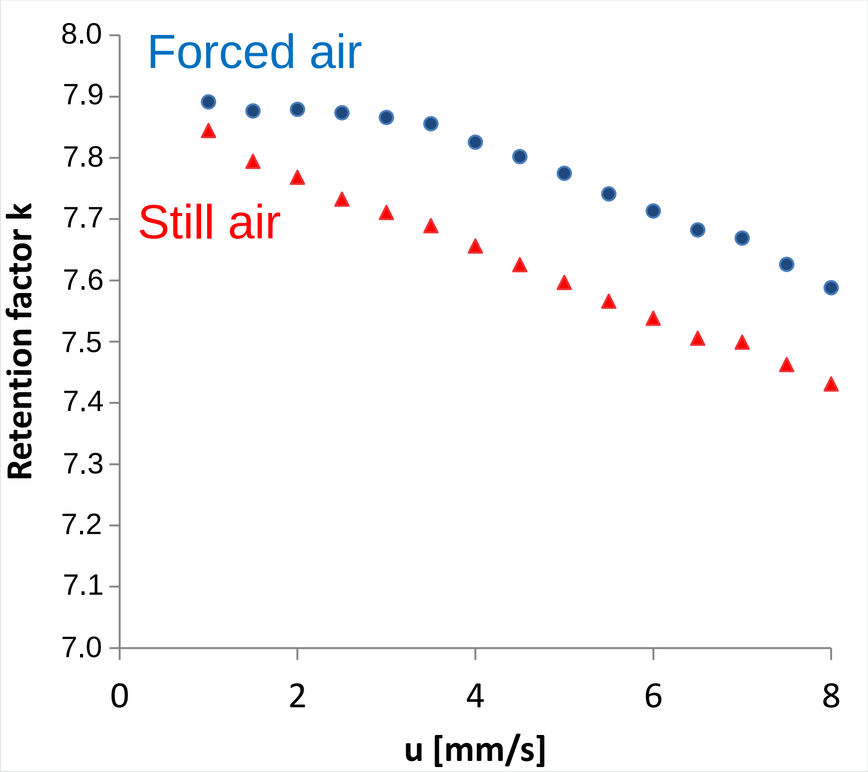
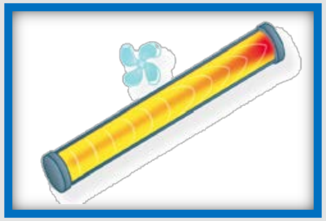
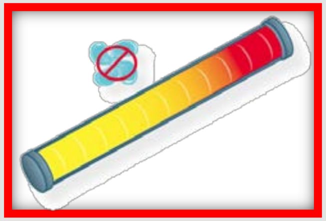



## Retention:





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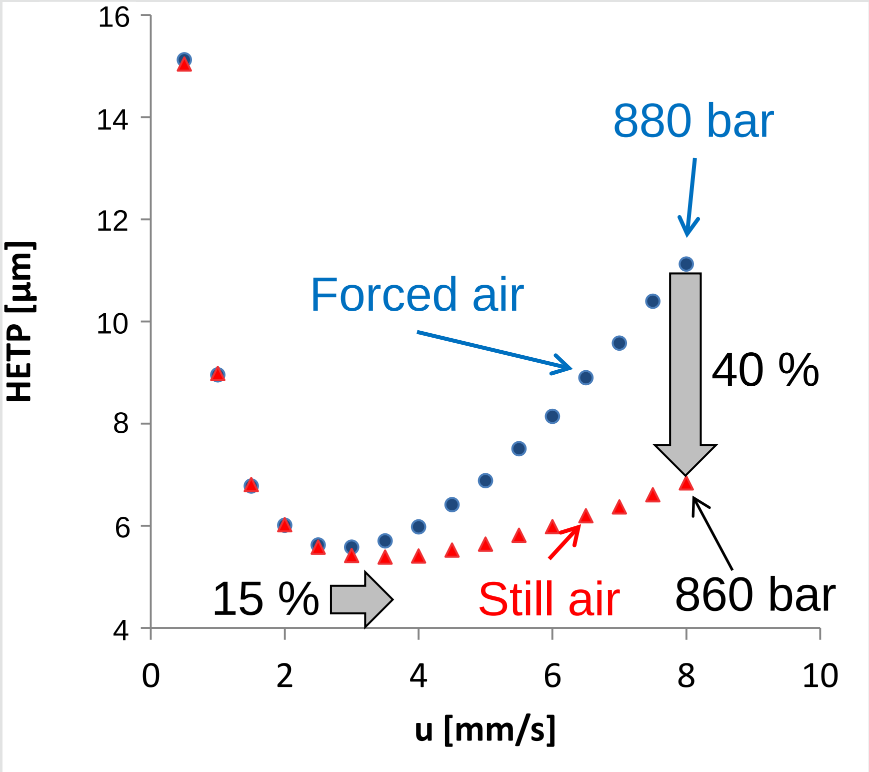


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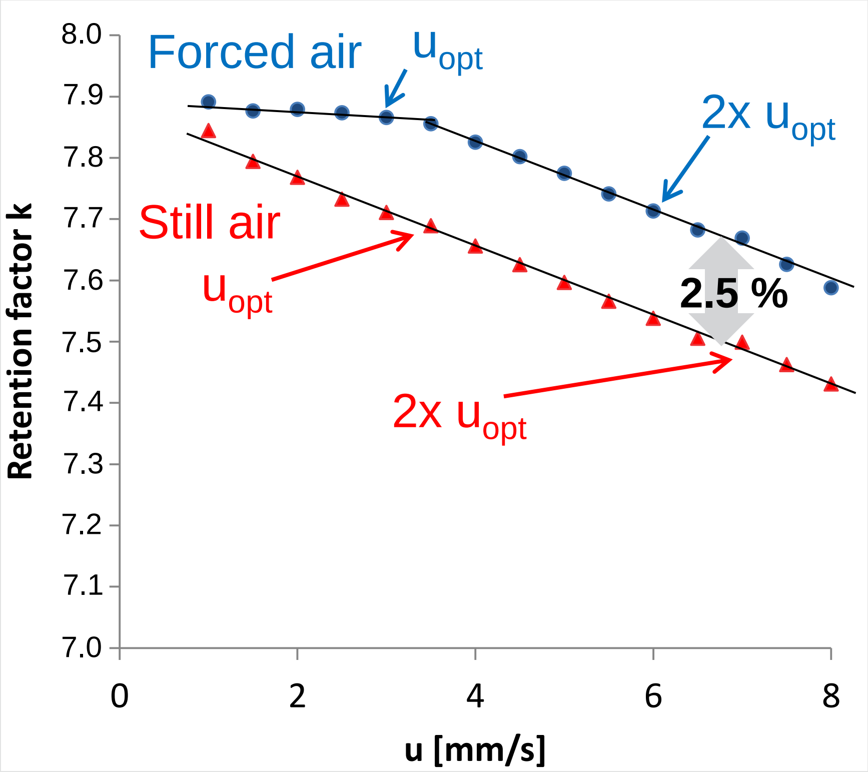


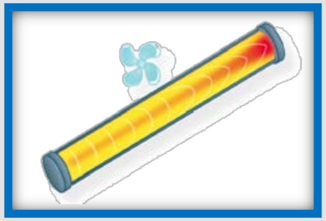

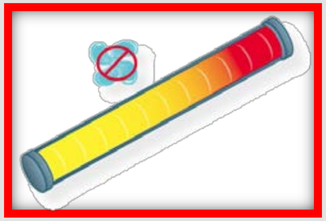

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# Measured Temperature Increase in Both Modes

**Experimental conditions:**

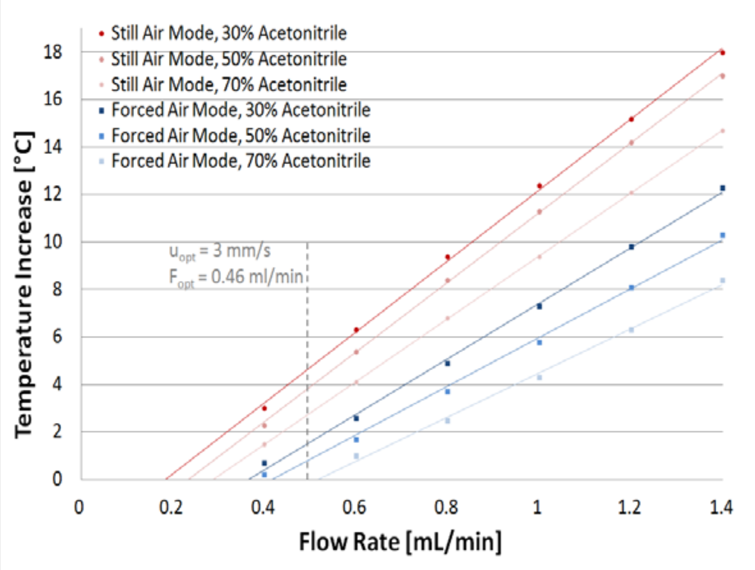
Instrument: Vanquish Horizon UHPLC System

Column: Hypersil GOLD, 1.9 µm,
2.1 x 100 mm

Eluent: $H_2O$/ACN

Flow rate: up to 1.40 mL/min

Temperature: 30 °C (Column compartment and eluent pre-heater)




| 50% acetonitrile | Still air mode | | Forced air mode | |
|---|---|---|---|---|
| Flow rate [mL/min] | Pressure [bar] | Temperature increase [°C] | Pressure [bar] | Temperature increase [°C] |
| 0.2 | 173 | 0.0 | 177 | 0.0 |
| 0.4 | 343 | 2.3 | 356 | 0.2 |
| 0.6 | 506 | 5.4 | 527 | 1.7 |
| 0.8 | 659 | 8.4 | 685 | 3.7 |
| 1.0 | 809 | 11.3 | 830 | 5.8 |
| 1.2 | 958 | 14.2 | 975 | 8.1 |
| 1.4 | 1079 | 17.0 | 1099 | 10.3 |



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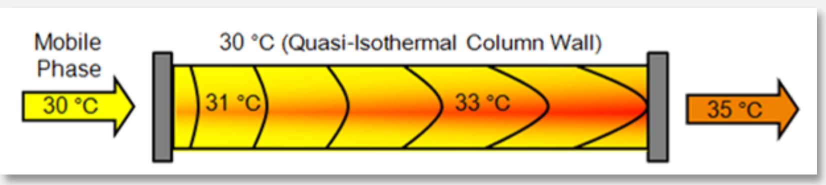
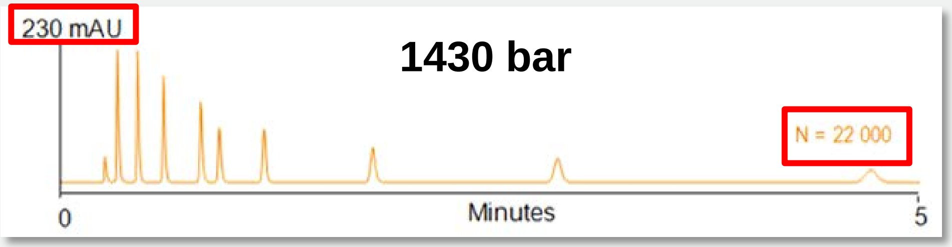
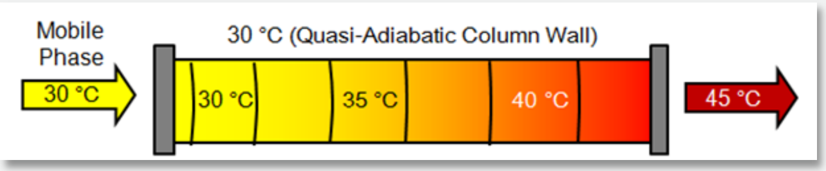
# Effects Resulting from Viscous Friction at 1400 bar

**Experimental conditions:**

| | |
|---|---|
| **Column:** | Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm |
| **Sample:** | Uracil, acetanilide and 8 alkylphenones |
| **Inj. volume:** | 1 µL |
| **Eluent:** | 45:55 $H_2O$:ACN (v/v) |
| **Flow rate:** | 0.65 mL/min |
| **Thermostatting:** | Compartment and active pre-heater at 30 ºC, fan speed 5 (forced air) or fan speed 0 (still air) |







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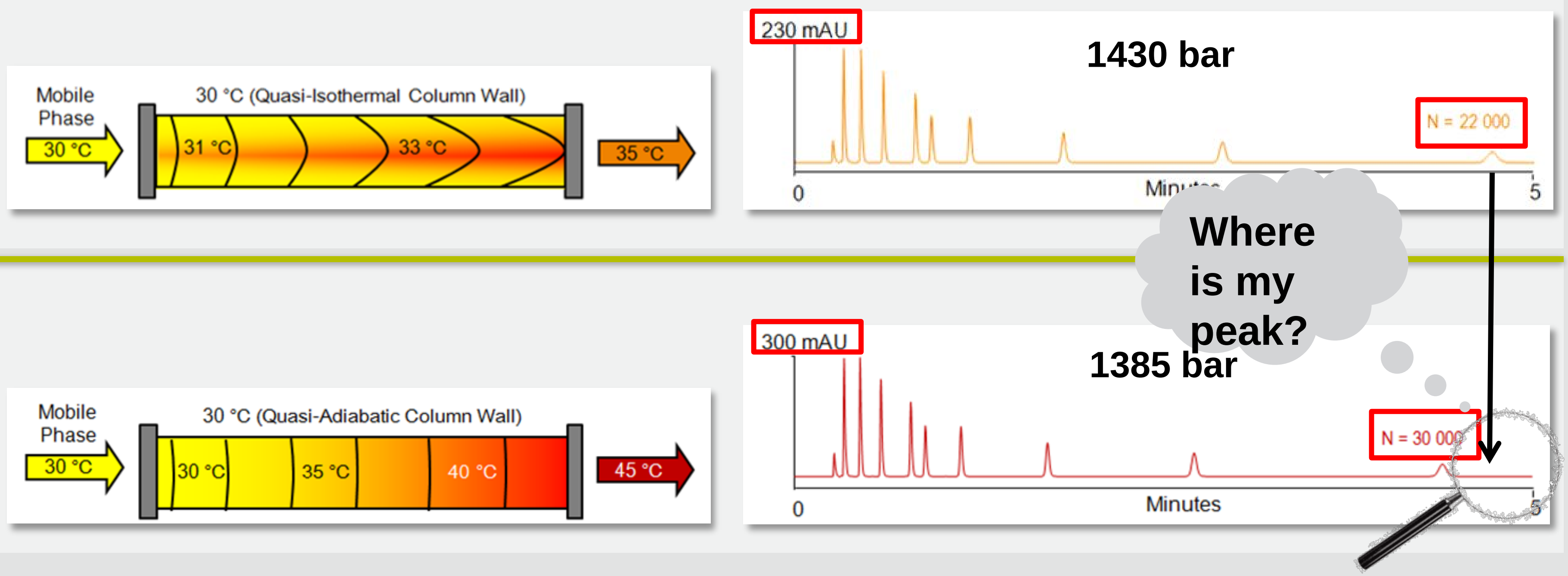

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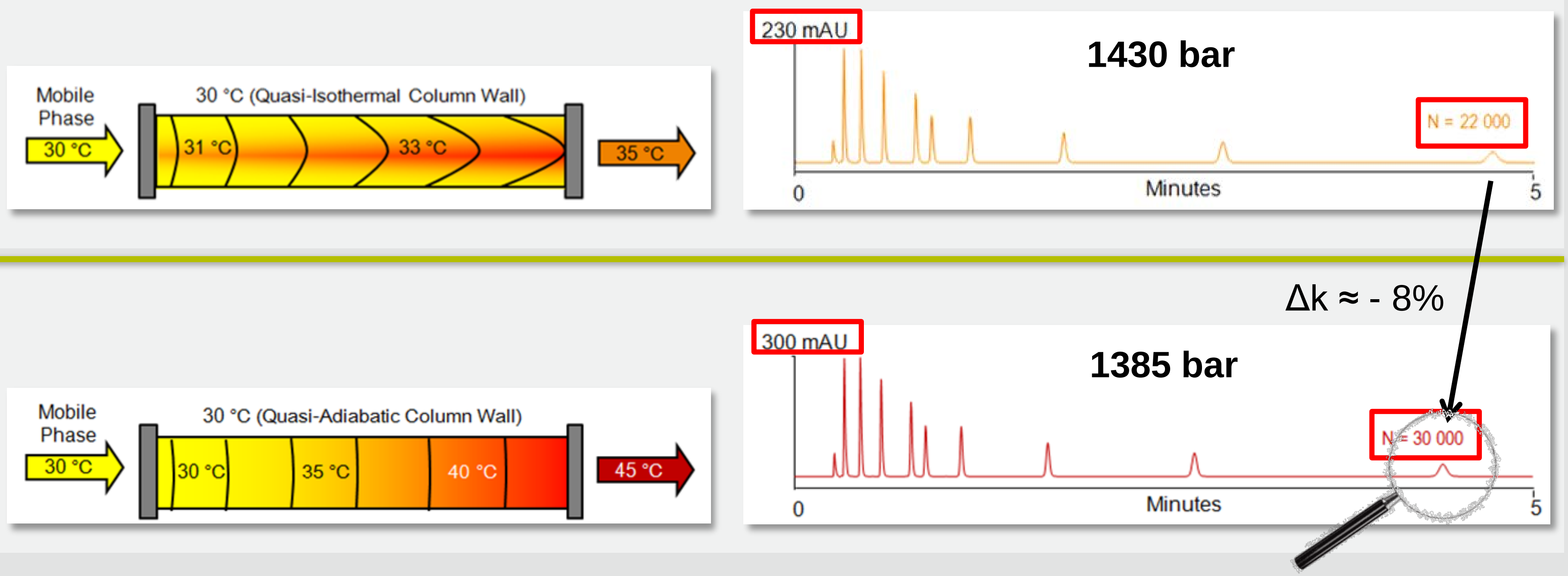
| **Sample:** | Uracil, acetanilide and 8 alkylphenones |
| **Inj. volume:** | 1 µL |
| **Eluent:** | 45:55 $H_2O$:ACN (v/v) |
| **Flow rate:** | 0.65 mL/min |
| **Thermostatting:** | Compartment and active pre-heater at 30 ºC, fan speed 5 (forced air) or fan speed 0 (still air) |



# Effects Resulting from Viscous Friction at 1400 bar

**Experimental conditions:**

| | |
|---|---|
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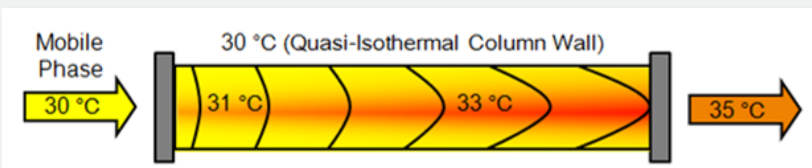
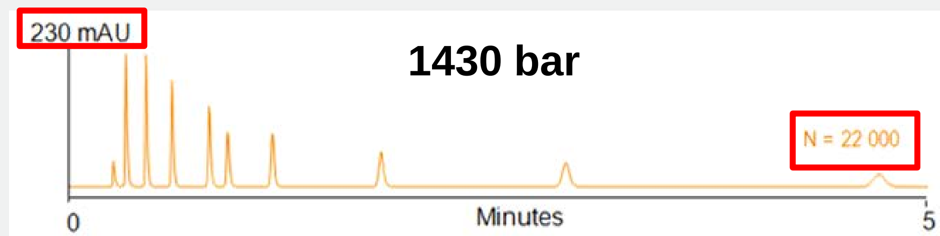
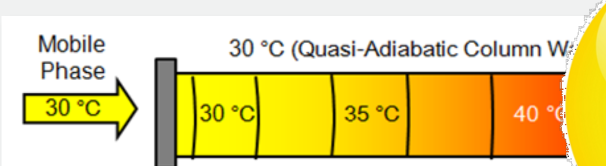
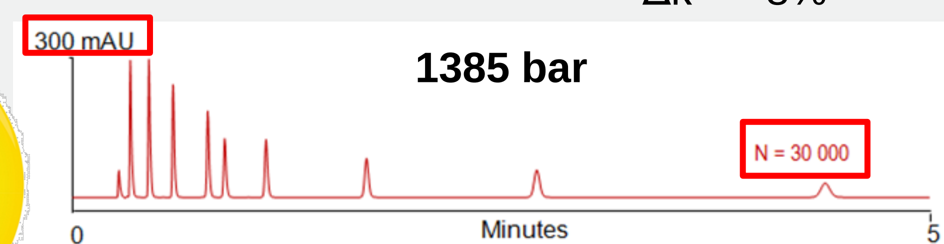


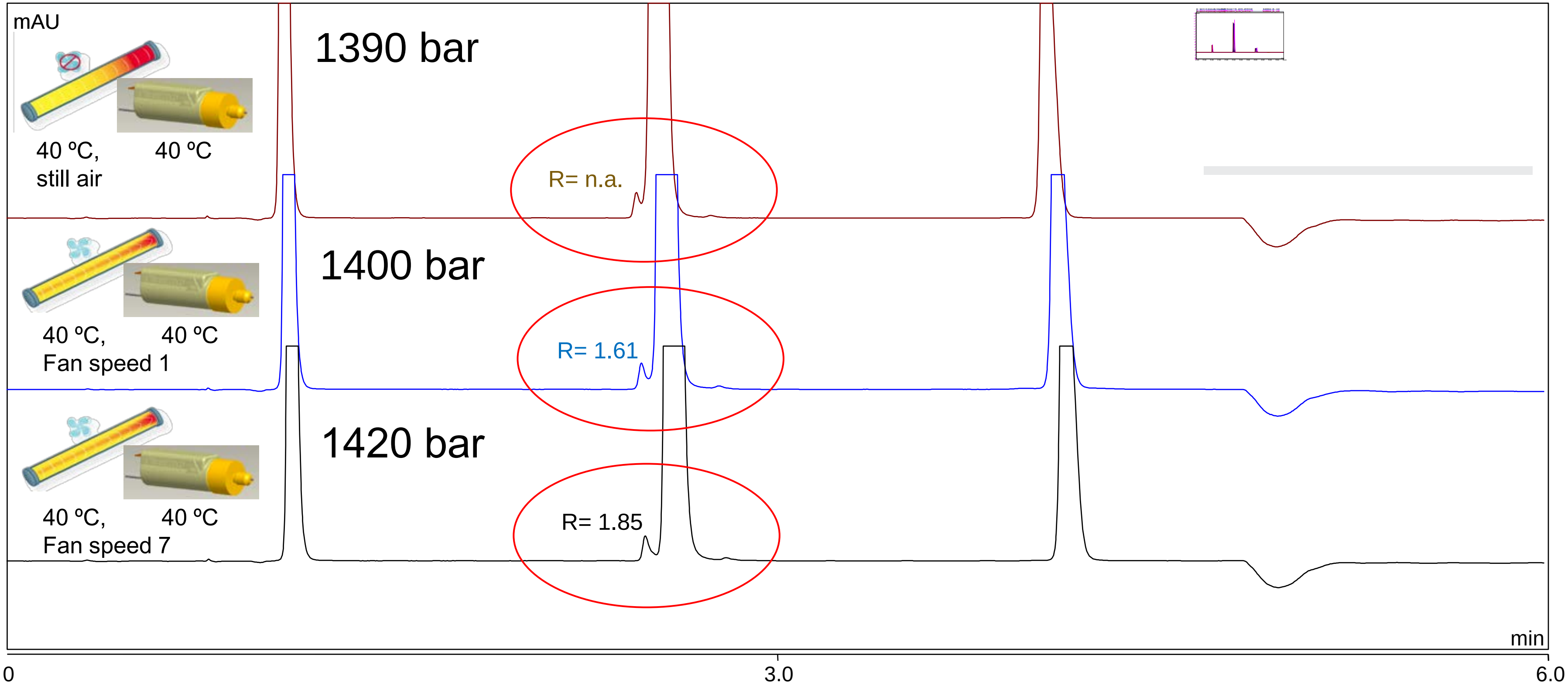

Δk ≈ - 8%




Still air is the better mode

# Fan Speed Variation with Another Separation Example




| | |
|---|---|
| **Instrument:** | Vanquish Horizon UHPLC system |
| **Gradient**: | From 5% to 30% B in 4 min, A: 0.1% formic acid in $H_2O$, B: ACN |
| **Column:** | Hypersil GOLD Vanquish, 1.9 µm, 2.1 x 200 mm |
| **Sample**: | Mix of gallic acid, caffeic acid, and salicylic acid |
| **Detection**: | UV 300 nm, 20 Hz, 0.2 s response time |
| **Flow rate**: | 0.8 mL/min |

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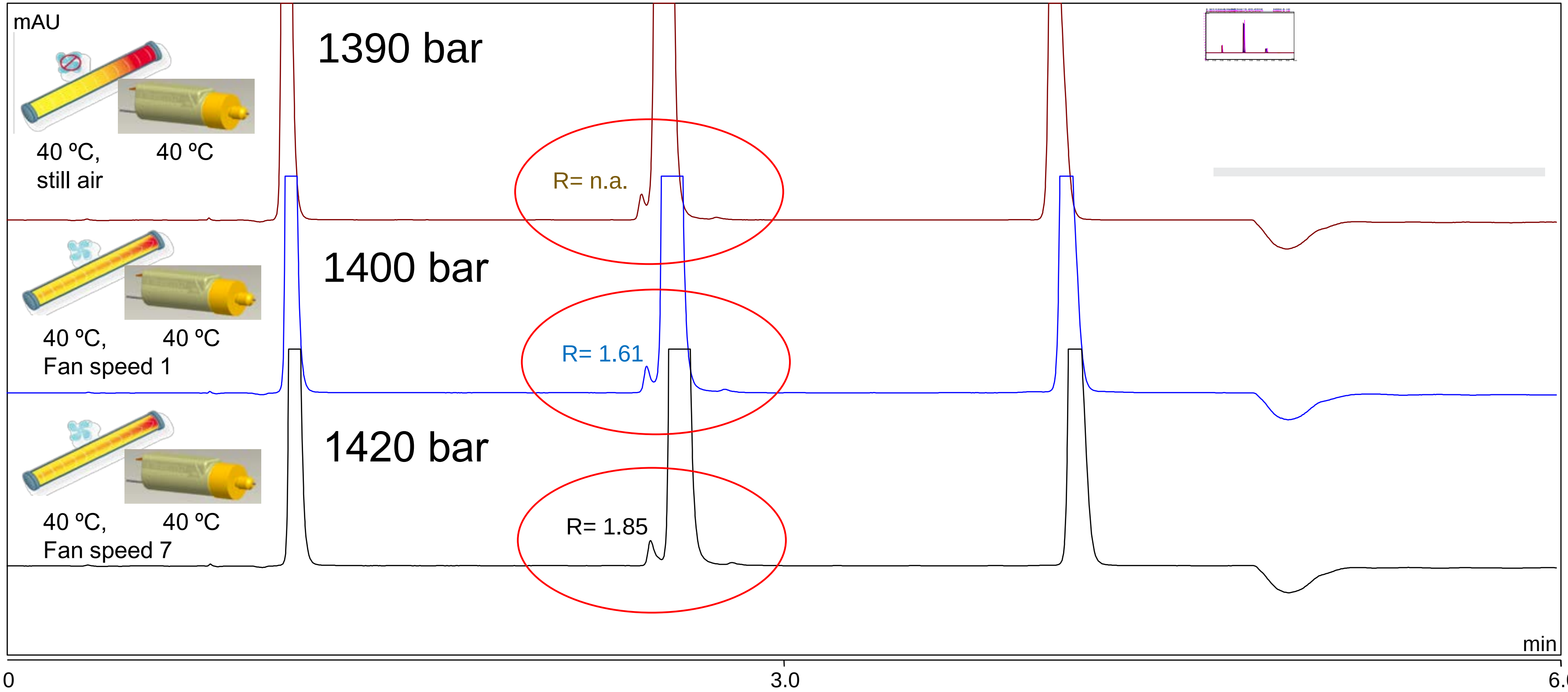


- Enhanced air circulation lowers the column temperature.
- The selectivity for the impurity peak improves.

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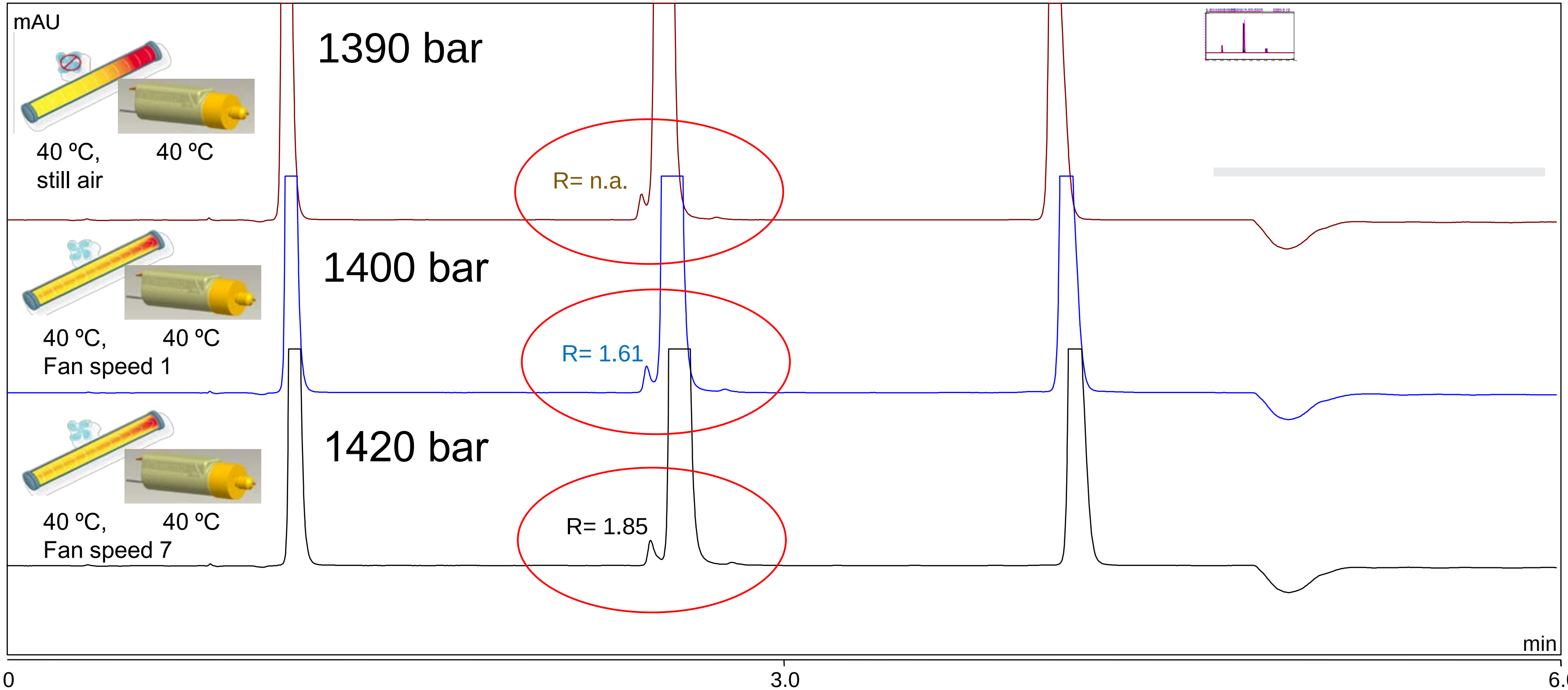


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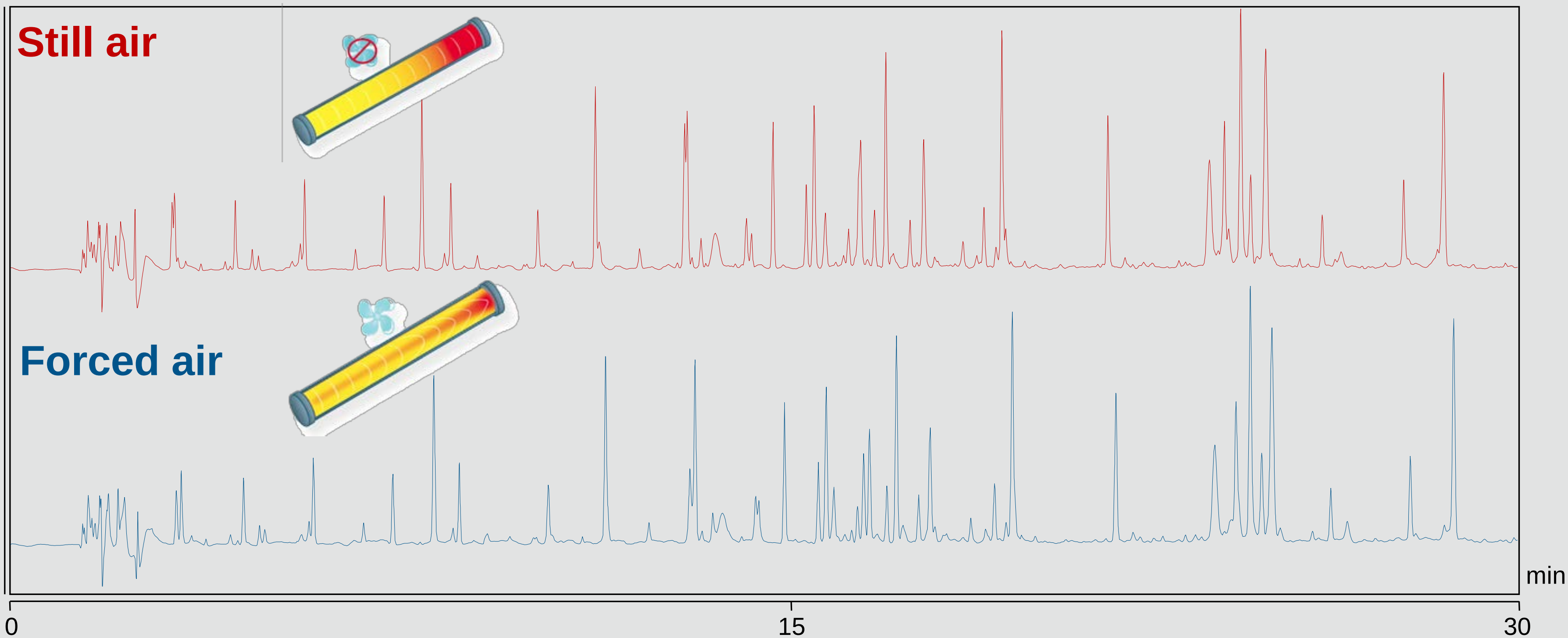
Forced air is the better mode

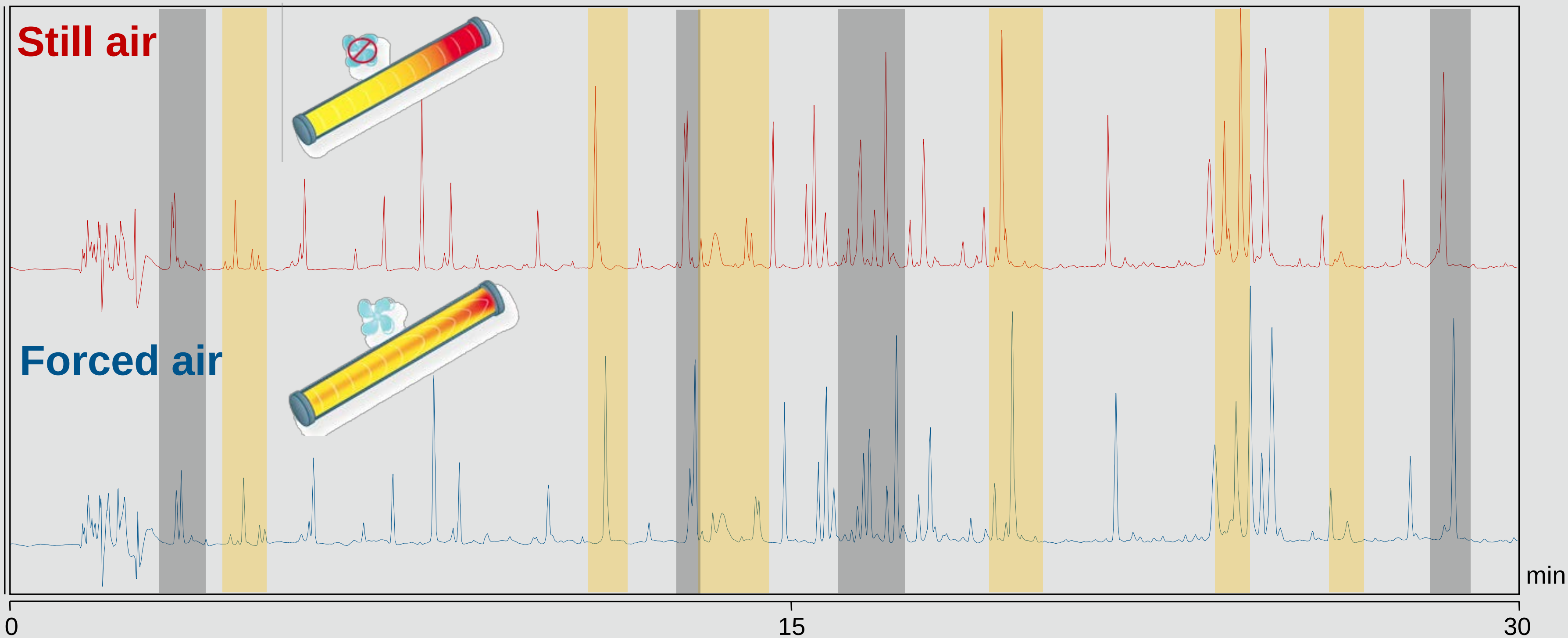
# Column Thermostatting for Peptide Mapping




**Vanquish Horizon UHPLC system** with 10 mm Thermo Scientific™ Lightpipe™ flow cell
Column: Thermo Scientific™ Acclaim™ RSLC 120 C18, 2.2 µm, 2.1 x 250 mm
Sample: 1 µL Monoclonal IgG Tryptic Digest (2 mg/mL)
Detection: 214 nm, 100 Hz
Temperature: CC and pre-heater 80 °C, **still air** and **forced air**, respectively

Mobile phase A: TFA 0.05% in water
Mobile phase B: TFA 0.05% in 2/8 water/ACN
Flow rate: 400 µL/min
Gradient: From 4% to 55% B in 30 min

# Column Thermostatting for Peptide Mapping

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Mobile phase A: TFA 0.05% in water

Mobile phase B: TFA 0.05% in 2/8 water/ACN

Flow rate: 400 µL/min

Gradient: From 4% to 55% B in 30 min

- Still air mode beneficial ➔ Partially better resolution due to enhanced efficiency
- Forced air mode beneficial ➔ Partially better resolution due to enhanced selectivity

➡ Both modes are complementary

# Summary: Variation of Frictional Heat Dissipation

**These effects are relevant with methods beyond 500 bar:**

- Air circulation in the column thermostat is hardware-depending.
- Still air thermostats typically provide better resolution with frictional heat.
- Different thermostats can lead to different chromatograms even with the same effective temperature.

**Hints:**

- Vary the forced air ventilation if feasible (Vanquish UHPLC systems).
- Try to insulate the column inside a forced-air thermostat.
- Try to vary the position of the column inside the thermostat.

# Conclusions

**Gradient delay volume (GDV)**

- The GDV decides on how long the sample is separated isocratically by the hold-up at the beginning of the gradient elution.
- Knowing and adjusting the GDV are decisive for the successful method transfer.
- Different GDVs between two systems can be adjusted
  - Either by the fluidics (e.g. by changing fluidic parts).
  - Or by changing the gradient program.
- An individually adjustable metering device in the autosampler of the Vanquish UHPLC systems allows an easy and automated GDV adjustment.

Vanquish **Horizon**

Vanquish **Flex**

UltiMate 3000 (Bio)RS

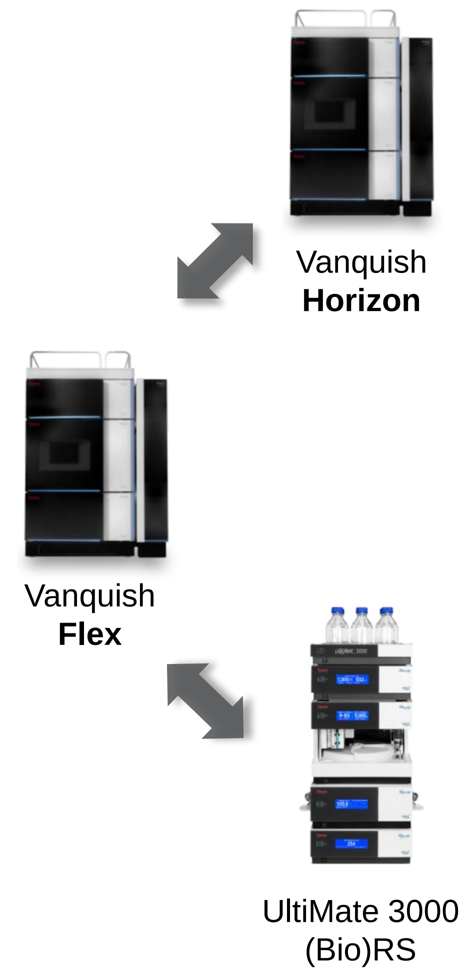


ThermoFisher SCIENTIFIC

# Conclusions

**Thermostatting**

- Column thermostats are frequently underrated – “no oven is like the other”
- The operation principle of the thermostat has a substantial impact on the success of a method transfer
- **Still air thermostats**
  - Lock the frictional heat in the column
  - Thus lead to better efficiencies and sharper peaks
  - But internal frictional heating has the potential to change selecitvities
- **Forced air thermostats**
  - Dissipate frictional heat from the column surface
  - Which may result in peak distortion due to a radial temperature gradient
- Both modes are complementary and can be used for fine-tuning of a separation



# 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.**