# ANALYTISCHE CHEMIE I

## Trennmethoden

## 2. Hochdruckflüssigkeitschromatographie / HPLC

**WS 2004/2005**

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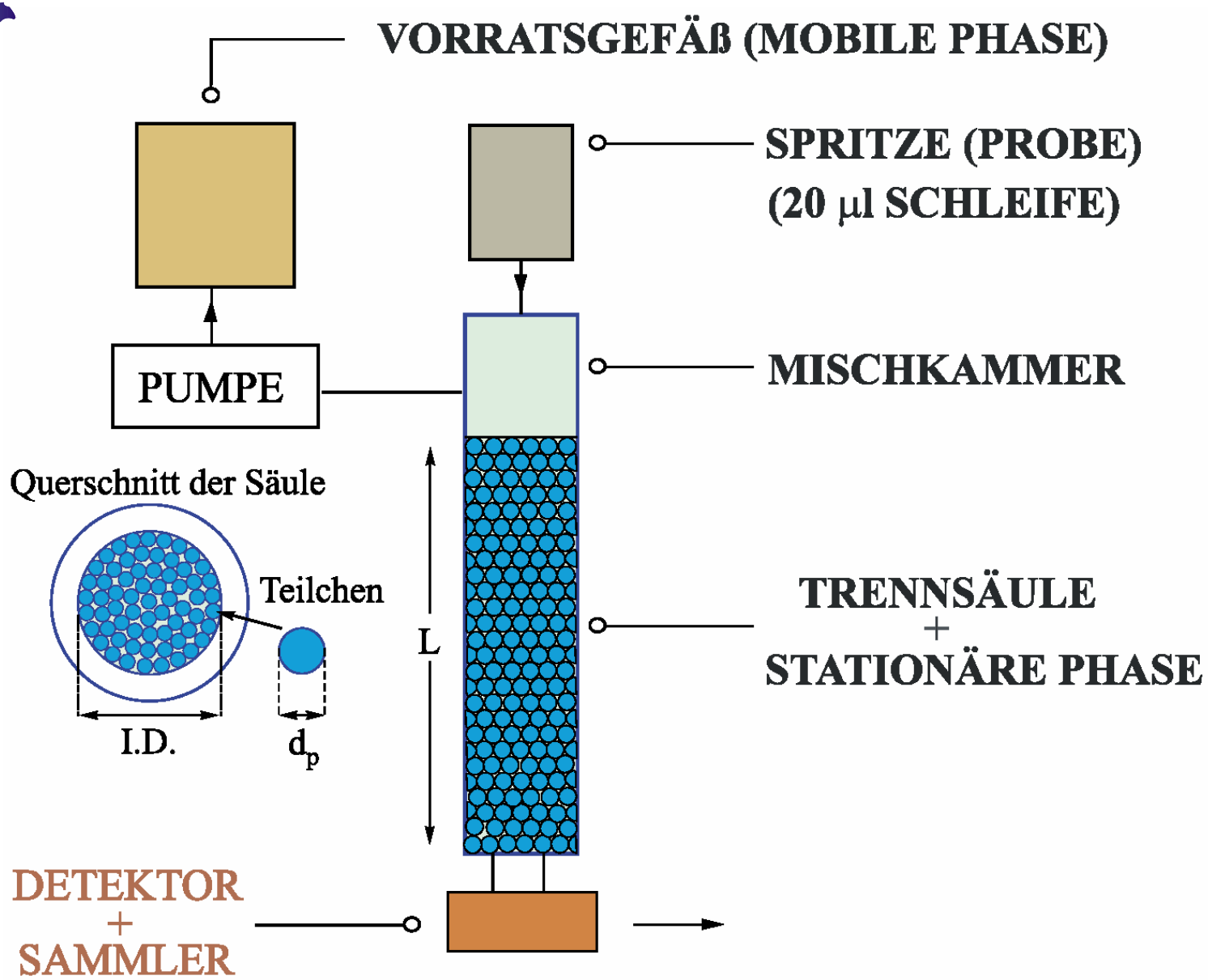
# High Performance Liquid Chromatography (HPLC)

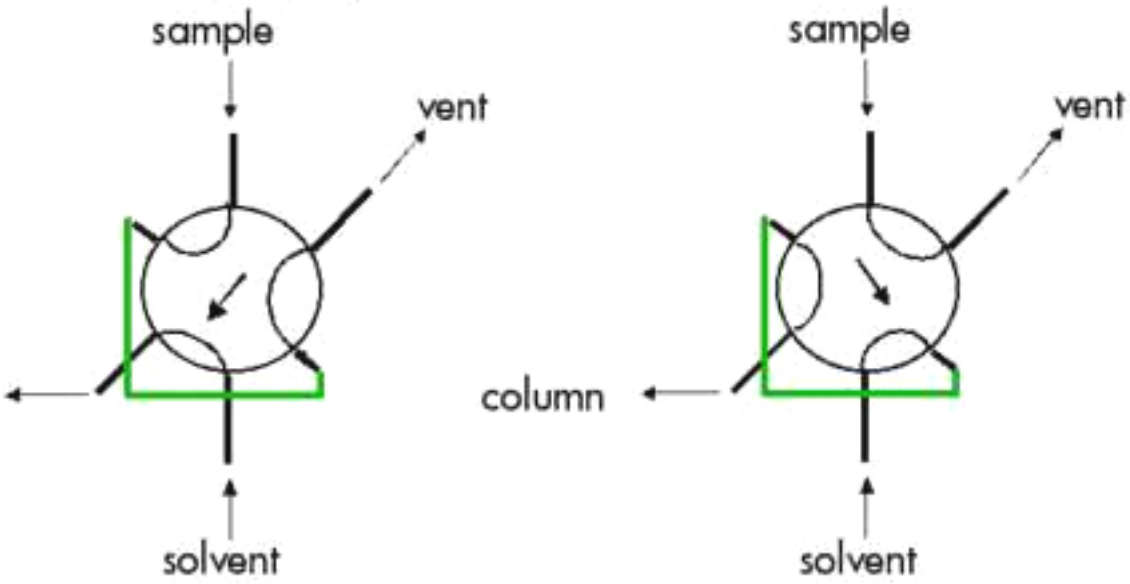


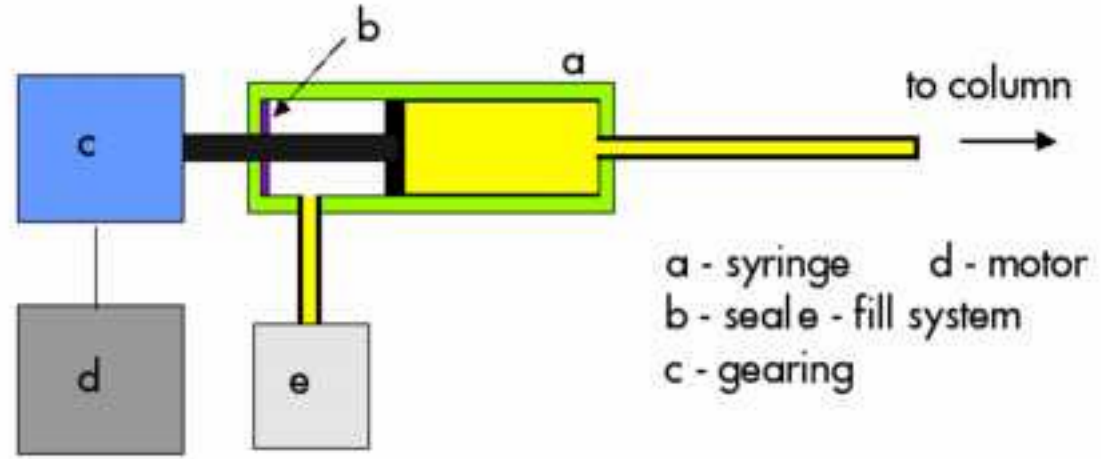
# Klassische SC und HPLC

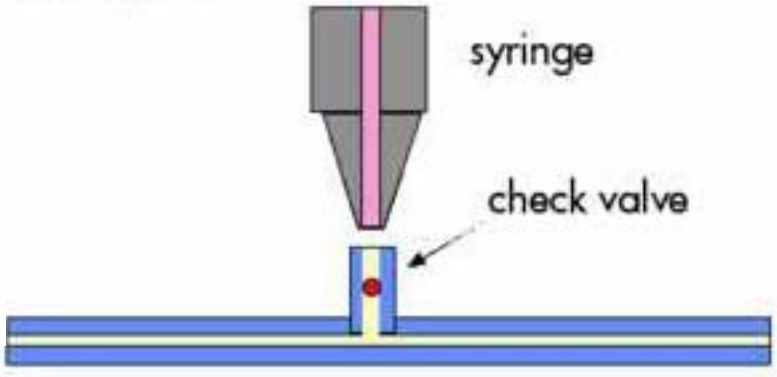
| KLASSISCHE SC | | HPLC | |
|---|---|---|---|
| I.D. | ~ 1 cm | I.D. | 2 - 5 mm |
| $d_p$ | 100 - 200 µm | $d_p$ | 5 - 10 µm |
| Druck | 1 - 5 bar | Druck | bis 400 bar |
| H | 1 - 10 mm | H | < 0.1 mm |
| N | $10^2$- $10^3$ | N | $10^4$- $10^5$ |
| L | bis 100 cm | L | 12.5, 25 cm |

| | | |
|---|---|---|
| Analytisch | 1 ml · $min^{-1}$ | } typische HPLC Fließgeschw. |
| Präparativ | 15 - 20 ml · $min^{-1}$ | |

# Proben-Injektion in der HPLC

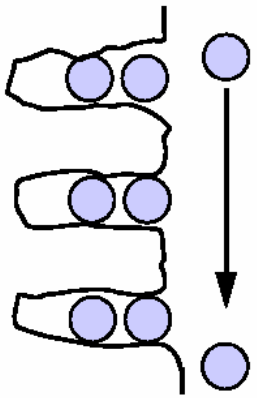


# Adsorption

Absorbens - poröse Beschaffenheit
große spezifische Oberfläche



Das Adsorptionsgleichgewicht ist von

(1) der Temperatur (T)

(2) der Konzentration des adsorbierten Stoffes in der mobilen Phase (c)

(3) Verhältnis adsorbierter Menge/Gesamtmenge an Adsorbens (x/m) abhängig

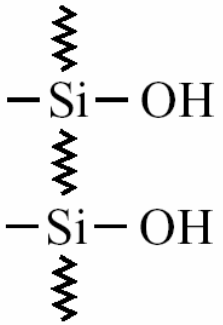
z.B. Aktivkohle

$10^3$ $m^2$ . $g^{-1}$ Oberfläche

# Stationäre Phasen

## Polare Normalphasen

Silikagel $SiO_2 \cdot xH_2O$




$CHCl_3 < CH_3CN < CH_3OH < H_2O$

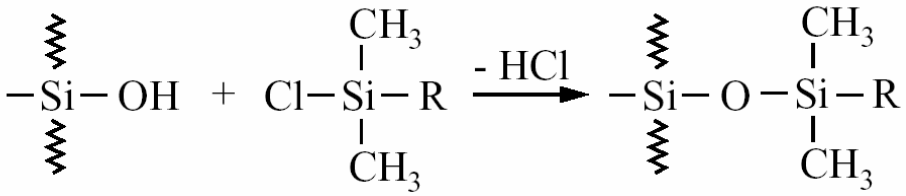
Eluotrope Reihe

Reihe der Fließmittel nach zunehmender Elutionskraft

Aluminiumoxid $Al_2O_3 \cdot xH_2O$

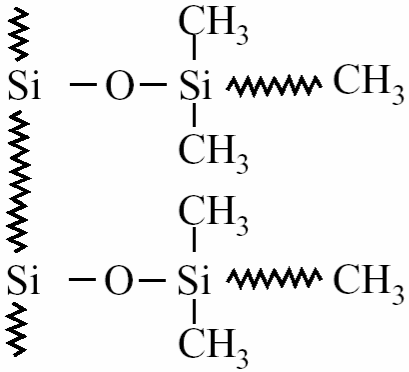
# Stationäre Phasen

## Gebundene Phasen




| polare Phasen R | | unpolare Phasen R | |
|---|---|---|---|
| $(CH_2)_3NH_2$ | Amino | $(CH_2)_3C_6H_5$ | Phenyl |
| $(CH_2)_3CN$ | Cyano | $(CH_2)_7CH_3$ | RP8 |
| $(CH_2)_2OCH_2CH(OH)CH_2OH$ | Diol | $(CH_2)_{17}CH_3$ | RP18 |

## Umkehrphasen (Reversed-phase) RP8, RP18

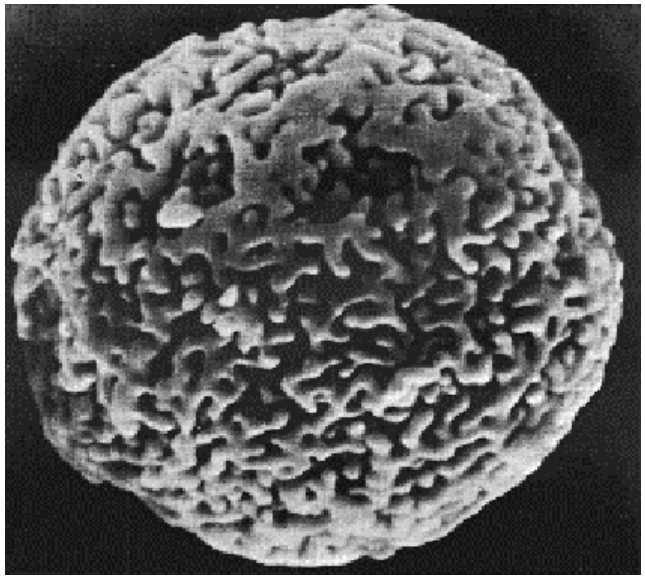



$H_2O < CH_3OH < CH_3CN < THF$

Eluotrope Reihe

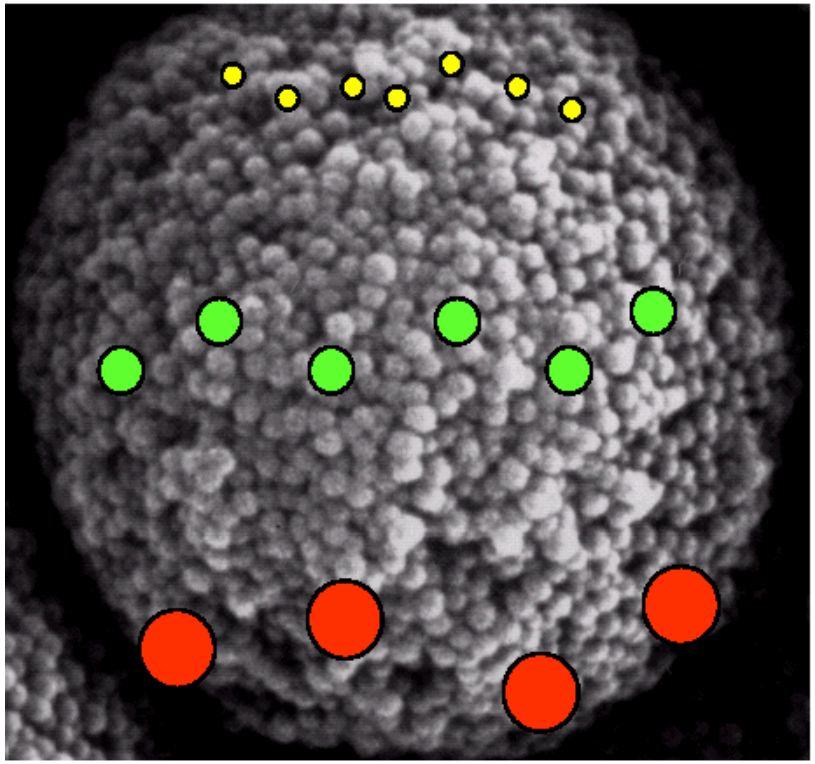
# RP-HPLC
## Pore size, shape and distribution

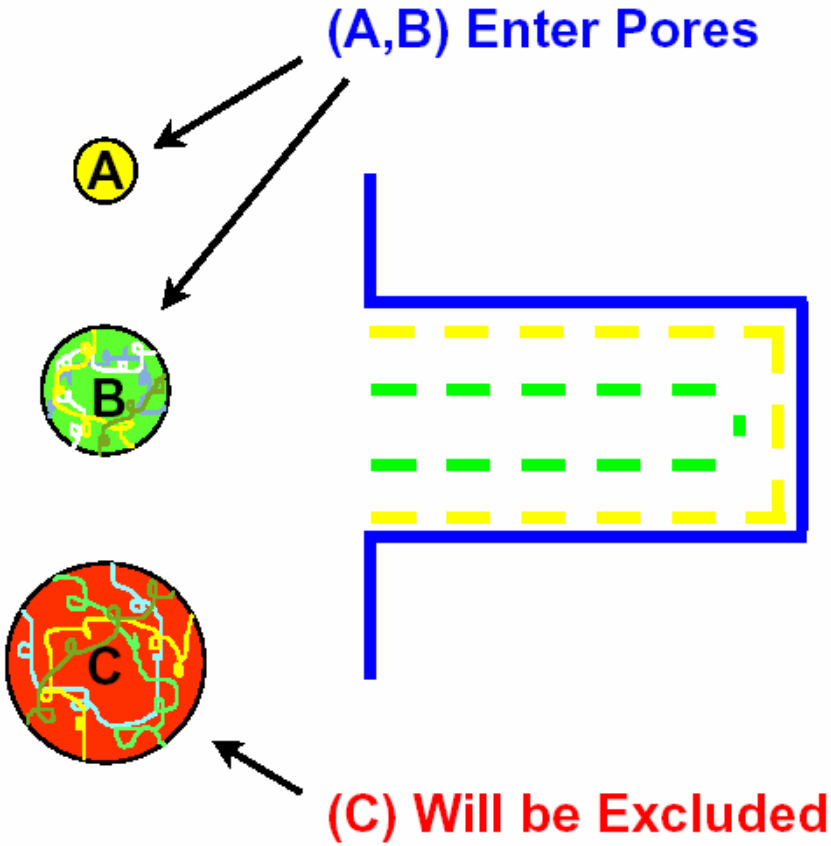
- Pore size defines an ability of the analyte molecules to penetrate inside the particle and interact with its inner surface
- The ratio of the outer particle surface to its inner one is about 1:1000
- The surface molecular interaction mainly occurs on the inner particle surface.

Macroporous spherical silica particle.
[K.K.Unger, Porous silica, Elsevier, 1979]

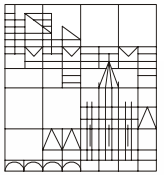
# RP-HPLC
## Pore size, shape and distribution






- Molecules must enter pores to interact with bonded-phase.
- Molecules must freely enter and exit pores to maximize efficiency.

# RP-HPLC

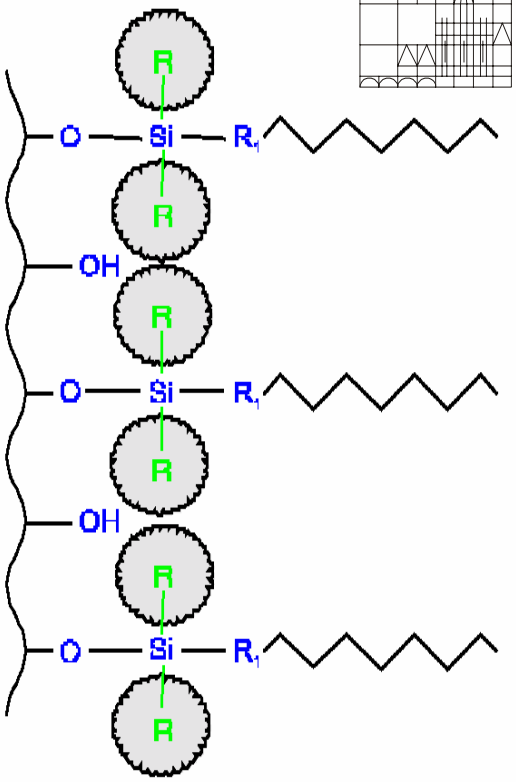


RP-HPLC separates molecules based on differences in hydrophobicity.

In RP-HPLC the polarity of the stationary phase is less than that of the mobile phase.

The bonded phase is an extremely hydrophobic or non-polar surface.

The mobile phase is polar, usually water.




**OPTIMIZATION**

- MOBILE PHASE
- TYPE OF MODIFIER (MeOH, ACN)
- SOLVENT STRENGTH (% modifier)
- pH
- TYPE OF BUFFER (phosphate, acetate)
- IONIC STRENGTH (Salts, buffer concentration)
- ION-PAIRING REAGENTS (TFA, alkyl-amines, -sulfonates)

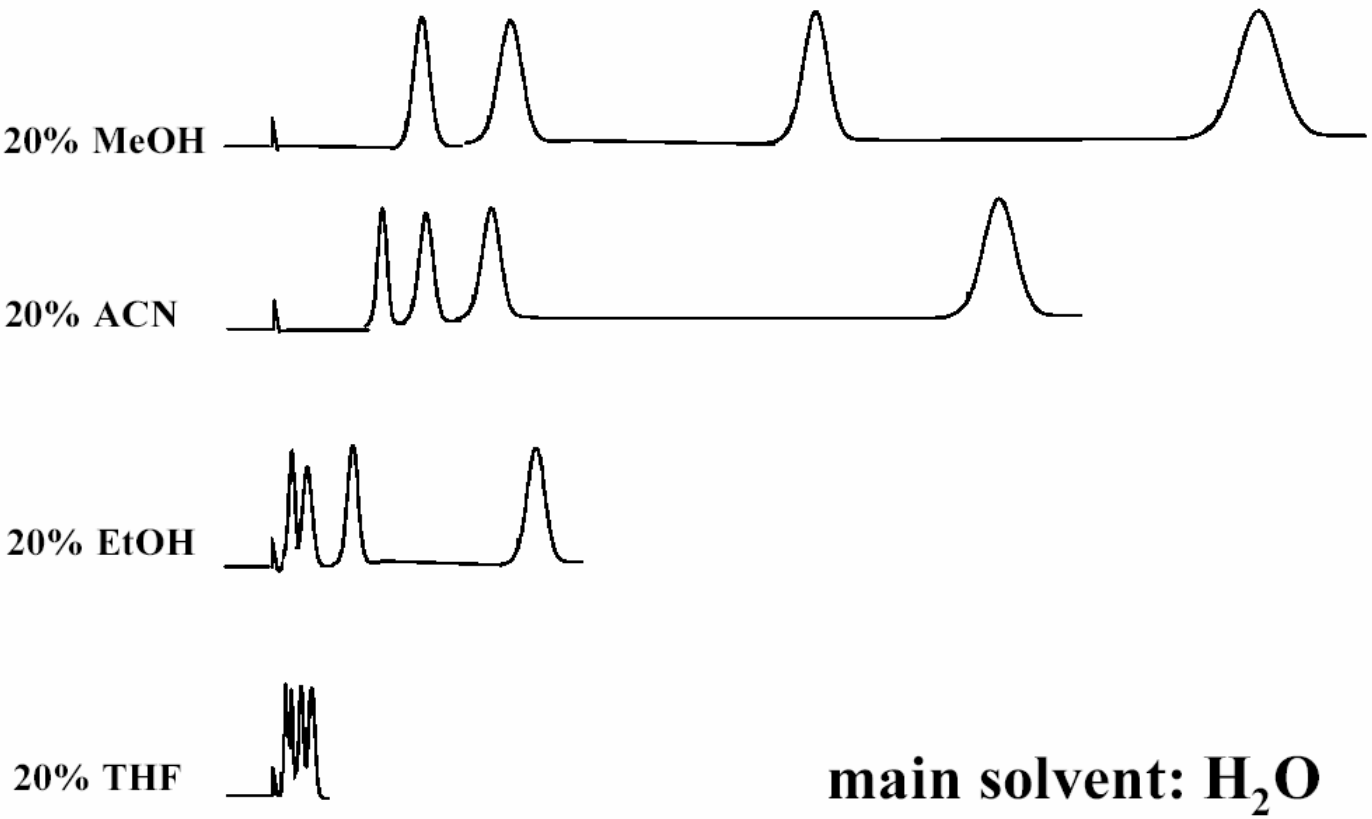
# RP-HPLC

## OPTIMIZATION: CHOICE OF SOLVENTS

Elutropic force

Water > Methanol > Acetonitrile > n-Propanol > THF

Polarity




**Mobile phase preconditions**

- **Water-miscible**
- **Low viscosity**
- **Low-UV detection**
- **Good solubilization**
- **Chemically unreactive**

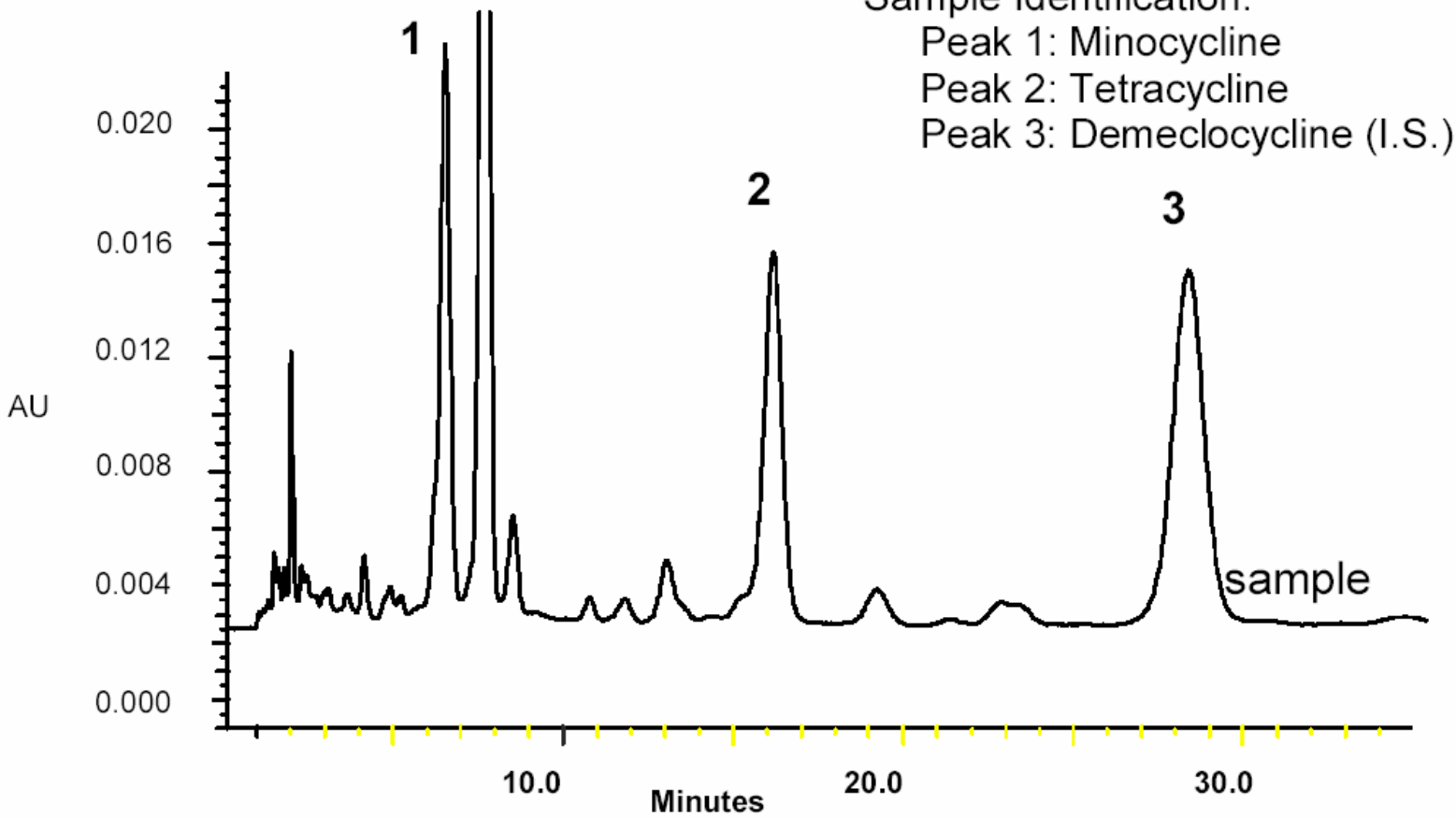
**main solvent: $H_2O$**

# RP-HPLC

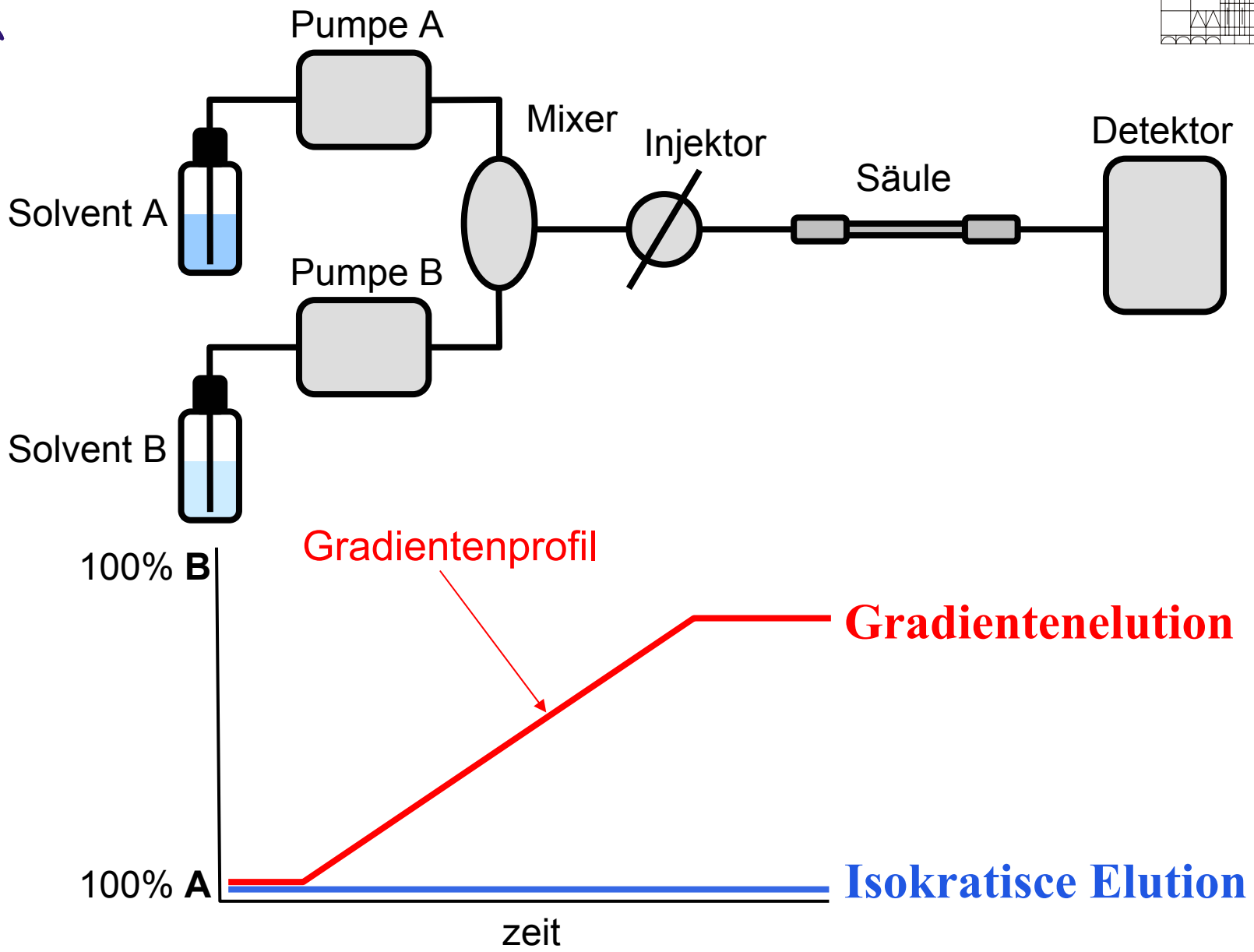
## Antibiotics

Column: SymmetryShield™ RP8, 5 µm, 3.0 x 150 mm
Mobile Phase: 0.1% TFA in Water : Acetonitrile: Methanol (91:7:2)
Detection: UV at 270 nm
Flow Rate: 0.9 mL/min.
Injection Volume: 20 µL

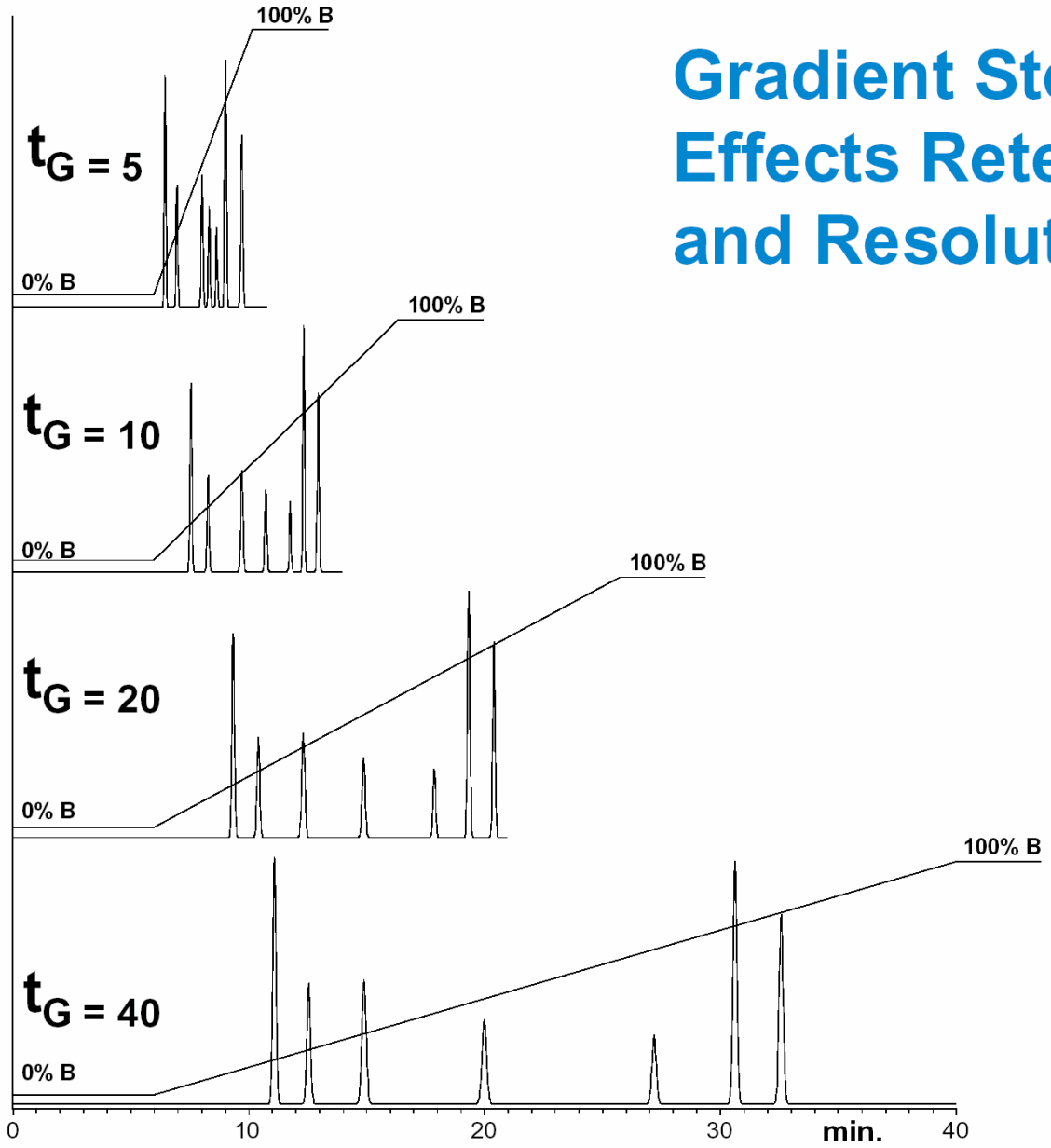
Sample Identification:
Peak 1: Minocycline
Peak 2: Tetracycline
Peak 3: Demeclocycline (I.S.)



# HPLC Gradientensystem



# Gradient effects




## Gradient Steepness Effects Retention ( k*) and Resolution

$$R \approx \frac{\sqrt{N}}{4} \alpha^* k^*$$

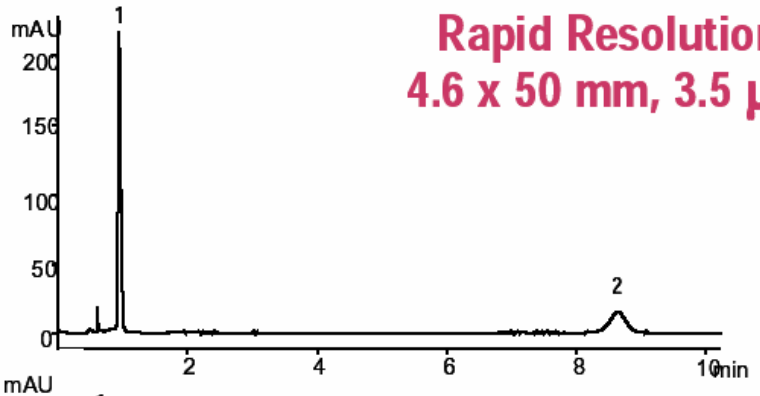
**k* (retention)**
**a* (selectivity)**
**N (column conditions)**

# RP-HPLC Separation of Lincomycin and Clindamycin

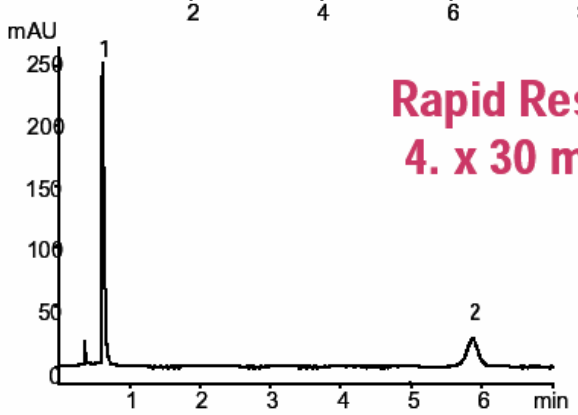
## Isocratic

Columns: ZORBAX Rapid ResolutionvSB-C18 Mobile Phase: 85% 20 mM $Na_2HPO_4$ pH, 2.8: 15% ACN Flow Rate: 1.0 mL/min

Temperature: Ambient Detection: UV 210 nm Sample: 1. Lincomycin 2. Clindamycin




Rapid Resolution
4.6 x 50 mm, 3.5 µm




Rapid Resolution HT
4. x 30 mm, 1.8 µm

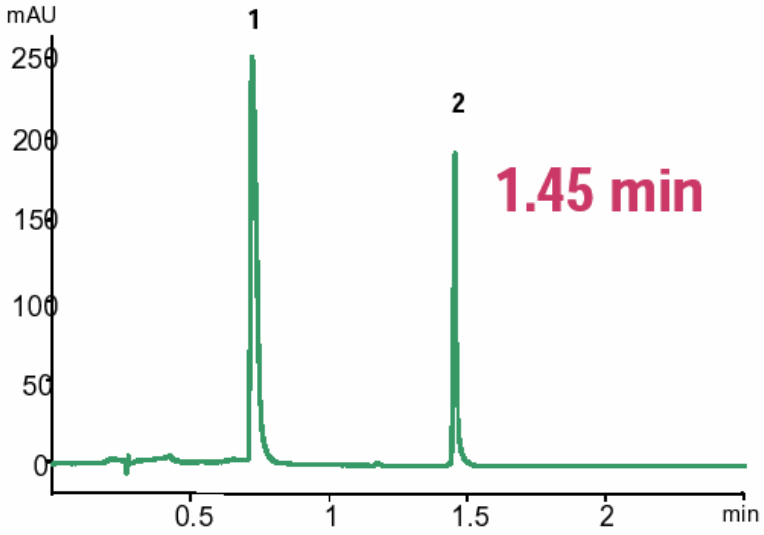
## Gradient

Column: ZORBAX Rapid Resolution HT SB-C18
Mobile Phase: Gradient: 10 – 40% B in 2 min

A: 20 mM $Na_2HPO_4$ pH, 2.8 B: ACN Flow Rate: 1.5 mL/min Temperature: Ambient
Detection: UV 210 nm

Sample: 1. Lincomycin 2. Clindamycin

Rapid Resolution HT
4. x 30 mm, 1.8 µm




1.45 min

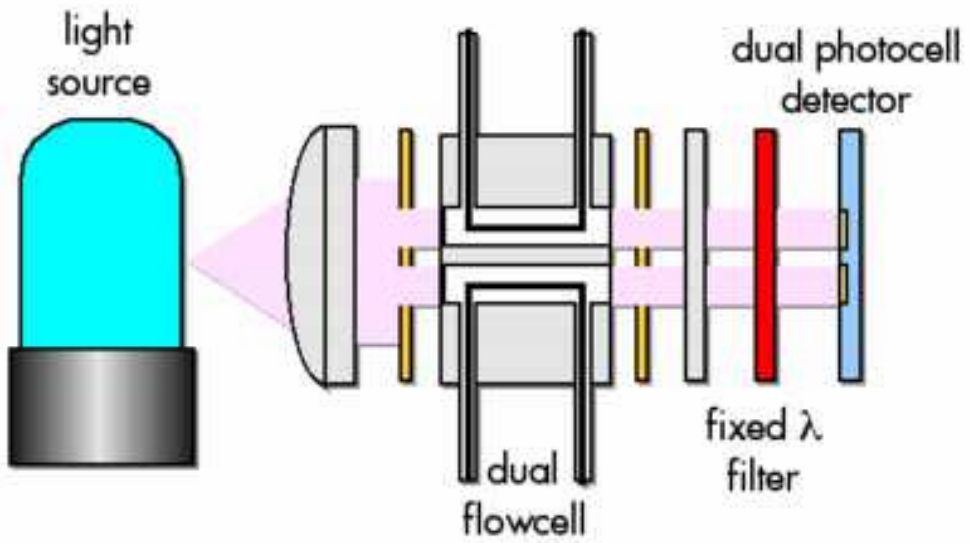
# Detektoren in der Flüssigkeitschromatographie

| Detektoren | Nachweisgrenze |
|---|---|
| UV/Vis- Absorption | 100pg – 1ng |
| Fluoreszenz | 1-10 pg |
| Elektrochemisch | 10pg – 1ng |
| Refraktions-index | 100ng – 1µg |
| Leitfähigkeit | 500pg – 1ng |
| Massenspektrometrie | 100pg – 1ng |

# HPLC Detektoren

## UV/Vis- Absorption

### Single Wavelength UV Detector




### Detector Optical Bench

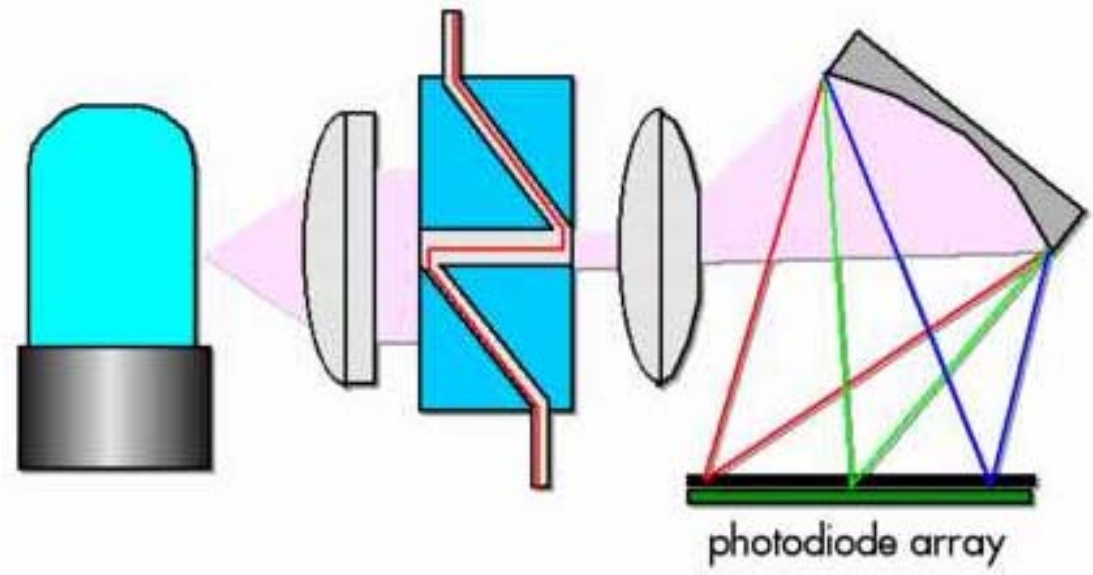
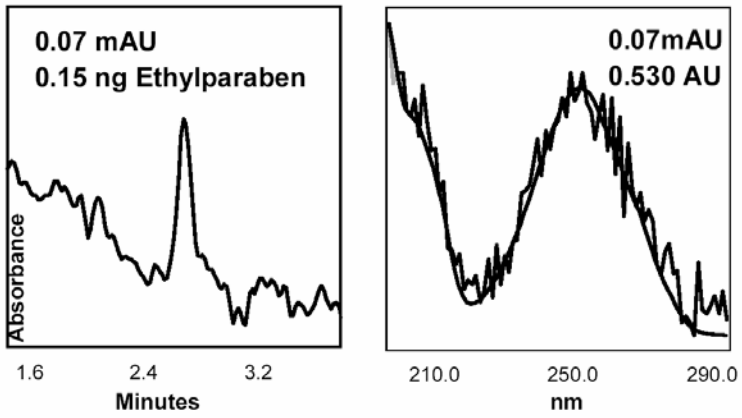


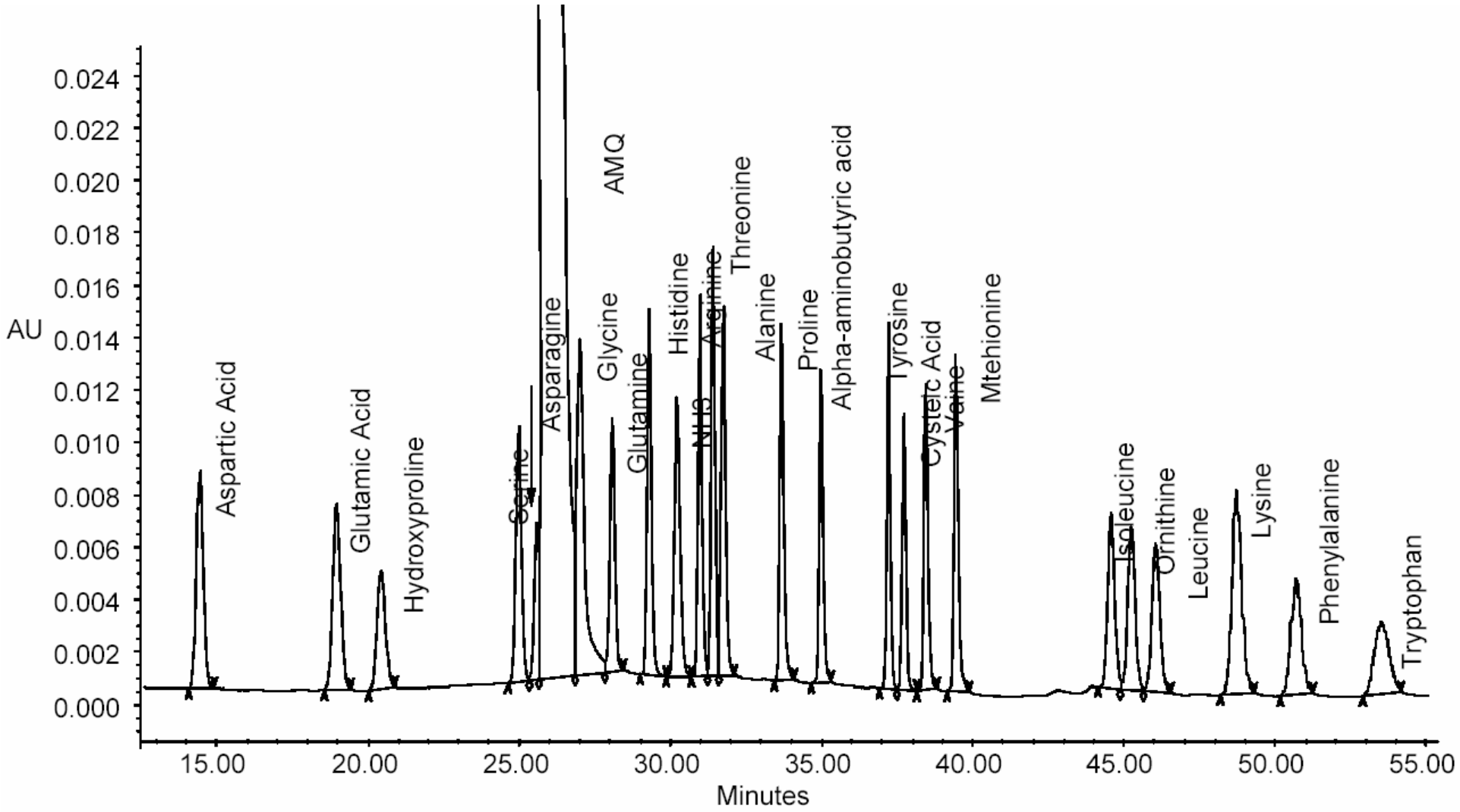

**Photo-diode array**

**Chromatographic and Spectral Sensitivity**



# HPLC Detektoren

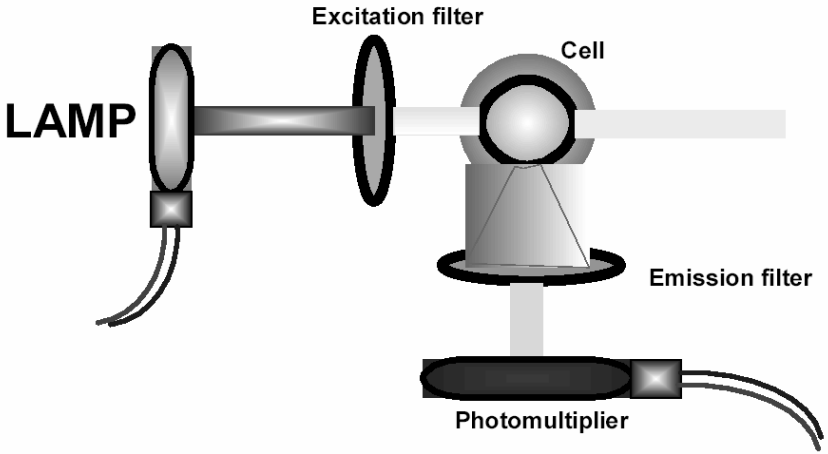
## UV Detection of AccQ-Tag Amino Acid Derivatives



# HPLC Detektoren

## Fluoreszenz

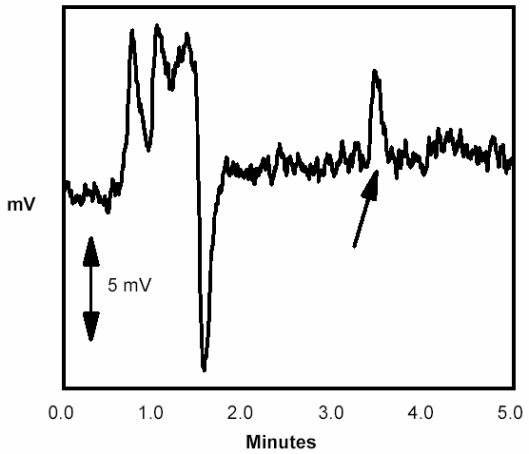
### Fluorescence Detectors




**Short pass - transmits all wavelengths below a specified cutoff**
**Long pass - transmits all wavelengths above a specified cutoff**
**Band pass - blocks all wavelengths outside a specified band**

### Sensitivity

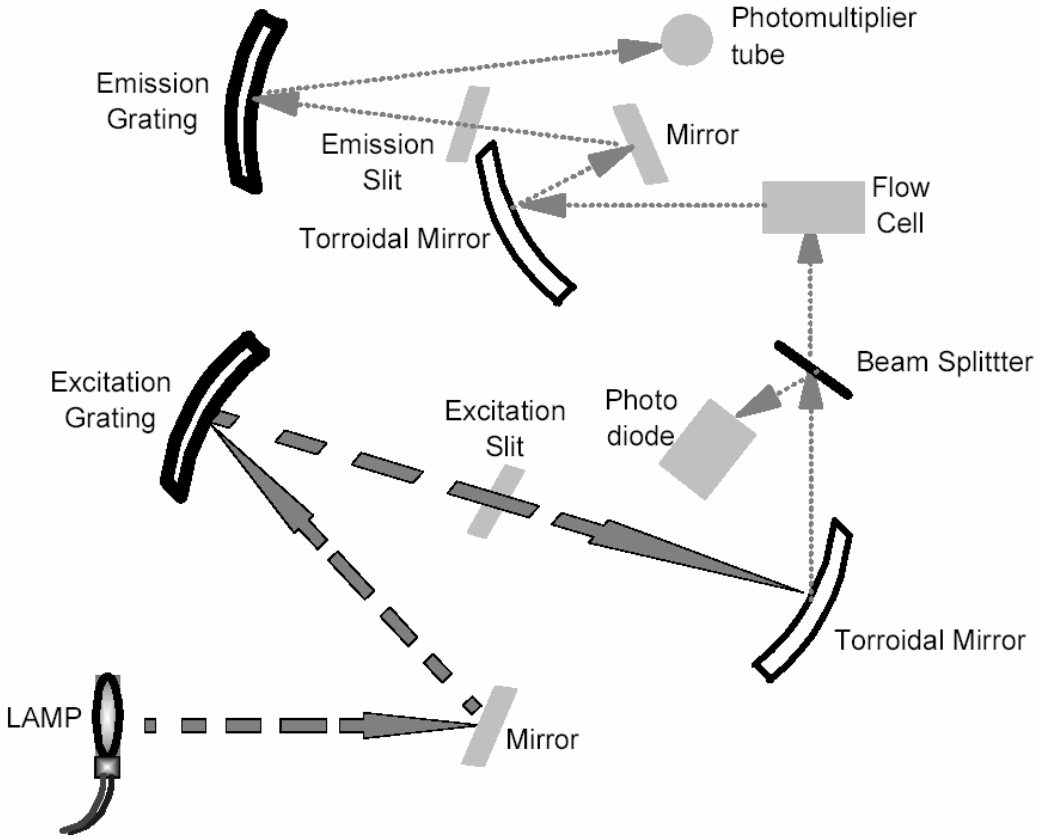
**Waters 474 Fluorescence Detector**




**0.1 pg Anthracene**

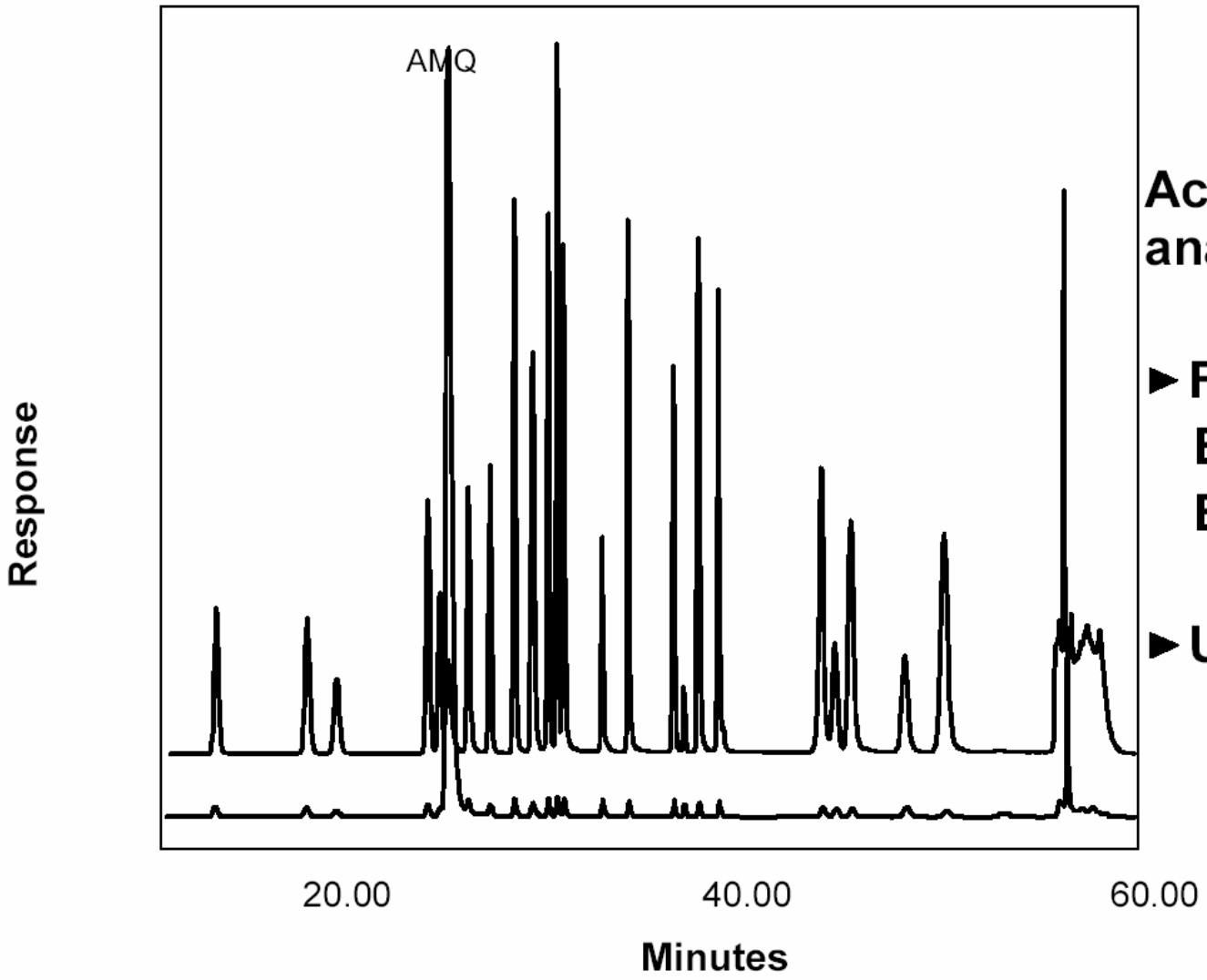
**Excitation = 251 nm**
**Emission = 406 nm**

### Detector Optical Bench



# HPLC Detektoren

## Fluoreszenz




**AccQ-Tag amino acid analysis**

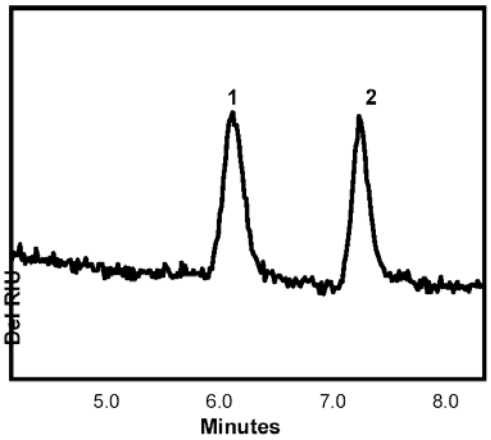
- **Fluorescence**
  **Excitation=250 nm**
  **Emission=395 nm**
- **UV 254 nm**

# HPLC Detektoren

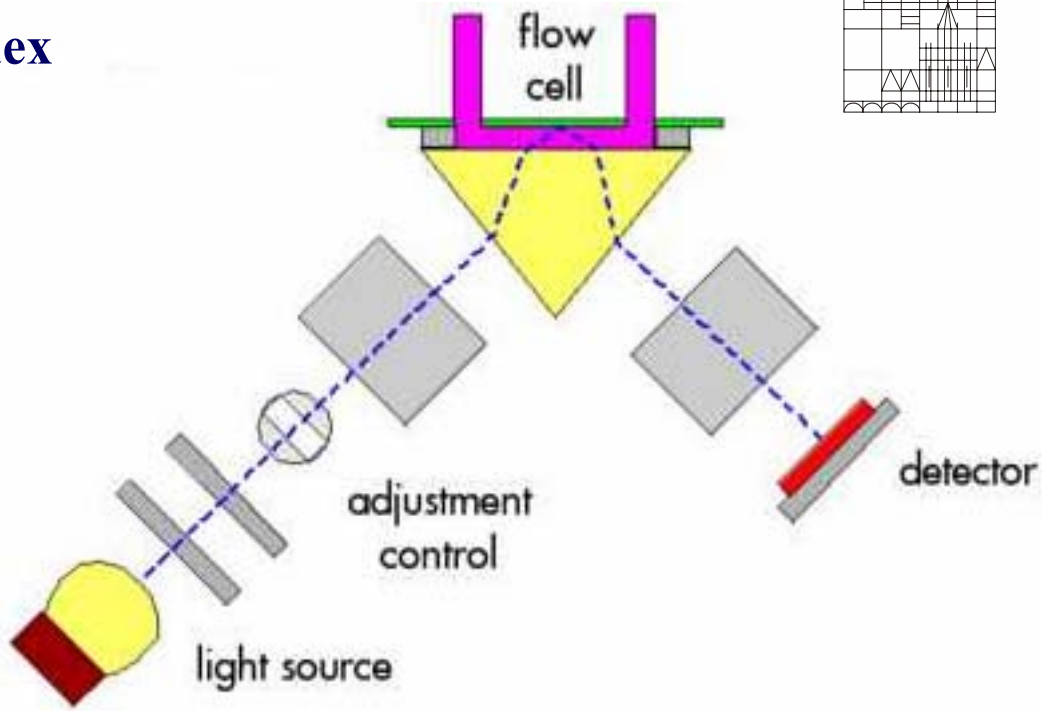
## Refraktions-Index

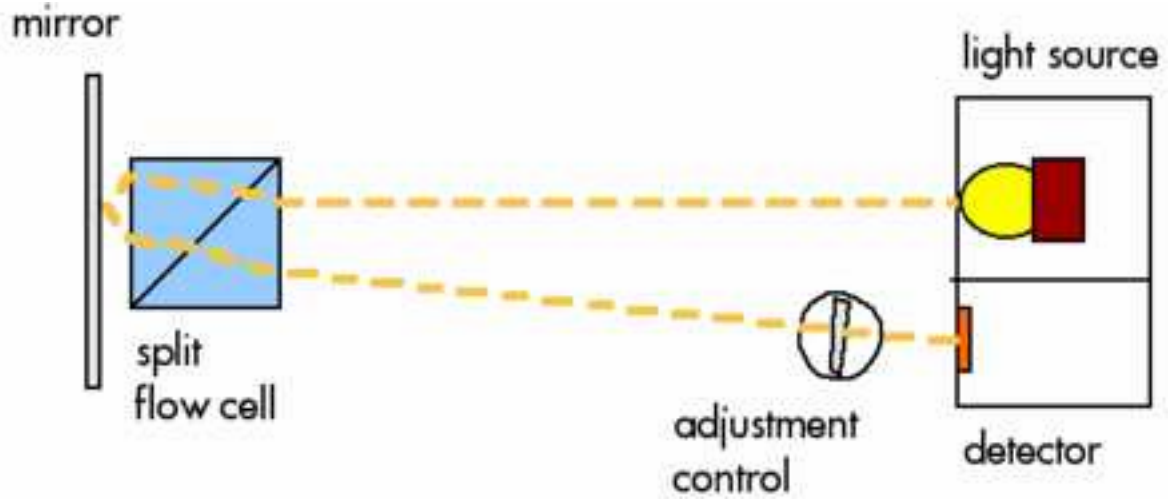
### Sensitivity

**Waters 410 Refractive Index Detector**



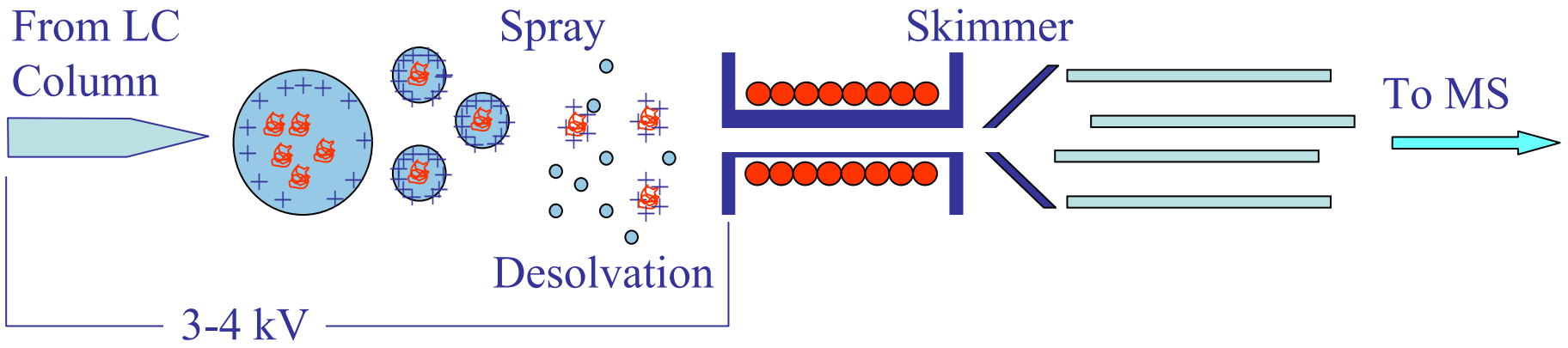

- 250 ng on column
  1=Tristearin
  2=Myristic acid
- Styragel HR 0.5, 4.6 x 300 mm, 35°C, 0.35 mL/min
- dRI sensitivity = 32X, 32°C



# HPLC Detektoren

## ESI-LC-MS




## LC-MS preconditions

| | |
|---|---|
| **Analyzed substances** | **ESI compatible, soluble** |
| **Mobile phase ESI** | **ESI compatible (water, MeOH, Acetonitril)** |
| **Flow rate** | **ESI compatible (best 2-10 µL/min)** |
| **Mobile phase modifiers** | **TFA, Formic acid, $NH_4OH$, $Et_3N$, $NH_4OAc$** |
| **Column materials** | **Stable at the separating conditions** |