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Method development of cytotoxic drugs – selectivity



IAC Seminar, 06.03.2015

**UNIVERSITÄT
DUISBURG
ESSEN**

Open-Minded


- Introduction
 - Definition of selectivity and efficiency
 - Influence parameters
 - Requirements
- Overall selectivity comparison
- Evaluation strategy
 - Critical peak pairs
 - Weighting system
 - Final choice
- Conclusion

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Definition:

- Two different ways are possible to optimize the chromatography

1. **Efficiency** describes the peak dispersion of a component

 The more efficient the better it is

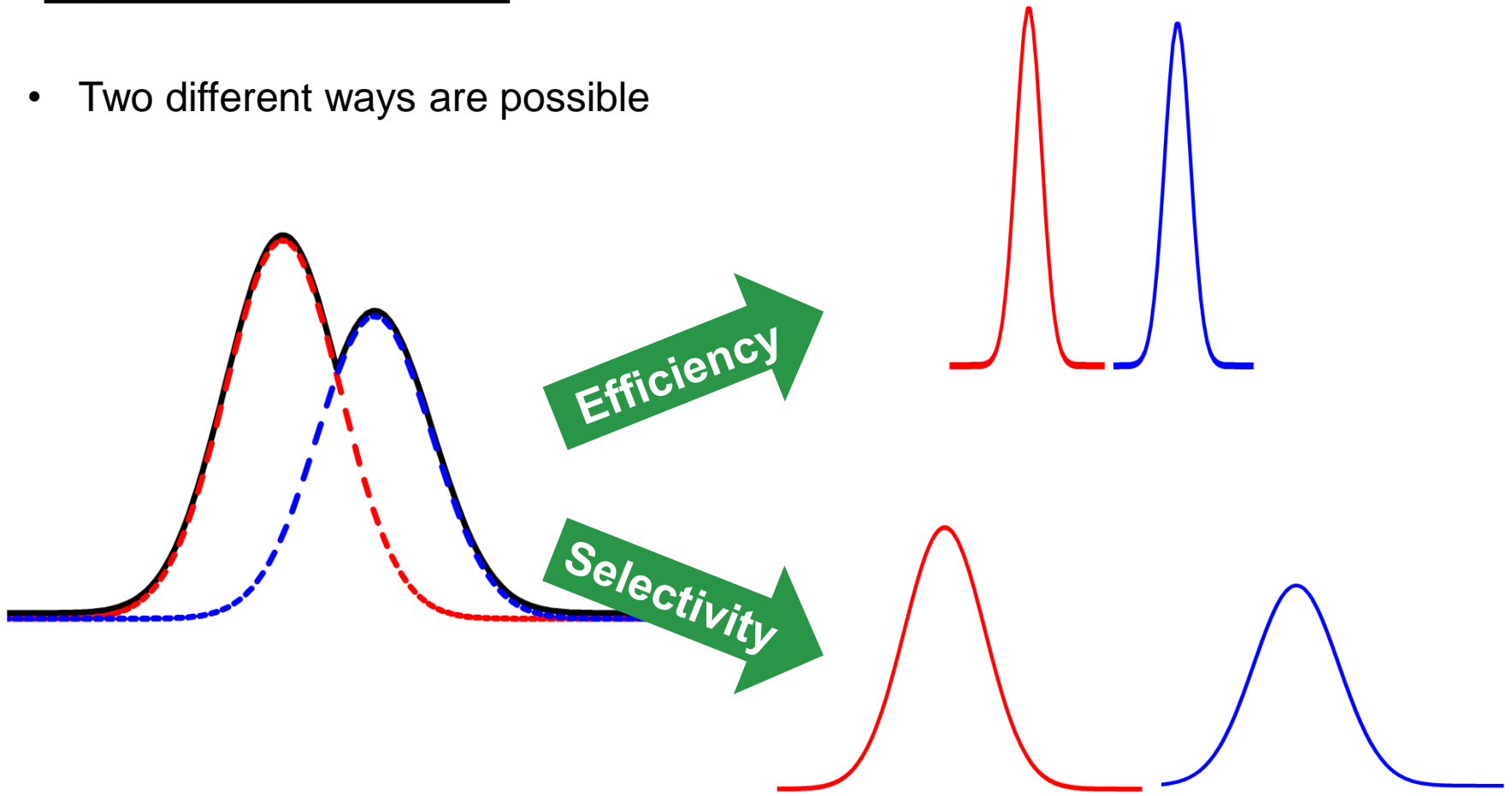
2. **Selectivity** describes the difference in retention of two components

 The greater the difference the more selective is the system

Sounds easy, right?

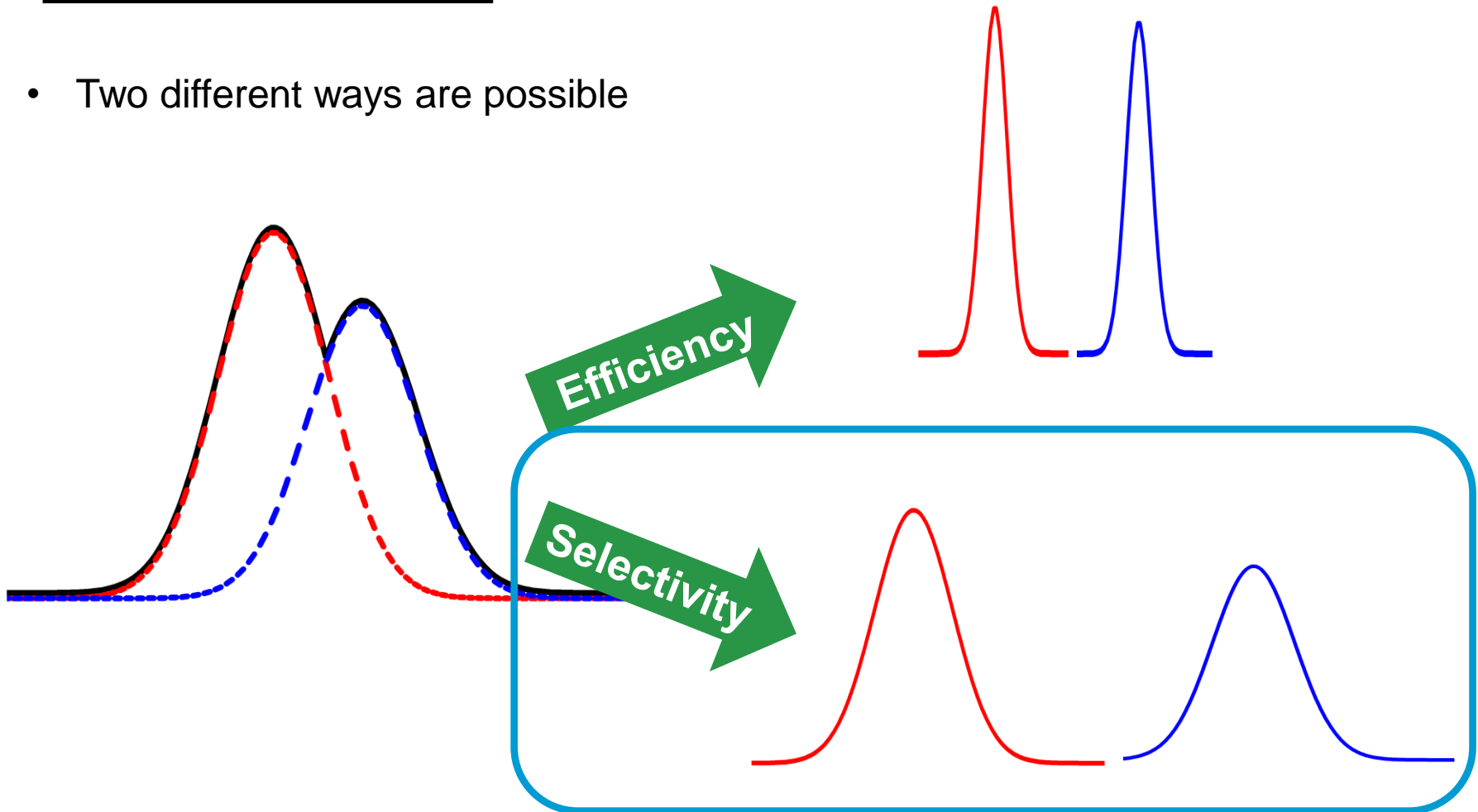
Separation optimization

- Two different ways are possible



Separation optimization

- Two different ways are possible



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

k : Retention

Value between 2 and 10 are preferred

N: Efficiency

Enters only with the square root

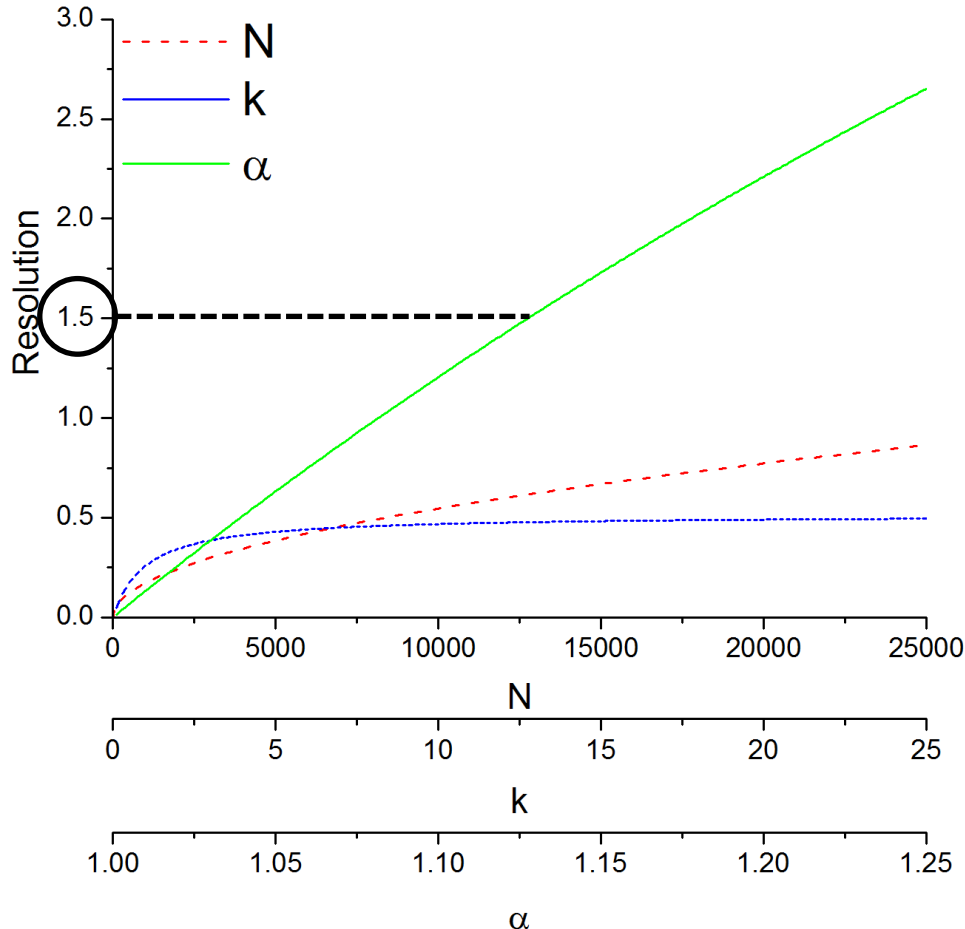
α : Selectivity

Parameter that should be optimized first

R: 1.5

Baseline separation

Influence on resolution



Parameter:

Retention k

- Runs asymptotically
- almost no influence

Efficiency N

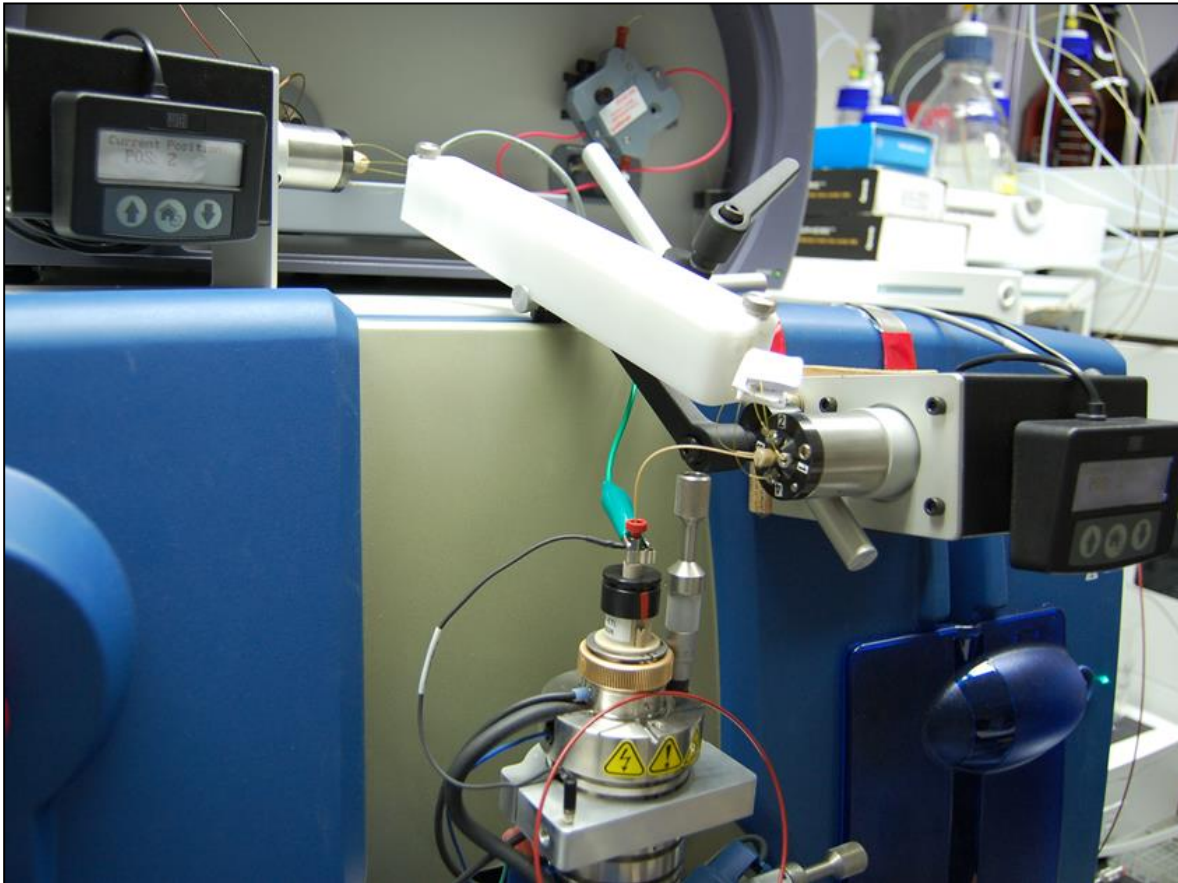
- Large differences
- Small influence

Selectivity α

- Small differences
- Large influence

Required resolution: 1.5

Application



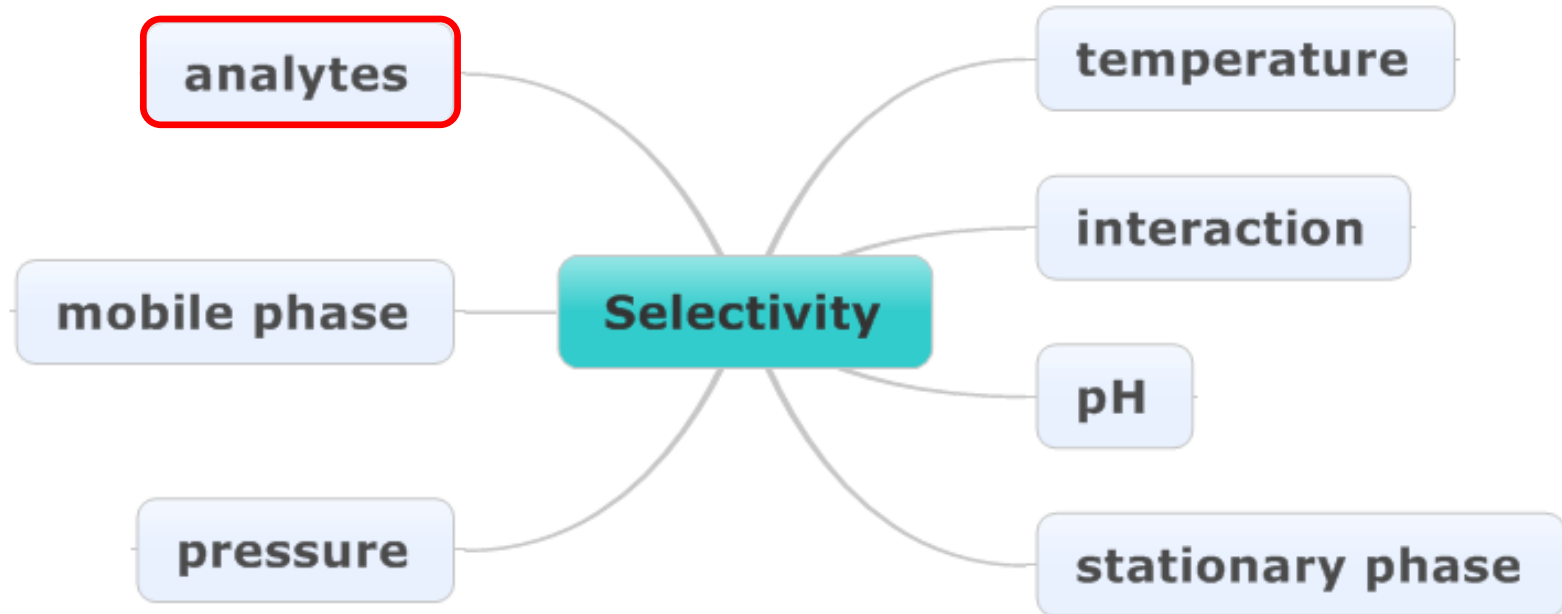
Perspective:

- Routine analysis
- Using μ LC-MS/MS

Study:

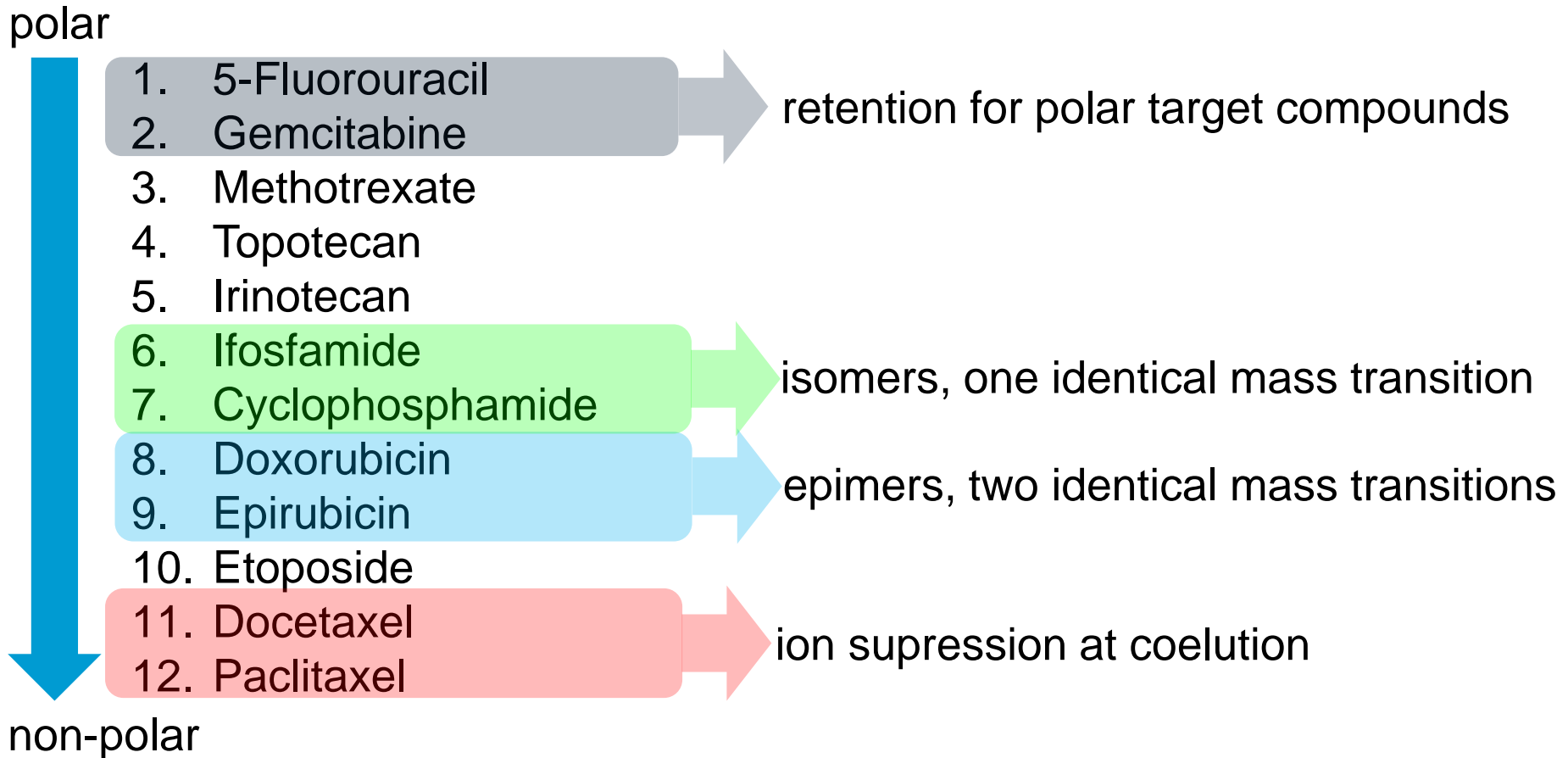
- Evaluation using UV-Detection

Influence parameters



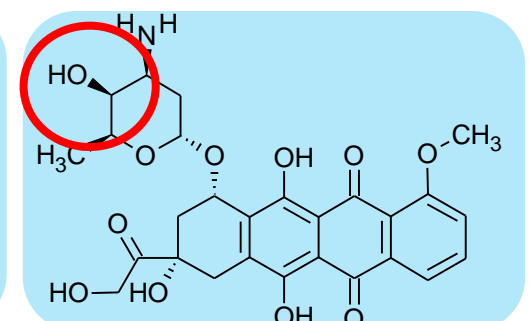
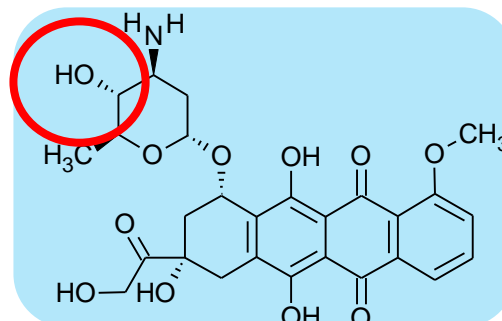
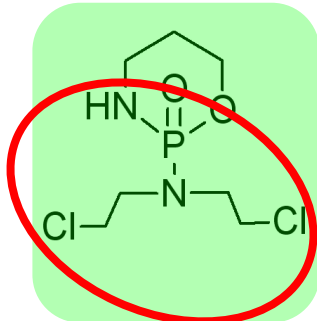
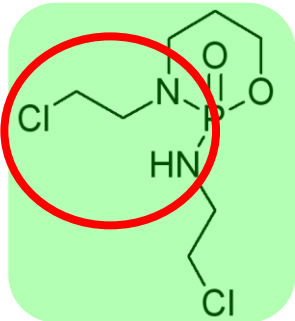
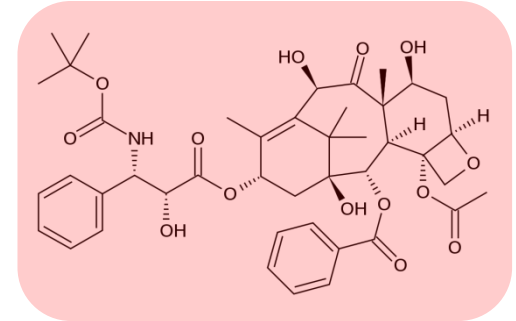
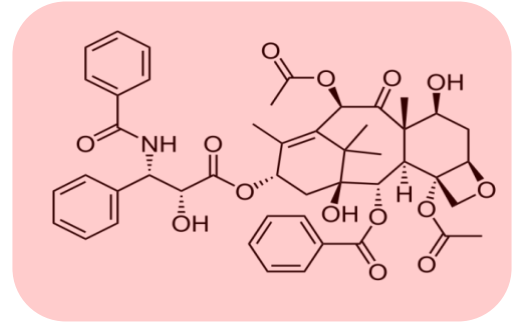
Analytes

chromatographic separation is necessary!!!

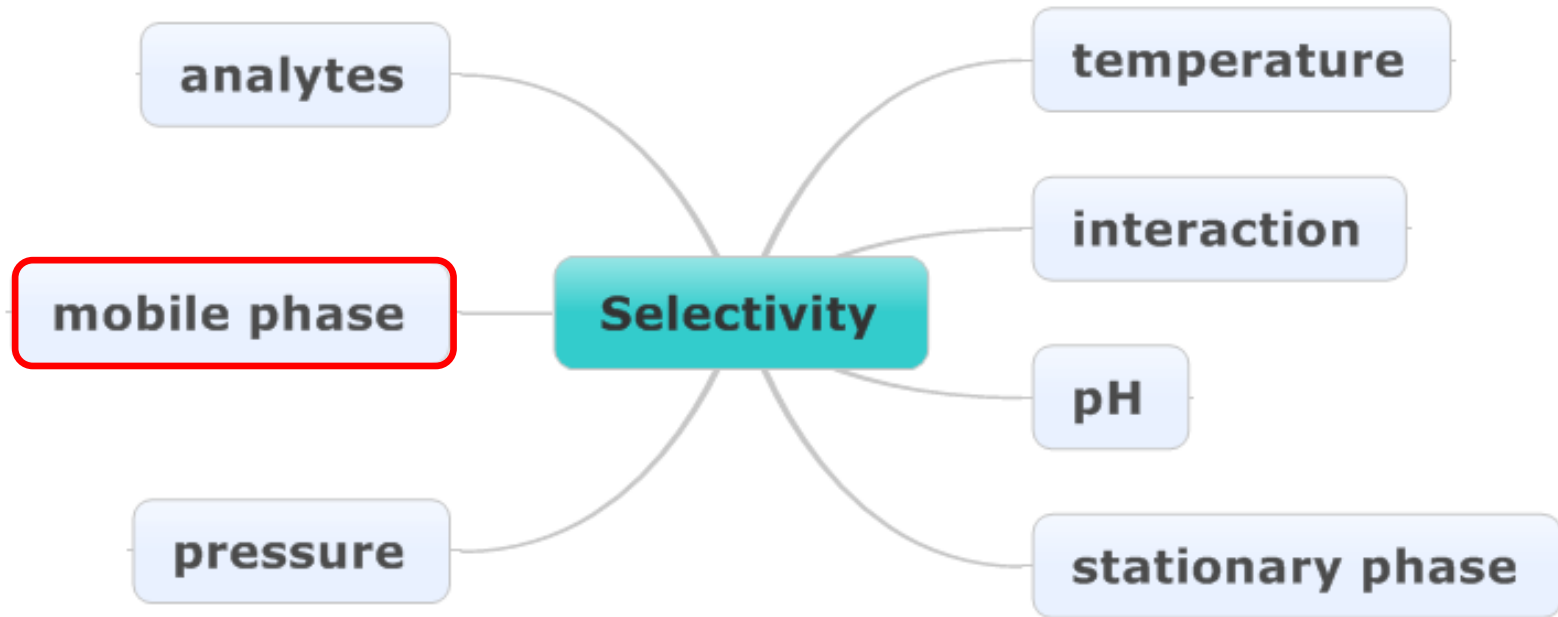


Requirements

- Retention factor ≥ 2 for polar target compounds
 - 5-fluorouracil**
 - gemcitabine**
- Separation of the three critical pairs
 - ifosfamide / cyclophosphamide**
 - doxorubicin/ epirubicin**
 - docetaxel / paclitaxel**



Influence parameters



Influence parameters

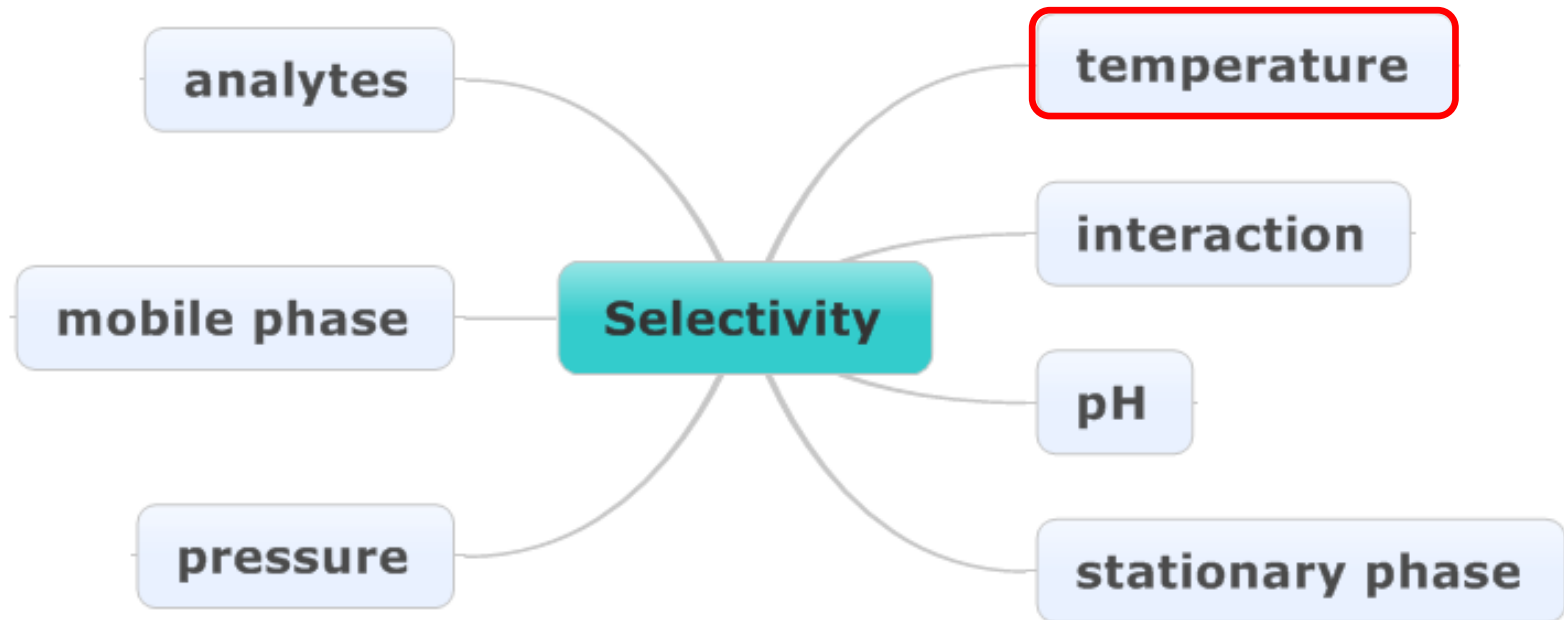
- Organic Modifier
 - Elution strength
 - Protic vs. Aprotic characteristic

Elution strength



- Water
- Methanol
- Acetonitrile

Influence parameters



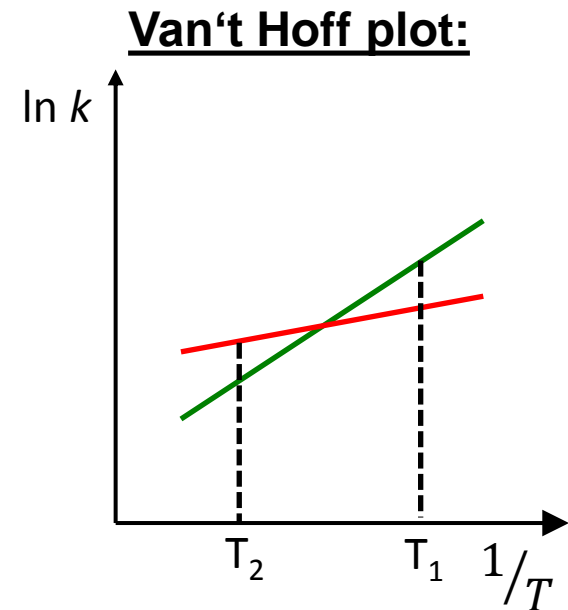
Influence parameters

- Temperature
 - Increased diffusion
 - Increased Elution strength
 - Resulting back pressure reduced

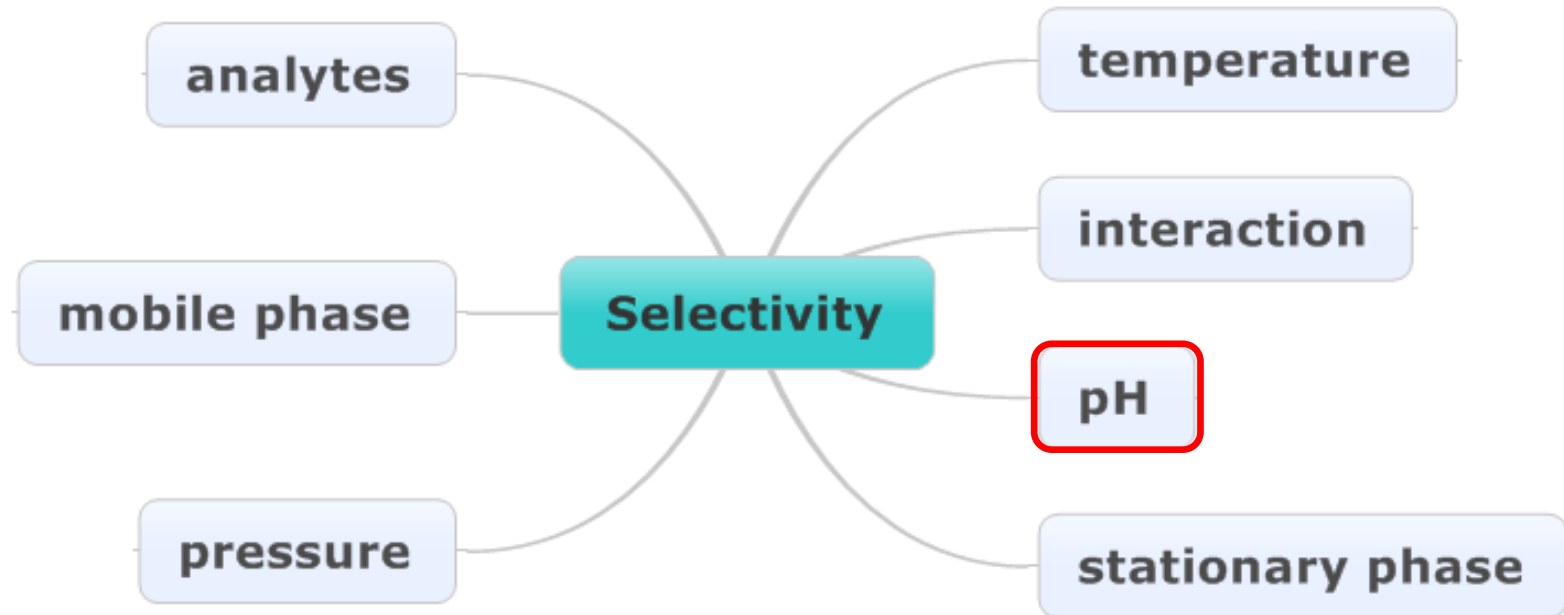
Compound A / Compound B

$$T_1: k_{\text{Compound A}} < k_{\text{Compound B}}$$

$$T_2: k_{\text{Compound A}} > k_{\text{Compound B}}$$



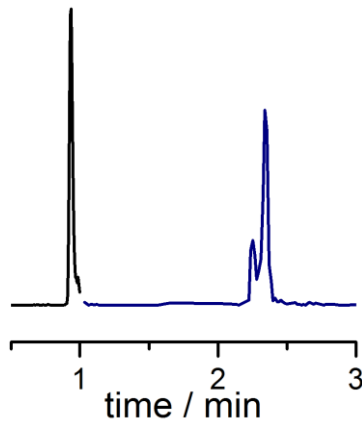
Influence parameters



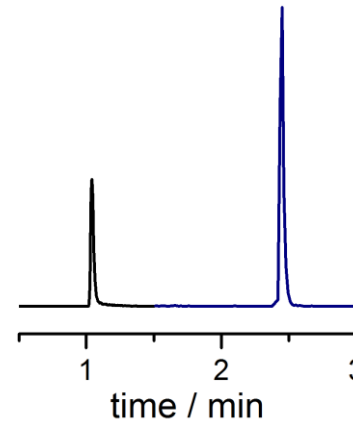
Influence parameters

- pH
 - pKs value of the substances
 - Defines the species
 - Secondary interactions are possible

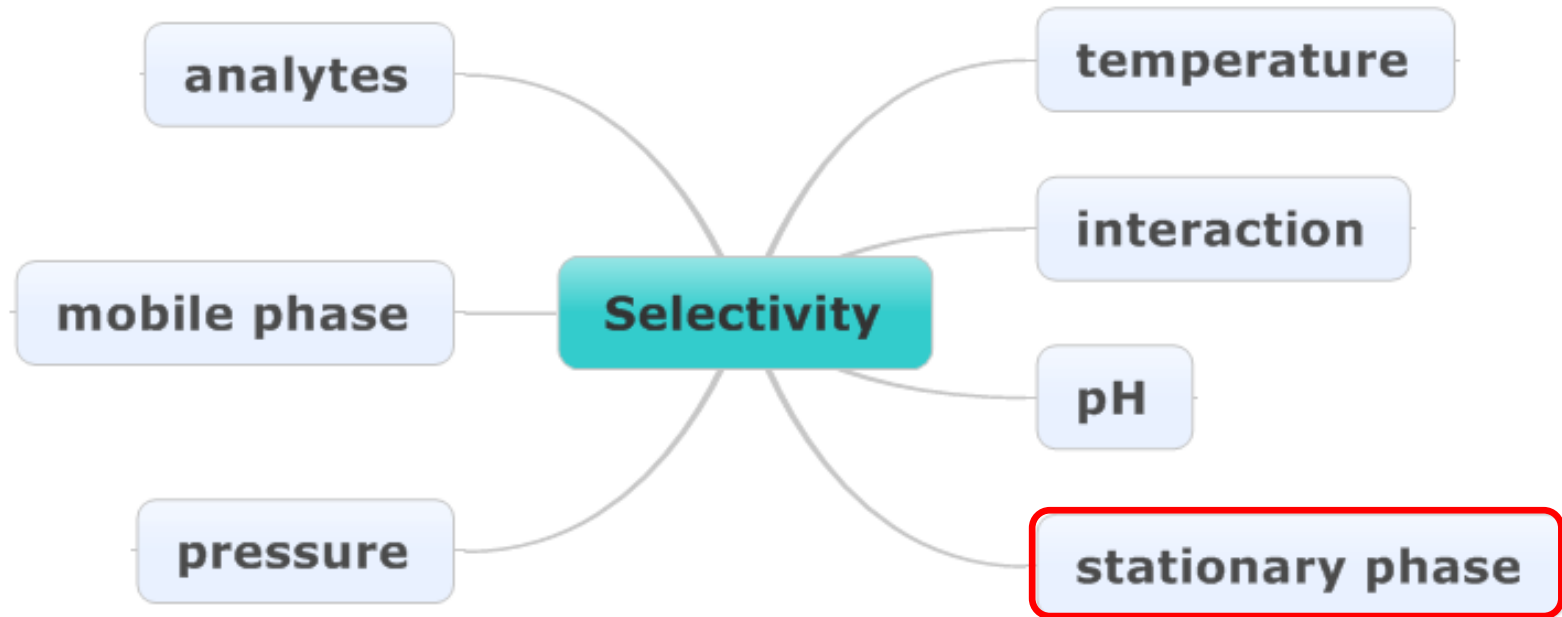
Acetic acid (pH = 3.5)



Formic acid (pH = 2.7)

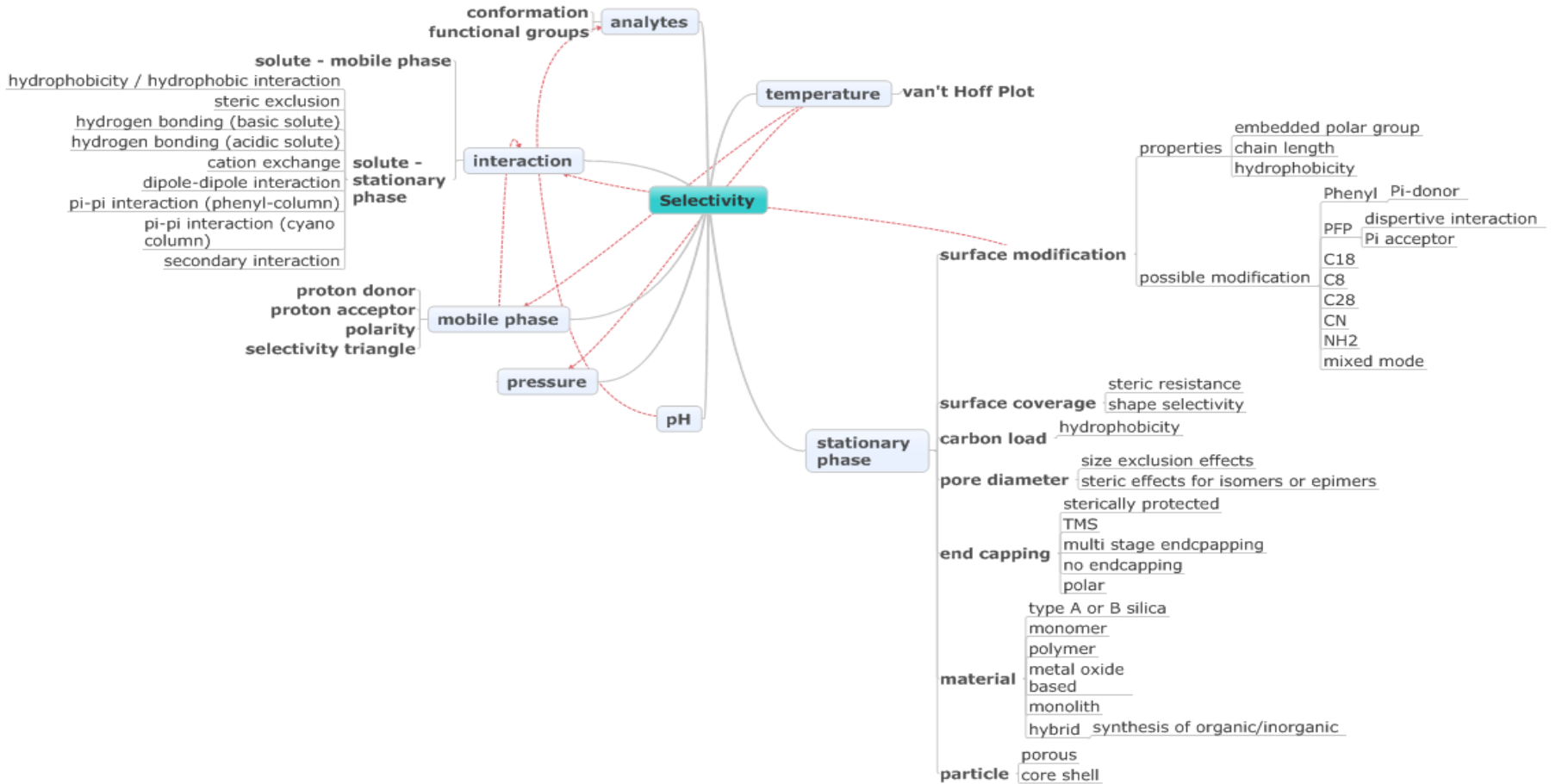


Influence parameters









Stationary phases

description	surface modification	end capping	particle size / µm	pore diameter / Å
Agilent Zorbax SB	C18	sterical protected	1.8	80
ChromaNik Sunshell RP-Aqua	C28	multistage	2.6	160
Macherey Nagel Nucleoshell RP 18plus	C18	multistage	2.7	90
Merck Chromolith FastGradient RP 18e	C18	fully endcapped	macro pores 1.5	meso pores 130
Phenomenex Kinetex	C18	trimethyl silane	2.6	100
Phenomenex Synergi RP polar	ether-linked phenyl phase	polar endcapped	2.5	
Restek Raptor ARC	C18	sterical protected	2.7	90
Restek Raptor	Biphenyl	fully endcapped		
Supelco Ascentis Express	C18	trimethyl silane	2.7	90
	C8			
	CN			



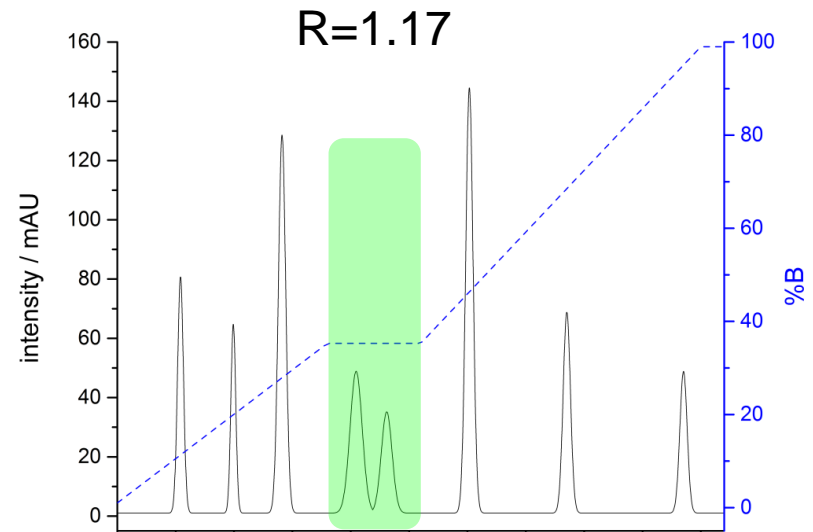
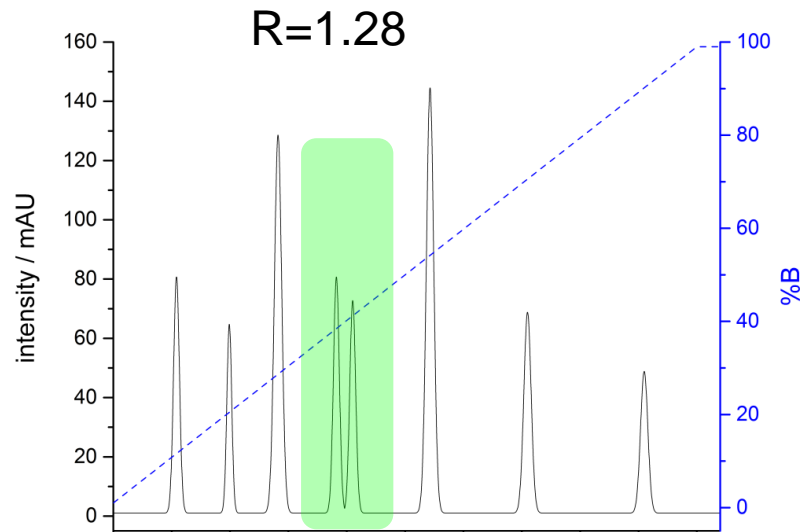
Final parameters

- Analysis of the target compounds on 20 different columns
- Organic modifier  Acetonitrile, Methanol
- pH  Acidic
- Temperature  30 °C, 50 °C
- Injection volume  2 µL
- Flow rate  350 µL min⁻¹
- Generic gradient  9.8 %B min⁻¹

Introduction

Isocratic plateaus during gradient elution

- Increasing peak width using isocratic plateau
- Loss of sensitivity
- Resolution seems to be improved



Selectivity test – How can we do it?

Selectivity tests

- Neue Test
- Engelhardt Test
- Tanaka Test
- Snyder et al. (Hydrophobic-subtraction model)

All these tests are performed under isocratic separation conditions

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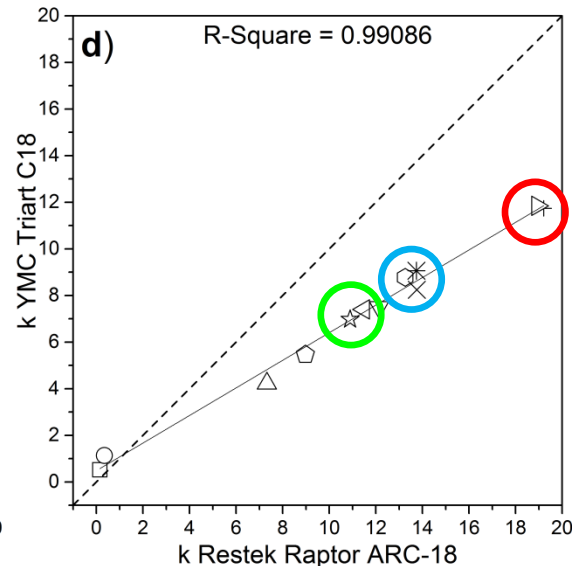
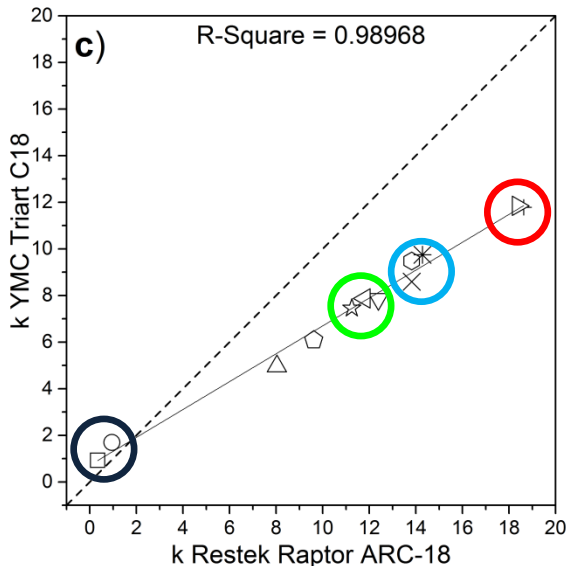
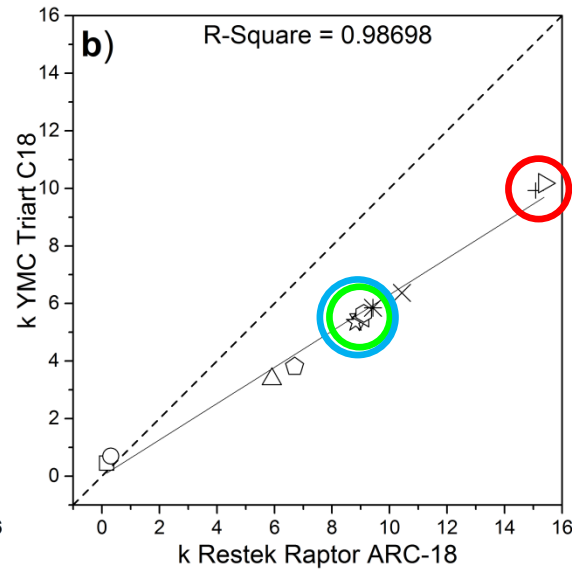
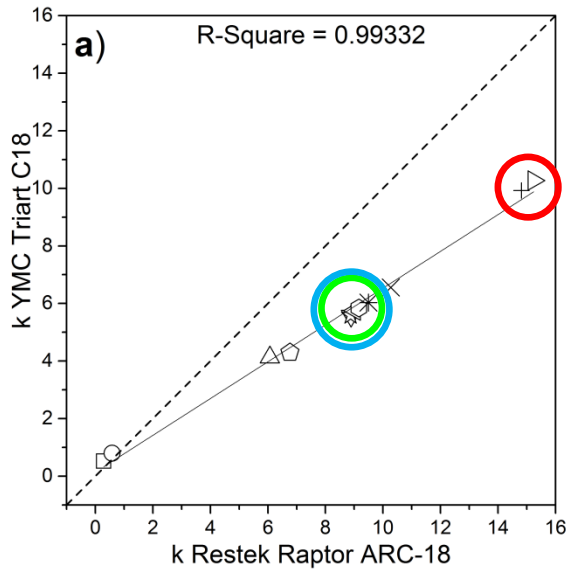
Approach

$$k = \frac{t_R - t_0}{t_0}$$

t_R : retention time
 t_0 : column dead time

- Neue et al.
- Retention factors for each target compound
- Under specific separation conditions
- For every column
- Plot of k vs k for two different columns
- Estimation of the correlation
- Variance is a quantity of the selectivity difference

$$s^2 = 1 - R^2$$



Properties:

a) ACN; 30 °C

b) ACN; 50 °C

c) MeOH; 30 °C

d) MeOH; 50 °C

- 5-fluorouracil
- gemcitabine
- methotrexat
- topotecan
- ifosfamide
- cyclophosphamide
- irinotecan
- doxorubicin
- epirubicin
- etoposide
- docetaxel
- paclitaxel

Utilization of the chromatographic space – equal band spacing

- Comparison between ACN and MeOH at 30 °C

YMC Triart C18 at 30 °C	ACN	MeOH
$\Delta(k_{\text{last}} - k_{\text{first}})$	0.98	2.30
Δk_{total}	9.74	10.92
$\Delta(k_{\text{last}} - k_{\text{first}}) / \Delta k_{\text{total}} / \%$	10.09	21.03

$$\Delta(k_{\text{last}} - k_{\text{first}}) = \Delta(k_{\text{epirubicin}} - k_{\text{ifosfamide}})$$

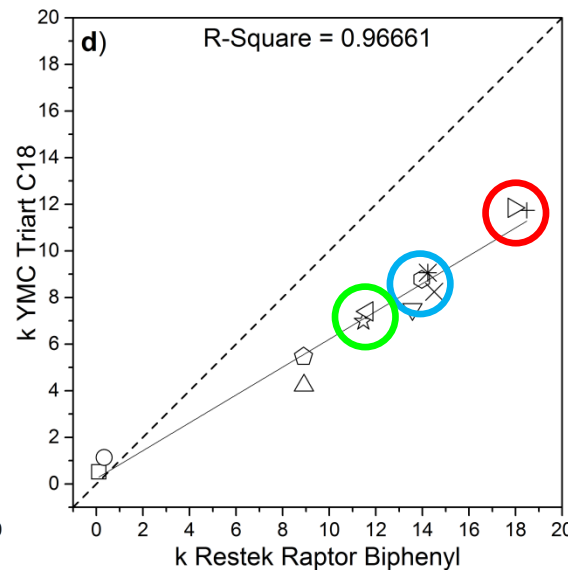
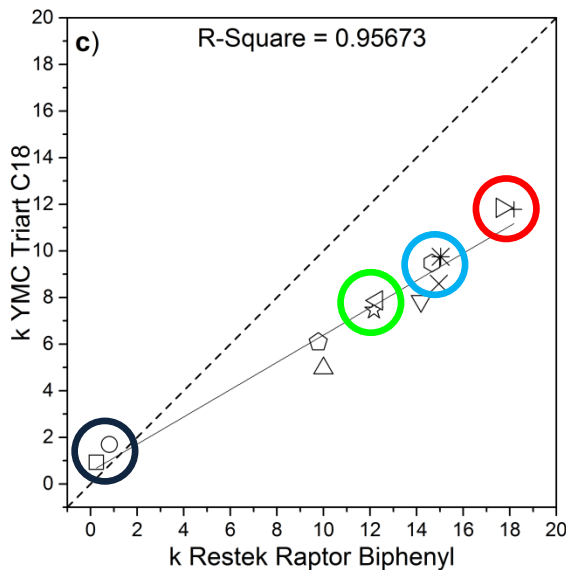
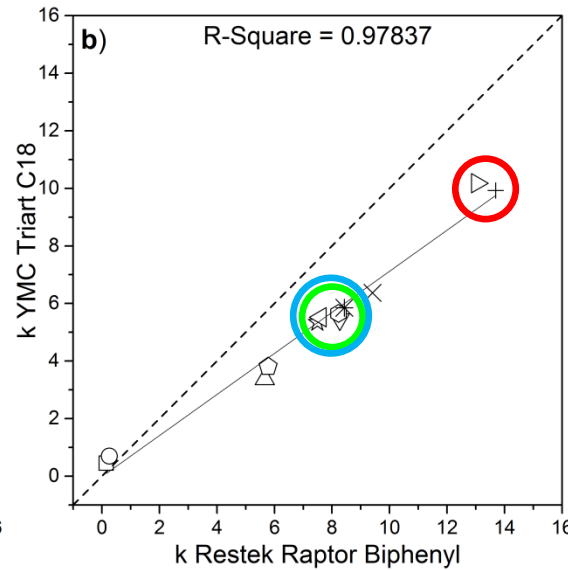
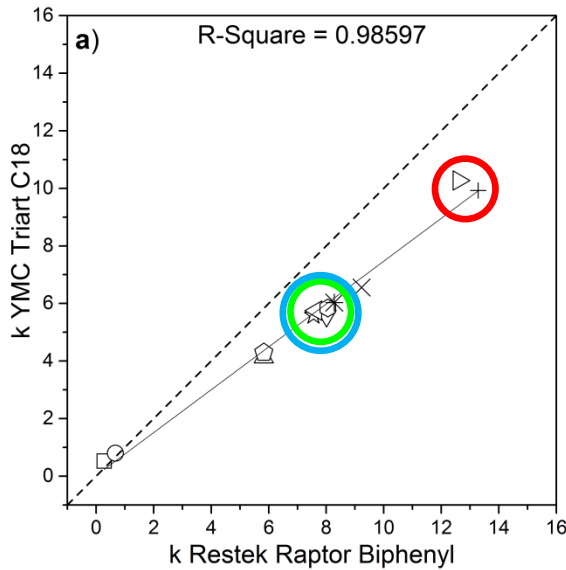
$$\Delta k_{\text{total}} = \Delta(k_{\text{docetaxel / paclitaxel}} - k_{\text{5-fluorouracil}})$$

Conclusion

- Higher retention factor using methanol instead of acetonitrile
- Higher retention factor for polar compounds using YMC Triart C18
- Higher retention factor for all other target compounds on Restek Raptor ARC-18
- Higher utilization of the chromatographic space using MeOH

Possible reason

- "Shape selectivity"
- Carbon load



Properties:

a) ACN; 30 °C

b) ACN; 50 °C

c) MeOH; 30 °C

d) MeOH; 50 °C

- 5-fluorouracil
- gemcitabine
- △ methotrexat
- ◡ topotecan
- ☆ ifosfamide
- ◁ cyclophosphamide
- ▽ irinotecan
- ◊ doxorubicin
- * epirubicin
- × etoposide
- + docetaxel
- ▷ paclitaxel

Neue et al. approach

$s^2 = 1-R^2$ Methanol 30 °C	Restek Raptor ARC-18	Restek Raptor Biphenyl	YMC Triart C18
Restek Raptor ARC-18	0	0.021	0.010
Restek Raptor Biphenyl	0.021	0	0.043
YMC Triart C18	0.010	0.043	0

Selectivity comparison in terms of the critical peak pairs – How can we do it?

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Quantitative value for describing selectivity

Selectivity factor α

$$\alpha = \frac{k_2}{k_1} \quad \text{with } k_2 \geq k_1$$

k_1, k_2 : retention factor

$$k = \frac{t_R - t_0}{t_0}$$

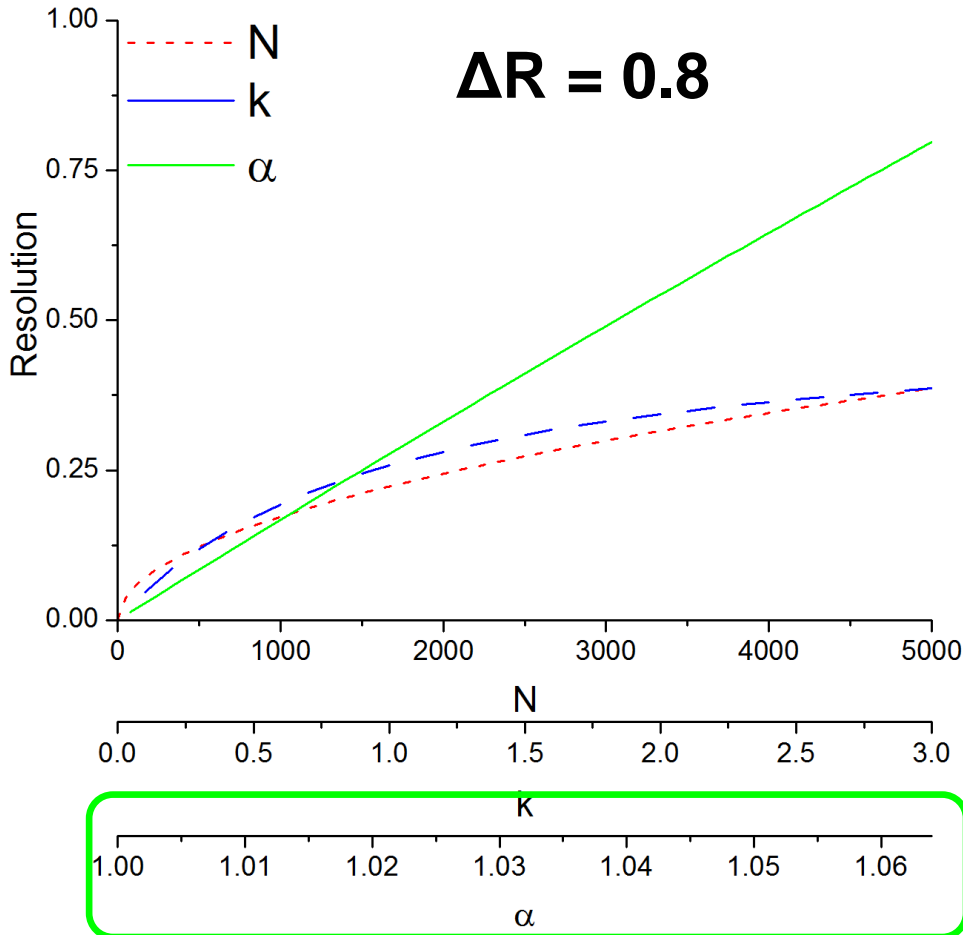
t_R : retention time
 t_0 : column dead time

	α Acetonitrile 50 °C			α Methanol 50 °C		
Critical peak pair	IF / CP	Doxo / Epi	Pac / Doc	IF / CP	Doxo / Epi	Pac / Doc
Restek Raptor ARC-18	1.030	1.035	1.019	1.055	1.036	1.014
Restek Raptor Biphenyl	1.014	1.025	1.049	1.014	1.019	1.031
YMC Triart C18	1.034	1.035	1.025	1.062	1.032	1.009

IF / CP : ifosfamide / cyclophosphamide

Different selectivity does not provide better separation and vice versa

Significance



Parameter:

Retention $k = 3$

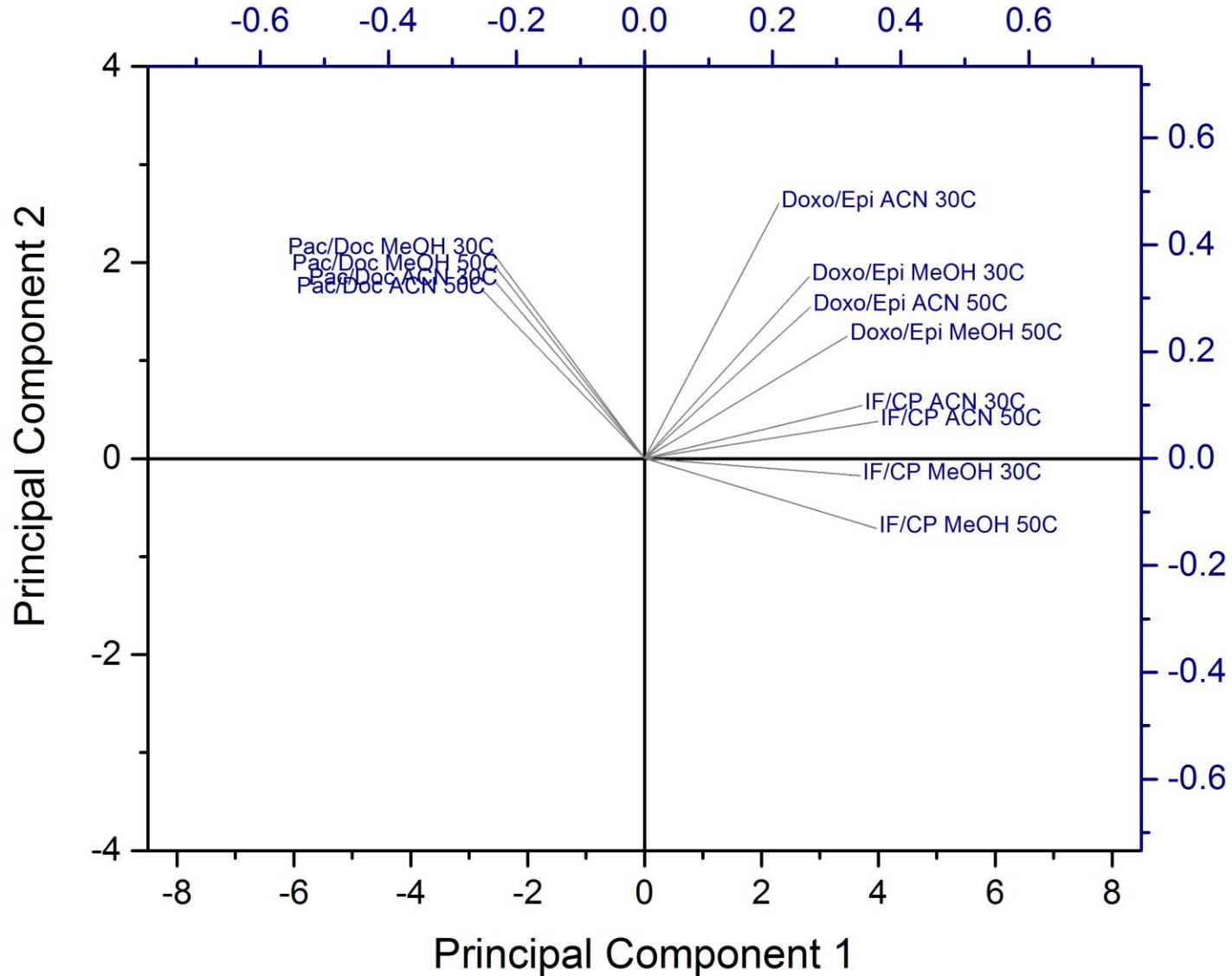
Efficiency $N = 5000$

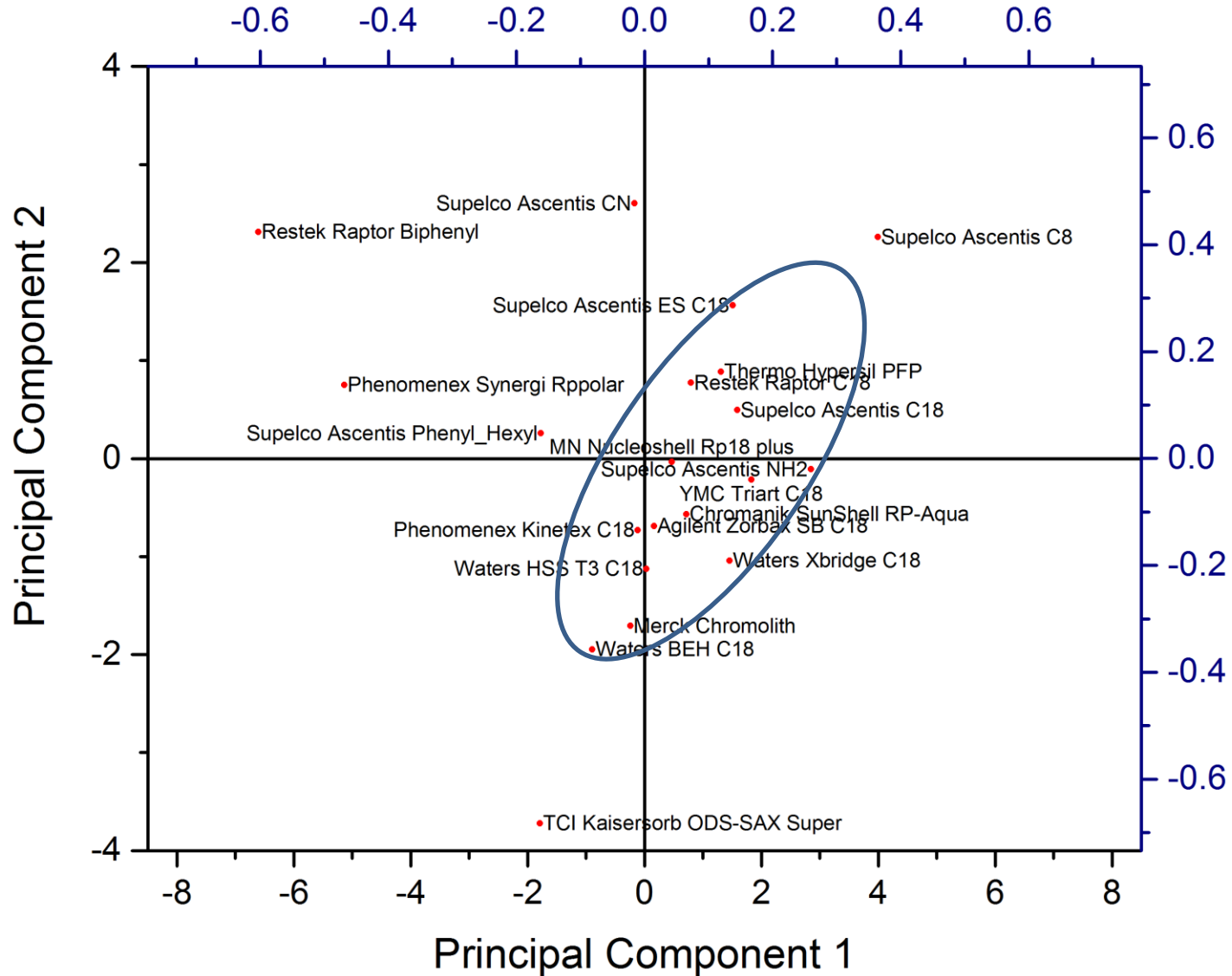
Selectivity α

- $\alpha_{\min} = 1.000$
- $\alpha_{\max} = 1.064$

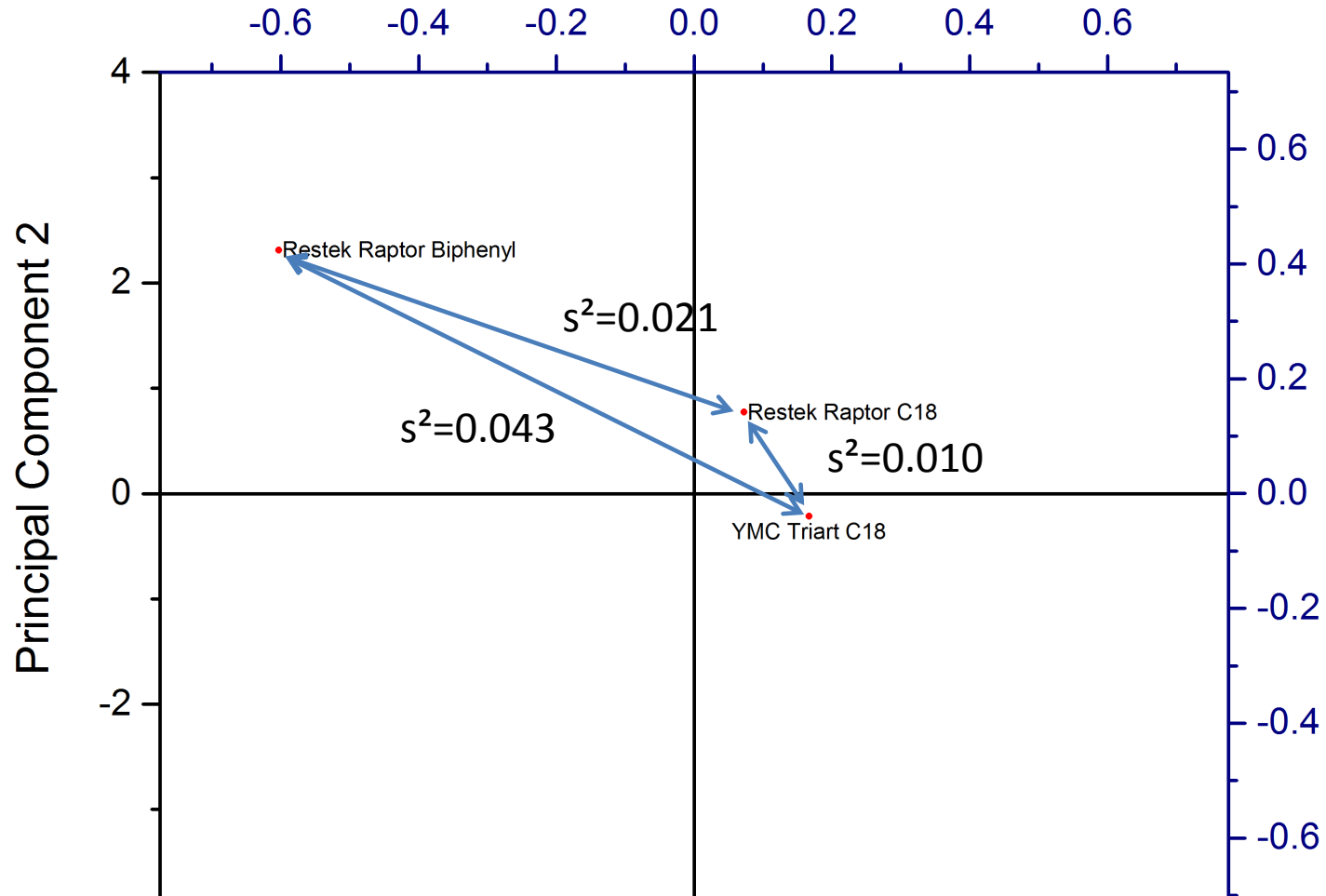
Quantitative value for describing selectivity

- Selectivity factor enables to classify columns
- Information about similarities and differences
- All substances = overall selectivity comparison
- Only for the three critical peak pairs
- Critical peak pairs = principal component analysis (PCA)
 - Multivariate data analysis
 - Simplification of large datasets





Evaluation strategy



Same results with the additional advantage of all information in one plot

Substantiation of the PCA

- Using the average selectivity factor for all critical peak pairs
- Classic calculation of the average selectivity factor could lead to false choice
- Weighting system is applied

Weighting system

Weighting factor

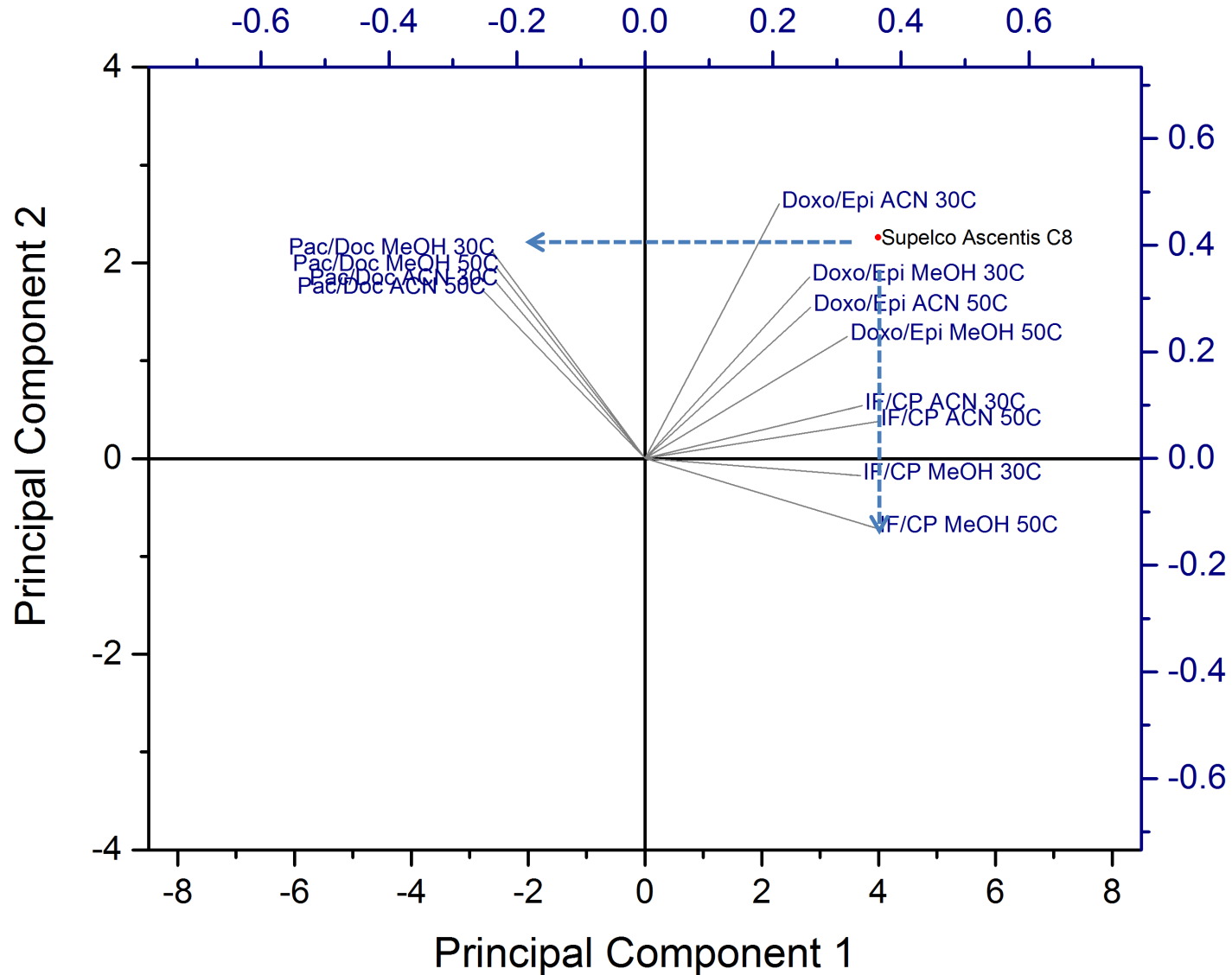
- | | |
|---------------------------------|---|
| • Docetaxel / paclitaxel | 1 |
| • Ifosfamide / cyclophosphamide | 2 |
| • Doxorubicin / epirubicin | 3 |

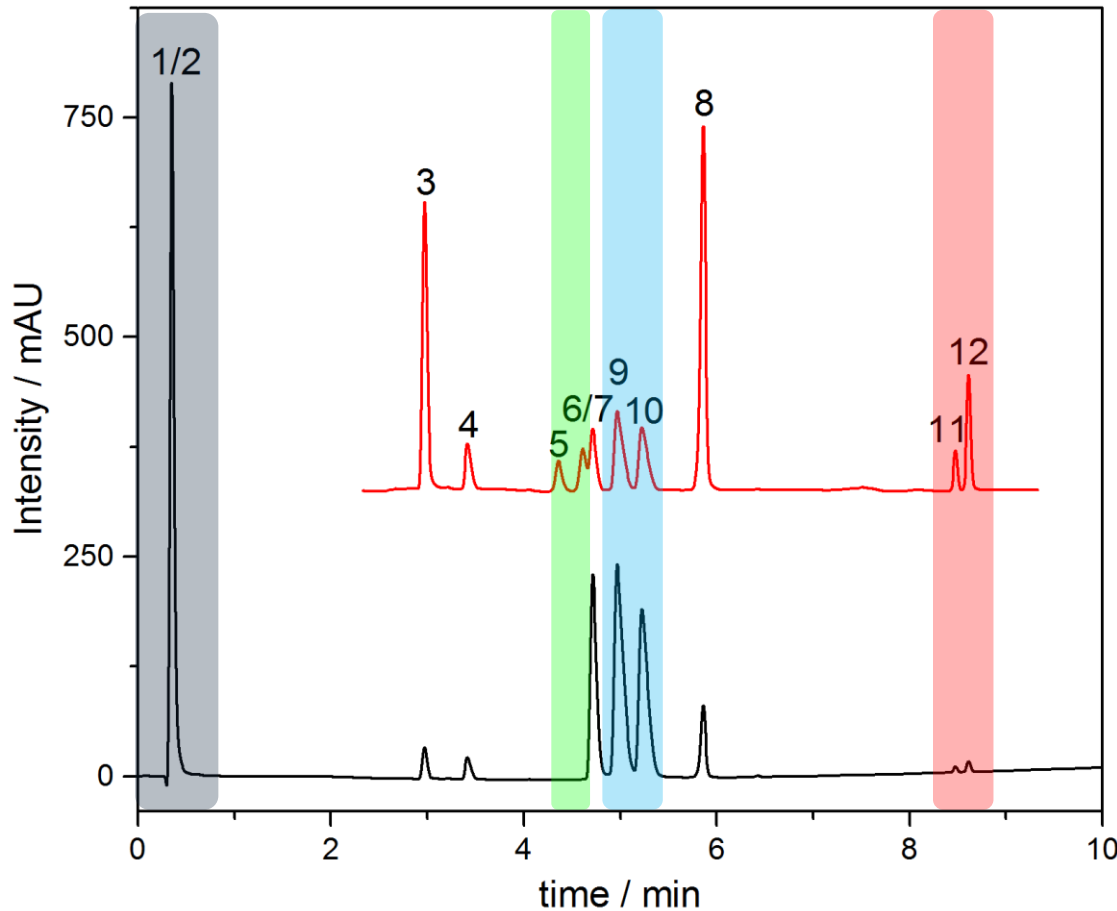
Influence of the weighting system

Column	Acetonitrile				Methanol			
	average α (30 °C)	<i>weighted</i> <i>average α</i> (30 °C)	average α (50 °C)	<i>weighted</i> <i>average α</i> (50 °C)	average α (30 °C)	<i>weighted</i> <i>average α</i> (30 °C)	average α (50 °C)	<i>weighted</i> <i>average α</i> (50 °C)
	Restek Raptor Biphenyl	1.031	1.027	1.030	1.025	1.022	1.021	1.021

Influence of the applied separation condition

mobile phase	temperature / °C	weighted average selectivity factor α (n=20)
Acetonitrile	30	1.027
	50	1.029
Methanol	30	1.031
	50	1.035





Chromatographic conditions

Column: Ascentis Express C8
(50 x 2.1 mm; 2.7 μ m)

Mobile phase:

A: H₂O + 0.1 % FA

B: MeOH + 0.1 % FA

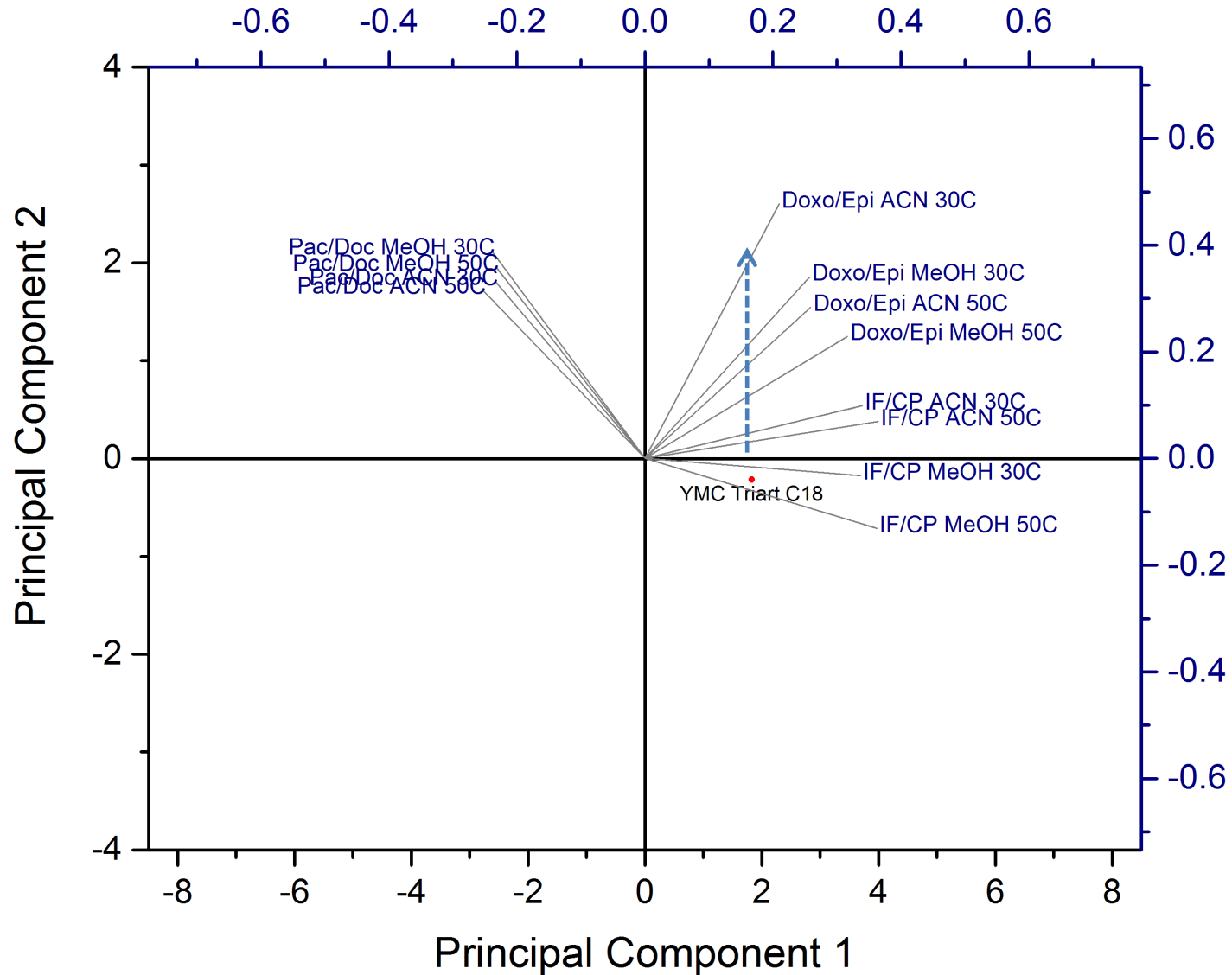
Temperature: 50 °C

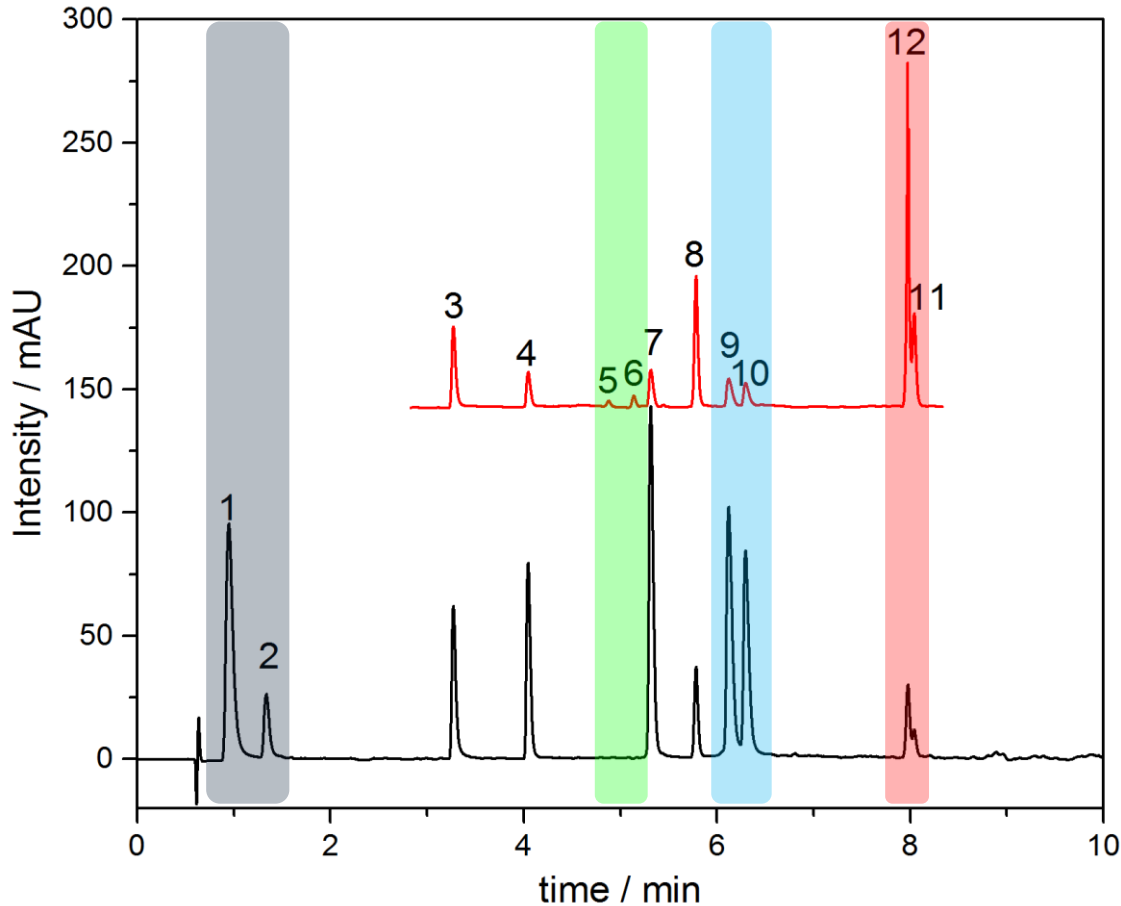
Injection volume: 2 μ L

Flow rate: 350 μ L min⁻¹

Gradient : 9.8 %B min⁻¹

UV — 254 nm
— 200 nm





Chromatographic conditions

Column: YMC Triart C18
(50 x 0.3 mm; 1.9 μm)

Mobile phase:

A: H_2O + 0.1 % FA

B: MeOH + 0.1 % FA

Temperature: 50 $^{\circ}\text{C}$

Injection volume: 0.04 μL

Flow rate: 7.14 $\mu\text{L min}^{-1}$

Gradient : 9.8 %B min^{-1}

UV — 254 nm
 — 200 nm

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- A lot of different influence factors must be taken into account
- Several requirements have to be considered
- The question of a suitable phase system cannot be answered unequivocally
- It is up to the user which requirements are preferred

Final Choice

- Using simulation software to improve chromatographic separation condition
- Investigation of column efficiency

Thank you for your attention!