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The Role of Temperature in HPLC –
How Column Thermostatting and Mobile Phase Pre-Conditioning Details enable to take full Advantage of It

Frank Steiner
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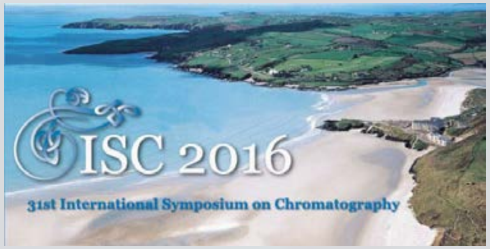
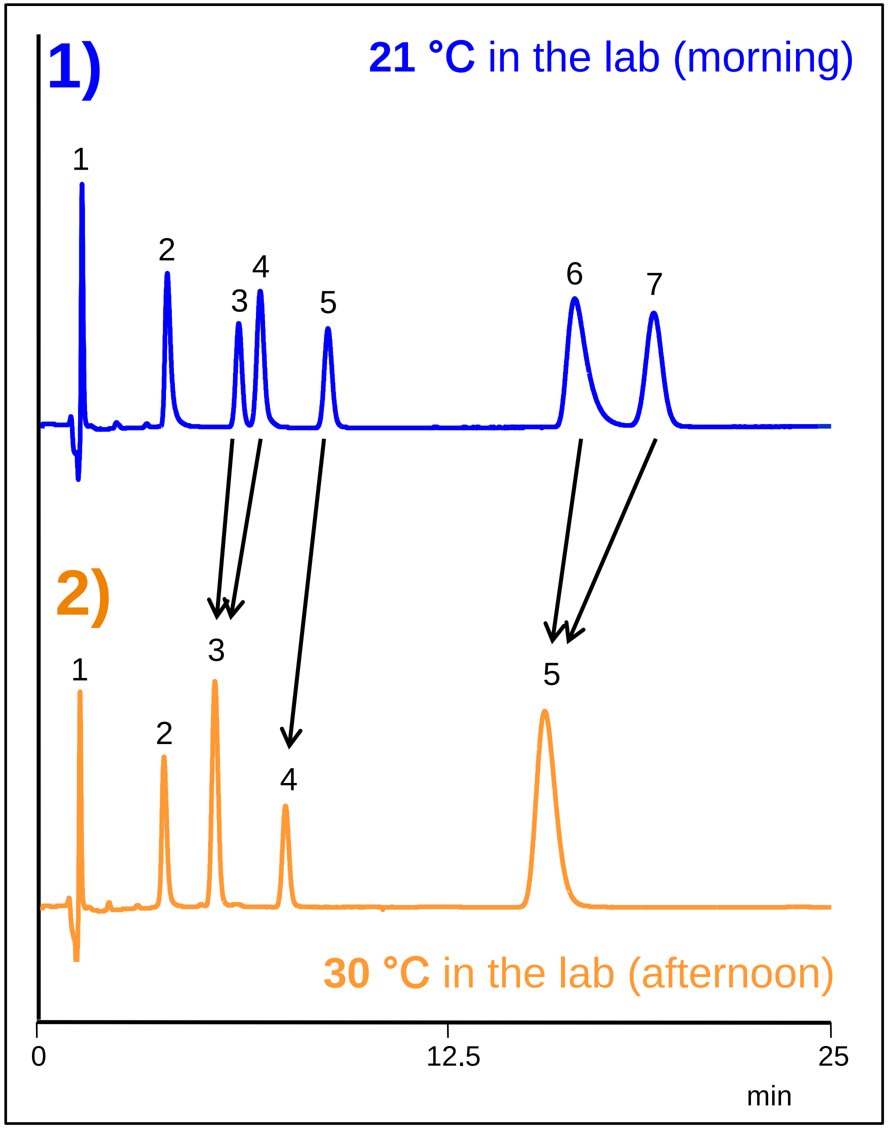


Outline

- How temperature rules retention and selectivity
- Temperature effects on peak shapes and analysis speed
- Importance of the specific thermostatting technique
- Separating eluent pre-heating from columns thermostatting

A Scenario in an HPLC-Lab without Column Thermostatting

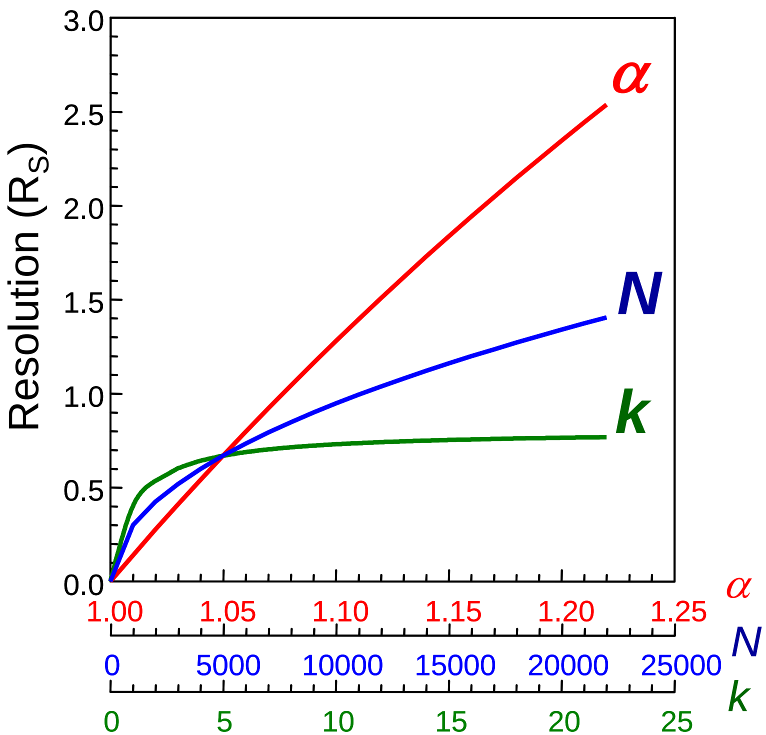


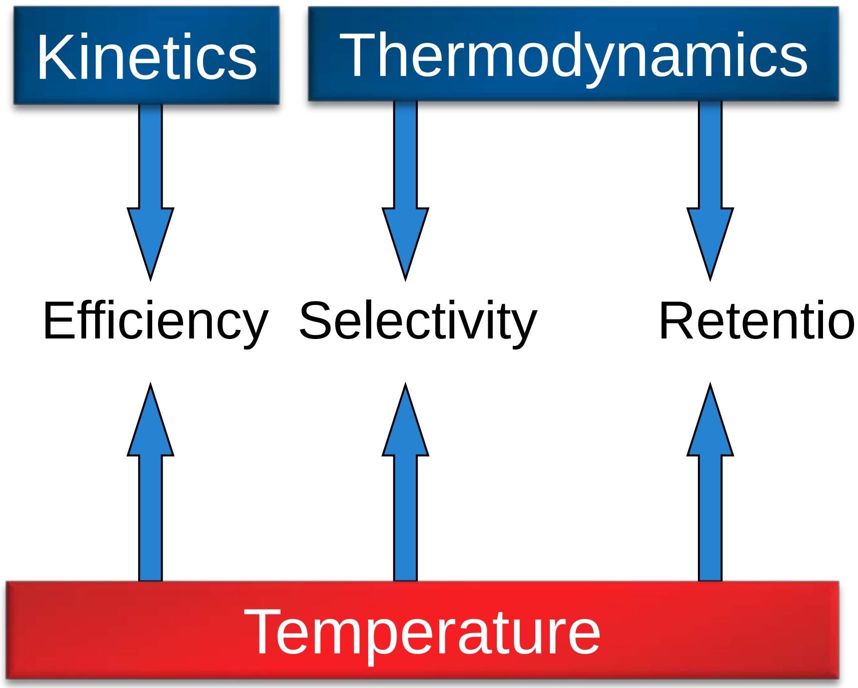
- We injected a standard test sample twice in a long sequence and the results were not anywhere near.
- No column temperature control and varying lab conditions morning to afternoon
- In some applications, temperature can have a dramatic influence on chromatographic results.

3 Parameters that Affect Peak Resolution in HPLC

Purnell equation:

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





- Most important parameter for resolution optimization in HPLC ➔ **Selectivity**
- Efficiency also plays a significant role (but much less than in GC)

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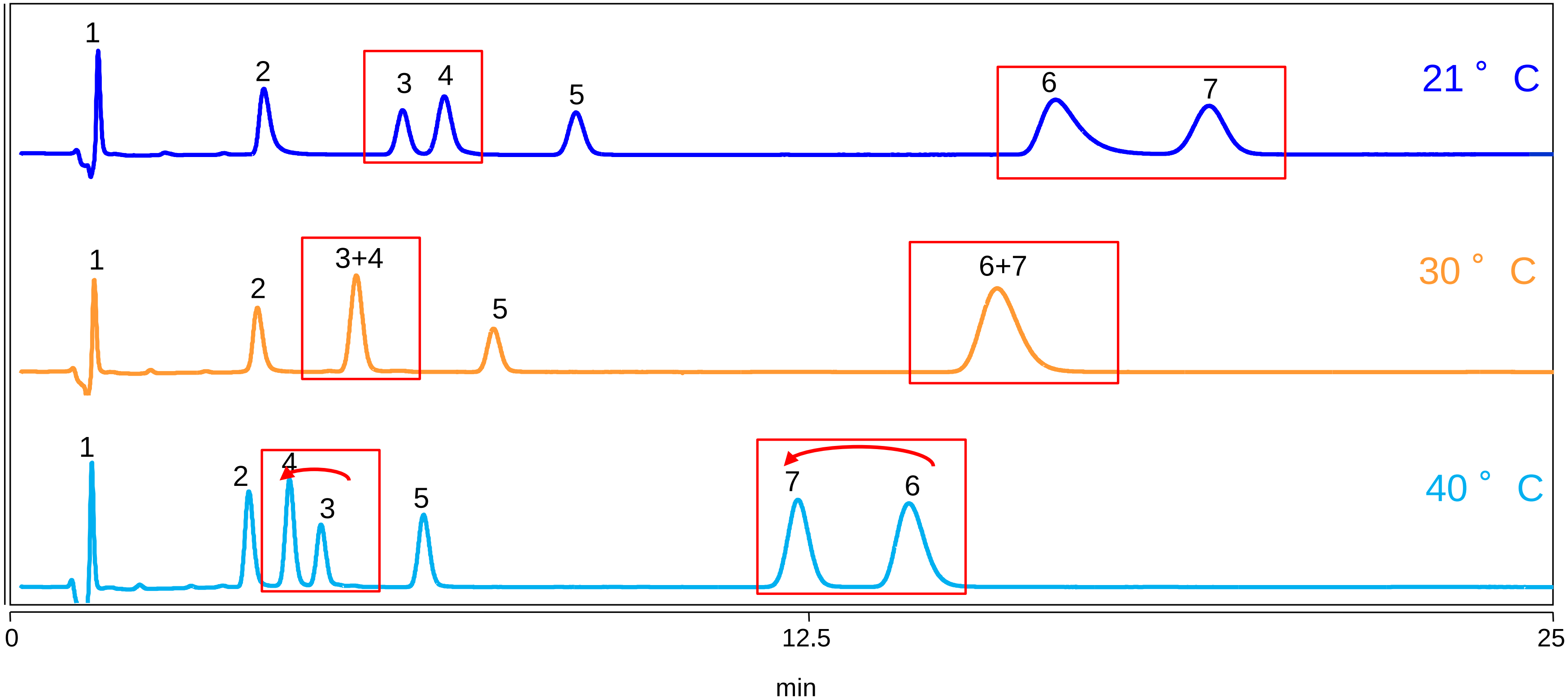
Temperature Impact on Chromatographic Selectivity



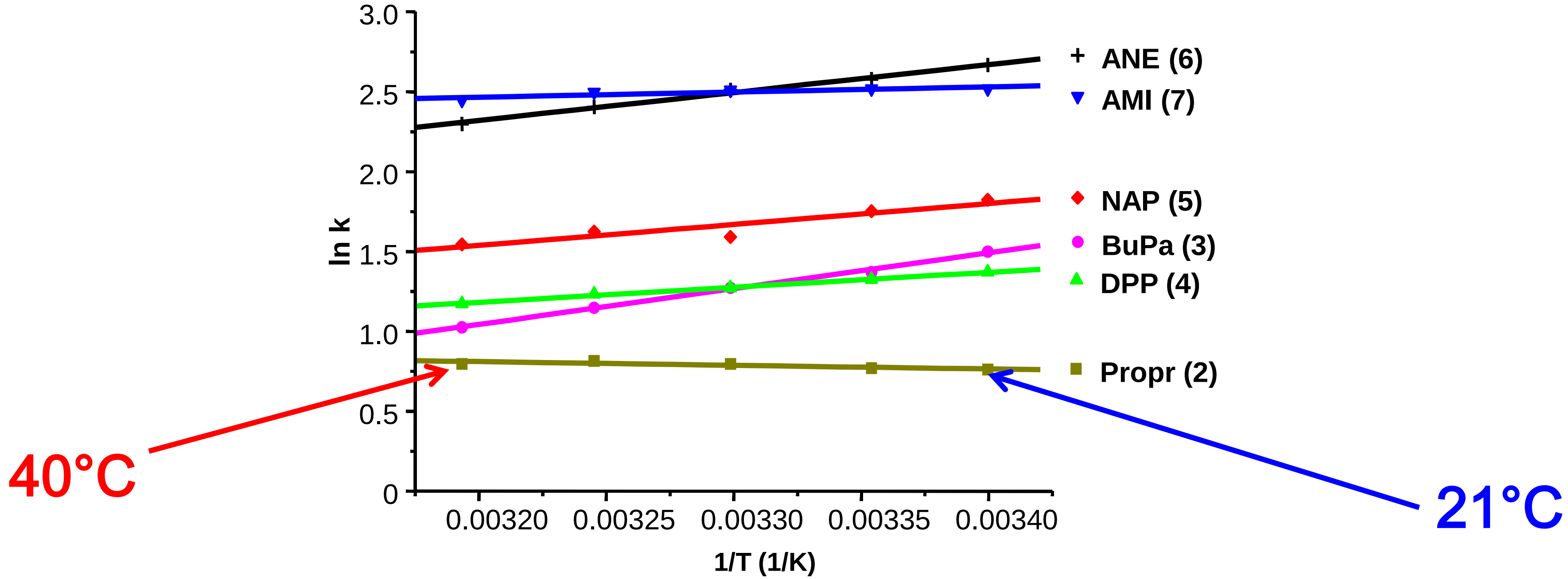
Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC System

Column:	ProntoSIL C18 ACE EPS, 125 x 4 mm (Bischoff)
Injection:	5 µL of a standard test sample (see below)
Detection:	210 nm, 100 Hz
Temperature:	see Figure
Mobile Phase:	20 mM phosphate buffer pH=7 / methanol 35/65 v/v
Isocratic:	mixed by pump (‘dial-a-mix’)
Flow rate:	1000 µL/min

Model sample: 1) Uracil, 2) Propranolol, 3) Dipropylphthalate, 4) Butylparaben, 5) Naphthalene, 6) Amitriptyline, and 7) Acenaphthene

Retention as Function of Temperature: Van't Hoff Plot

Retention data from method in slide 5 recorded at 21°C, 25 °C, 30°C, 35°C, 40°C:



- Thermodynamic data can be derived from chromatographic experiments
- Van't Hoff equation: $$lnk = -\frac{\Delta H^0}{R} \cdot \frac{1}{T} + \frac{\Delta S^0}{R} + ln\beta$$
- Rule of thumb: **Δ*k* equals approx. 3 % per Δ°C**

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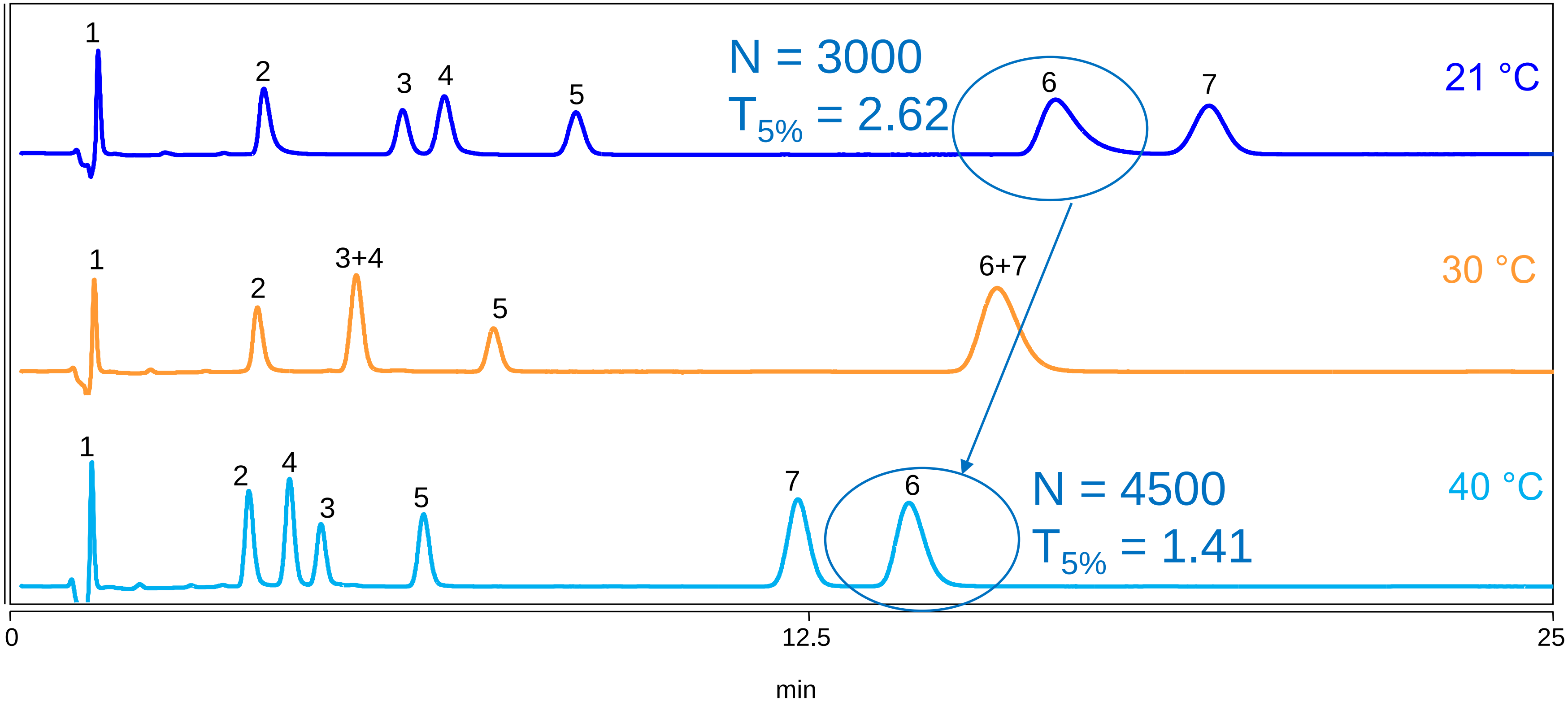
Temperature Impact on Peak Shape

Amitriptyline (6) is a strong base and mainly retained through cation exchange at silanols



UltiMate 3000 RSLC System

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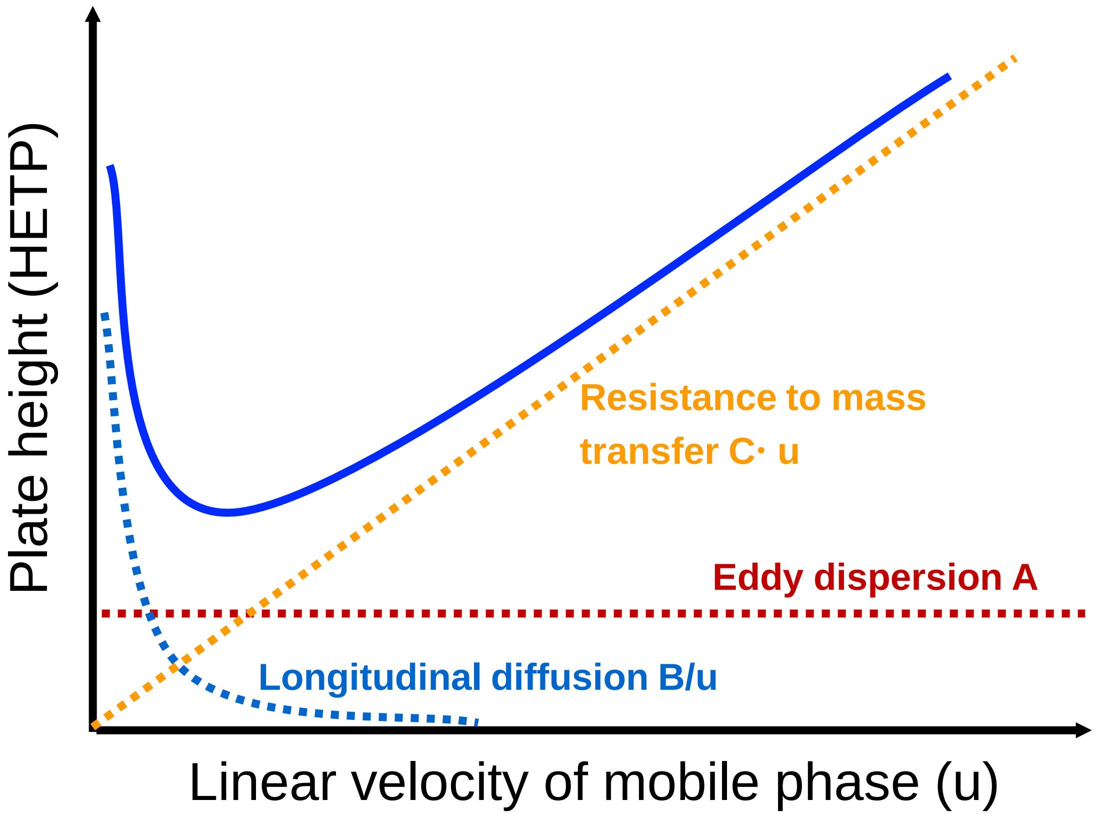
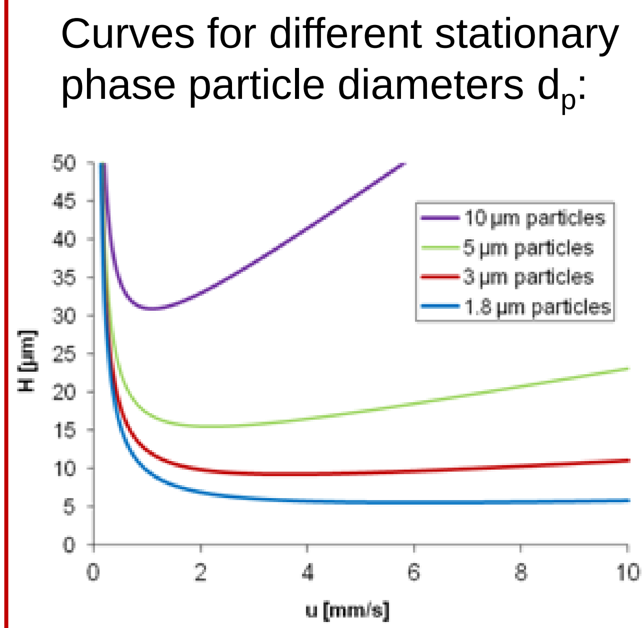
Van Deemter Curves to Discuss HPLC Kinetics





$$H = A + \frac{B}{u} + (C_m + C_s)u = 2\lambda d_p + \frac{\gamma D_m}{u} + \frac{\omega d_p^2}{D_m}u + \frac{c\, d_f^2}{D_s}u$$

u: **linear velocity;** λ, γ, ω, **c: constants;** D_m, D_s: **diffusion coefficients in mobile/stationary phase;** d_p: **particle diameter;** d_f: **thickness of stationary phase**

More Influence Parameters on van Deemter Curves

The solute diffusion coefficients matter as well (but let's skip C_s for simplification):

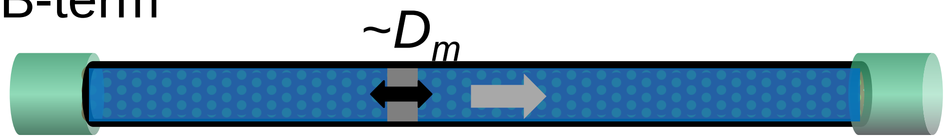
$$H = A + \frac{B}{u} + C \cdot u = 2\lambda dp + \frac{2\gamma D_m}{u} + \frac{\omega dp^2}{D_m} \cdot u$$

van Deemter

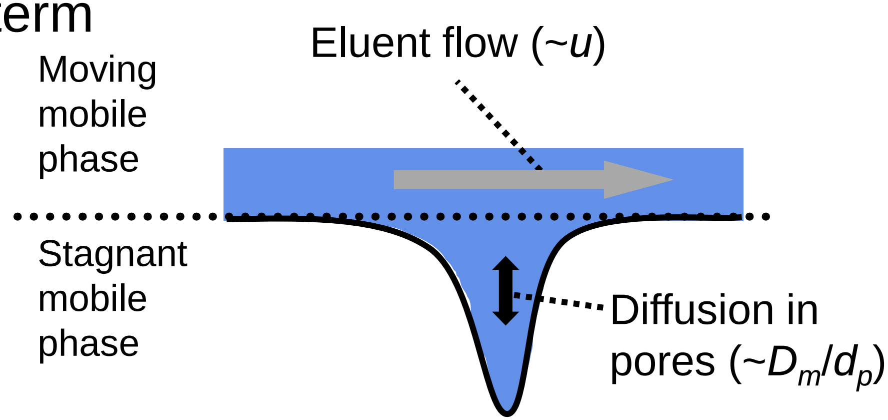
$$D_m = \frac{7.4 \cdot 10^{-15} \cdot \sqrt{\Psi \cdot M} \cdot T}{V_s^{0.6}\, \eta}$$

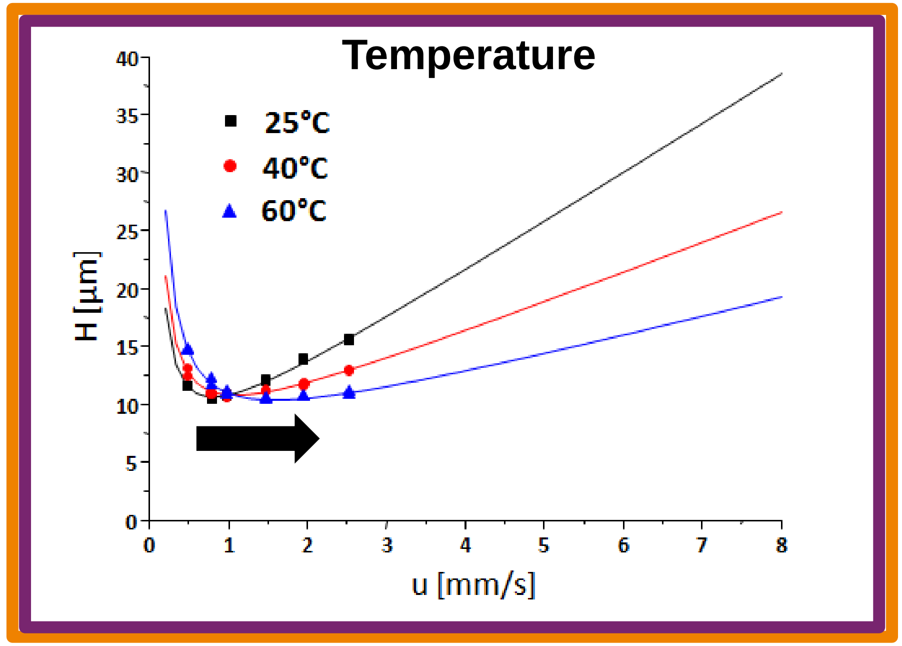
Wilke-Chang

B-term



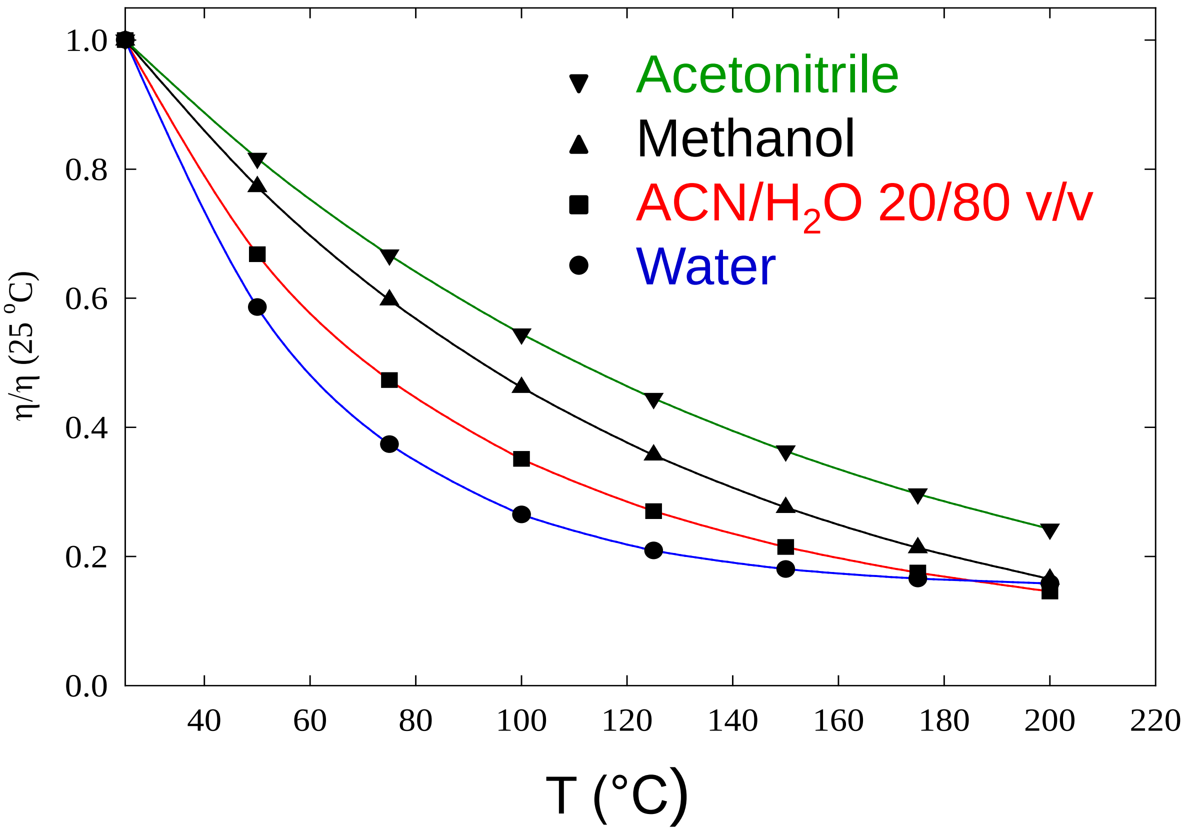
C-term





Increasing mobile phase temperature

Viscosity of RP Eluents at Different Temperatures



- Viscosity decreases with increasing temperature (non-linear)
- Curves change strongly with mobile phase composition
- Aqueous mobile phases deviate the most from linearity

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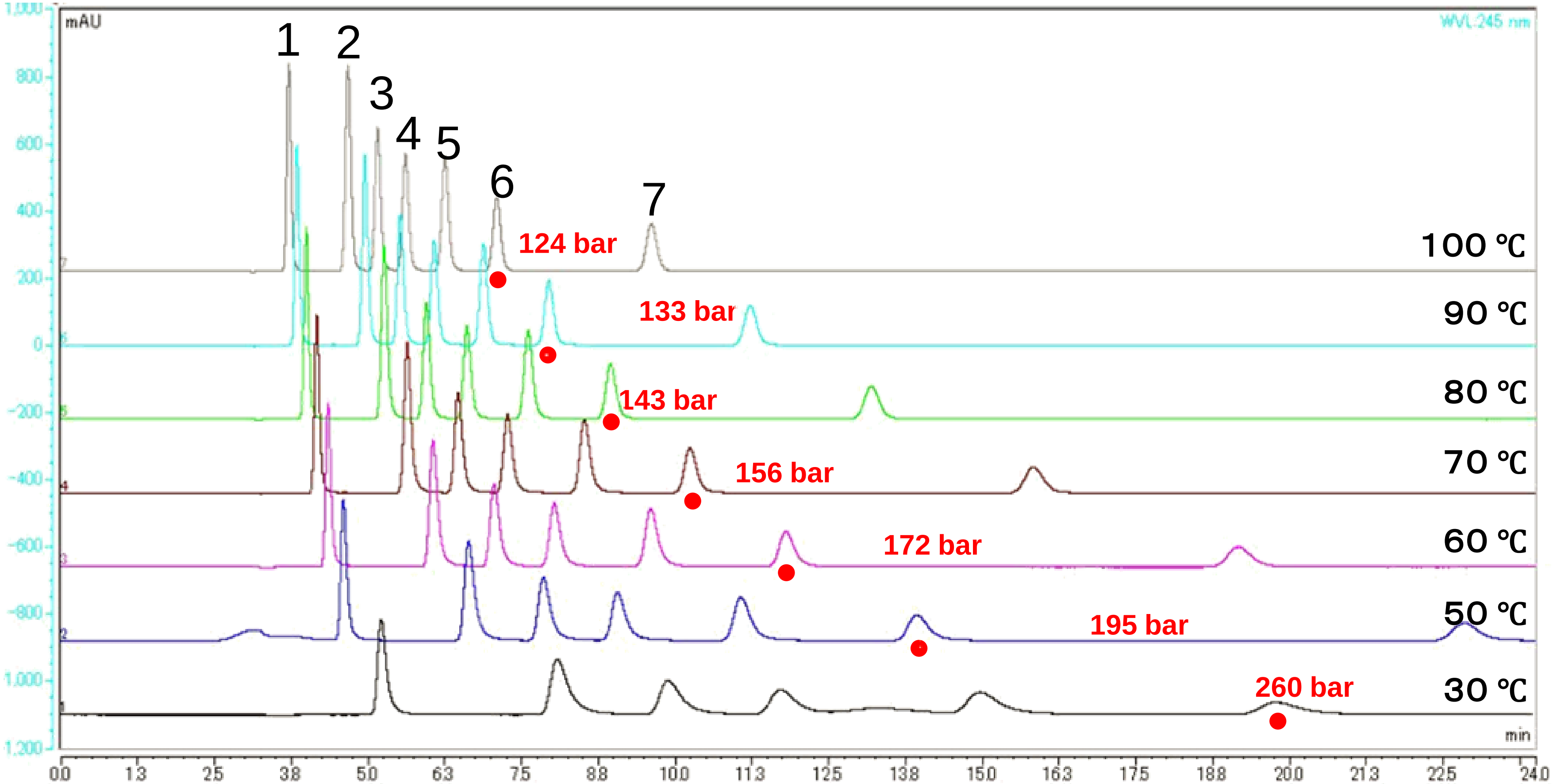
Effect of Temperature Increase Up to 100 °C

Separation of alkylphenones on a C18-modified PVA phase:



Column: Shodex® ET-RP1 4D 5 µm, 250 x 3 mm; Eluent: H_2O / MeOH / 25/75 v/v; Flow: 0.5 mL/min

Peaks: 1 Acetophenone, 2 Propiophenone, 3 Butyrophenone, 4 Pentanophenone, 5 Hexanophenone, 6 Heptanophenone, 7 Octanophenone

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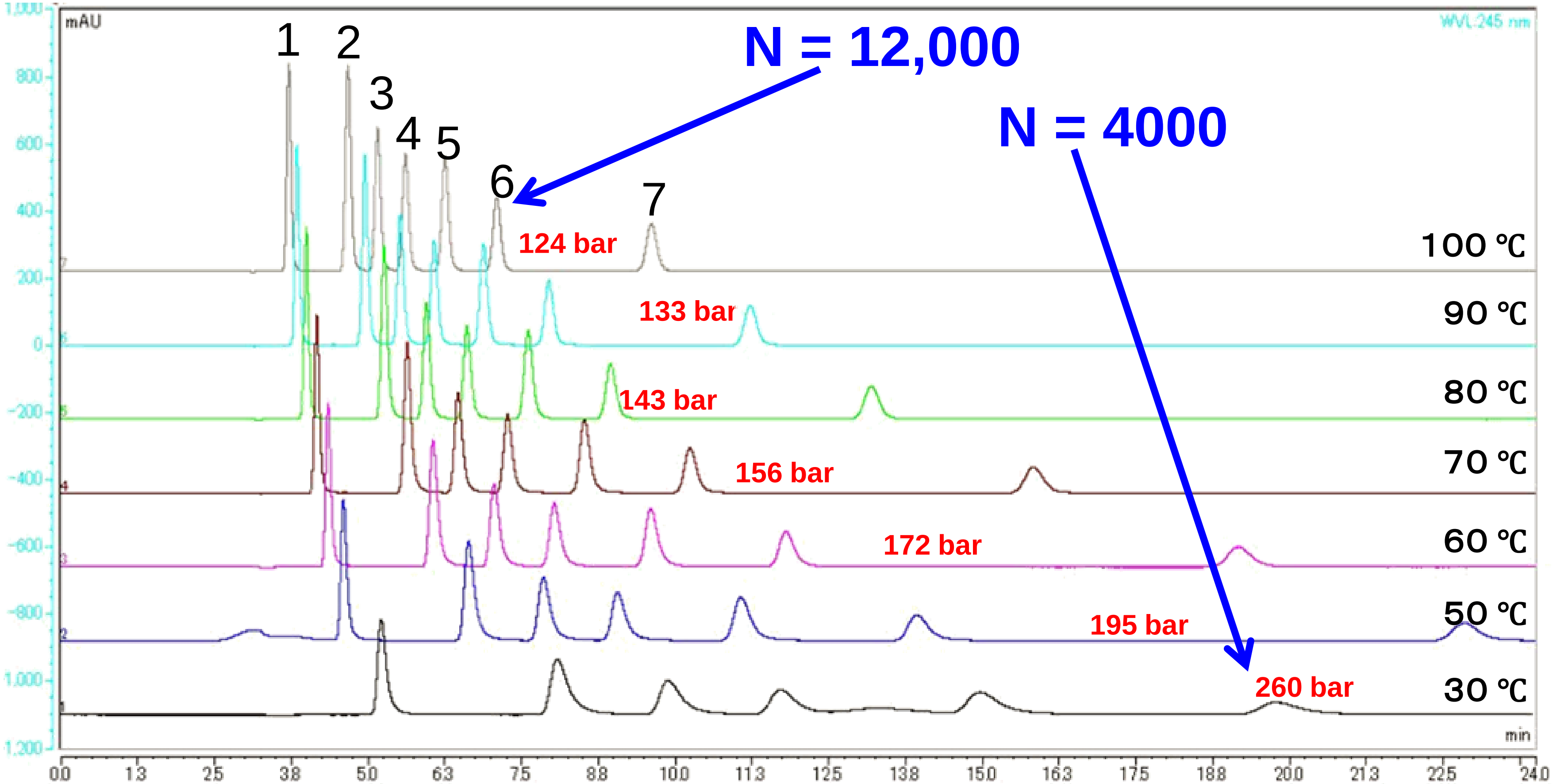

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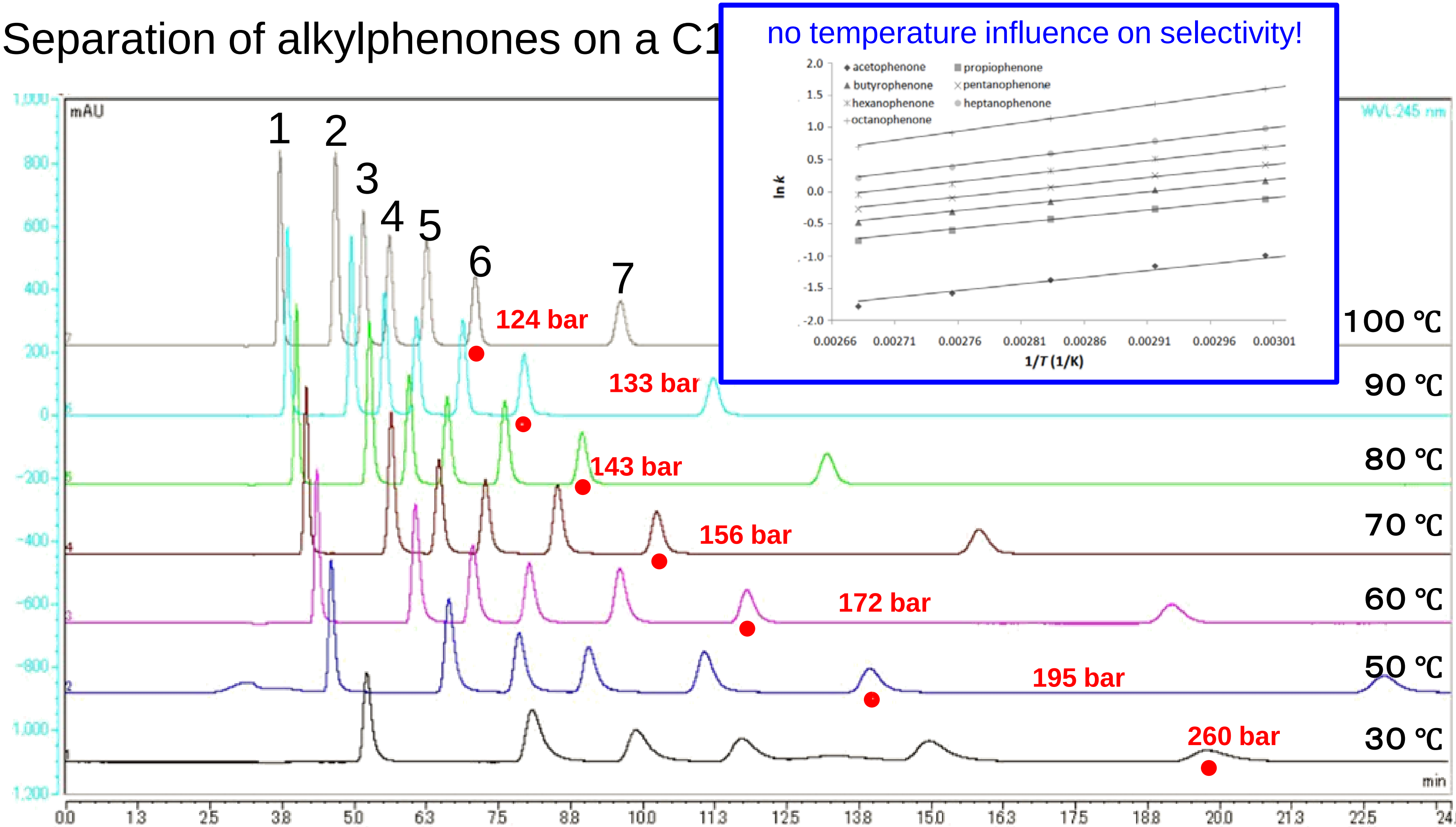
Peaks: 1 Acetophenone, 2 Propiophenone, 3 Butyrophenone, 4 Pentanophenone, 5 Hexanophenone, 6 Heptanophenone, 7 Octanophenone

Effect of Temperature Increase Up to 100 °C

Separation of alkylphenones on a C1



Column: Shodex® ET-RP1 4D 5 µm, 250 x 3 mm; Eluent: H_2O / MeOH / 25/75 v/v; Flow: 0.5 mL/min

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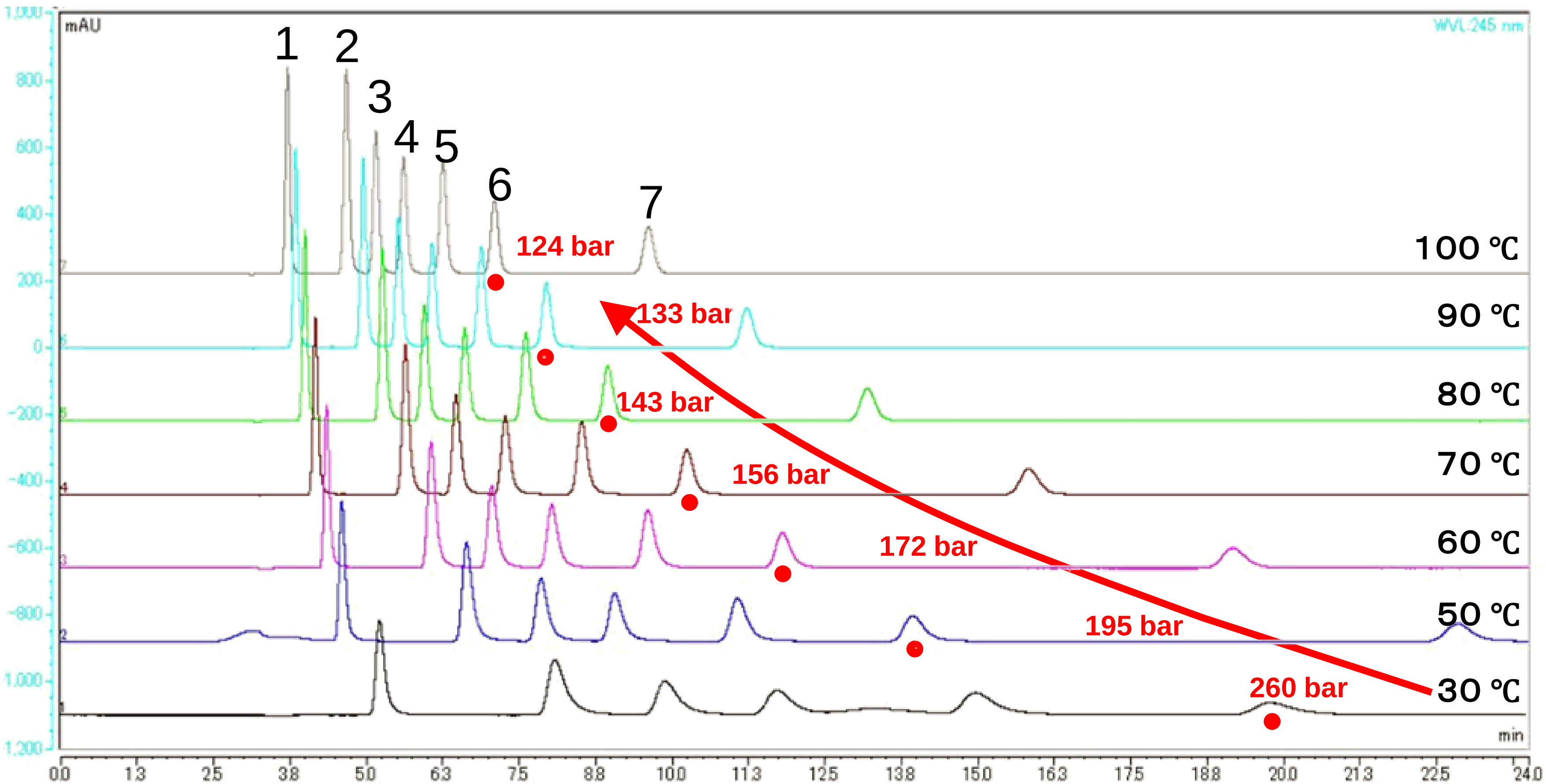
Effect of Temperature Increase Up to 100 °C

Separation of alkylphenones on a C18-modified PVA phase:



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Effect of Temperature Increase Up to 100 °C

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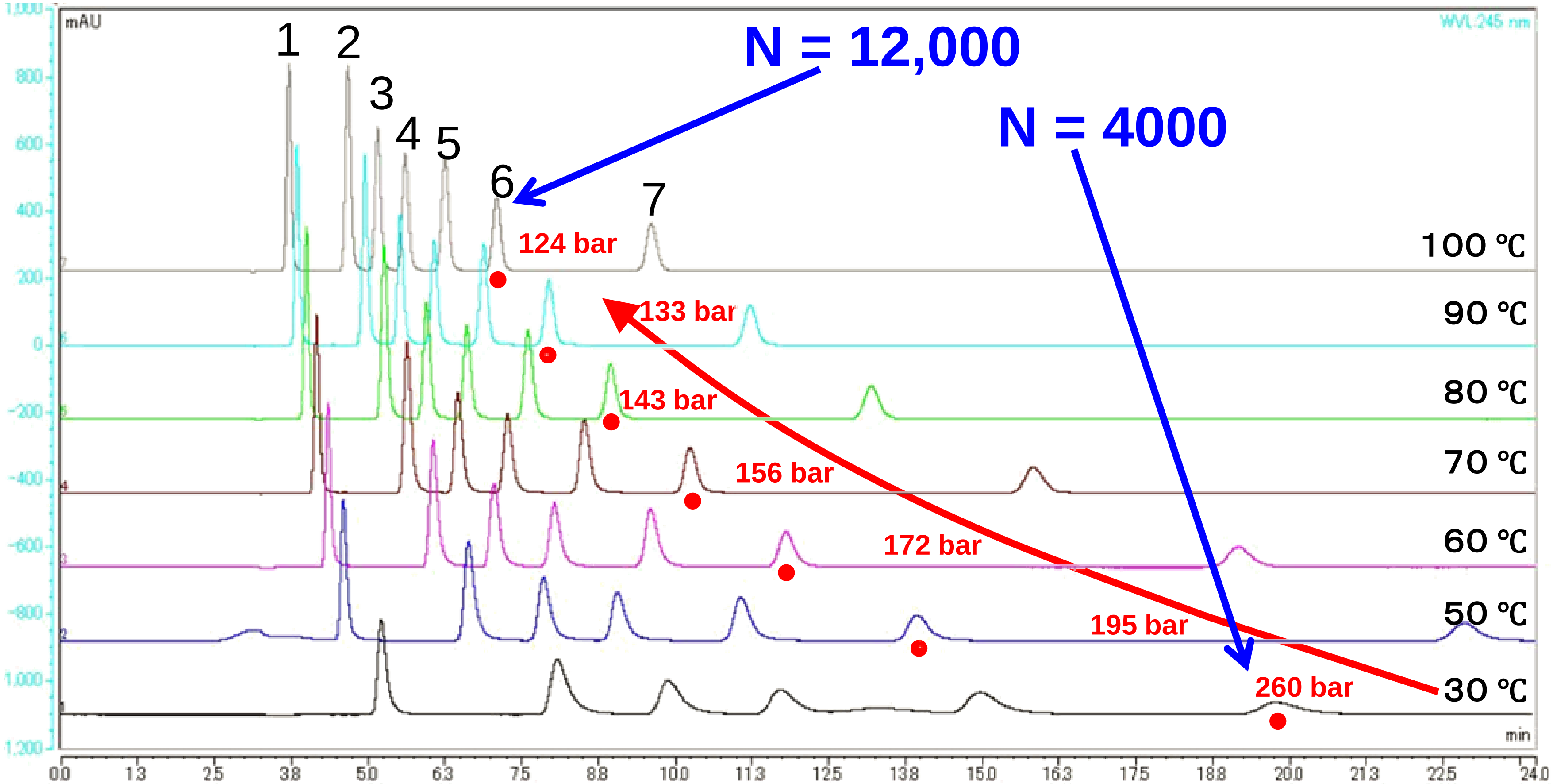

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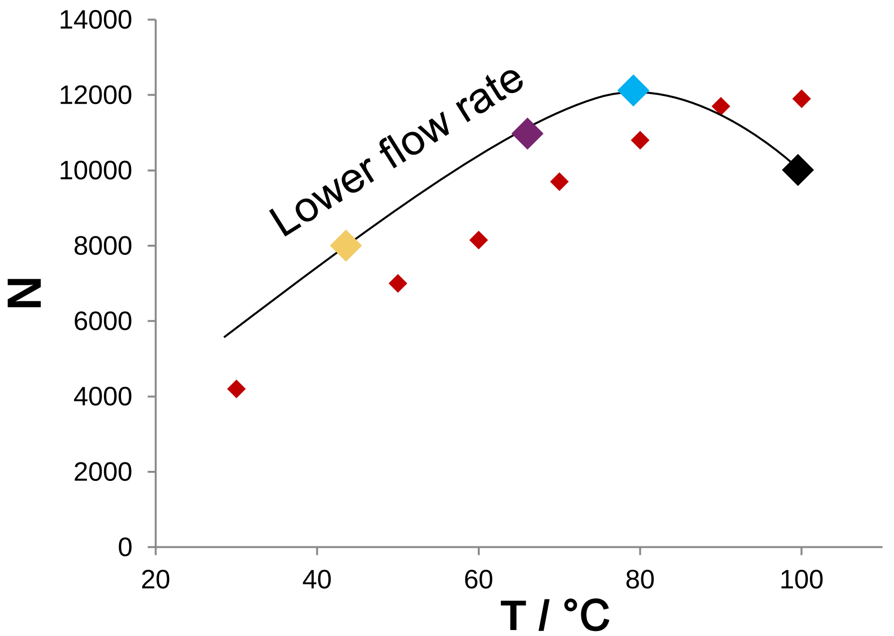
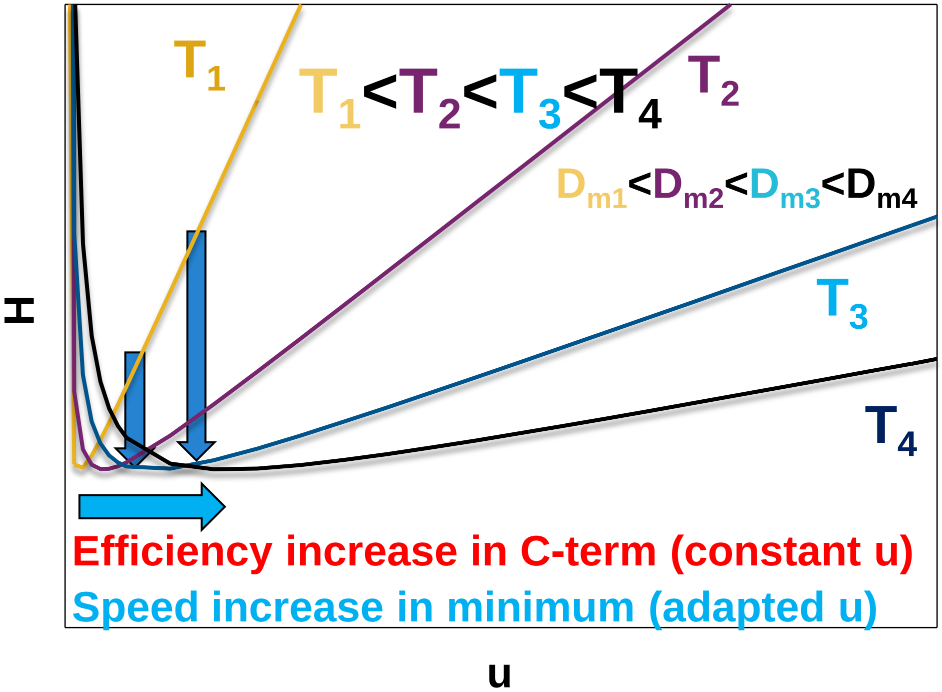
Peaks: 1 Acetophenone, 2 Propiophenone, 3 Butyrophenone, 4 Pentanophenone, 5 Hexanophenone, 6 Heptanophenone, 7 Octanophenone

Increase of N with Increasing T – Why is this?



Temperature dependence of the heptanophenone plate number on C18 modified PVA phase



- Obviously the separation at 0.5 mL/min and 20 °C ran far in the C-term.
- With increasing T the C-term shrinks (increasing D_m) and N increases (at higher u).
- The curve minimum moves to the right with increasing T and at 90 °C the flow of 0.5 mL/min seems to eventually approach it.
- Another flow rate would give a completely different picture.

Rule of Thumb for Temperature Increase and Flow Rate

1) $$D_m(T) = \Psi \cdot \frac{T}{\eta(T)}$$

Simplified Wilke Chang: D_m changes reciprocally with η, while T (Kelvin) has only minor impact (30 °C to 100 °C ➔ 303 K to 373 K)

2) $$u_{\min} \sim \frac{D_m}{dp}$$

u_{min} linearly increases with D_m as this evolves with T

3) $$\Delta p \sim \frac{\eta \cdot L \cdot u}{dp^2}$$

Δp is also a strict function of η and respectively decreasing with T

Example scenario:

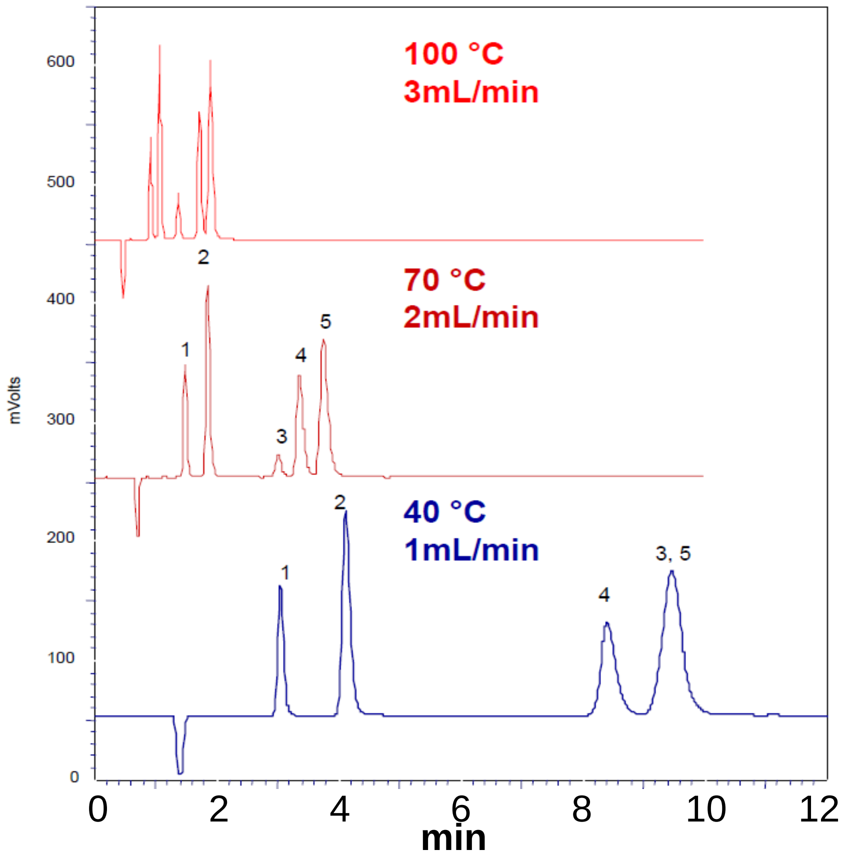
- T increased by 30 °C ➔ halved η ➔ halved Δp observed at constant F
- halved η ➔ approximately doubled D_m ➔ u_{min} will be at double F
- doubled F ➔ doubled Δp ➔ pressure is back to original value

Rules:

- Make sure you work at optimum efficiency (van Deemter minimum) at the original T
- Change of T ➔ adapt F to keep pressure constant (and stick to optimum N)
- You cannot improve N at u_{min} (N_{opt}) by T increase, but speed-up separations at N_{opt}

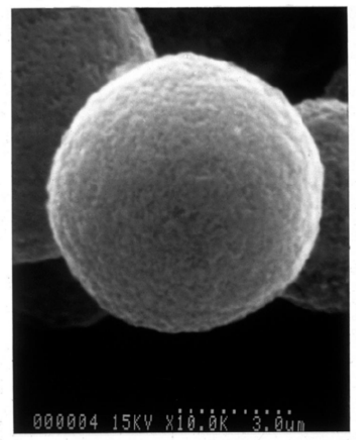
Hippuric Acid and Metabolites

On Thermo Scientific™ Hypercarb™ porous graphitic carbon column at elevated temperature



Hypercarb column:

100% porous graphitic carbon (PGC)



UltiMate 3000 RSLC System

Column:	Hypercarb 5 µm, 100 x 4.6 mm
Detection:	225 nm
Eluent:	H_2O/MeCN 50/50 v/v 0.1% formic acid
Flow rate:	see figure
Temperature:	see figure
Analytes:	2-Methyl Hippuric Acid (1), Hippuric Acid (2), Xylene (3), 3-Methyl Hippuric Acid (4), 4-Methyl Hippuric Acid (5)

- Selectivity improved (see xylene, peak 3) and mass transport increase
- Constant peak height indicates constant N when F is adapted
- Combination of reduced retention and elevated flow yields speed increase of factor 5

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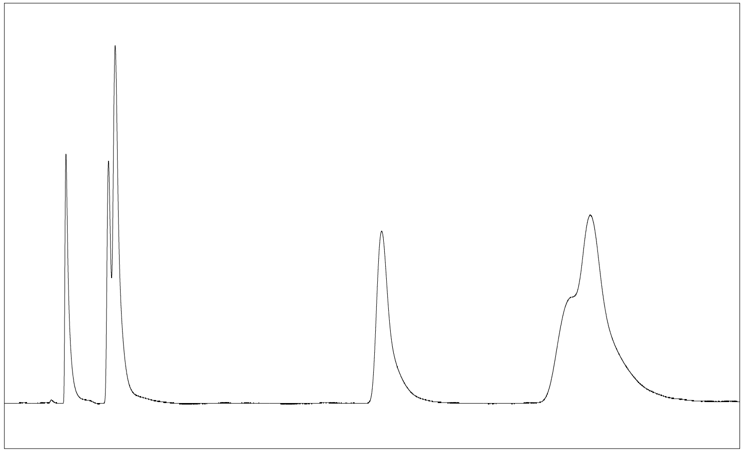
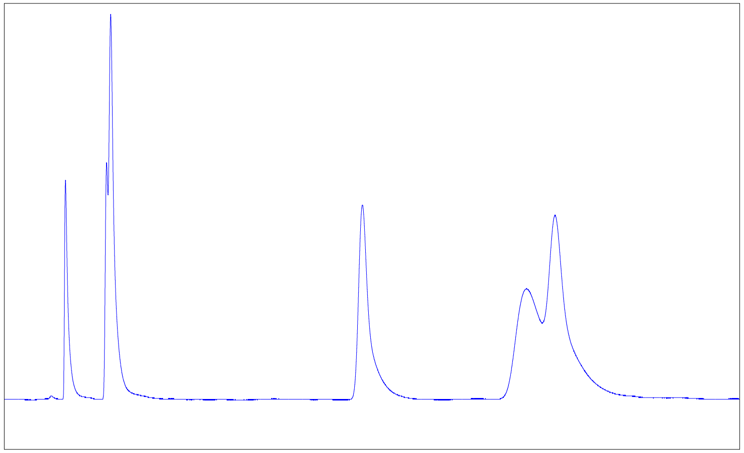
Another Scenario in the Lab with Different Column Thermostats

Chromatogram run on system X with given column and eluting conditions



Chromatogram run on system Y with same column and eluting conditions



- We had to change the column compartment during a method development process (routine application was due on it)

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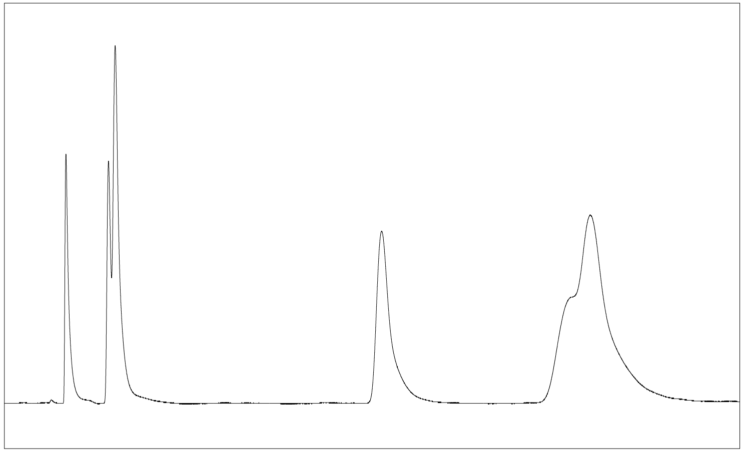

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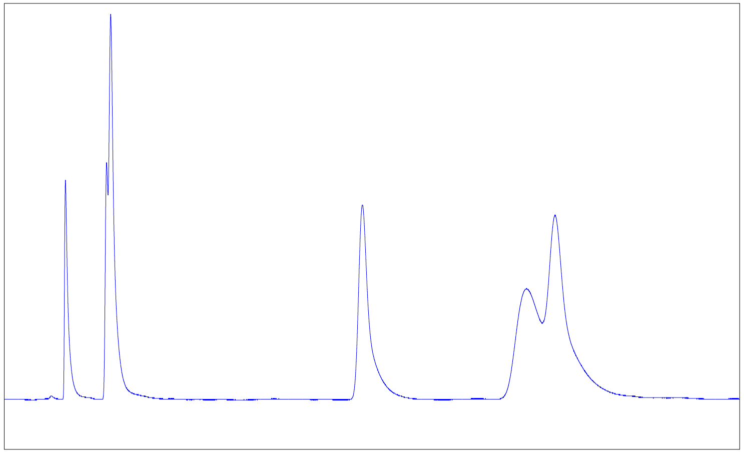

- We had to change the column compartment during a method development process (routine application was due on it)
- By just running the same (not yet finished) method on the same system but with another column oven, we made this weird observation…

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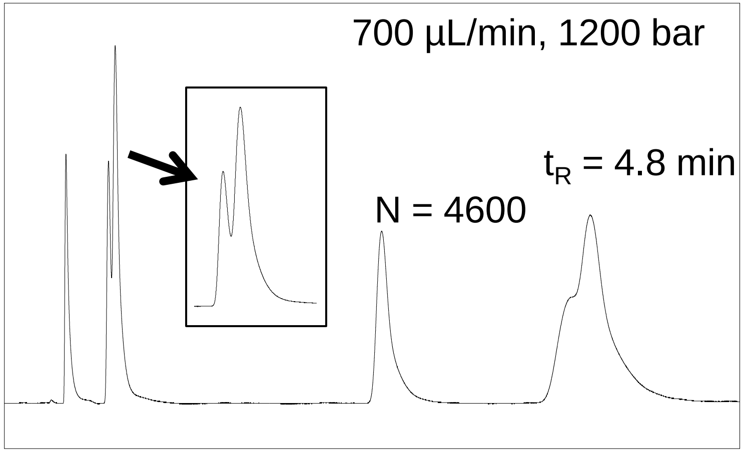
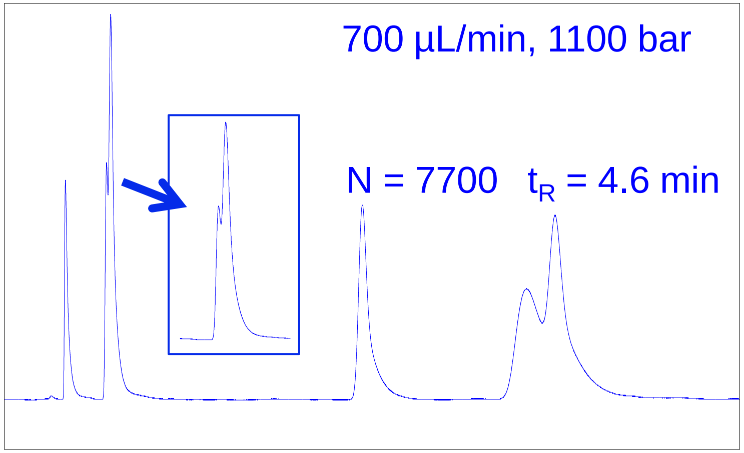
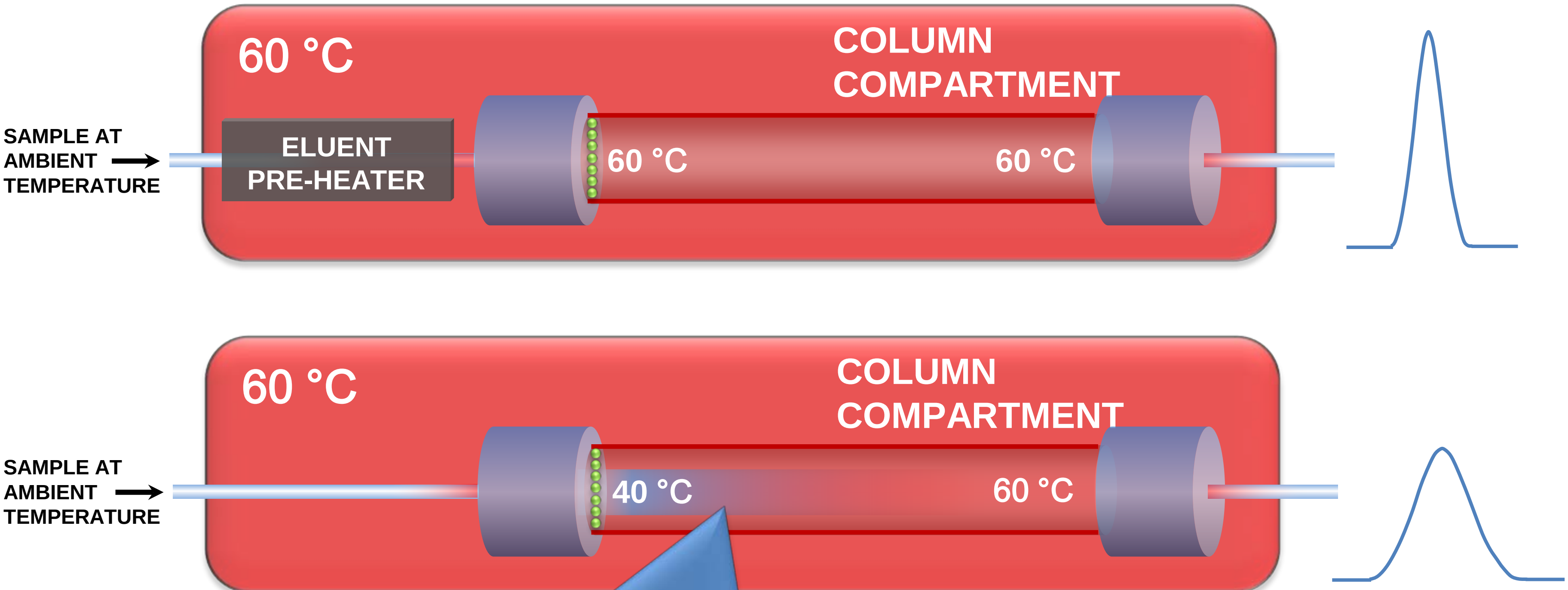


Chromatogram run on system Y with same column and eluting conditions



- We had to change the column compartment during a method development process (routine application was due on it)
- By just running the same (not yet finished) method on the same system but with another column oven, we made this weird observation…
- Thermostats can differ in heat dissipation and eluent-preheating

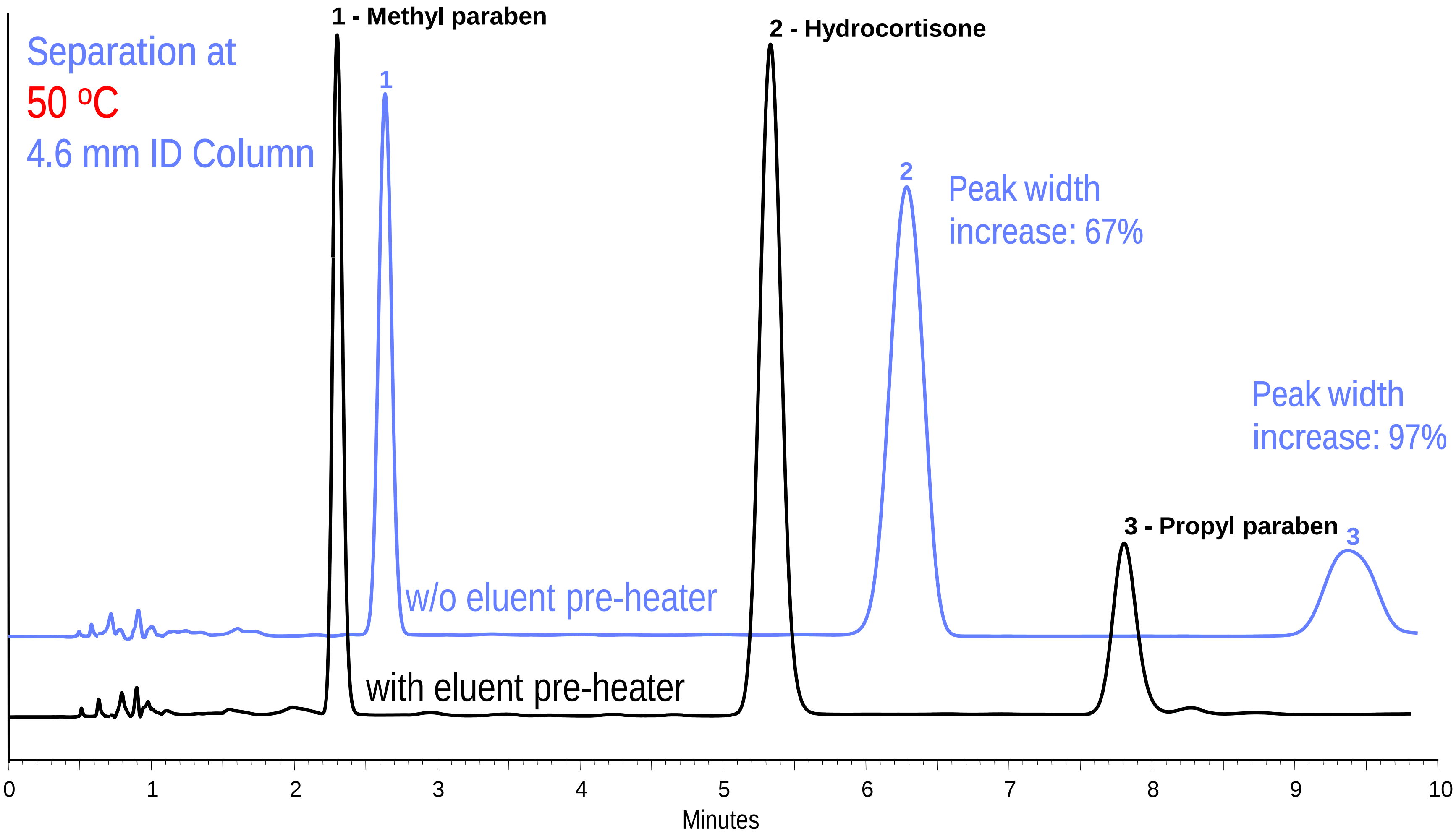
Effects From Non-Matching Eluent Temperature



Mismatch:

- Centre of column below oven temperature
  - Higher viscosity, lower linear velocity in centre
  - Higher retention in centre

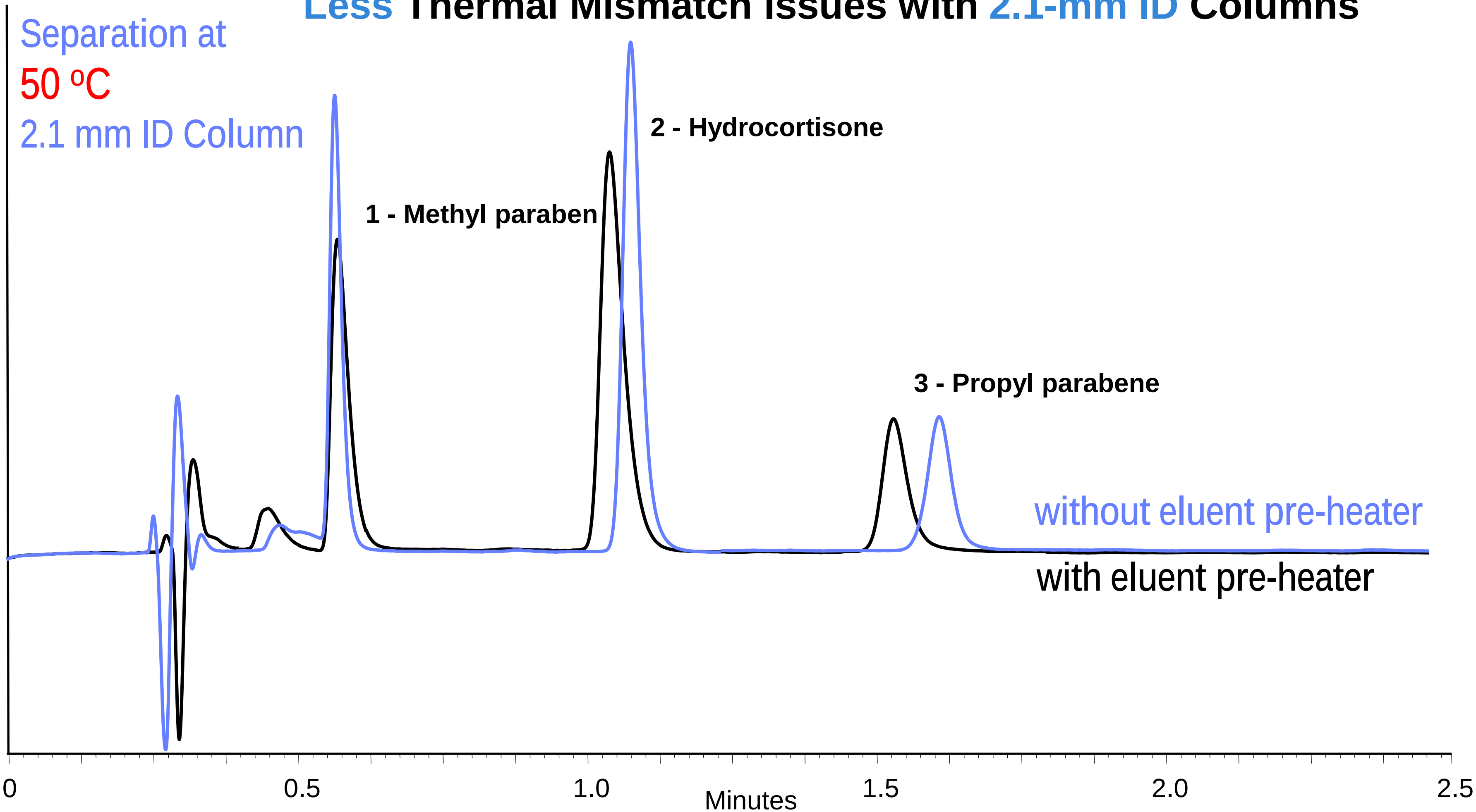
Thermal Mismatch Issues (Ointment Application)



Lower internal column temperature and thermal mismatch

Thermal Mismatch Issues (Ointment Application)

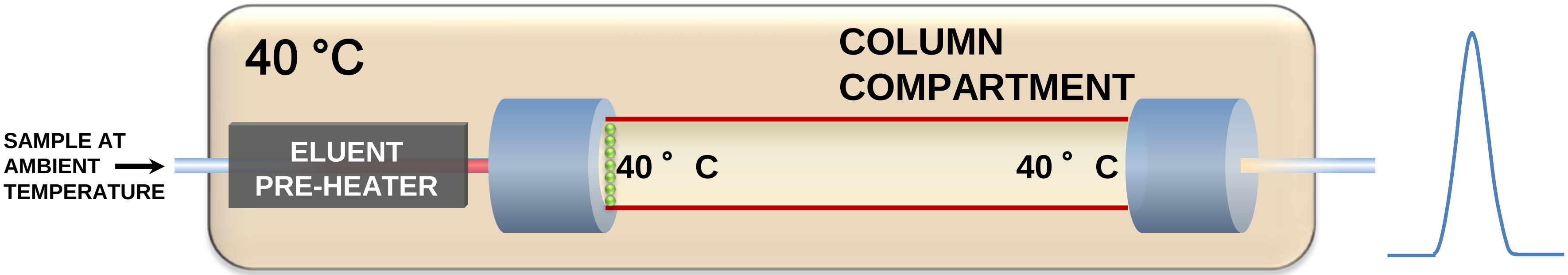
Less Thermal Mismatch Issues with 2.1-mm ID Columns



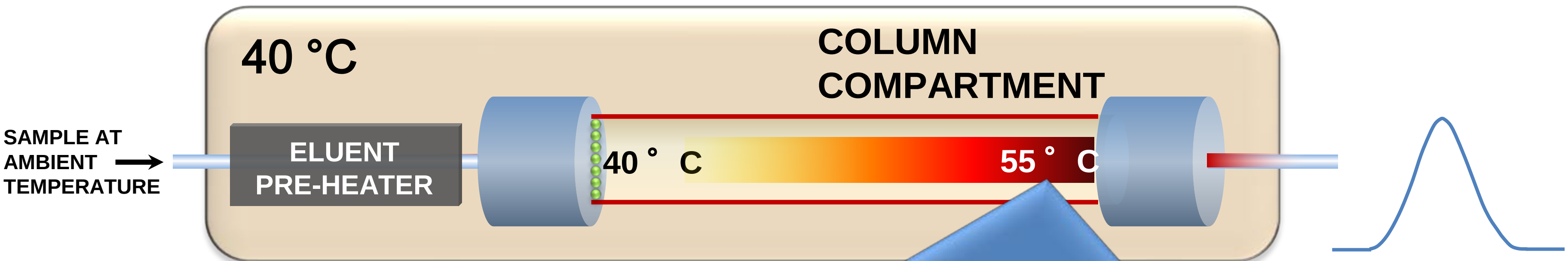
Pre-heater leads to extra column band broadening, and is sometimes not needed for micro-column applications

Mismatch Effects From Frictional Heating in Column

HPLC column operated at non-viscous heating conditions:



UHPLC column operated at extreme pressure:

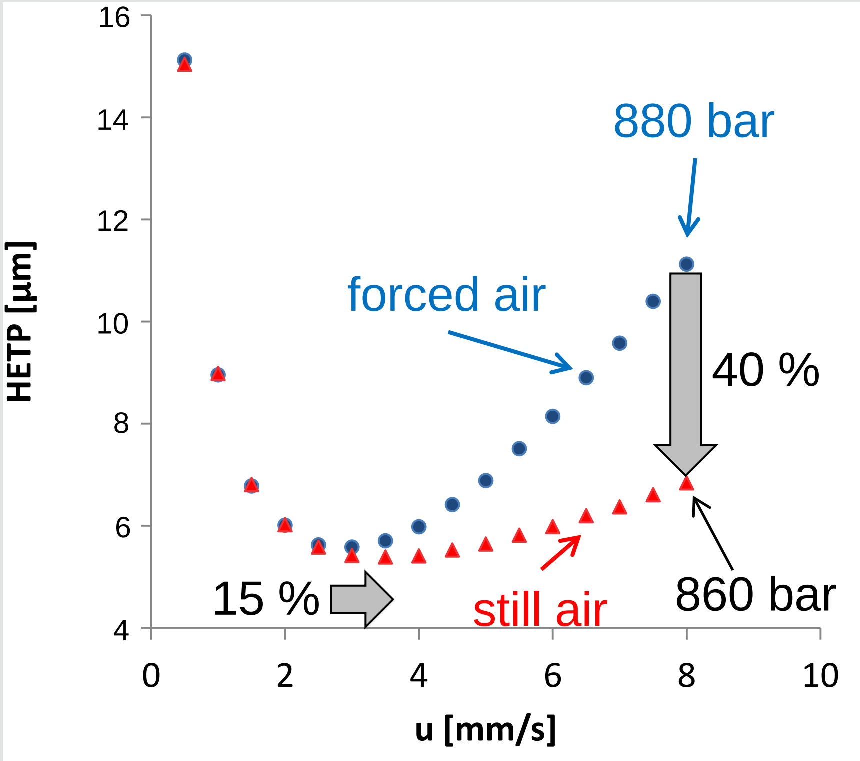


Mismatch due to viscous heating:

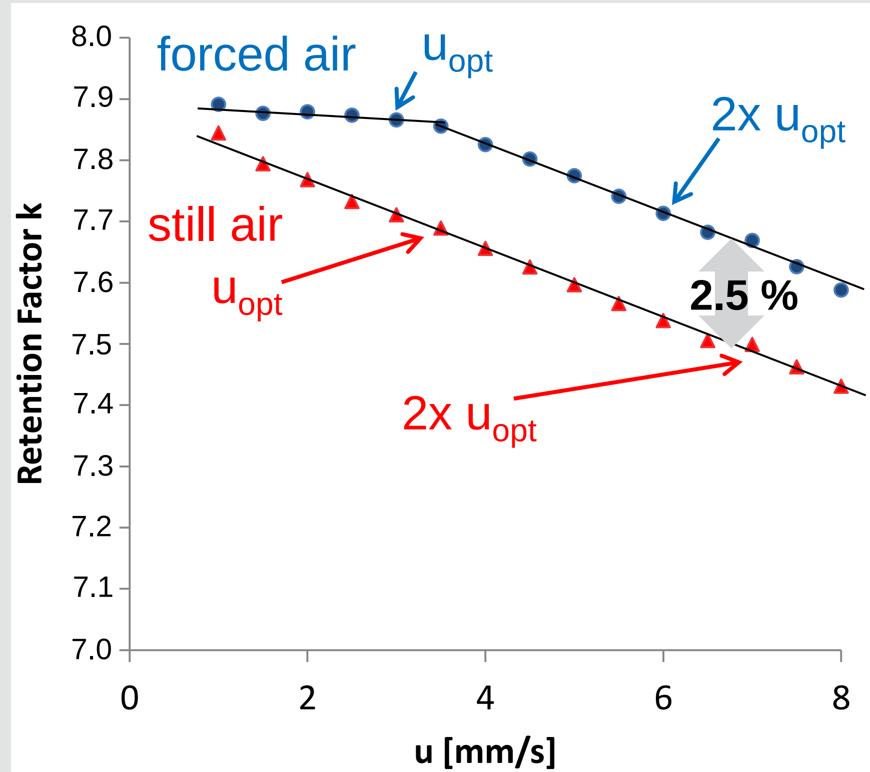
- Centre of column towards outlet above oven temperature
  - Lower viscosity, higher linear velocity in center
  - Lower retention in center

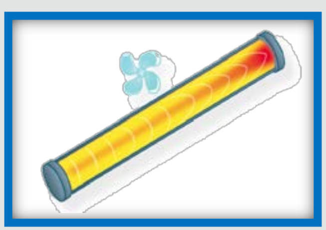
Impact of Viscous Heating on Efficiency and Retention

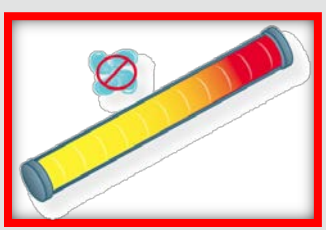
Efficiency:



Retention:







Column:	Thermo Scientific™ Hypersil GOLD™, 1.9 µm, 2.1 x 100 mm
Mobile Phase:	50% water / 50% acetonitrile,
Sample:	Hexanophenone
Temperature:	CC and Pre-Heater 30 °C, forced air or still air mode, respectively

Measured Temperature Increase from Frictional Heating

Experimental conditions:

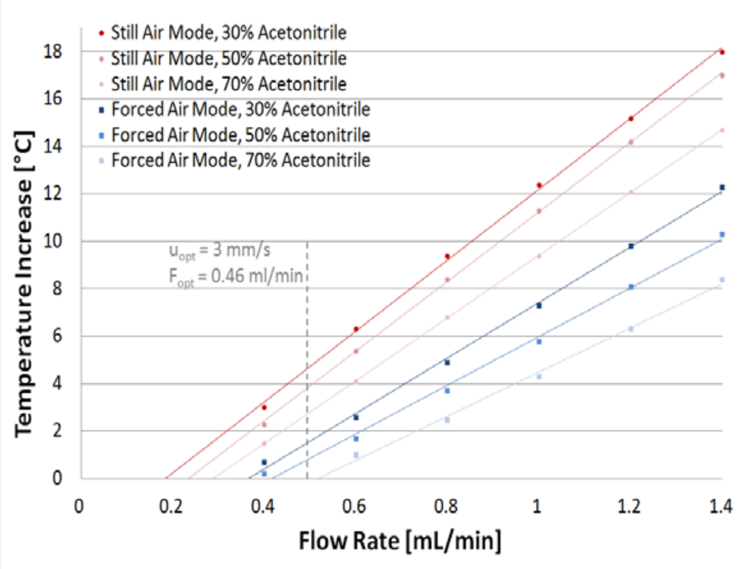
Instrument: Thermo Scientific™ Vanquish™ Horizon UHPLC System

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

Eluent: $CH_3CN:H_2O$

Flow Rate: Up to 1.40 mL/min

Temperature: 30 °C (column compartment and mobile phase pre-heater)



50% Acetonitrile	Still Air Mode		Forced Air Mode	
Flow Rate [mL/min]	System Backpressure [bar]	Axial Temperature Increase [°C]	System Backpressure [bar]	Axial 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

11.5-fold ΔT with still air

2.3-fold ΔT with still air

1.7-fold ΔT with still air

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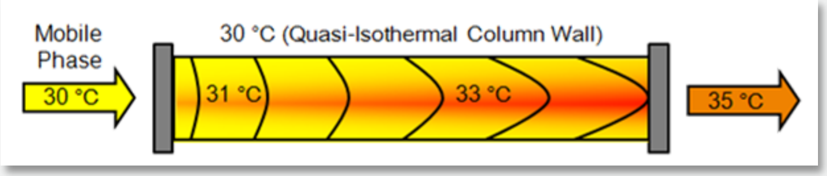
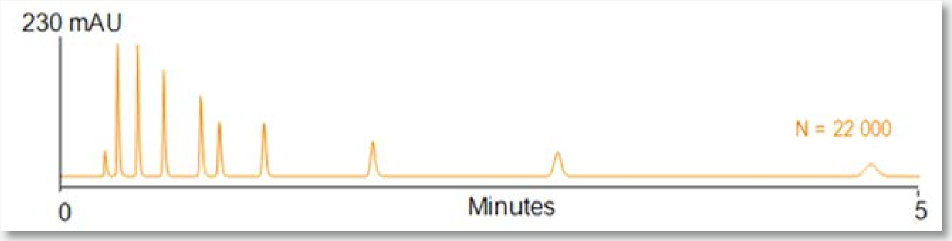
Viscous Heating and Thermostatting at 1400 bar Level

Experimental conditions:

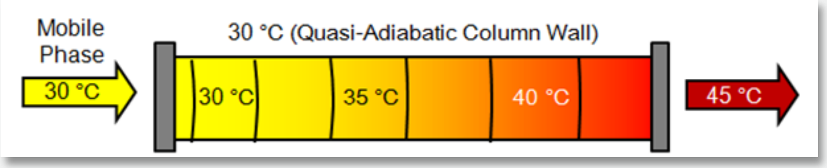
Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 H_2O:CH_3CN (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

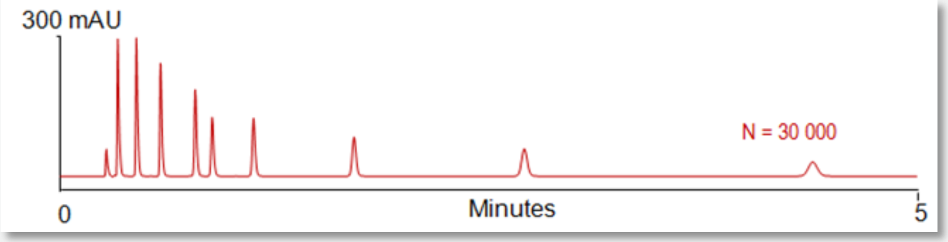
Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.





Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.





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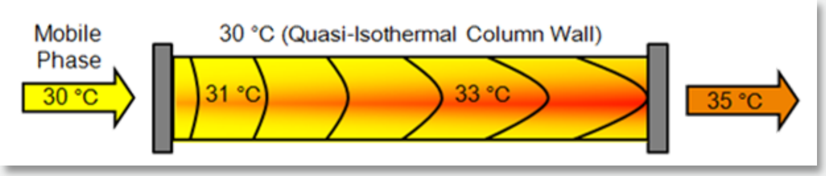
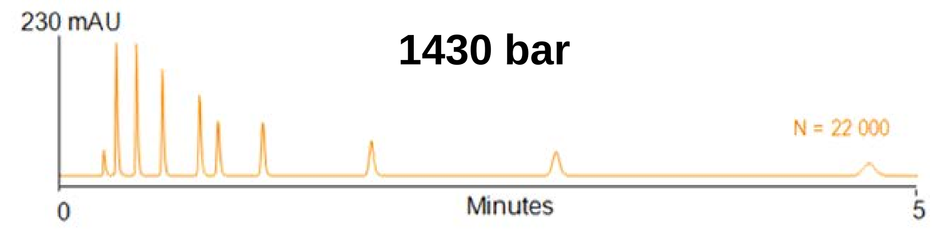
Viscous Heating and Thermostatting at 1400 bar Level

Experimental conditions:

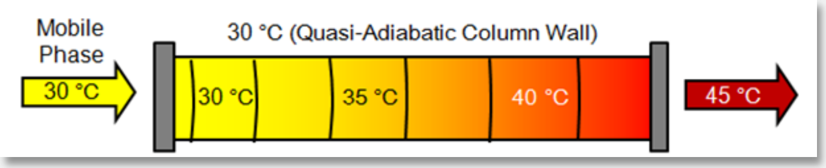
Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 H_2O:CH_3CN (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.





Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.

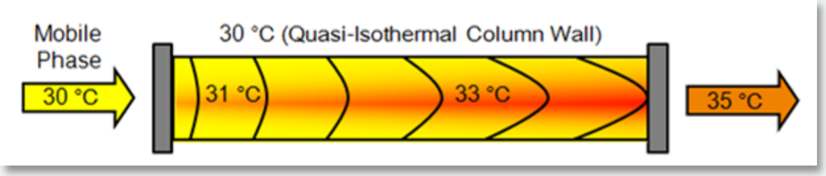


Viscous Heating and Thermostatting at 1400 bar Level

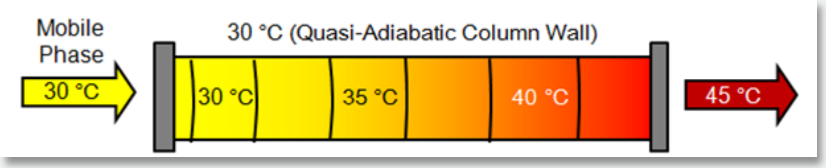
Experimental conditions:

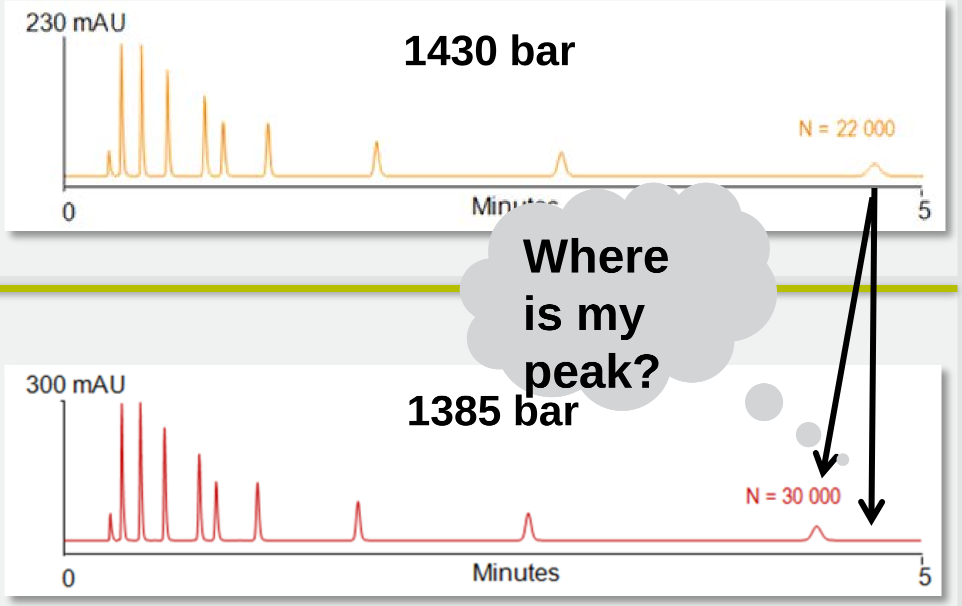
Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 H_2O:CH_3CN (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.



Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.



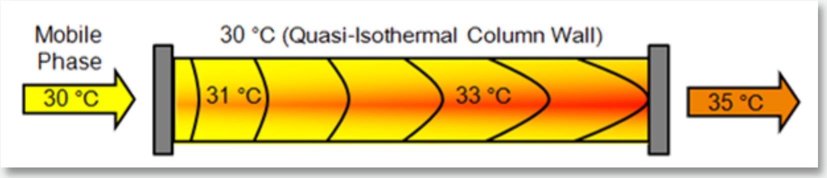


Viscous Heating and Thermostatting at 1400 bar Level

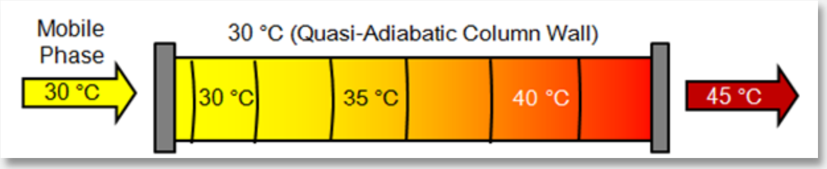
Experimental conditions:

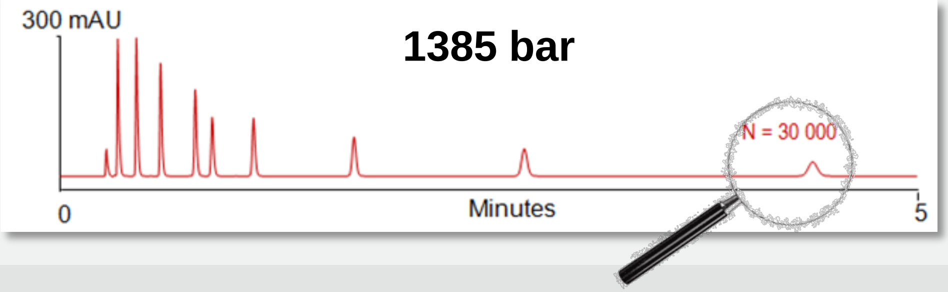
Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 $H_2O:CH_3CN$ (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.



Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.



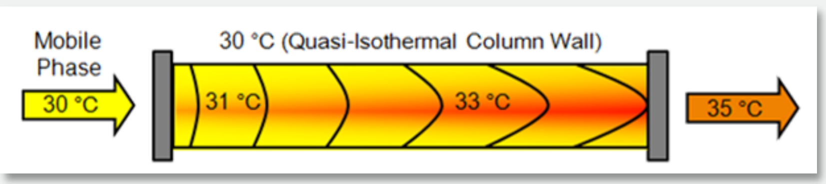


Viscous Heating and Thermostatting at 1400 bar Level

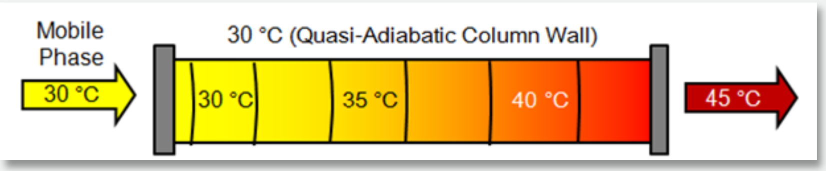
Experimental conditions:

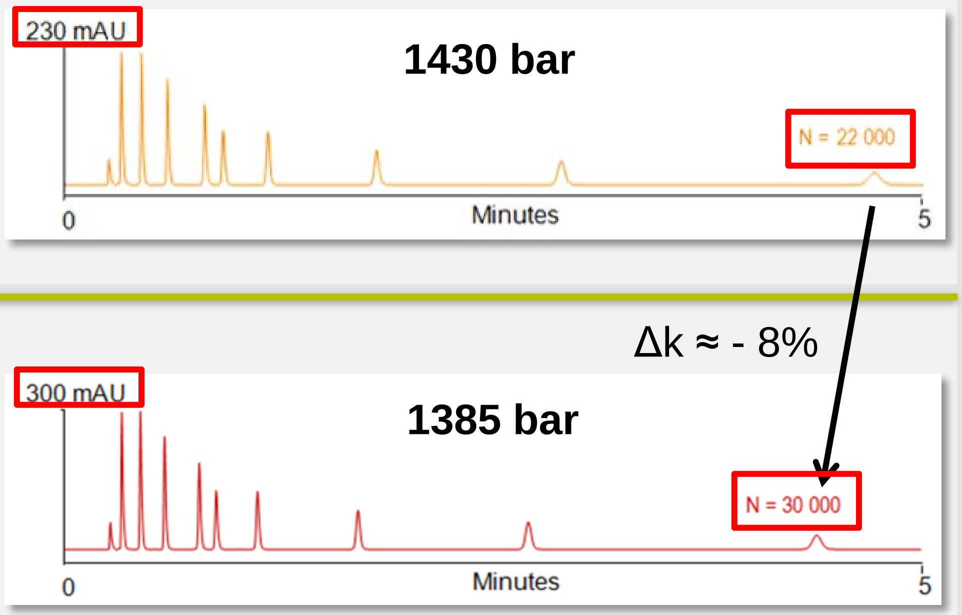
Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 H_2O:CH_3CN (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.



Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.



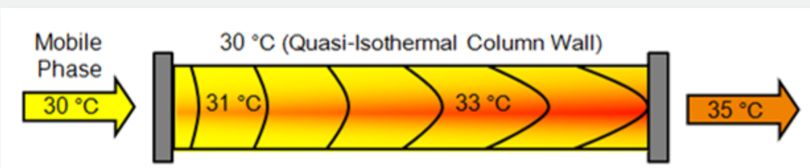


Viscous Heating and Thermostatting at 1400 bar Level

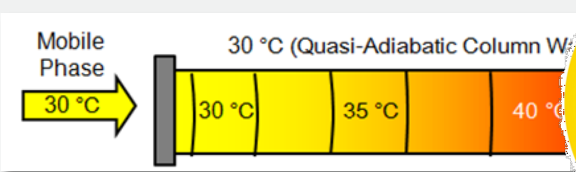
Experimental conditions:

Column:	Thermo Scientific™ Accucore™ Vanquish™ C18, 1.5 µm, 2.1 x 100 mm
Sample:	Uracil, acetanilide, and 8 phenones
Inj. Volume:	1 µL
Eluent:	45:55 H_2O:CH_3CN (v/v)
Flow Rate:	0.65 mL/min
Thermostatting:	Compartment and active pre-heater at 30 °C, fan speed 5 (forced air) or fan speed 0 (still air)

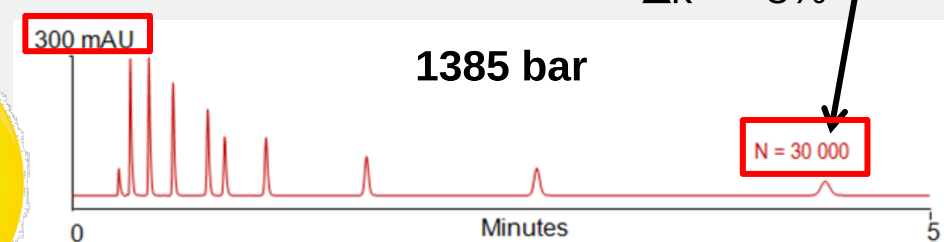
Influence of frictional heat under quasi-isothermal conditions (forced air mode) on temperature distribution and resulting chromatogram.



Influence of frictional heat under quasi-adiabatic conditions (still air mode) on temperature distribution and resulting chromatogram.



Δk ≈ - 8%



Still air is the better mode

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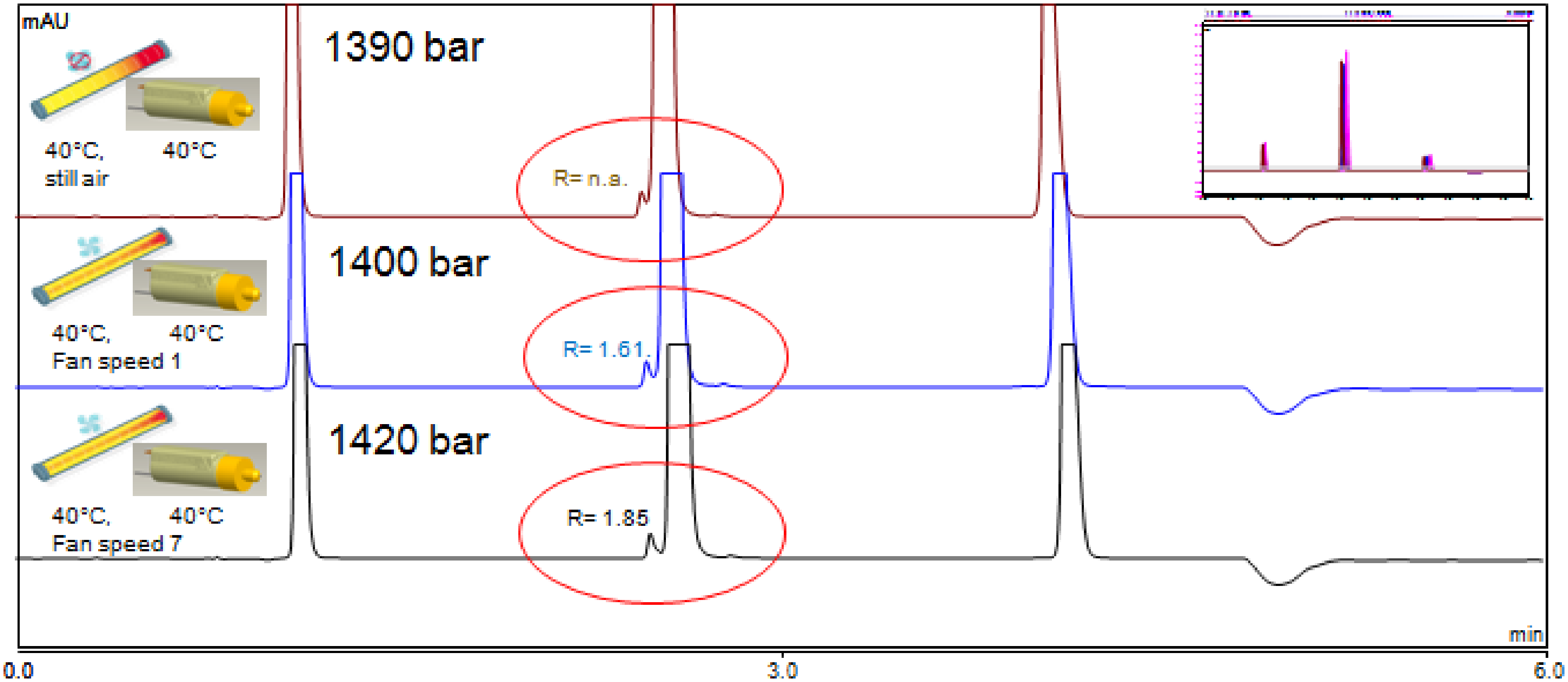
Fan Speed Options – Method Optimization

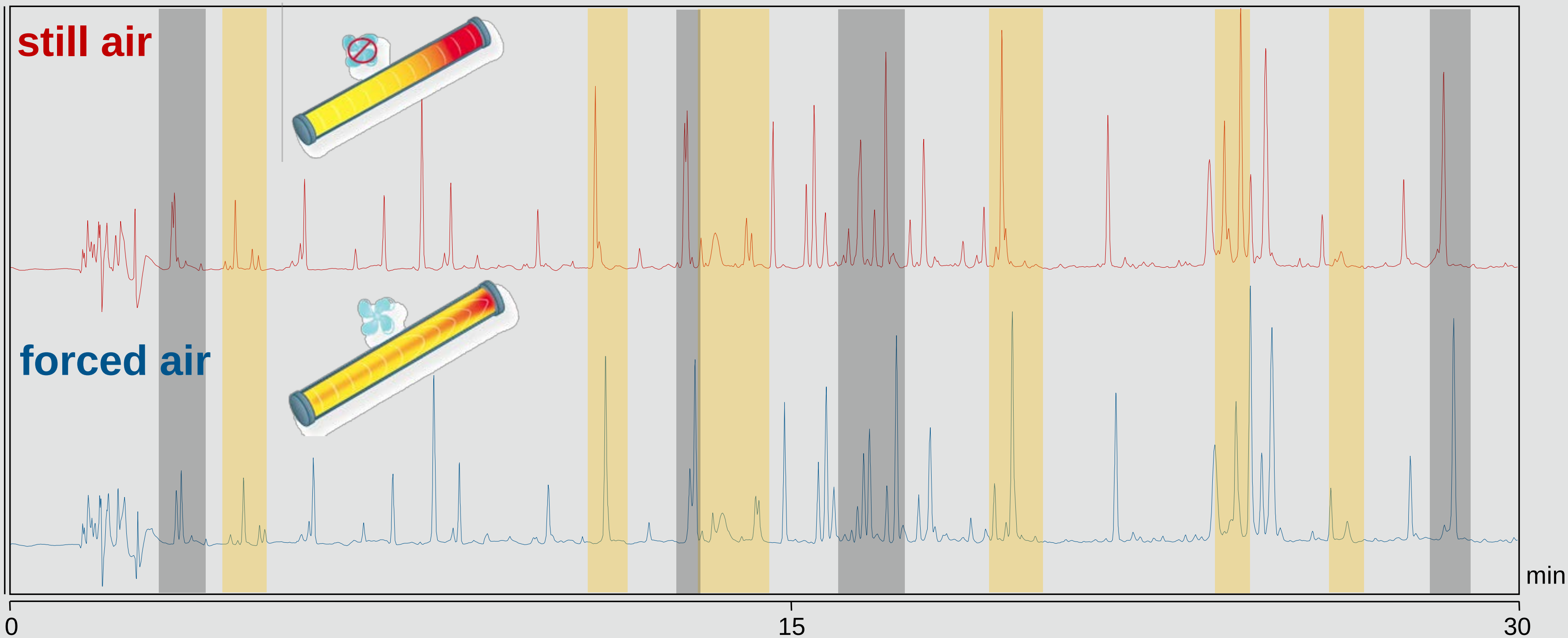


- Fan speed increase enables to reduce column temperature
- Selectivity for impurity peak increases with decreasing temperature

Instrument:	Vanquish Horizon UHPLC system
Gradient:	From 5% to 30% B in 4 min, A: 0.1% formic acid in H_2O, B: MeCN
Columns:	2 x Hypersil GOLD, 1.9 µm, 2.1x100 mm coupled in series
Sample:	Mixture of gallic acid, caffeine, caffeic acid, and salicylic acid
Detection:	UV 300 nm, 20 Hz, 0.2 s response time
Flow:	0.8 mL/min

Forced air is the better mode

Column Thermostatting in High Resolution Peptide Mapping



Vanquish Horizon UHPLC System with 10 mm Lightpipe™ Flow Cell

Column: Thermo Scientific™ Acclaim™ RSLC 120 C18, 2.2 µm, 2.1 x 250 mm
Injection: 1 µL Monoclonal IgG Tryptic Digest (2 mg/mL)
Detection: 214 nm, 100 Hz, Easy Mode
Temperature: CC and Pre-heater 80 °C, **still air** or **forced air**, Easy Mode

Mobile Phase A: TFA 0.05% in water
Mobile Phase B: TFA 0.05% in 2/8 water/acetonitrile
Flow rate: 400 µL/min
Gradient: from 4% to 55% B in 30 min

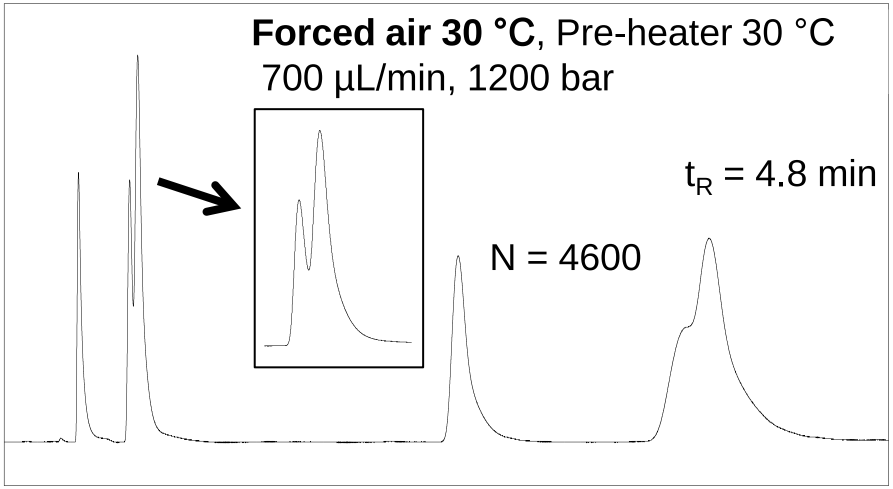
- Advantage of still air mode ➔ better resolution due to better efficiency
- Advantage of forced air mode ➔ better resolution due to more suited selectivity

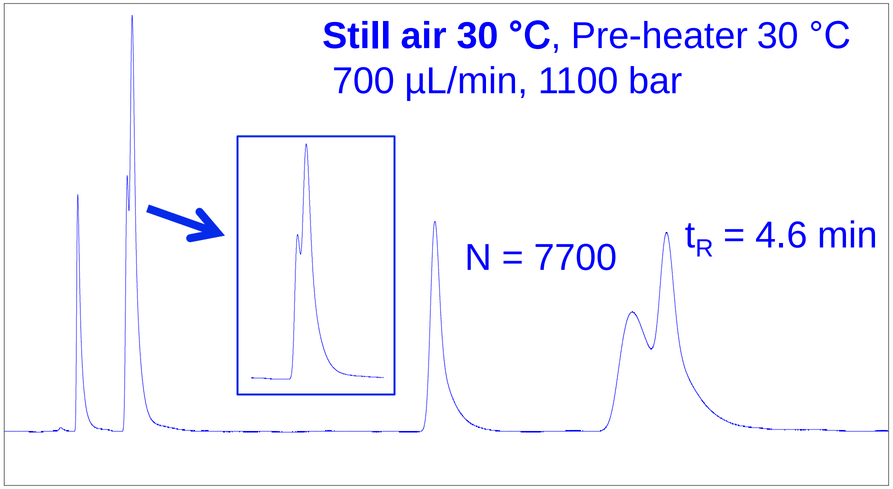
➡ Both modes are complimentary

Outline

- How temperature rules retention and selectivity
- Temperature effects on peak shapes and analysis speed
- Importance of the specific thermostatting technique
- Separating eluent pre-heating from columns thermostatting

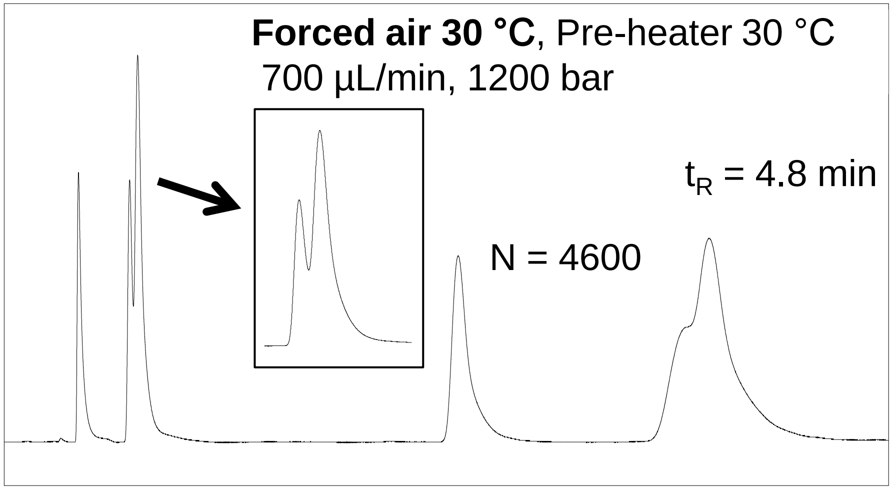
Where Forced Air Selectivity Meets Still Air Efficiency

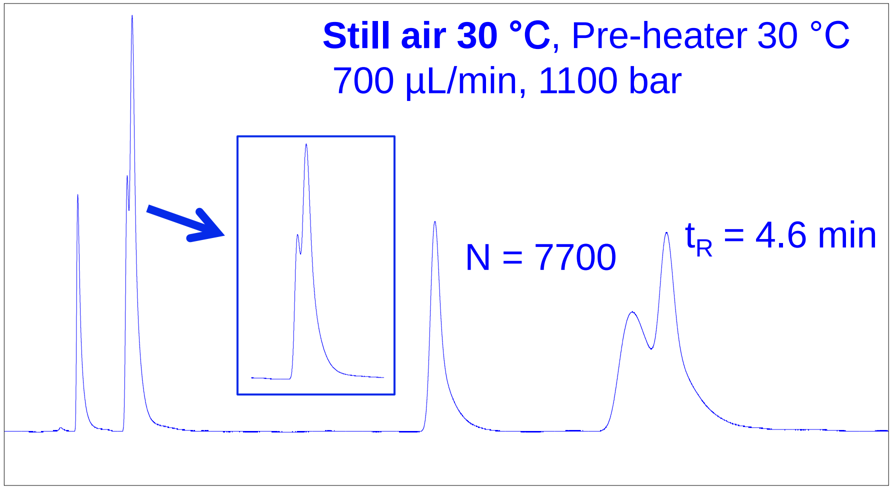


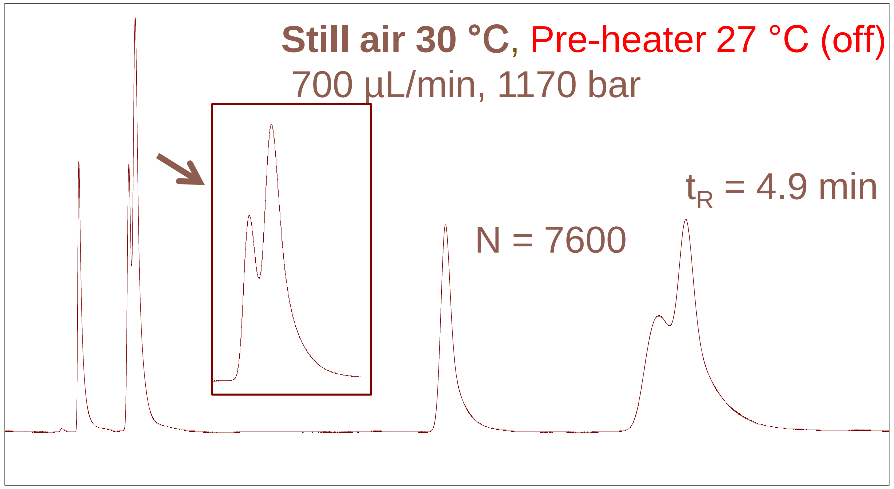


- Extreme pressure UHPLC method with MeOH mobile phase generates strong T-effects
- Thermostatting mode affects column-T and column-p ➔ impacts on selectivity and efficiency

Where Forced Air Selectivity Meets Still Air Efficiency

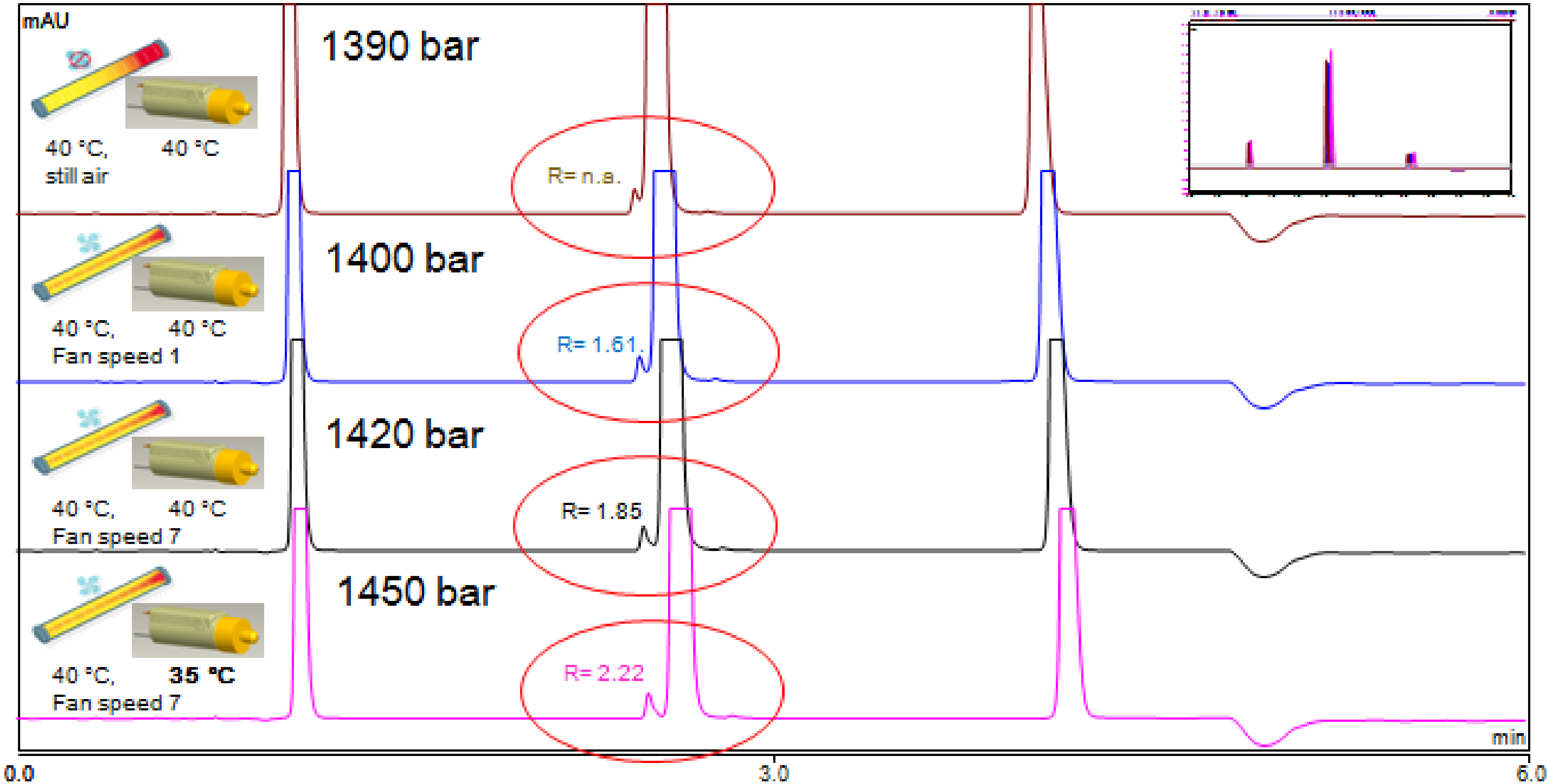






- Extreme pressure UHPLC method with MeOH mobile phase generates strong T-effects
- Thermostatting mode affects column-T and column-p ➔ impacts on selectivity and efficiency
- Pre-heater to a lower T than the compartment-T➔ forced air column-T with still air efficiency

Multiple Temperature Control Options – Method Optimization



- Fan speed and active pre-heater temp. enable to reduce column temp.
- **2 additional settings** help to resolve impurity peak

Instrument: Vanquish Horizon UHPLC system
Gradient: From 5% to 30% B in 4 min, A: 0.1% formic acid in H_2O, B: MeCN
Columns: 2 x Hypersil GOLD, 1.9 µm, 2.1x100 mm coupled in series
Sample: Mixture of gallic acid, caffeine, caffeic acid, and salicylic acid
Detection: UV 300 nm, 20 Hz, 0.2 s response time
Flow: 0.8 mL/min

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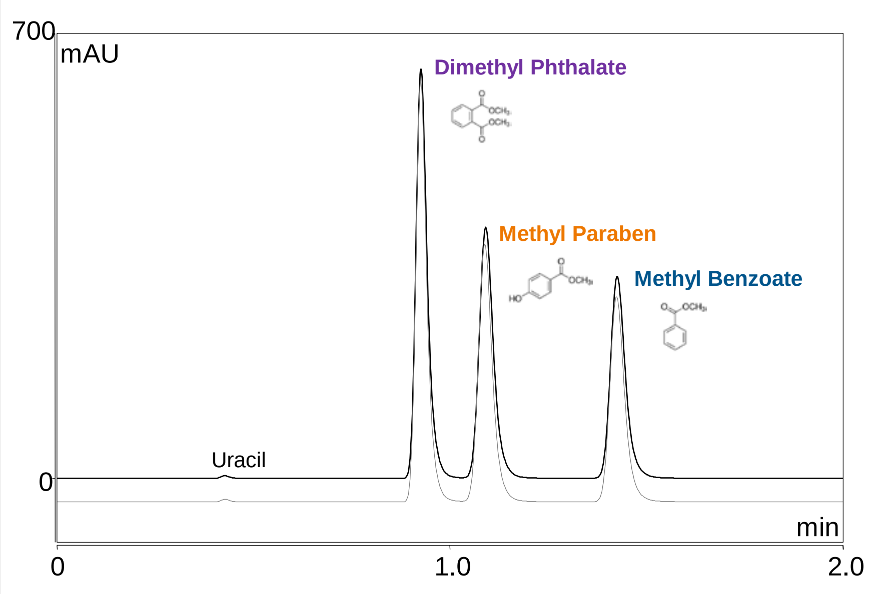
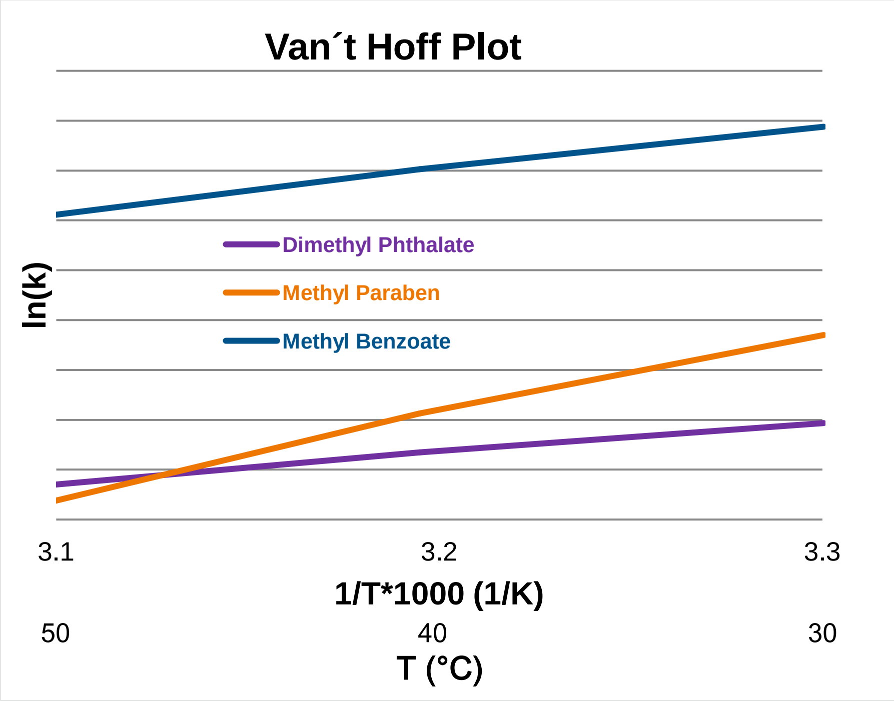
Temperature Dependent Selectivity – Van´t Hoff Plot

Application:
Phthalates and parabenes are potential endocrine disruptors and are used, next to benzoates, in personal care and beauty products



Vanquish Flex Quaternary UHPLC system

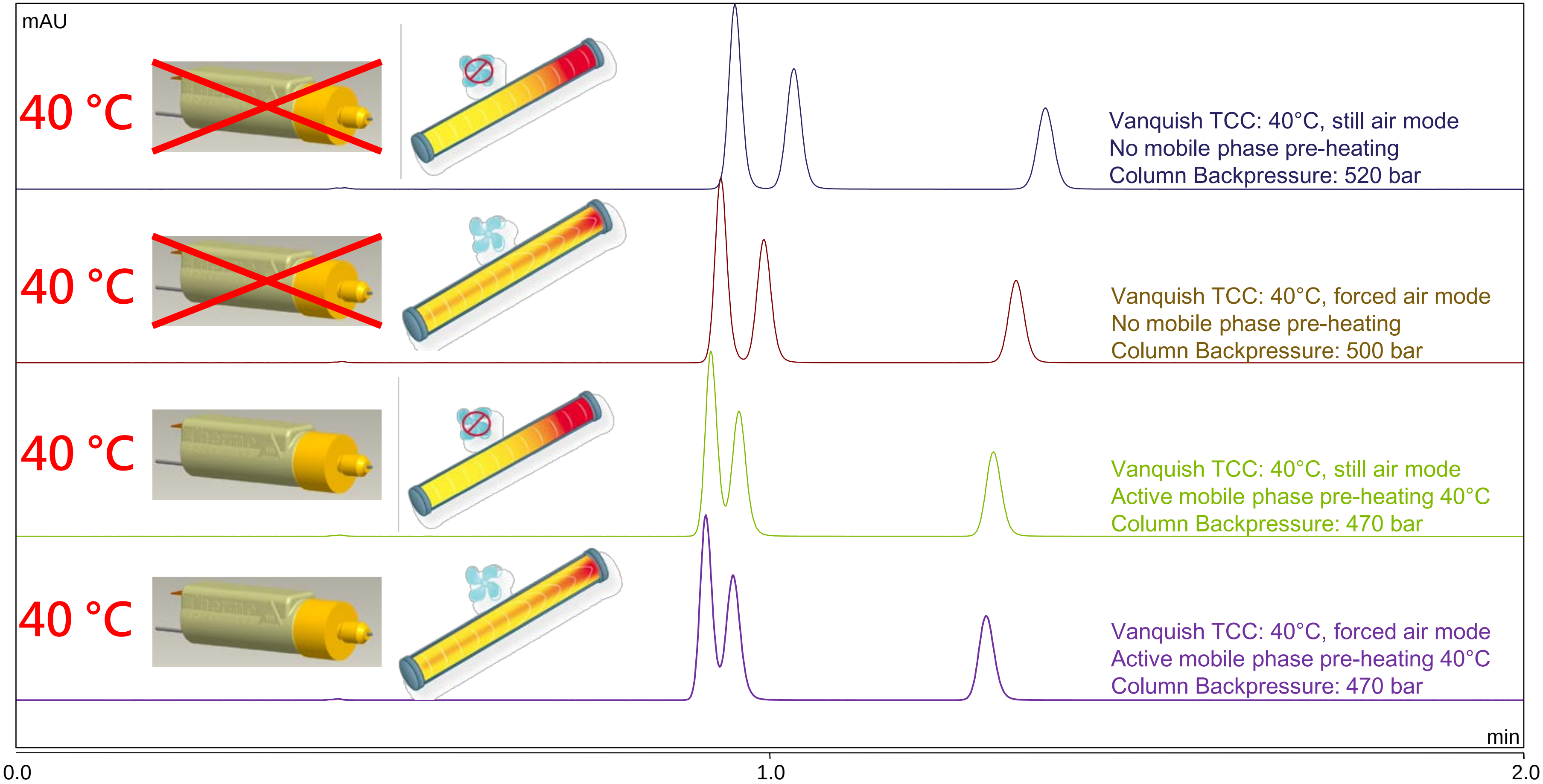
Mobile phase:	20 mM Phosphate Buffer pH 7 / Methanol (35v/65v) dial-a-mix
Column:	Thermo Scientific™ Acclaim™ RSLC PA2, 2.2 µm, 3x100 mm
Sample:	Mixture of dimethyl phthalate, methyl parabene, and methyl benzoate
Detection:	UV 210 nm, 50 Hz, 0.04 s response time
Temperature:	**30°C** (**forced air mode** or **still air mode** and pre-heater at 30°C)
Flow:	0.85 mL/min; pressure: 550 bar



Column thermostatting (see overlaid chromatograms):

- Retention and efficiency very similar in forced air and still air mode
- No indication for relevant viscous heating
- Assumption: $T_{Column} \approx T_{Thermostat}$

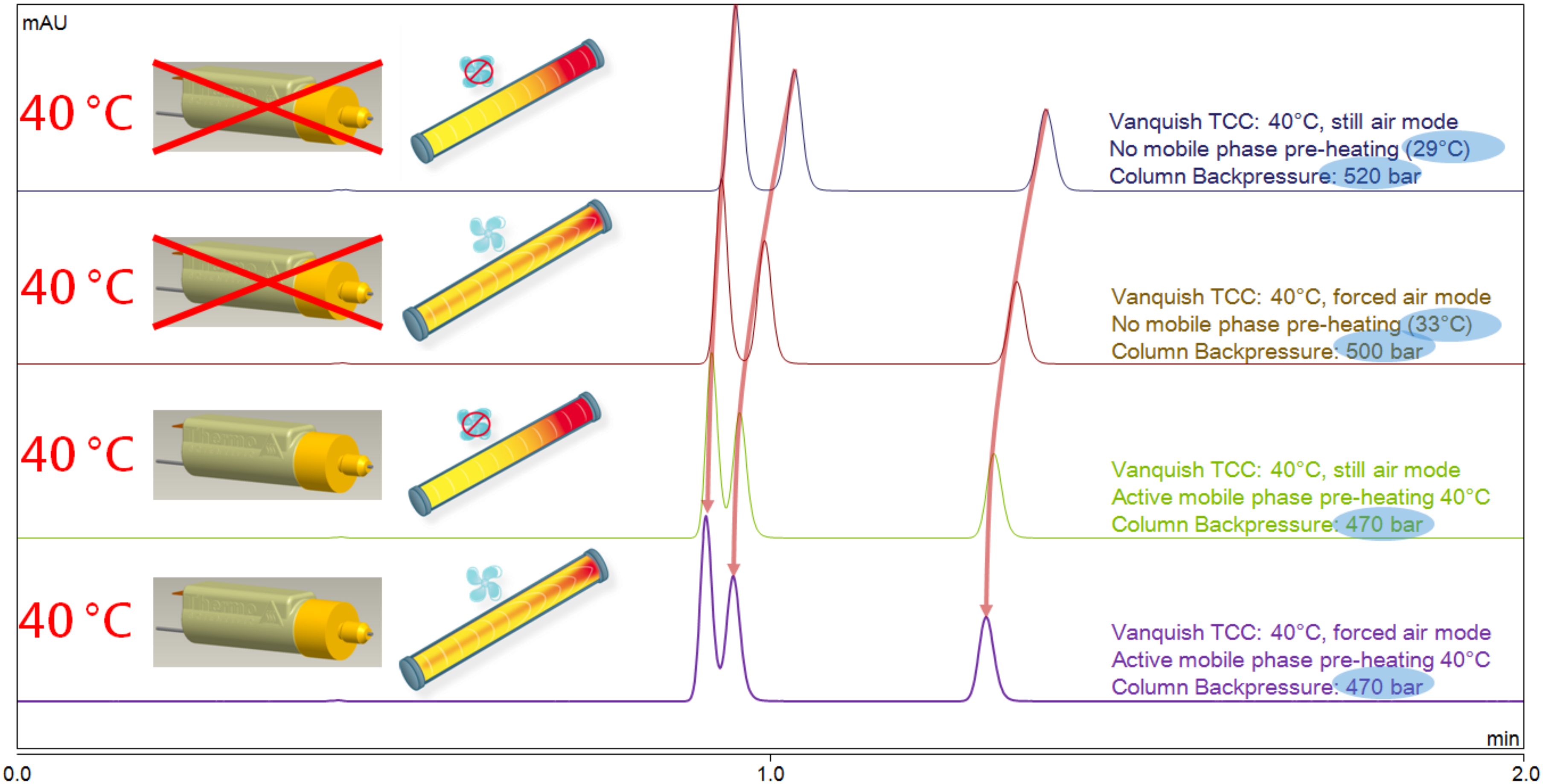
Effect of Mobile Phase Pre-Heating (Active Pre-heater off)



Vanquish Flex Quaternary UHPLC system

Mobile phase:	20 mM Phosphate Buffer pH 7 / Methanol (35v/65v) dial-a-mix
Column:	Acclaim RSLC PA2, 2.2 µm, 3x100 mm
Sample:	Mixture of dimethyl phthalate, methyl parabene, and methyl benzoate
Detection:	UV 210 nm, 50 Hz, 0.04 s response time
Temperature:	40°C (forced air mode or still air mode and pre-heater at 40°C or non-active)
Flow:	0.85 mL/min

Effect of Mobile Phase Pre-Heating (Active Pre-heater off)



Vanquish Flex Quaternary UHPLC system

Mobile phase:	20 mM Phosphate Buffer pH 7 / Methanol (35v/65v) dial-a-mix
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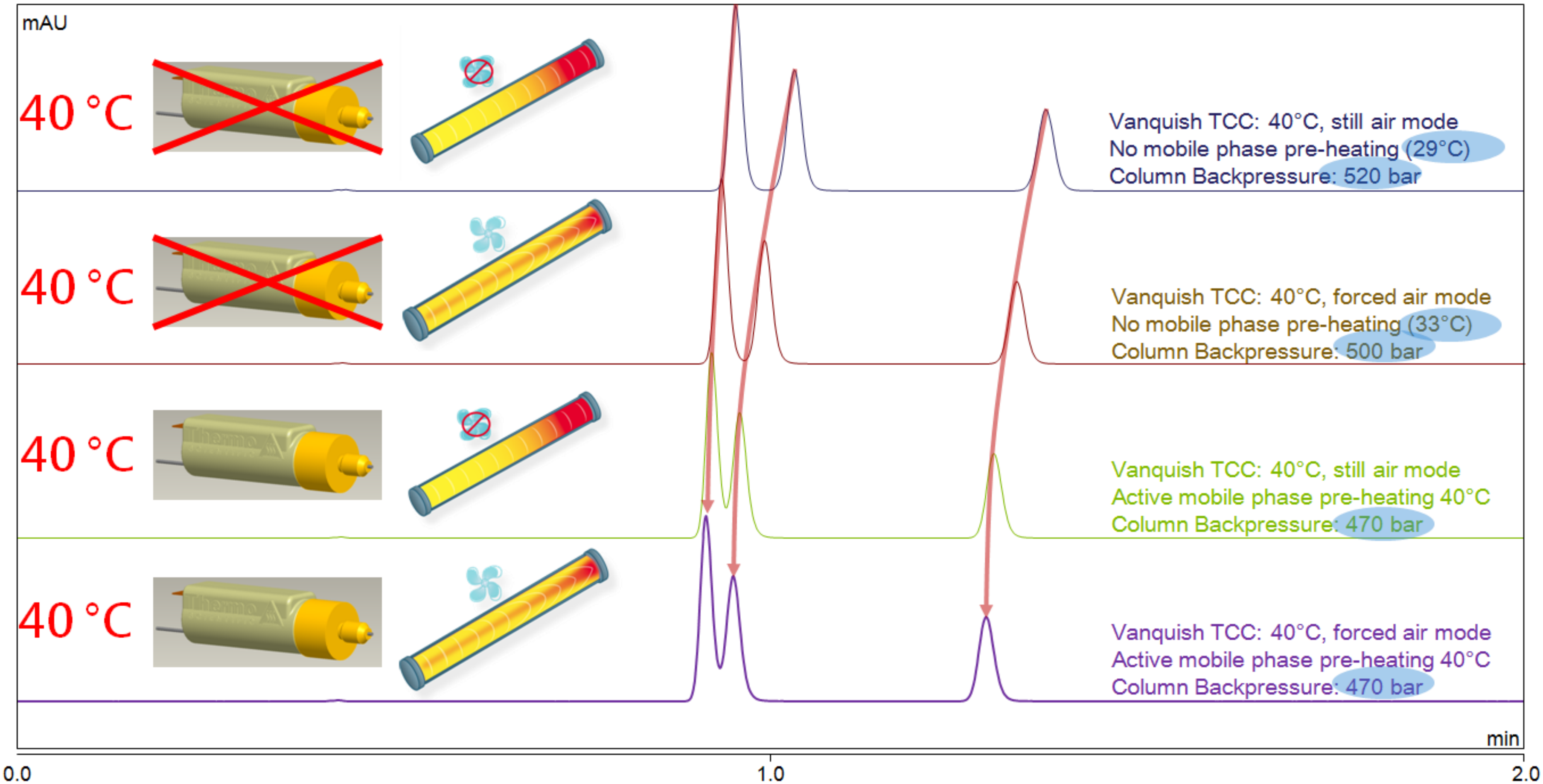
Effect of Mobile Phase Pre-Heating (Active Pre-heater off)



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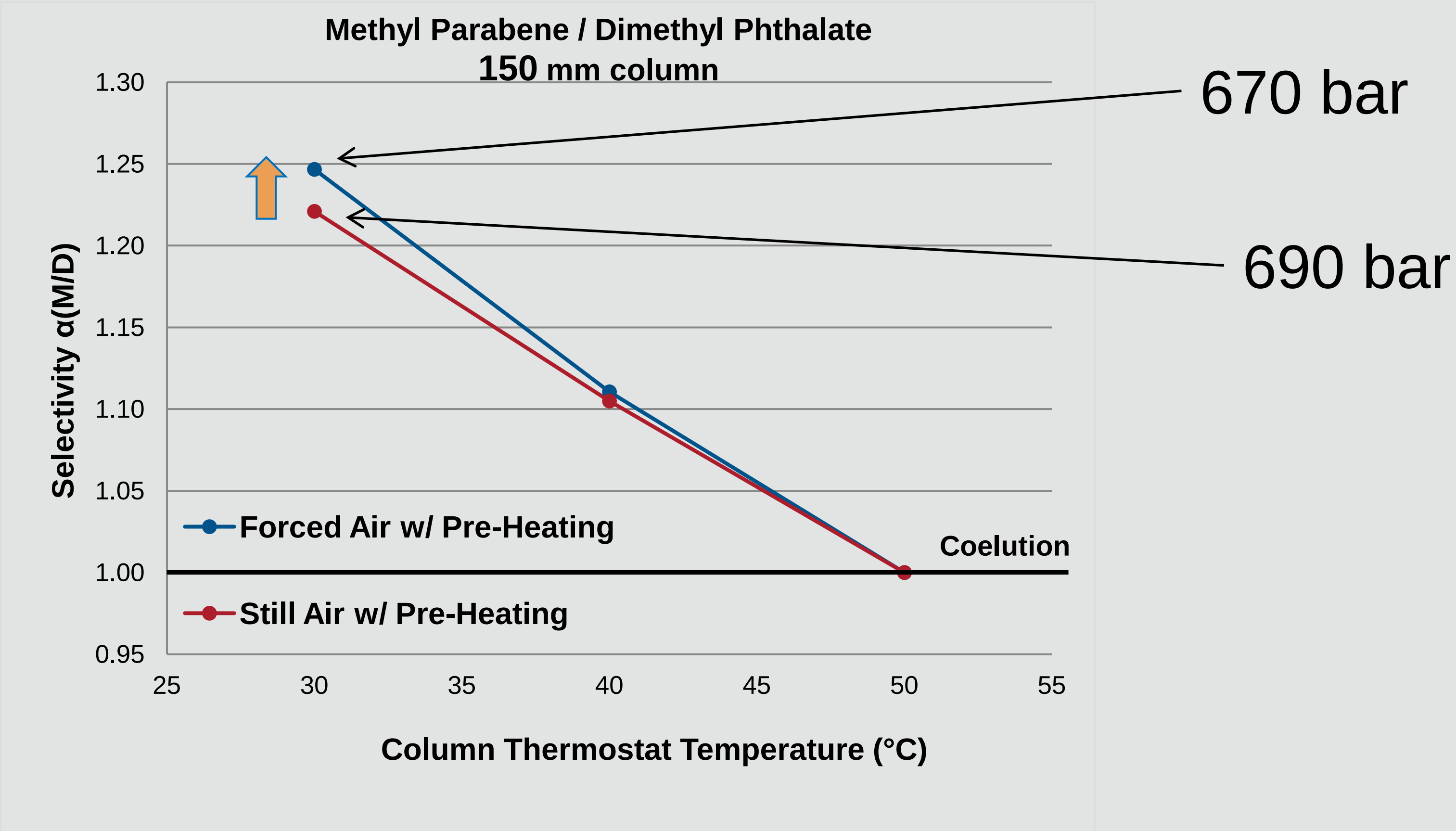
Column:	Acclaim RSLC PA2, 2.2 µm, 3x100 mm
Sample:	Mixture of dimethyl phthalate, methyl parabene, and methyl benzoate
Detection:	UV 210 nm, 50 Hz, 0.04 s response time
Temperature:	40°C (forced air mode or still air mode and pre-heater at 40°C or non-active)
Flow:	0.85 mL/min

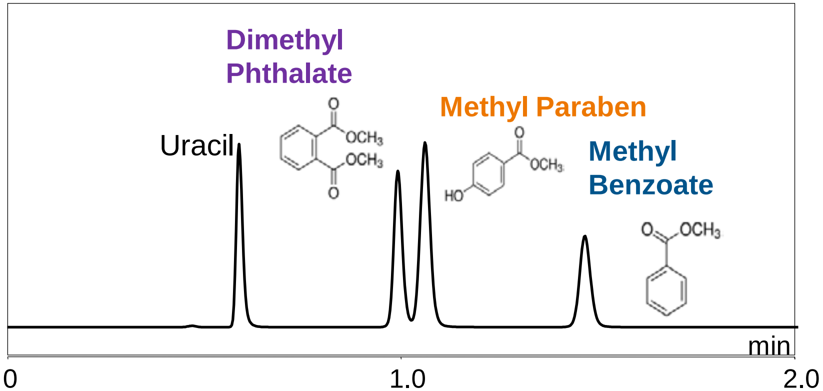
- Forced air strongly supports passive eluent pre-heating
- No relevant viscous heating as identical pressure with active pre-heating indicates

Impact on Selectivity with Longer Column and Higher Pressure

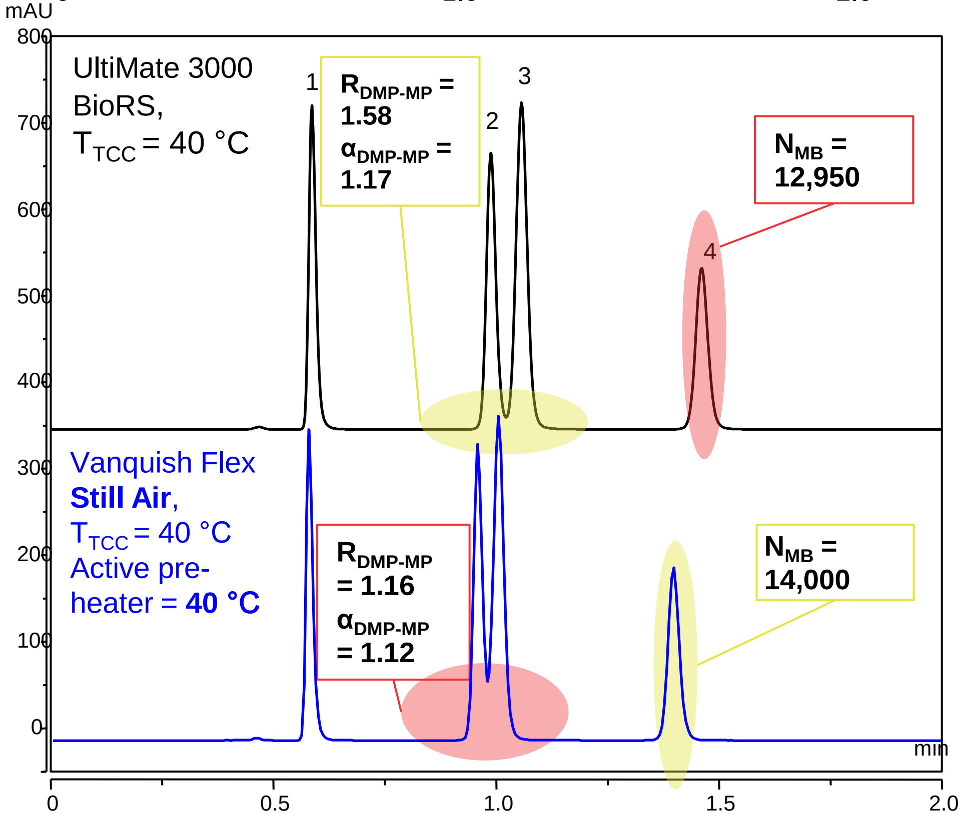


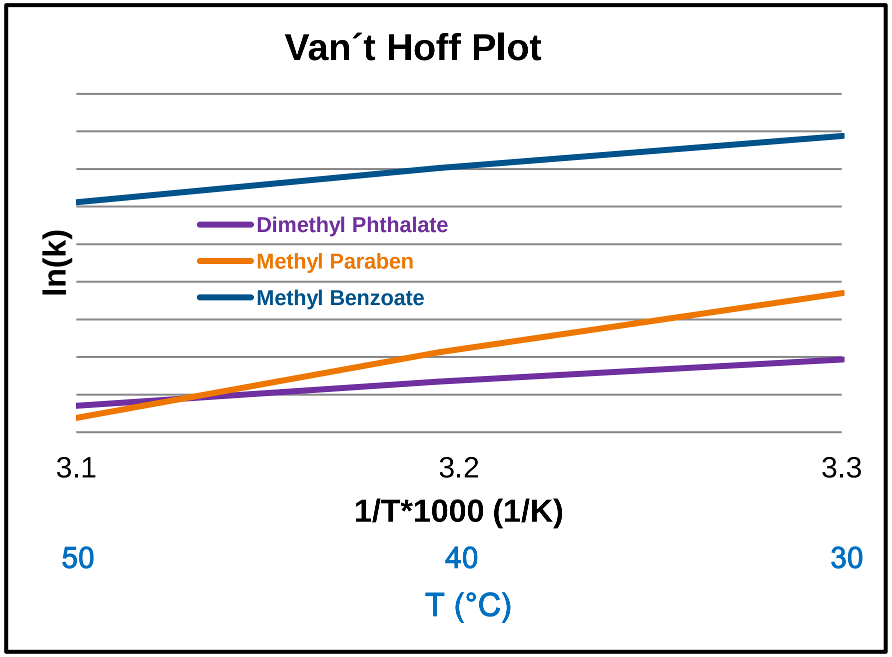
- Strongest viscous heating at 30 °C (highest viscosity)
- Forced air mode removes viscous heat, lowers T, and increases α

Column Thermostatting Effects – Analysis of Preservatives



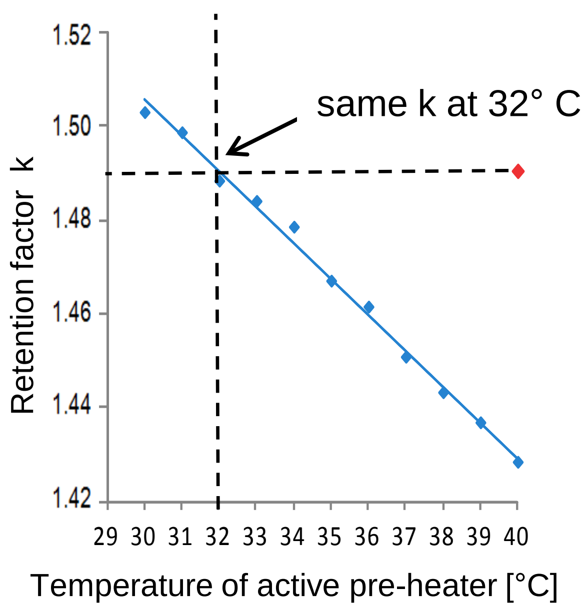
Column:	Acclaim RSLC PA2, 2.1 x 150 mm, 2.2 µm
Mobile Phase:	isocratic 20 mM phosphate buffer pH 7/methanol (35/65, v/v, dial-a-mix)
Flow rate:	0.55 mL/min, resulting in 760 bar back pressure
Temperature:	40 ºC
Injection:	1 µL
Detection:	UV, 2.5 µL flow cell, 254 nm, 50 Hz (VWD)
Sample:	Uracil and 3 preservatives



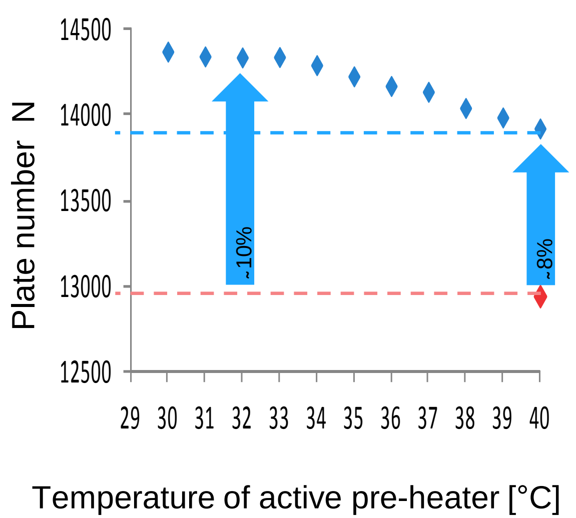


Setting Eluent Pre-heater Temperature Different to the Column Compartment

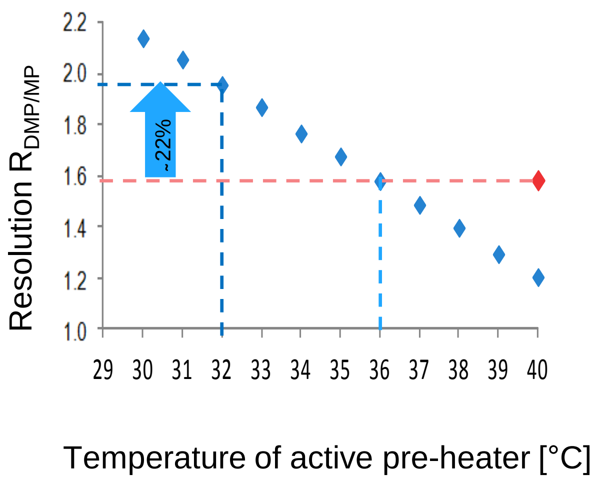
Retention of methylbenzoate



Efficiency of methylbenzoate



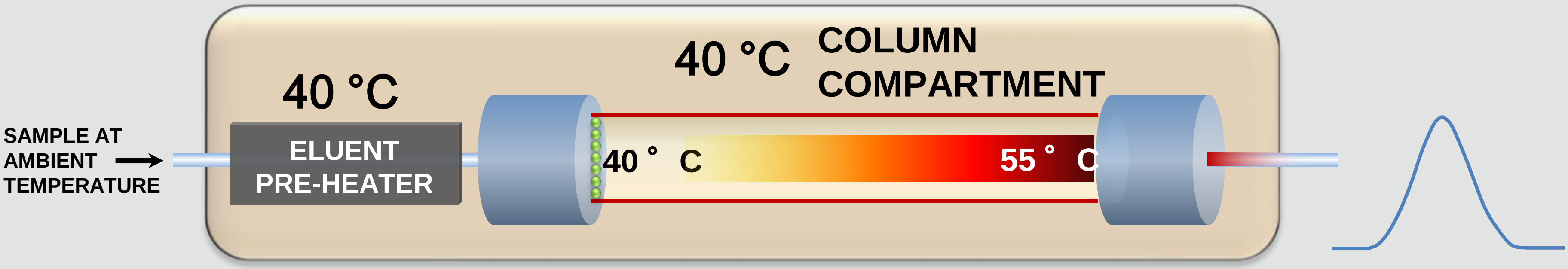
Resolution of critical pair



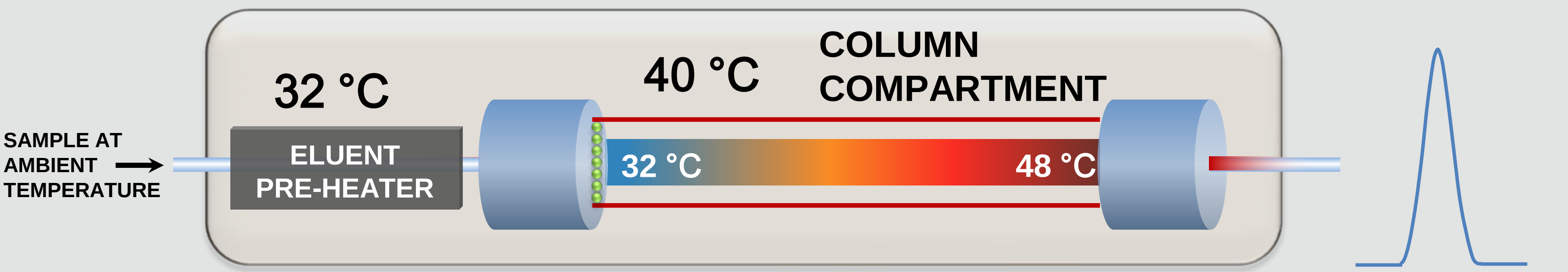
- **UltiMate 3000 BioRS** @ T_{TCC} = 40 °C (forced air, passive pre-heating by default)
- **Vanquish Flex** @ T_{TCC} = 40 °C, still air mode, active pre-heater temperature varied

Efficiency Increase with Lowering Incoming Eluent Temperature

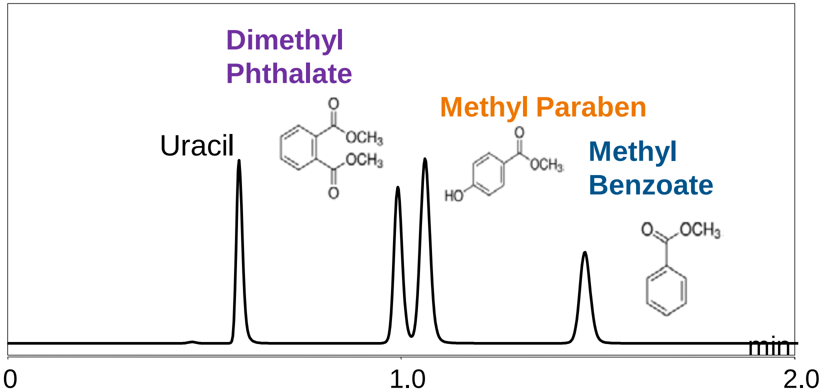
Viscous heating and pre-heater at compartment temperature:



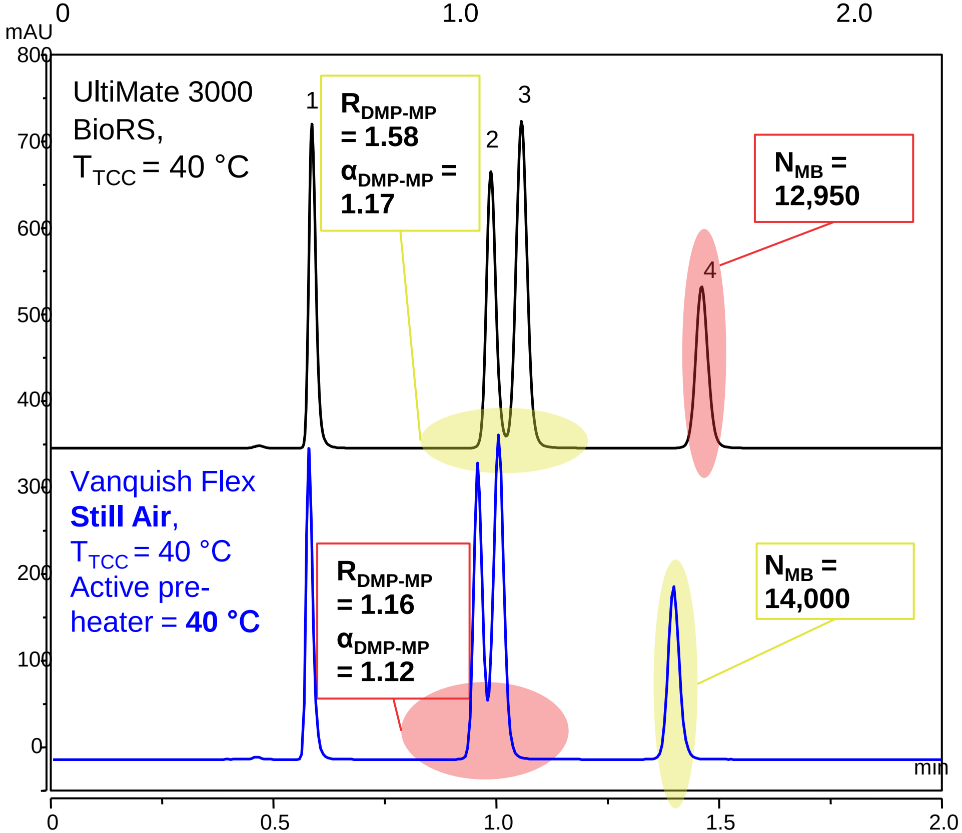
Viscous heating and pre-heater below compartment temperature :

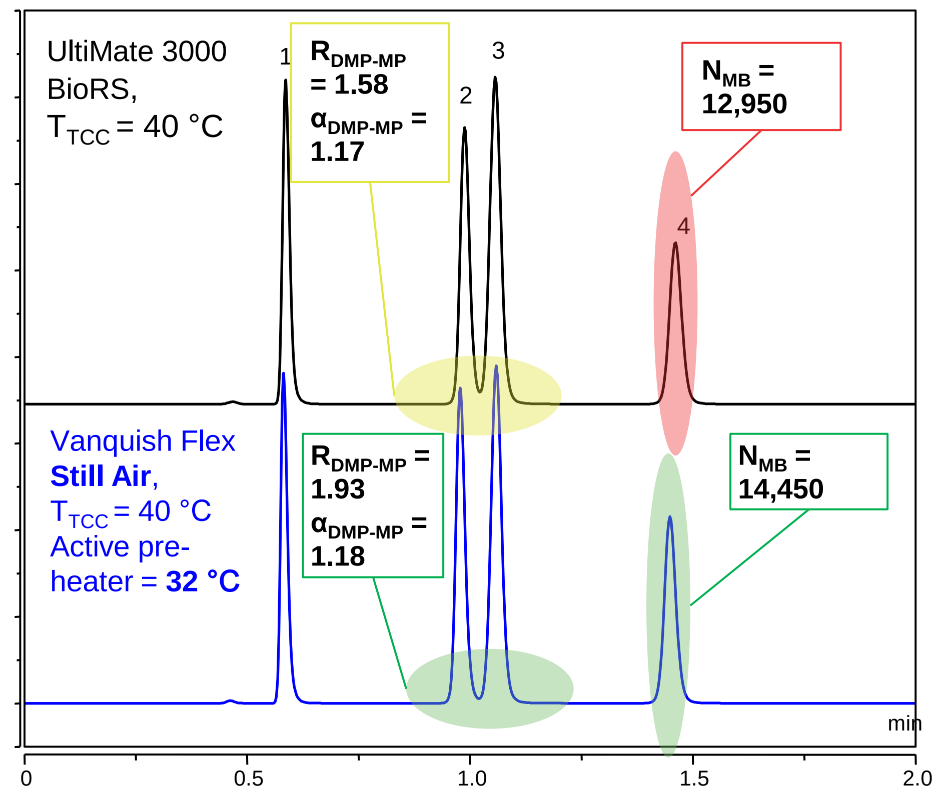


Column Thermostatting Effects – Analysis of Preservatives



Column:	Acclaim RSLC PA2, 2.1 x 150 mm, 2.2 µm
Mobile Phase:	isocratic 20 mM phosphate buffer pH 7/methanol (35/65, v/v, dial-a-mix)
Flow rate:	0.55 mL/min, resulting in 760 bar back pressure
Temperature:	40 ºC
Injection:	1 µL
Detection:	UV, 2.5 µL flow cell, 254 nm, 50 Hz (VWD)
Sample:	Uracil and 3 preservatives





In a Nutshell…

- Temperature can be used for tuning selectivity if $\alpha = f(T)$
- Temperature increase can speed-up separations, and in some applications even improve peak shapes (this is not true when only the effect on D_m is relevant).
- Under viscous heating: still air thermostatting improves efficiency, but changes retention relative to forced air thermostatting, and this can affect selectivity when $\alpha = f(T)$
- Lowering column eluent pre-heater temperature to compensate viscous heating effects enables selectivity and efficiency optimization and helps in method transfer