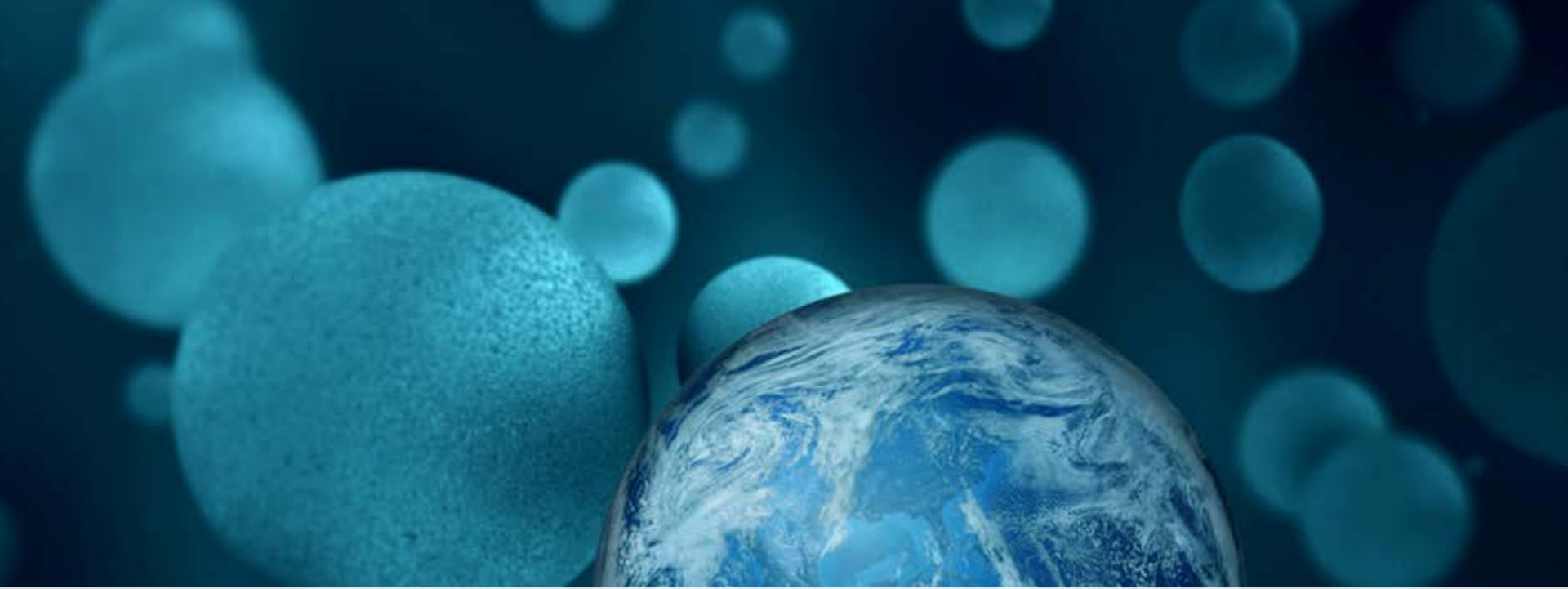

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Tips and Tricks for HPLC and UHPLC

Jan Pettersson
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Thermo Fisher Scientific, Hägersten/Sweden

Overview

Thermo Scientific™
Vanquish™ UHPLC system

Thermo Scientific™
UltiMate™ 3000 UHPLC system

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Mobile Phase

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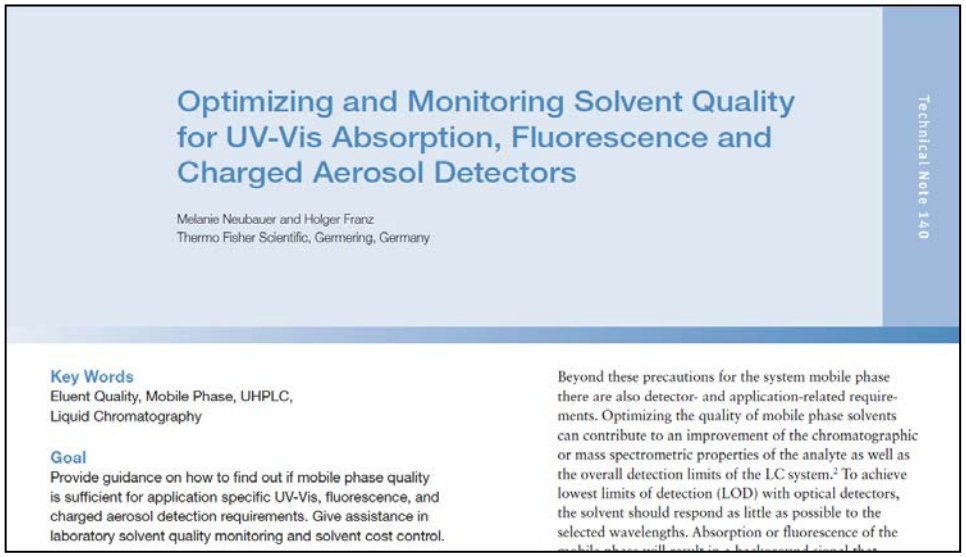
Common Problems: Mobile Phase

- **Solvent compatibility**
  - Try to use pre-mixed solvents
    - Add 5-10% of organic eluent to the aqueous eluent
    - Add 5-10% aqueous eluent to the organic eluent
    - Avoids local crystallization in the pump (with buffers)
  - Eluents with salt buffers
    - Change eluents with salt buffers regularly
    - Filtrate buffers
    - Use water with 18,2M Ohm AND <5ppb TOC

Solvent Quality

- **Technical note**
  TN140: Solvent quality

- The quality of the eluent is very important to keep the noise as low as possible.
- Make sure that the eluents are good by running them without injection (Sample type "Blank“).
- For MS and Thermo Scientific™ Corona™ charged aerosol detector only use volatile buffers.



Technical Note 140

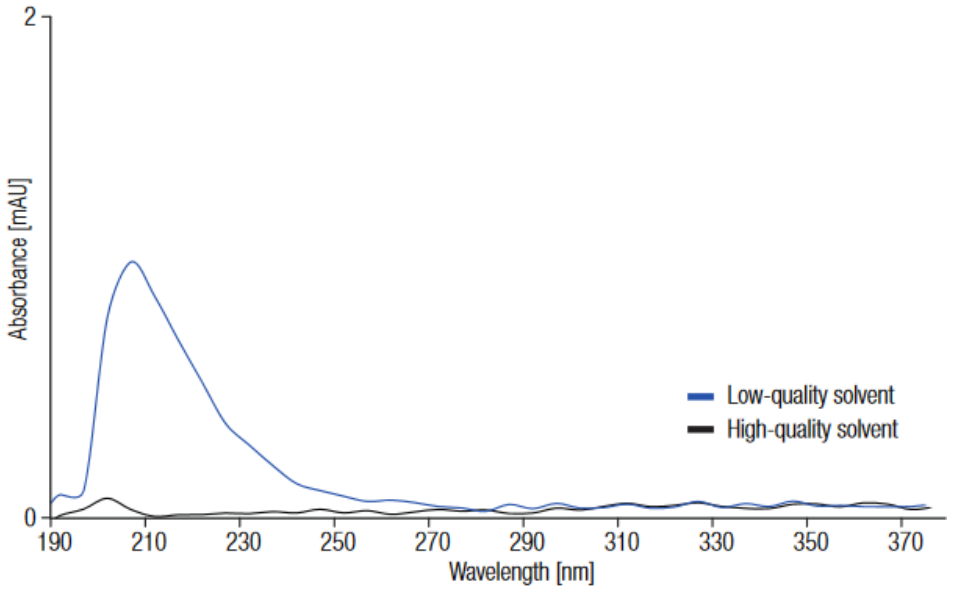
Optimizing and Monitoring Solvent Quality for UV-Vis Absorption, Fluorescence and Charged Aerosol Detectors

Melanie Neubauer and Holger Franz
Thermo Fisher Scientific, Germering, Germany

Key Words
Eluent Quality, Mobile Phase, UHPLC, Liquid Chromatography

Goal
Provide guidance on how to find out if mobile phase quality is sufficient for application specific UV-Vis, fluorescence, and charged aerosol detection requirements. Give assistance in laboratory solvent quality monitoring and solvent cost control.

Beyond these precautions for the system mobile phase there are also detector- and application-related requirements. Optimizing the quality of mobile phase solvents can contribute to an improvement of the chromatographic or mass spectrometric properties of the analyte as well as the overall detection limits of the LC system.[2] To achieve lowest limits of detection (LOD) with optical detectors, the solvent should respond as little as possible to the selected wavelengths. Absorption or fluorescence of the



UV-spectra at 200–250 nm of two methanol samples (both LC/MS grade)

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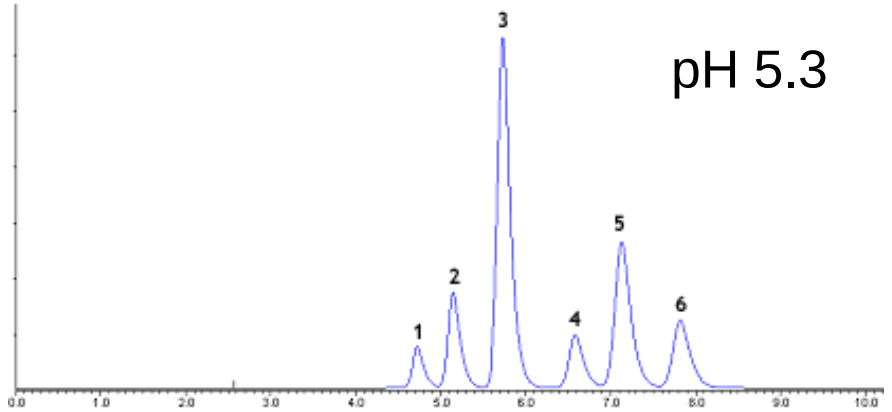
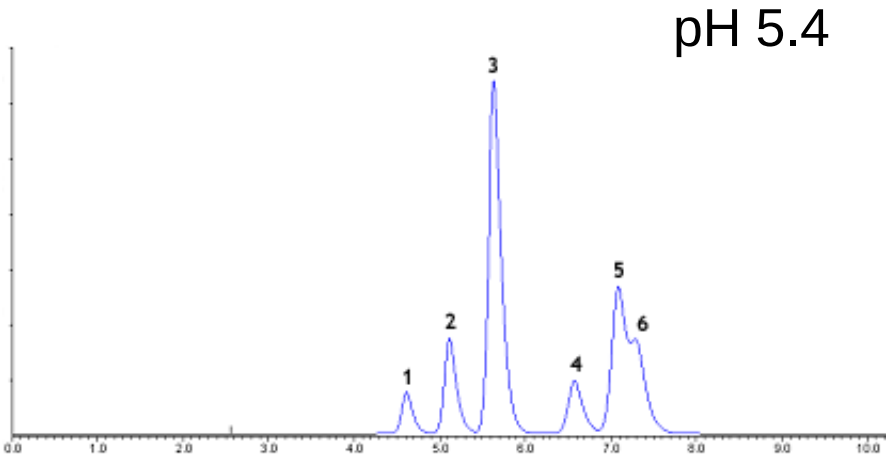
Common Recommendations: Mobile Phase

- Symptoms
  - Peaks shift
  - Loss of resolution
- Causes
  - Mobile phase pH changes
- Prevention
  - Use correct buffer for pH range.
  - Control pH of mobile phase.
  - Maintain buffer strength in aqueous phase.
  - Control temperature.



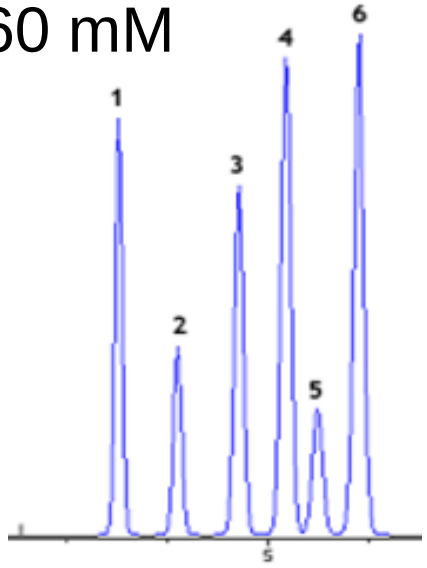


Common Buffers and their Effective pH Range

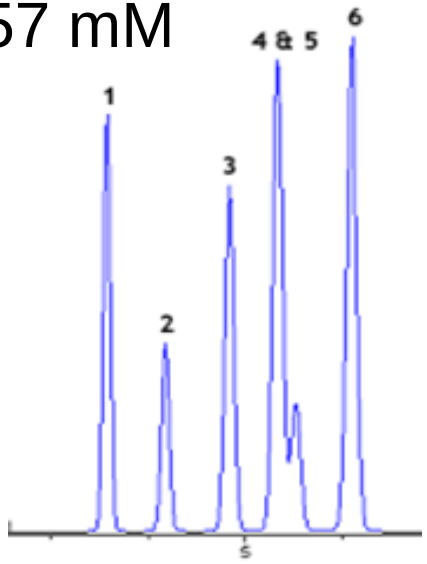
Buffer	pKa	pH range
Phosphate	2.1, 7.2, 12.3	1. -3.1, 6.2-8.2, 11.3.13.3
Citrate	3.1, 4.7, 6.4	2.1- 4.1, 3.7-5.7, 5.4-7.4
Carbonate	6.1, 10.3	5.1-7.1, 9.3-11.3
Formate	3.8	2.8-4.8
Acetate	4..8	3.8-5.8
Ammonia	9.3	8.3-10.3
Borate	9.2	8.2-10.2

Common Problems: Mobile Phase

60 mM



57 mM



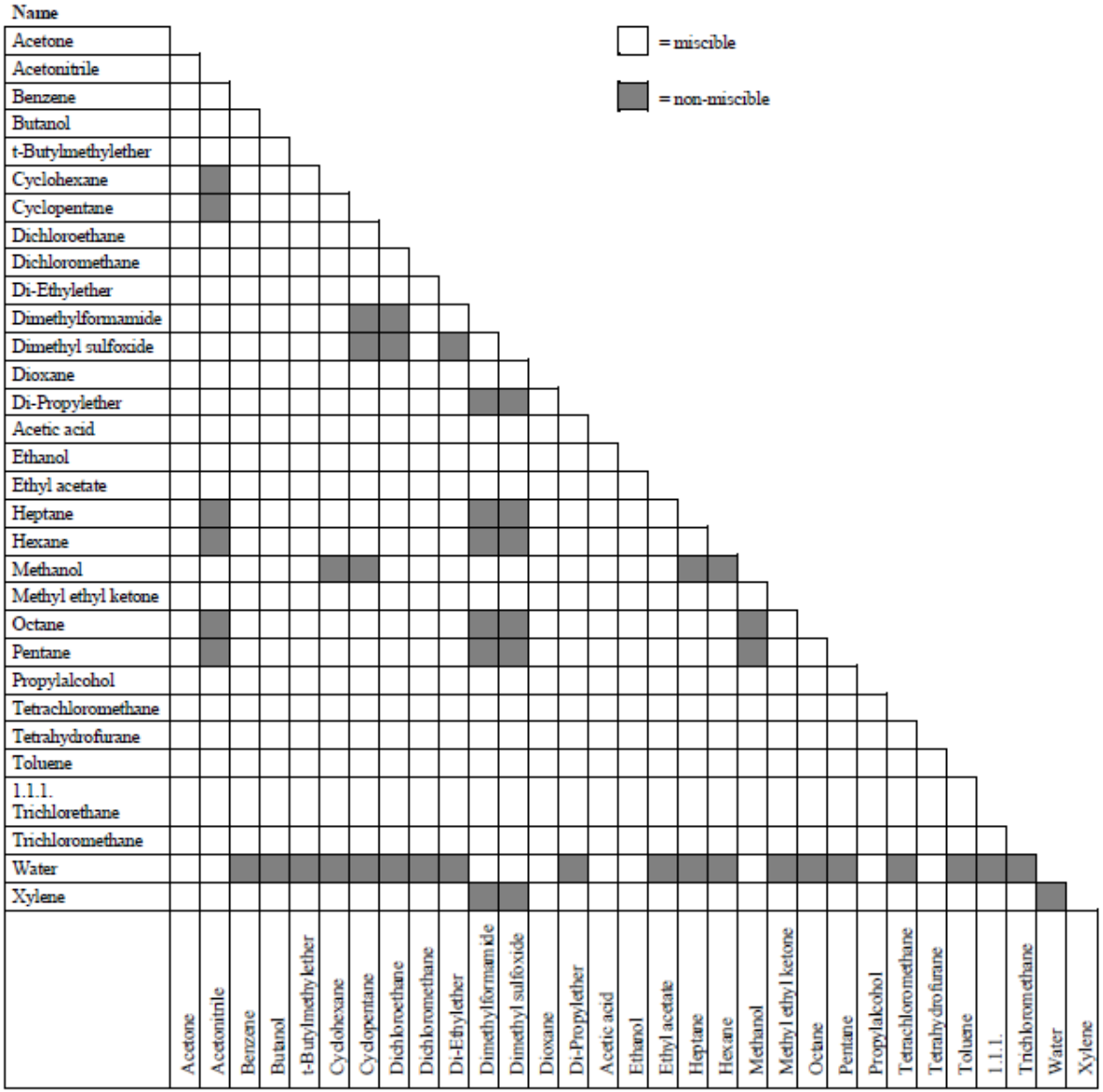
- The two chromatograms illustrate the effect of a small reduction (3 mM) in buffer concentration on the separation of six food additives:
  - Symptoms
    - Peaks shift
    - Loss of resolution
  - Causes
    - Mobile phase buffer strength changes
    - Prevention
      - Maintain buffer strength in aqueous phase.
      - Control temperature.
      - Filter solvents rather than using vacuum degassing.

Solvent Effects – Why Water and AcN are Popular

Solvent (nm)	Minimum wavelength
Acetonitrile	190
Water	191
Cyclohexane	195
Hexane	201
Methanol	203
Ethanol	204
Ethoxyethane	215
Dichloromethane	220
Trichloromethane	237
Tetrachloromethane	257

- Inappropriate solvent choice can cause issues with reduced sensitivity, noise and rising baselines in gradient analysis.

Common Problems: Solvent Mixing



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The Pump

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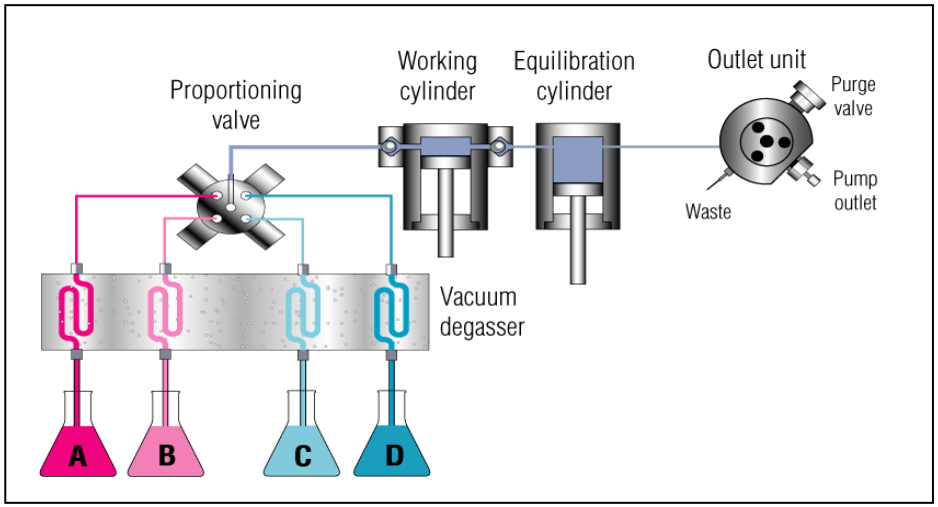
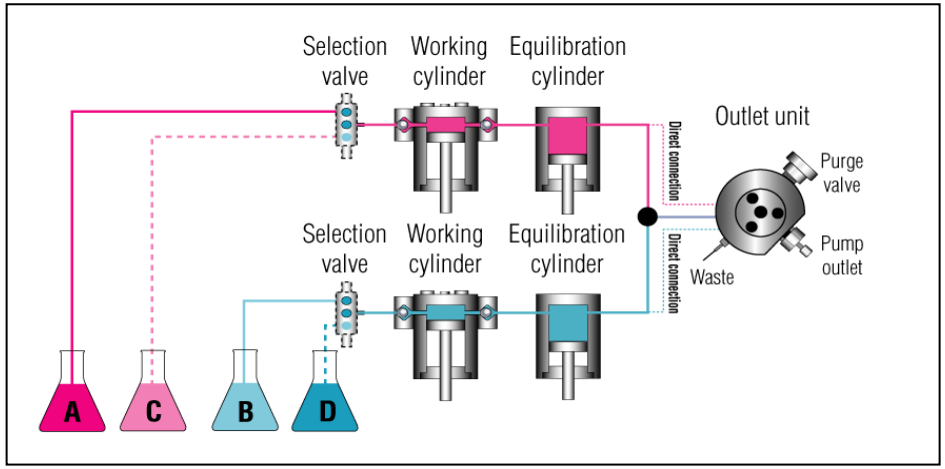
Pump Types



LPG pump type

- Eluent composition at the low pressure side
  => Before the pump head
- Eluent is composed through proportioning valves
- Eluent segments pass working and equilibration cylinder
- Up to four solvents can be mixed

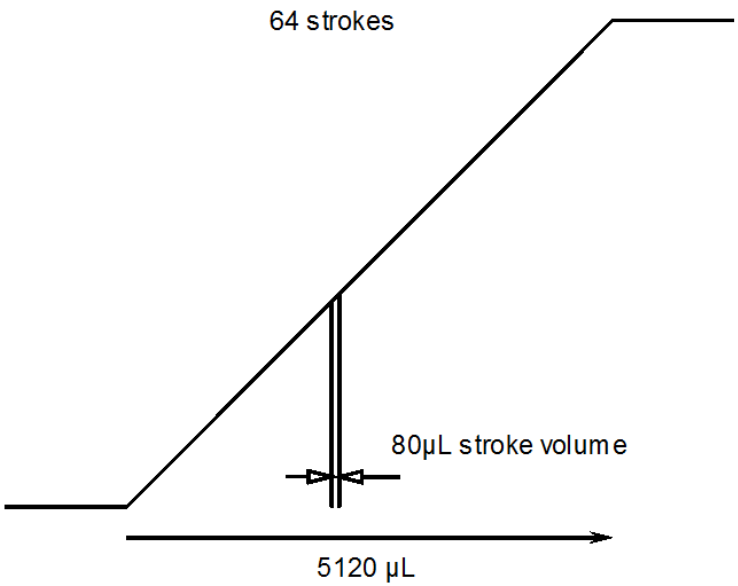


HPG pump type

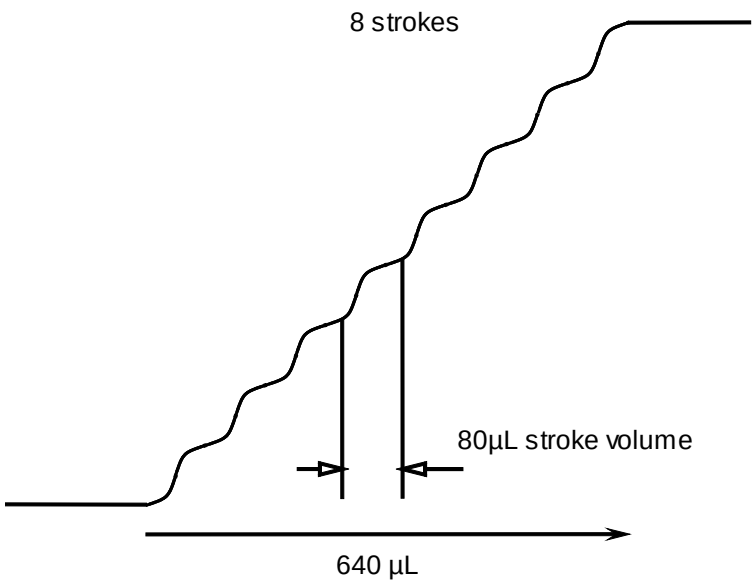
- Eluent composition at the high pressure side
  => Behind the pump head
- Pure solvents pass working and equilibration cylinder
- Eluent mixture is prepared with two pump blocks
  => Binary eluent mixture only

LPG and Ballistic Gradients

- Speed of ballistic gradients is limited by the composition change per stroke. This should not higher be than 2.0% per stroke.



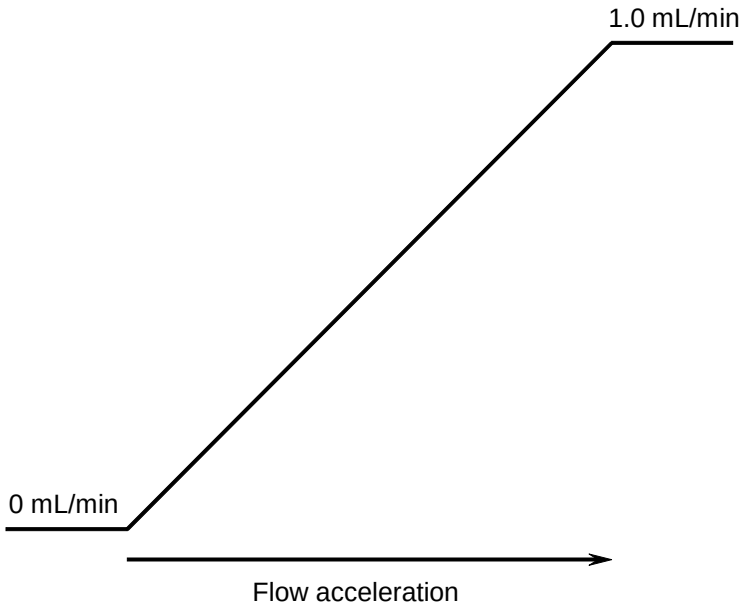
5.0 min gradient time @ 1 mL/min
Gradient volume = 5120 µL
Piston strokes for gradient = 64
For 0-50% gradient the composition changer per stroke is **0.8%**



38 s gradient time @ 1 mL/min
Gradient volume = 640 µL
Piston strokes for gradient = 8
For 0-50% gradient the composition changer per stroke is **6.3%**

HPG and Ballistic Gradients

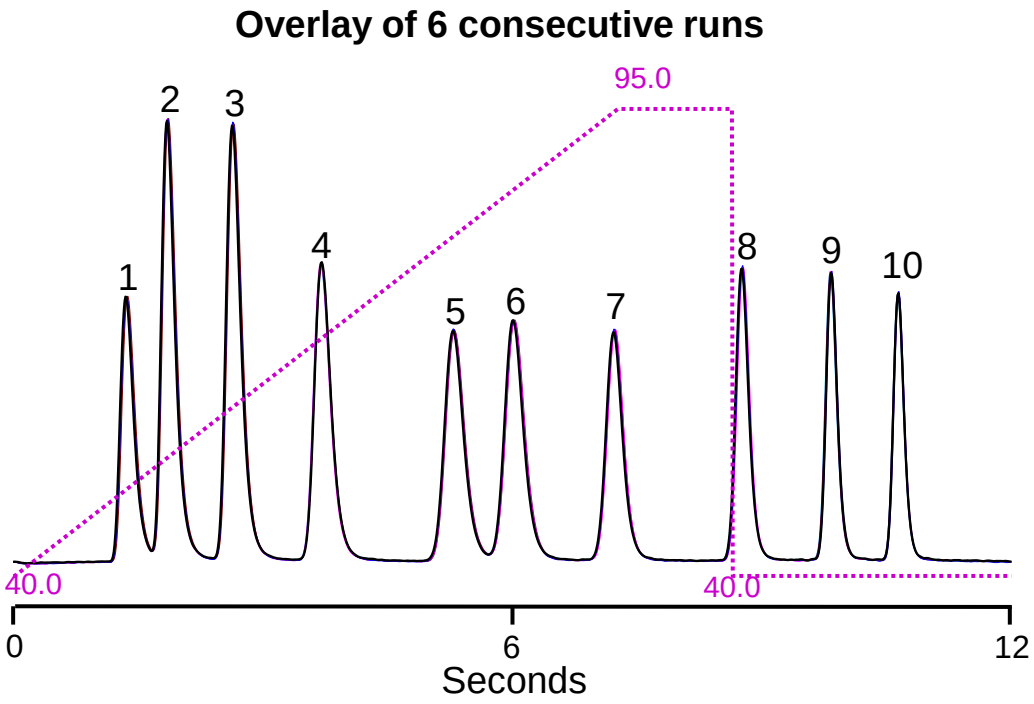
- Speed of ballistic gradients limited by the flow acceleration and deceleration of the pump



Ballistic gradient formation via ultra-precise acceleration and deceleration with an internal resolution of 125 Hz

→ **Ballistic gradients faster than a second are theoretically possible**

10 Peaks in 10 Seconds with a Ballistic Gradient



Test conditions

Column:	C18, 30 x 2.1 mm, 1.8 µm
Eluents:	A: Water B: Acetonitrile
Flow:	3.70 mL/min @ 725 bar (10,500 psi)
Temperature:	100 ° C
Inj. volume:	1 µL
Test mixture:	Uracil and 9 alkylphenones

Resolution (Critical peak pair): 1.7

	Peak number									
	1	2	3	4	5	6	7	8	9	10
Retention time RSD [%]	0.76	0.41	0.29	0.14	0.15	0.14	0.11	0.09	0.08	0.05
Retention time SD [ms]	8.54	8.81	8.61	9.14	17.00	18.34	16.26	15.25	11.09	10.97

HPG-3200RS

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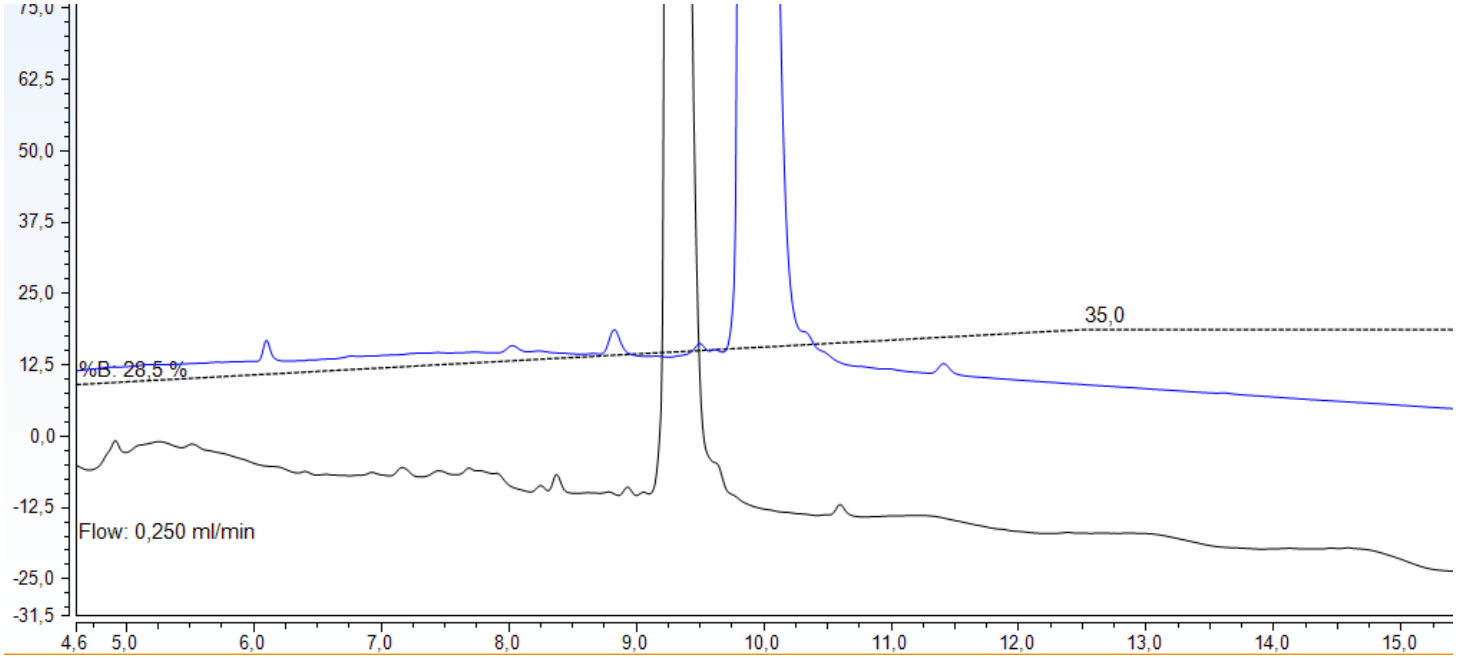
The Comprehensive SpinFlow Mixer Portfolio

- Range of static mixers suitable from low-GDV LC/MS application to high-sensitive TFA application

Thermo Scientific™ SpinFlow™ technology

Effects of Different Mixers

- These samples were run with the same Vanquish Horizon system, the only change was the size of the mixer
- Flow 0,250µl/min
- A: (0.1% TFA, 50 mM NaCl):MeCN 95:5
- B: (0.1% TFA, 50 mM NaCl):MeCN 30:70
  - Black 10µl mixer
  - Blue 350µl mixer



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Easy Tunable for Optimum Application Performance

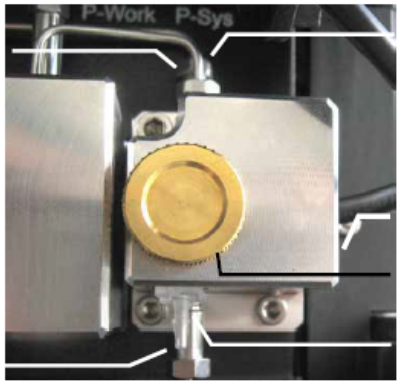
- For fast separations where the mixing ripple does not interfere with the detection (e.g., Corona charged aerosol detector or MS detectors), use the low mixer volumes (35 µL, 100 µL).
- Use the medium sized mixers (200 µL, 400 µL) as the best balance between fast separation and low mixing ripple in UV detection.
- For highest sensitivity and when mixing ripples interfere with the detection (e.g., due to use of UV-absorbing solvents), use a larger mixer volume (400 µL, 800 µL).
- For UV-absorbing solvent additives that amplify the mixing ripples by interaction with the stationary phase (e.g., TFA application), use for highest sensitivity the largest mixer volumes (800 µL, 1550 µL).

Practical Use of a HPLC Pump

- Prime the pump – but be sure that there is eluent in the pump

- Manually or with the autosampler

Pump Type	Purge Flow	Purge Time
Analytical pump	3 mL/min	5 min
Micro pump	2 mL/min	5 min
Semipreparative pump	30 mL/min	5 min



Connection port for the right pump head (if available)

Connection port for the left pump head

Pressure transducer for the system pressure

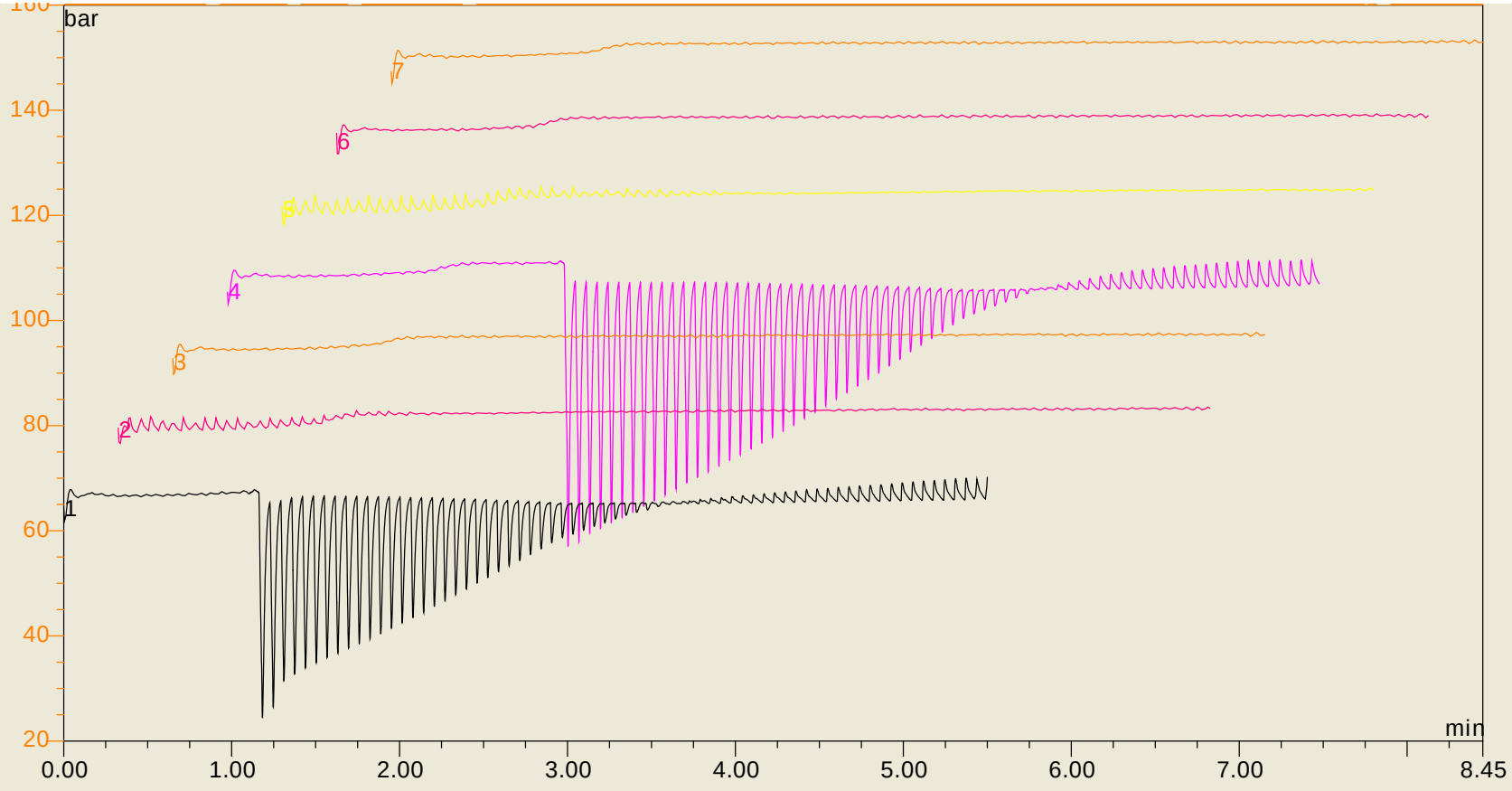
Purge valve knob

Connection port (capillary mixer/ inline filter/pulse damper)

Purge outlet nozzle

Prime and Degas

- Air bubble stuck in the pump
- Prime the pump
- Degas the eluents 5 min in an ultrasonic water bath



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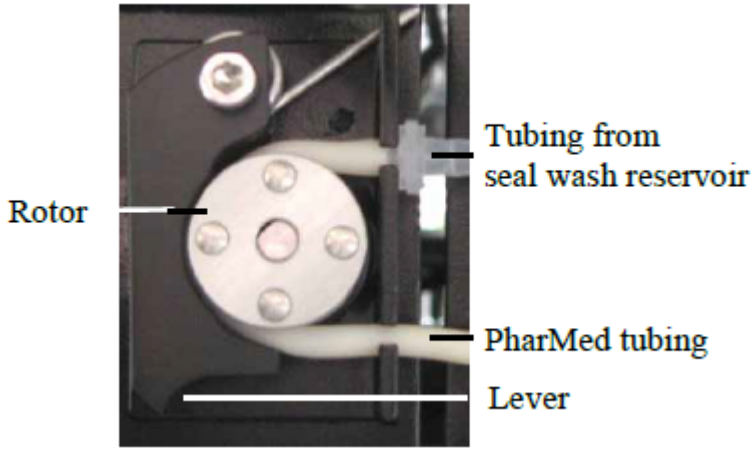
Practical Use of a HPLC Pump

- Use eluent filters if necessary
- Use a degasser – it might be a good idea to degas in an ultrasonic water bath for 5 min

- Seal wash – helps the pump seal to survive
- If the pump is out of wash solution it should not start
- If the pump starts to leak the Thermo Scientific™ Chromeleon™ chromatography data software will give a warning



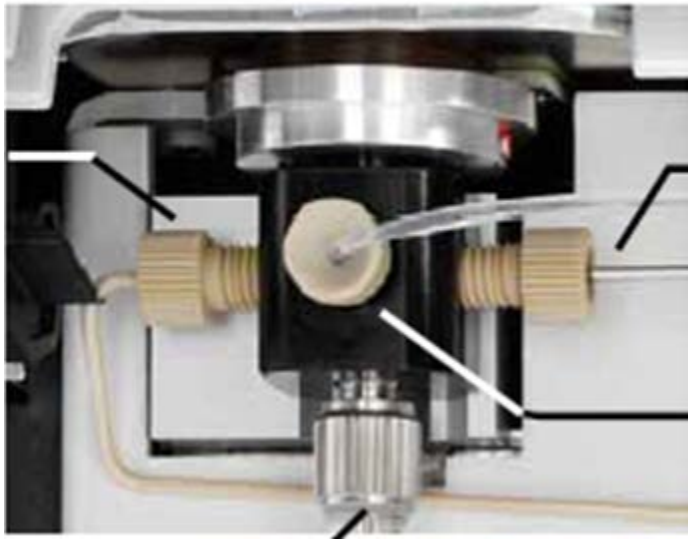
Autosampler

Autosamplers

- Modern autosamplers should be considered as pipetting robots
- Other than injecting the sample they can:
  - Dilute samples
  - Do pre-column derivatization
  - Spike the samples

Autosamplers

- Before you run samples flush the syringe
  - Even a tiny air bubble ruins the performance
  - Autosamplers need a transport liquid
  - Usually eluent A is connected to the sampler and the pump
  - It is possible to use a separate bottle of transport liquid
  - The transport liquid is used to wash the needle

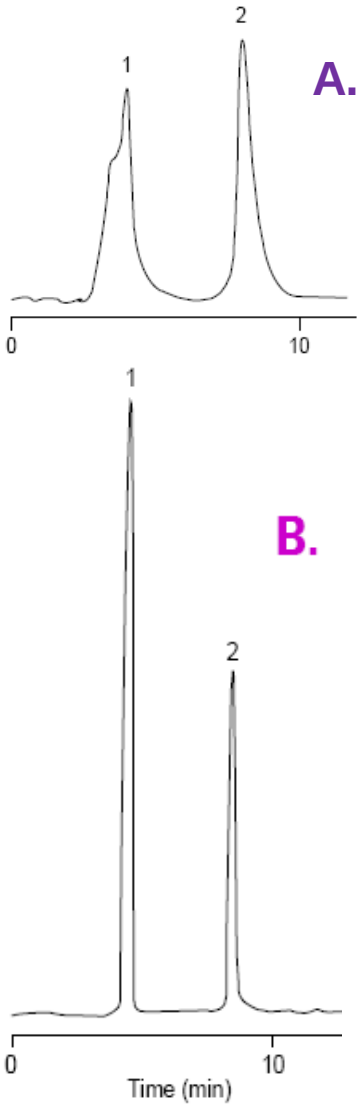


Important Parameters

- **DrawSpeed**
  - Defines the speed at which the sample is drawn by the syringe
  - In analytical range (5 - 100 µL) a draw cycle should normally take 3 - 4 seconds.
    At lower volumes, a draw cycle should take approximately 10 times longer

    Examples *(normal HPLC eluents and samples dissolved in starting eluent)*
    - => 10 µL injection volume -> Recommended draw speed: 2 - 3 µL/sec
    - => 2 µL injection volume -> Recommended draw speed: 0.2 - 0.3 µL/sec
  - Draw speed has to be adapted in case of samples and eluents with higher viscosity.
  - Incorrect setting is frequently responsible for area precision problems.

Sample Solvent



- Matching injection volume with solvent strength
  - Ideally the sample solvent will not affect chromatographic separation
  - If a stronger sample solvent is required, injection volumes should be kept to a minimum
  - In a strong solvent, the sample moves more quickly through the mobile phase and often split or distorted peak shapes are observed as in chromatogram A
  - The syringe is usually connected to eluent A

Vials and Septa

- Do not overfill the vial – the septum is used to clean the needle. Fill 2/3 of the volume
- If you use inserts make sure there are no airbubbles in them
- Autosamplers with PEEK needle must have caps with slit septa
- Never shake the vial. If you do there is going to be sample on the underside of the septum which gives carry over
- Adjust the needle height according to the vial dimensions
- Rubber septas get dry and can block the injector

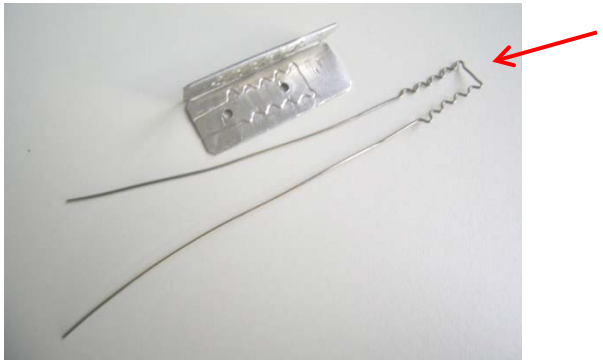
... And use the vial and septum only once!

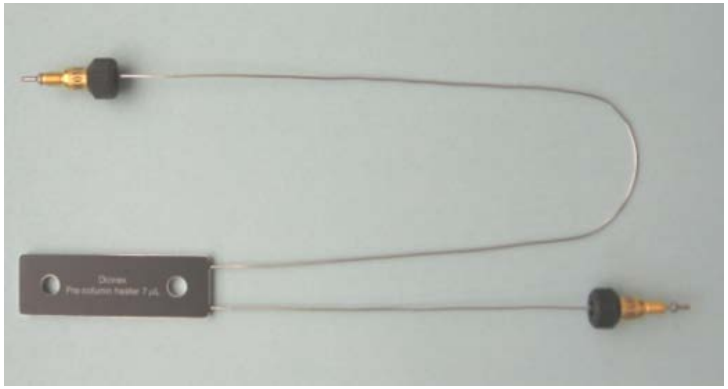
Column Compartment

Pre-Column Heater

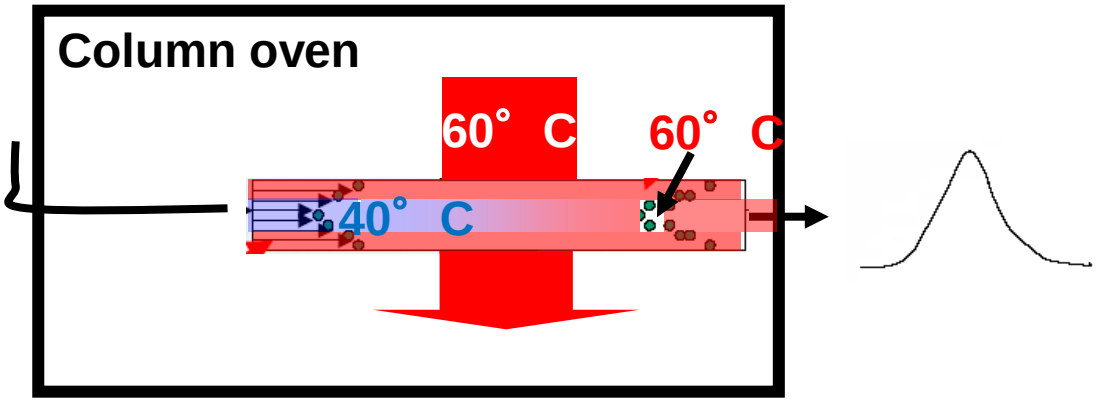
- Serpentineshaped capillary embedded in aluminum block
- Different types available

2µL,	0.12mm ID
7µL,	0.18mm ID
11µL,	0.25mm ID



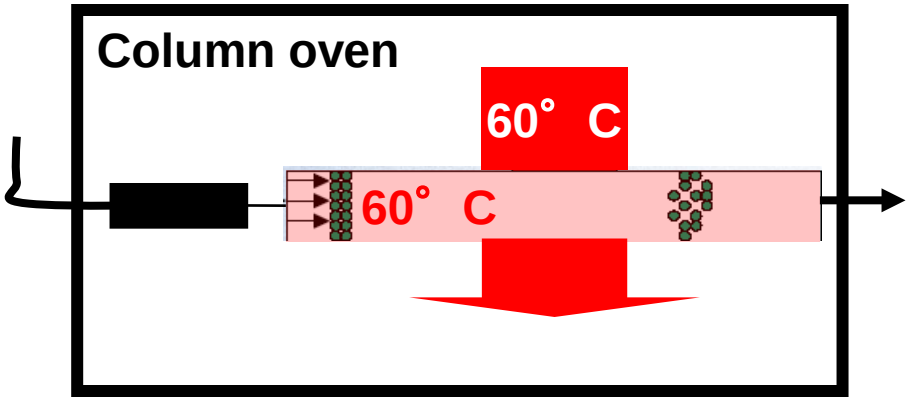


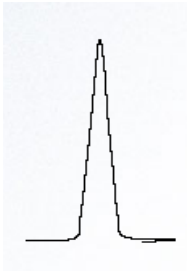
Pre-Column Heater



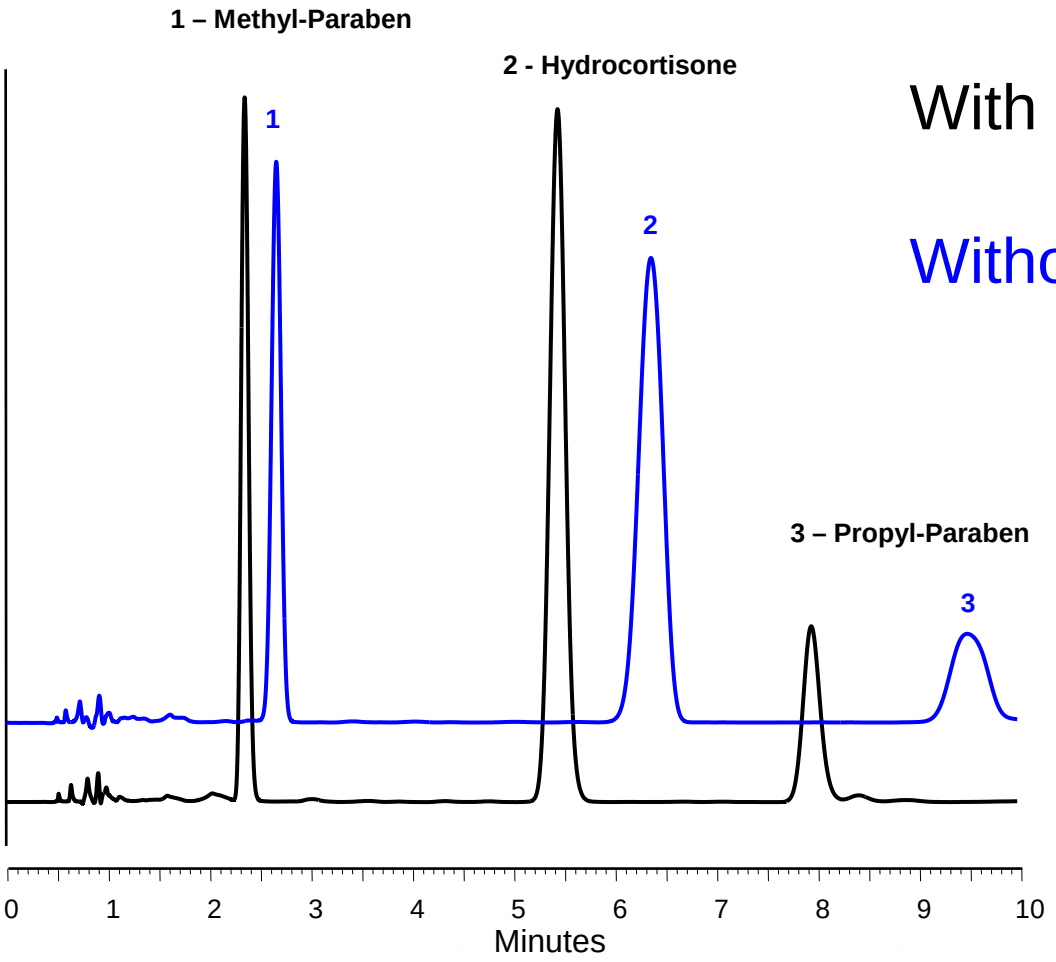
Without pre-heating:

- Poor peak resolution
- Peak broadening
- Peak splitting
- Extended analysis time





Pre-Column Heater



With pre-column heater

Without pre-column heater

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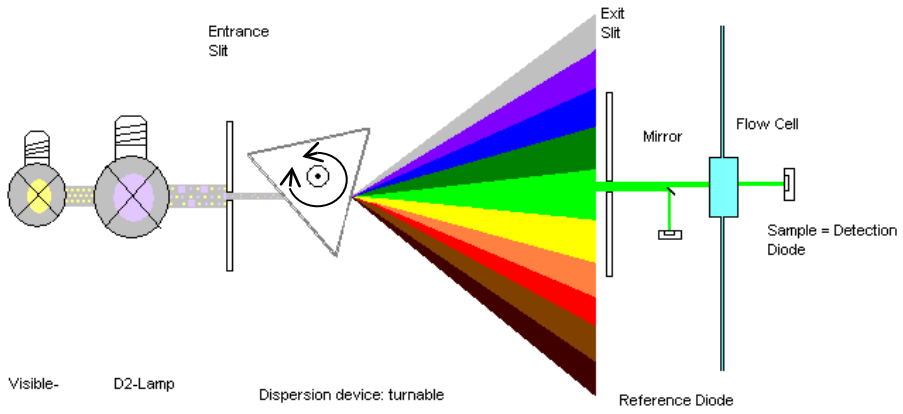
UV-Detectors

Operating Principle Variable Wavelength Detector (VWD)

Forward optics design

- Only the selected wavelength passes the flow cell and is detected by the sample diode
- A part of the light beam is redirected to the reference diode
- Reference signal is not influenced by the content of the flow cell
  - ‘True’ reference

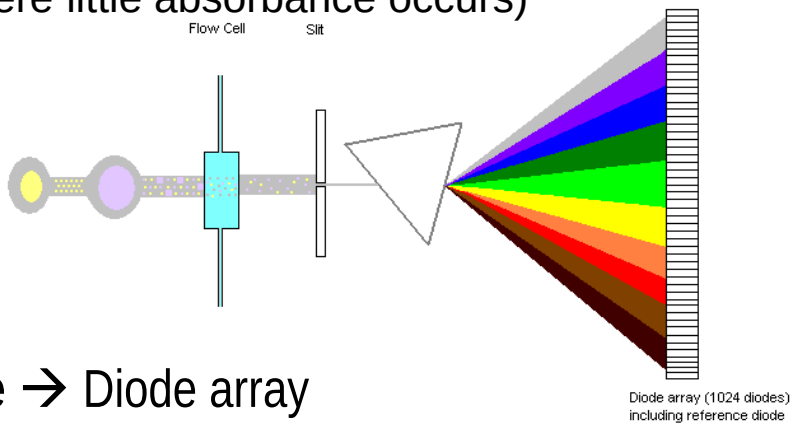


Light source → Dispersion device → Flow cell → Sample diode

Recommended Parameters: Reference Wavelength DAD

Reversed optics design

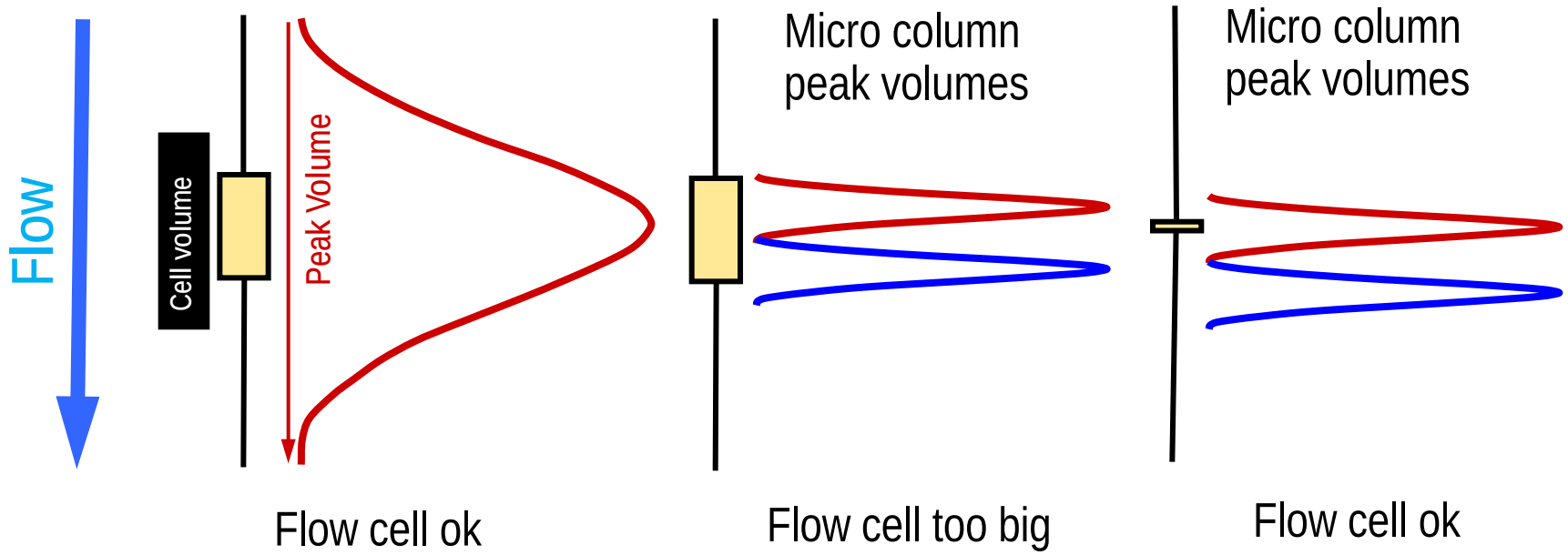
- Light beam passes the flow cell before being diffracted
  - No true reference signal can be obtained
- Instead *-and with limitations-*, any diode or bunch of diodes can be selected as a reference
  - If selected reference and acquisition wavelength are the same, the resulting signal would be zero (0)
  - As a consequence either don‘t use a reference (preferred) or select a reference wavelength in a ‘quiet’ area of the spectrum (where little absorbance occurs)
  - Reference wavelength range should not interfere with absorbance range of any compound of interest



Light source → Flow cell → Dispersion device → Diode array

Flow Cell Volume

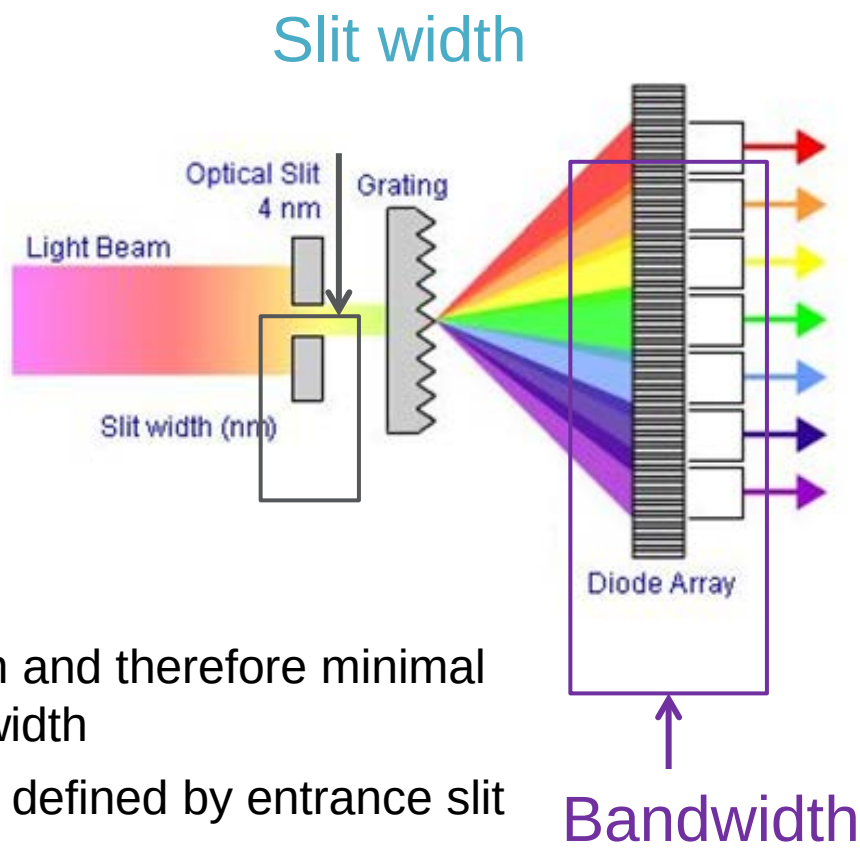
- Flow cell volume depends on peak volume
  - Rule: Flow cell volume should not exceed 10% of the peak volume



Note: Besides lamp age the light intensity is highly dependent on the installed flow cell

Smaller cell volume → Less light is passing through the flow cell

Bandwidth and slit width



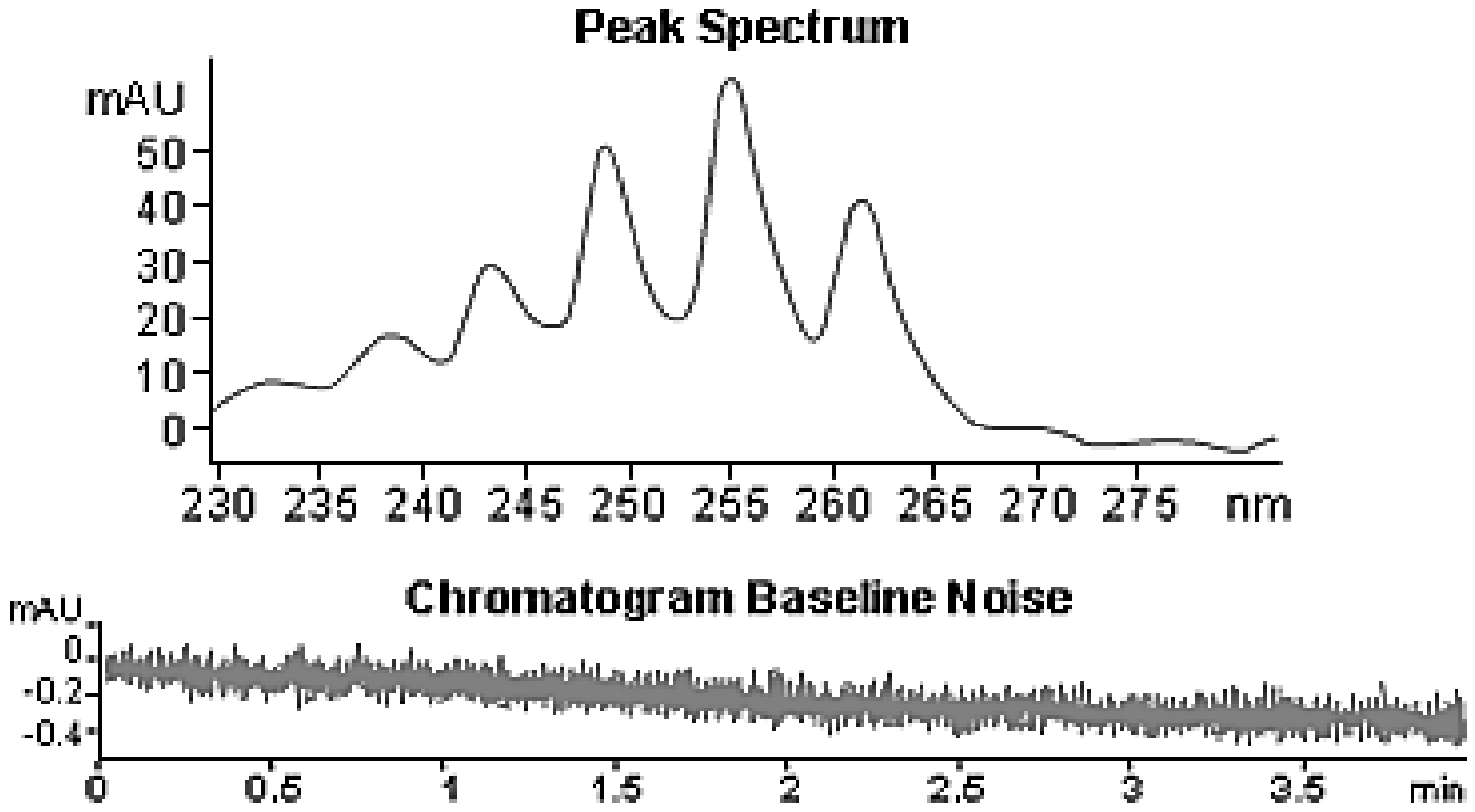
- Slit defines optical resolution and therefore minimal physically meaningful bandwidth
- VWD detector: Bandwidth is defined by entrance slit

Slit width	Baseline noise	Spectral resolution
↓	↑	↑
↑	↓	↓

Bandwidth	S/N ratio	Spectral resolution
↑	↑	↓
↓	↓	↑

Effects of Slit Width

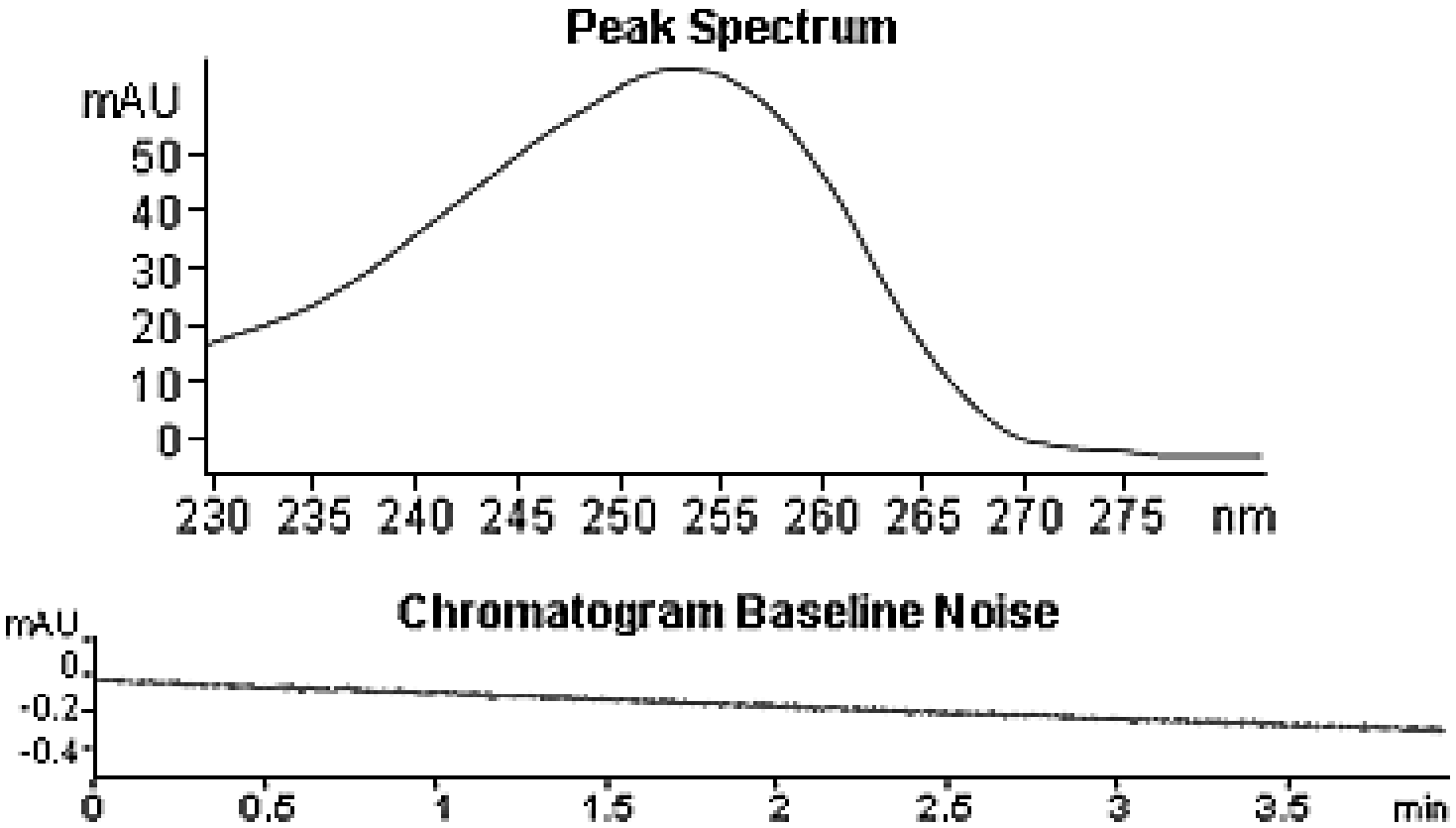
Slit width - 1nm



4 x light > 0.5 x noise

Effects of Slit Width

Slit width - 16nm

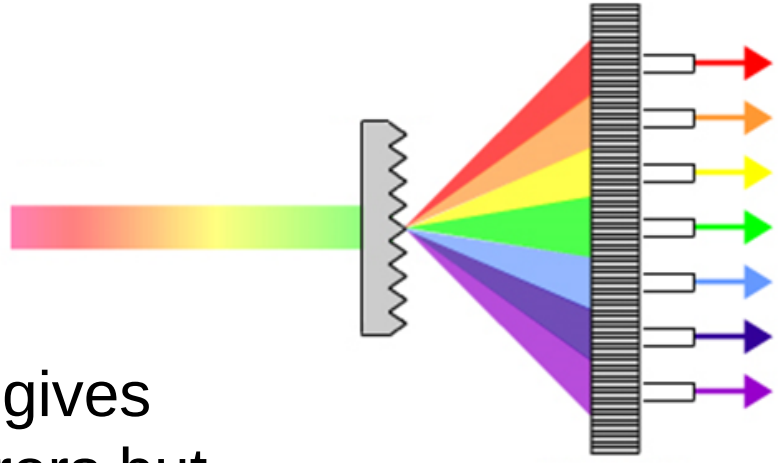


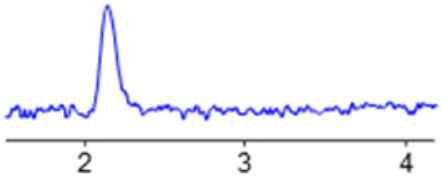
4 x light > 0.5 x noise

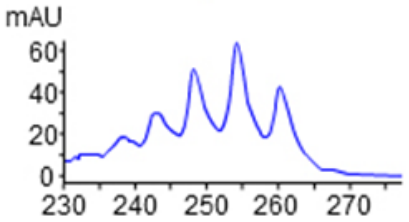
Setting Bandwidth

Bandwidth 4 nm

s/n = 5



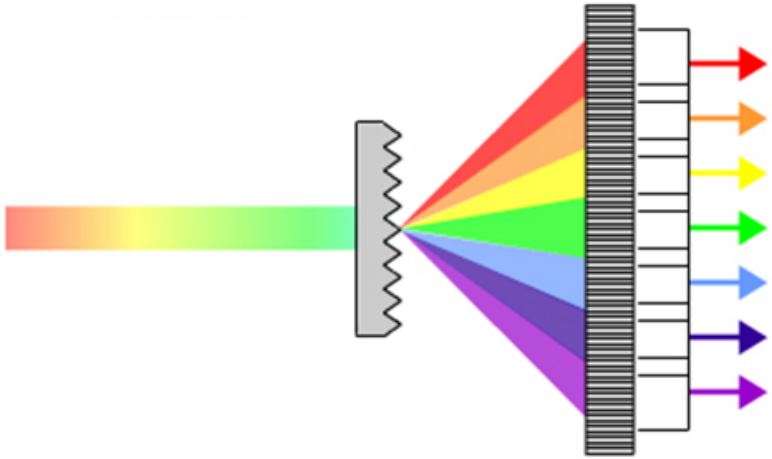


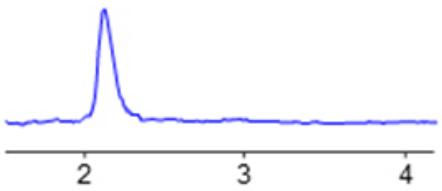


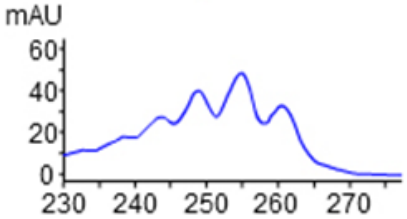
Narrow bandwidth gives fewer stray light errors but lower sensitivity

Bandwidth 30 nm

s/n = 25

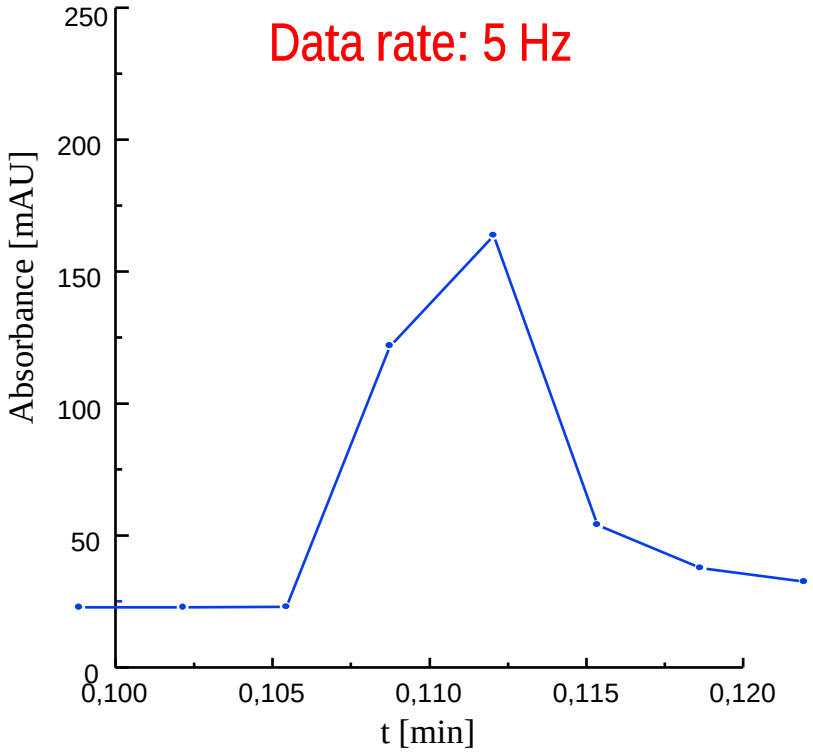


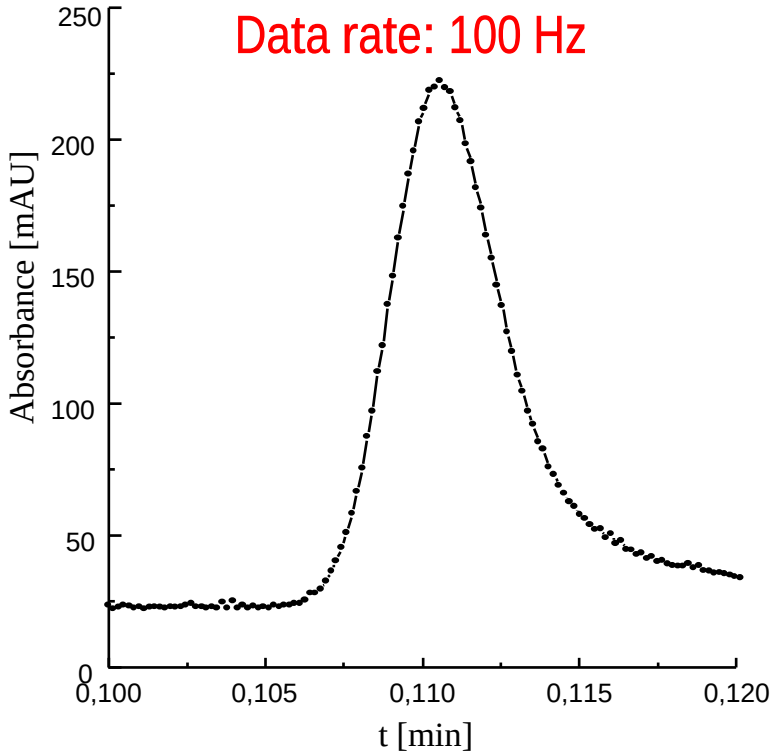




4 x light = < 0.5 noise

Recommended Parameters: Data Acquisition

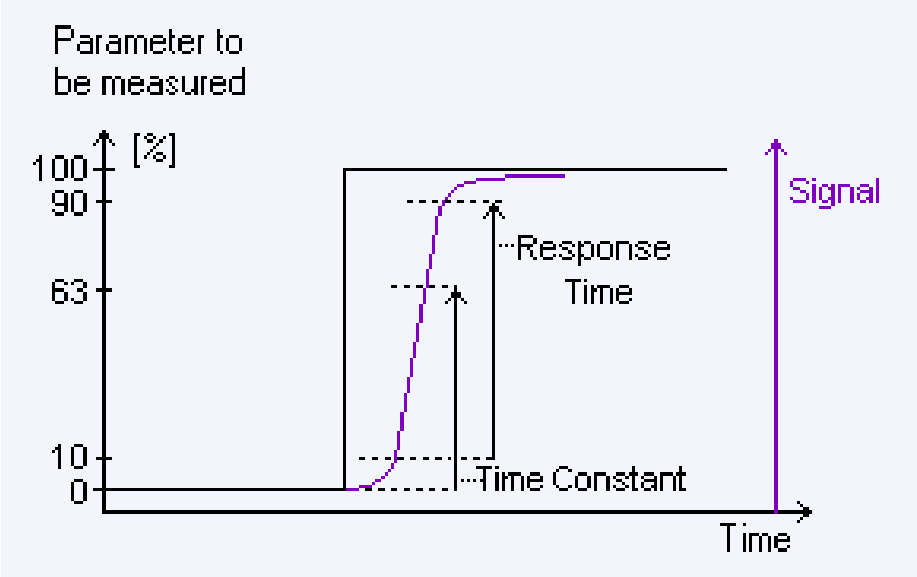




- Too few data points effect peak form, reproducibility and area precision
  - A minimum of 20, ideally 30-40 data points/peak is required

Time Constant

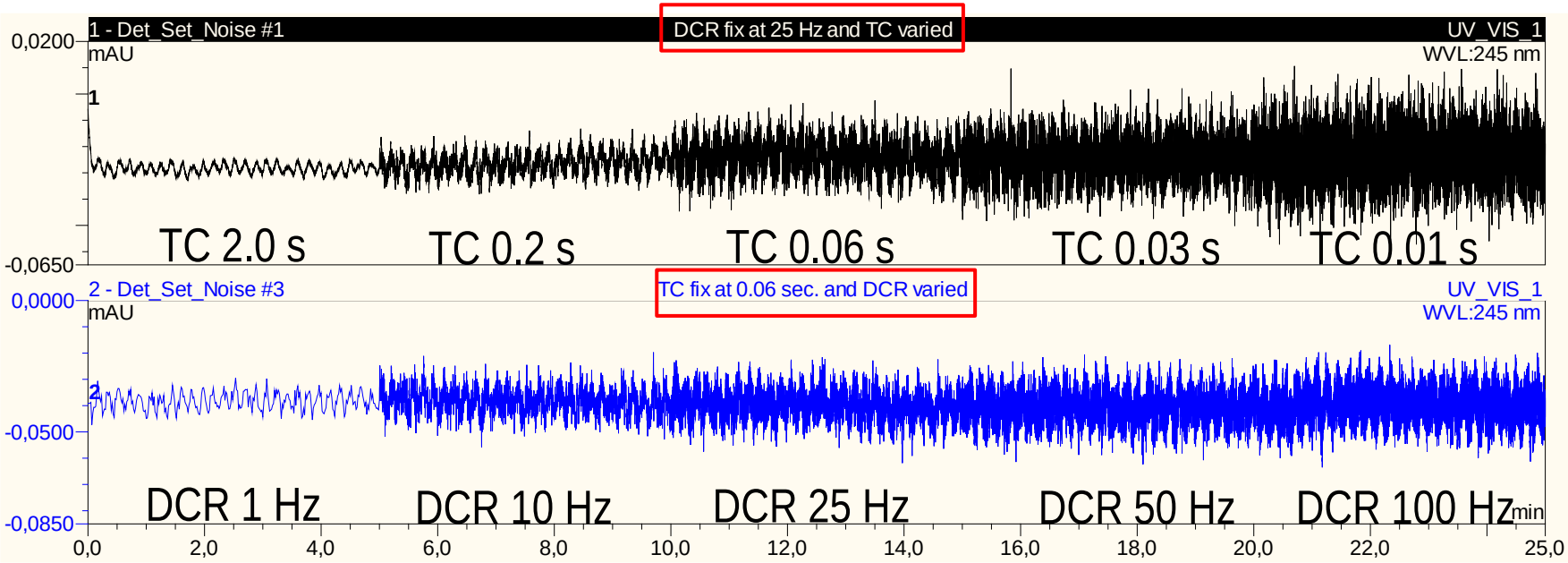
- The parameter is a measure of how quickly the detector responds to a change in signal
- Defined as the time it takes the detectors output signal to rise from 10% of its final value to 90%



- The Rise Time (Response Time) is closely releated to the time constant:

  Rise time = 2,2 x Time constant

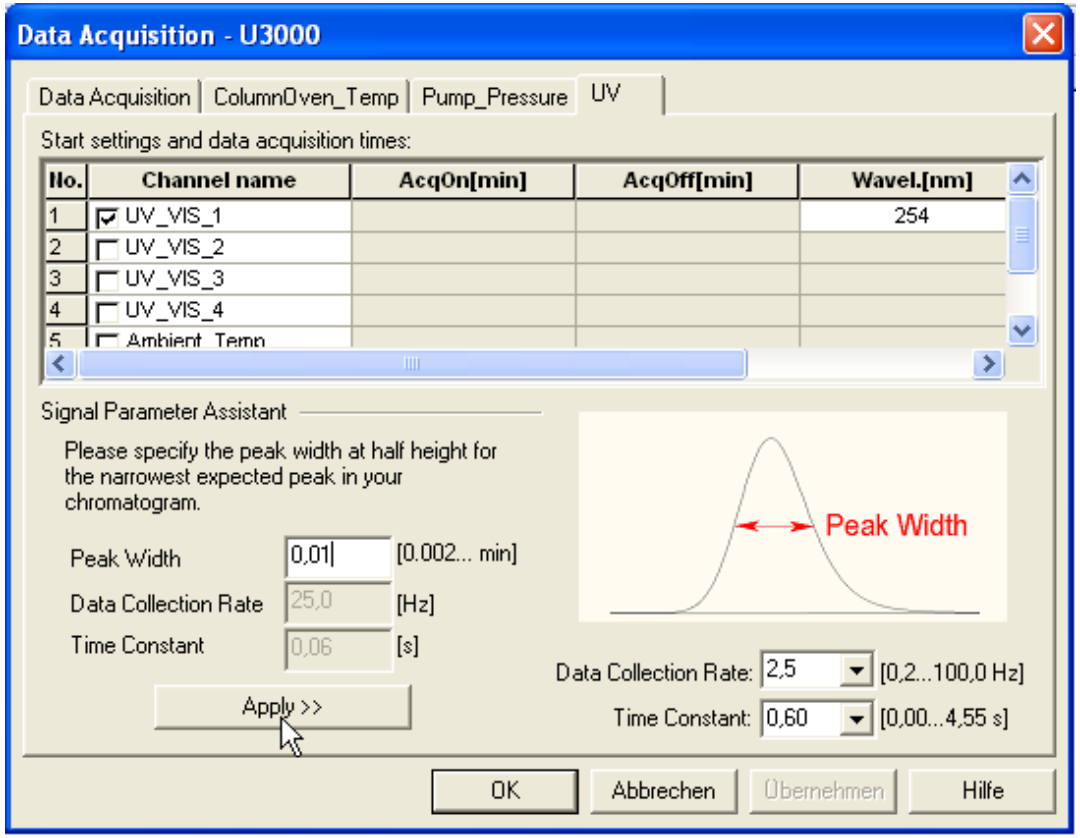
Recommended Parameters: Data Acquisition



- Noise is much more influenced by time constant (TC) than by data collection rate (DCR)

Recommended Parameters: Data Acquisition

- The Program Wizard of Chromeleon has a dedicated step for setting the correct '**Data Collection Rate'** and '**Time Constant'**
- The internal calculation is based on the peak width at half peak height of the slimmest peak in the chromatogram

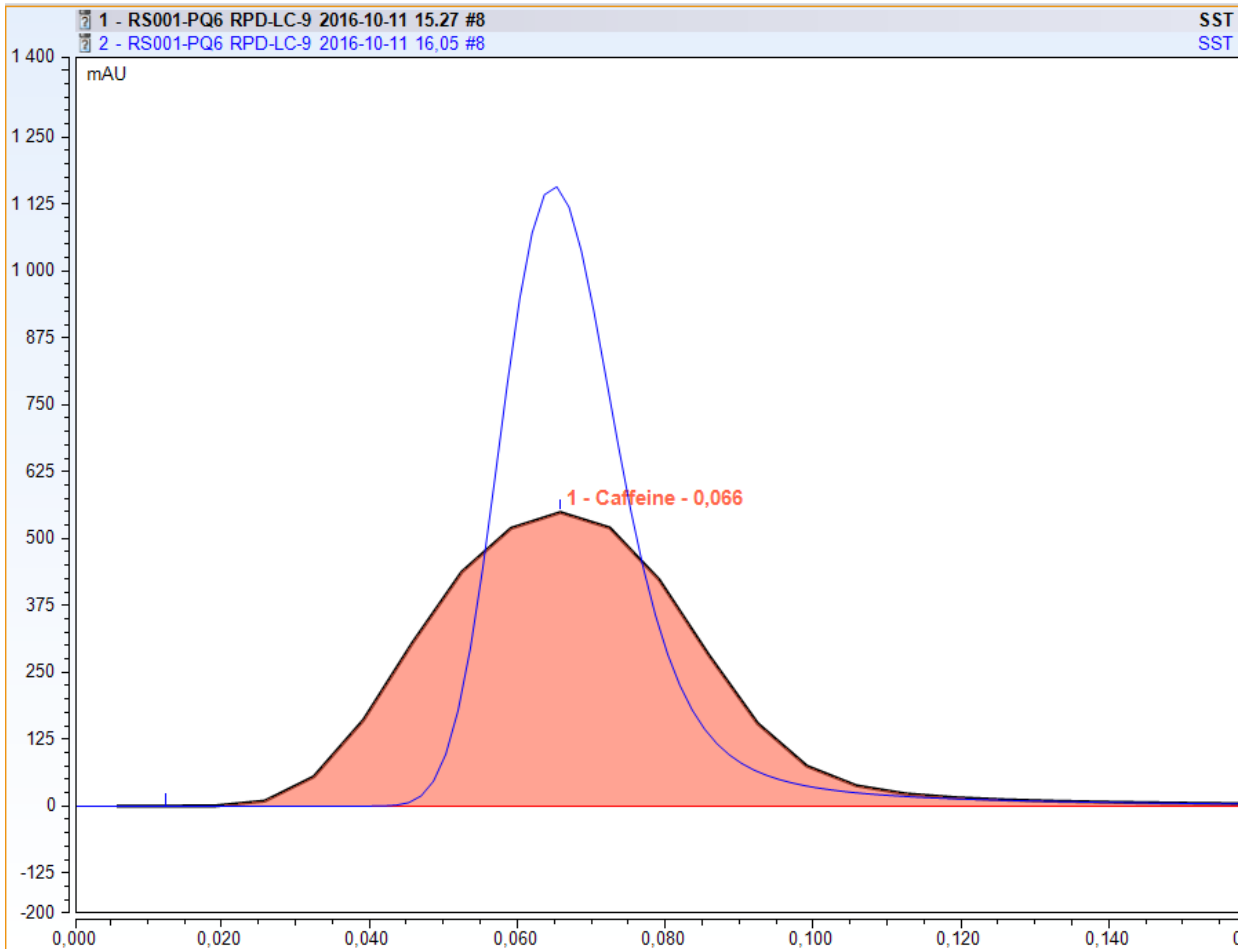


Sampling and Rise Time

- The same instrument, back pressure loop, eluent and sample. The area is the same – the peakshape is very different.

- 2,5 Hz 2s response time

- 10 Hz 0,5s response time



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THANK YOU!

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- Applications Library Resource www.thermoscientific.com/AppsLab

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.