The right solvent for the best separation

Eike Reich
CAMAG Labor
Sonnenmattstrasse 11
4132 Muttenz/Schweiz

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The analytical goal

Identification	→ specificity
Adulteration/falsification	→ specificity
Detection of mixtures	→ specificity, LOD
Impurities	→ specificity, range
Limit test	→ precision, LOD
Assay	→ accuracy, precision

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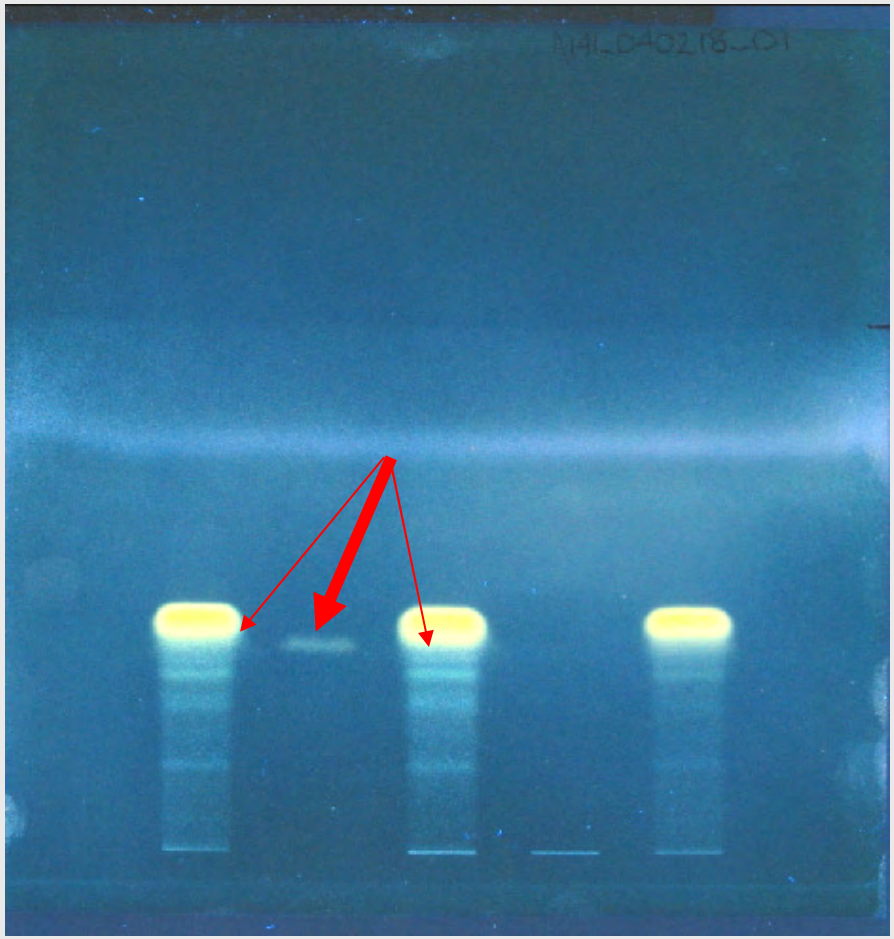
Impurity in tail of principal component



Oxymetholone

TLC 20 x 20 cm

Silica gel 60 F_{254}
Cyclohexane : ethyl acetate : acetic acid 50:48:2
development over 2/3 of the plate, air dried, sprayed with vanillin reagent, heated at 130 °C for 5 min

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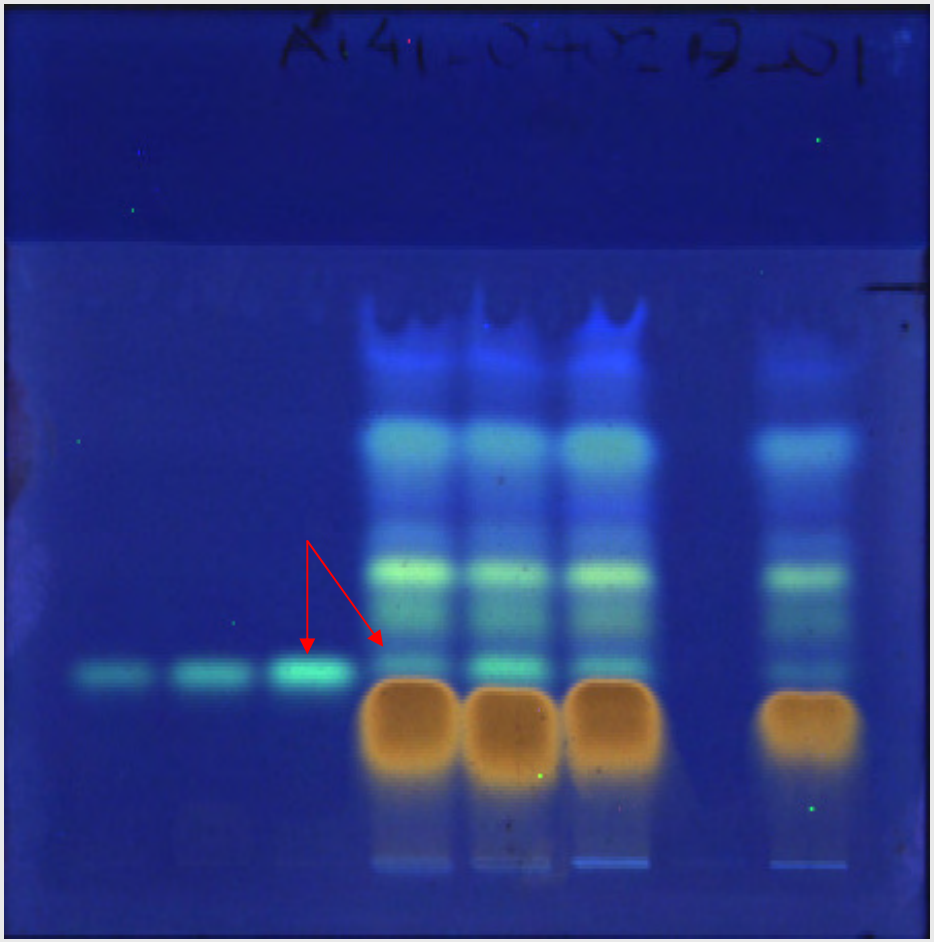
Inversed elution order (RP - mode)



HPTLC 10 x 10 cm

RP-18 F_{254s} (bands: 8 mm).
Development ACN:H_2O 80:20 to 70 mm, air dried.

Derivatization: dipping in H_2SO_4 reagent, followed by heating for 3 minutes at 100 °C on plate heater.

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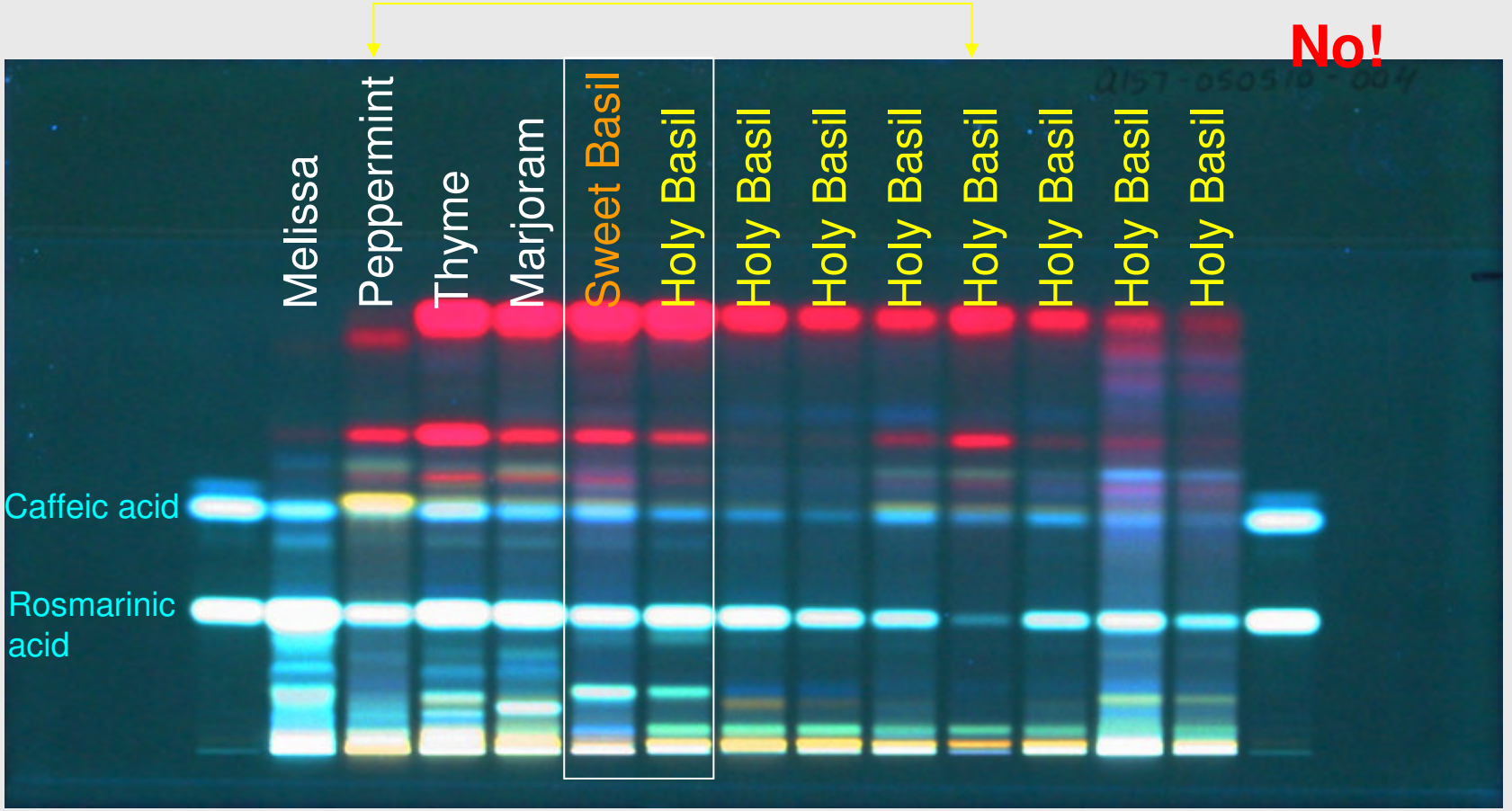
Meaningful fingerprint?



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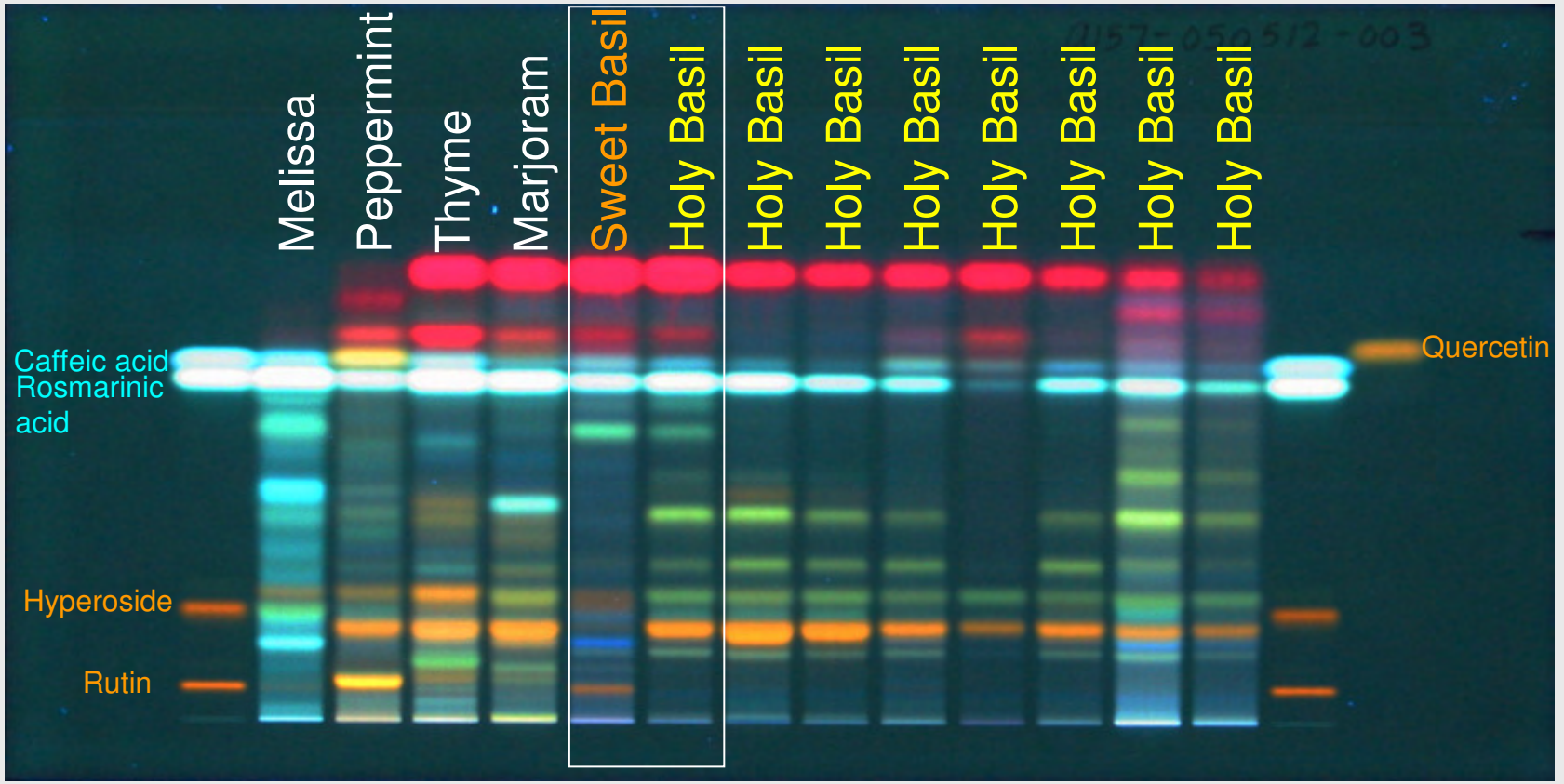
Meaningful fingerprint?

YES!



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Some basic theory

- How TLC works
- What separation depends on
- How separation can be described
- How separation can be affected

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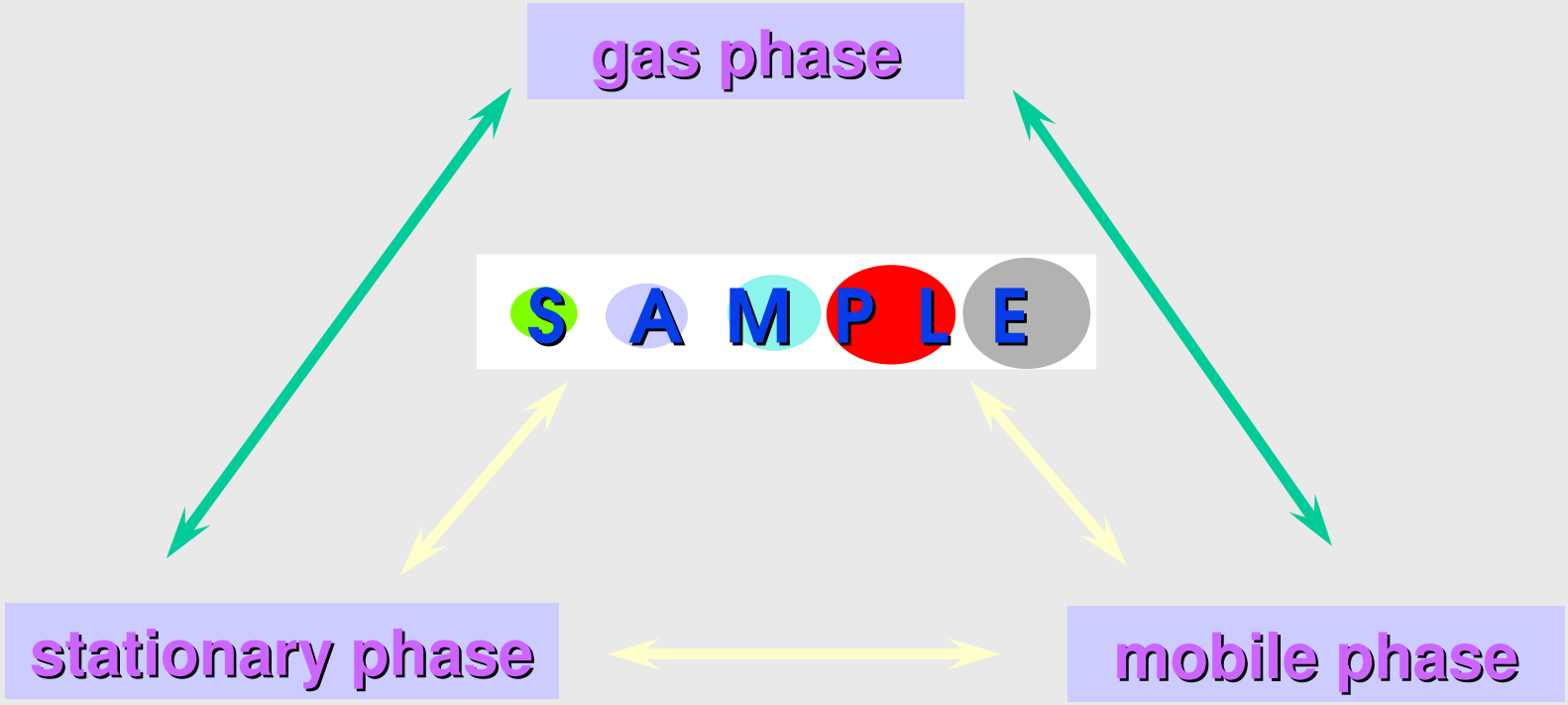
The TLC system



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The gas phase

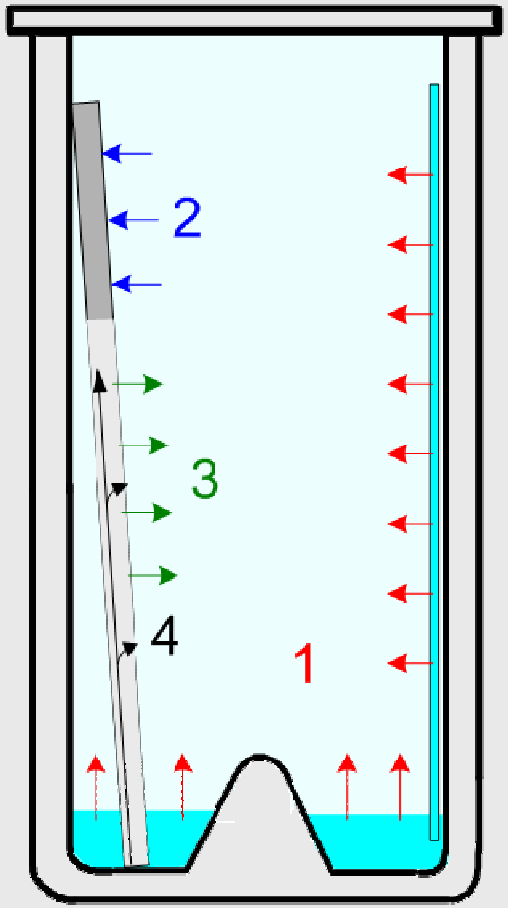
- Affects:
  - Layer activity
  - R_F
  - Separation
  - Mobile phase

1: (Chamber) Saturation

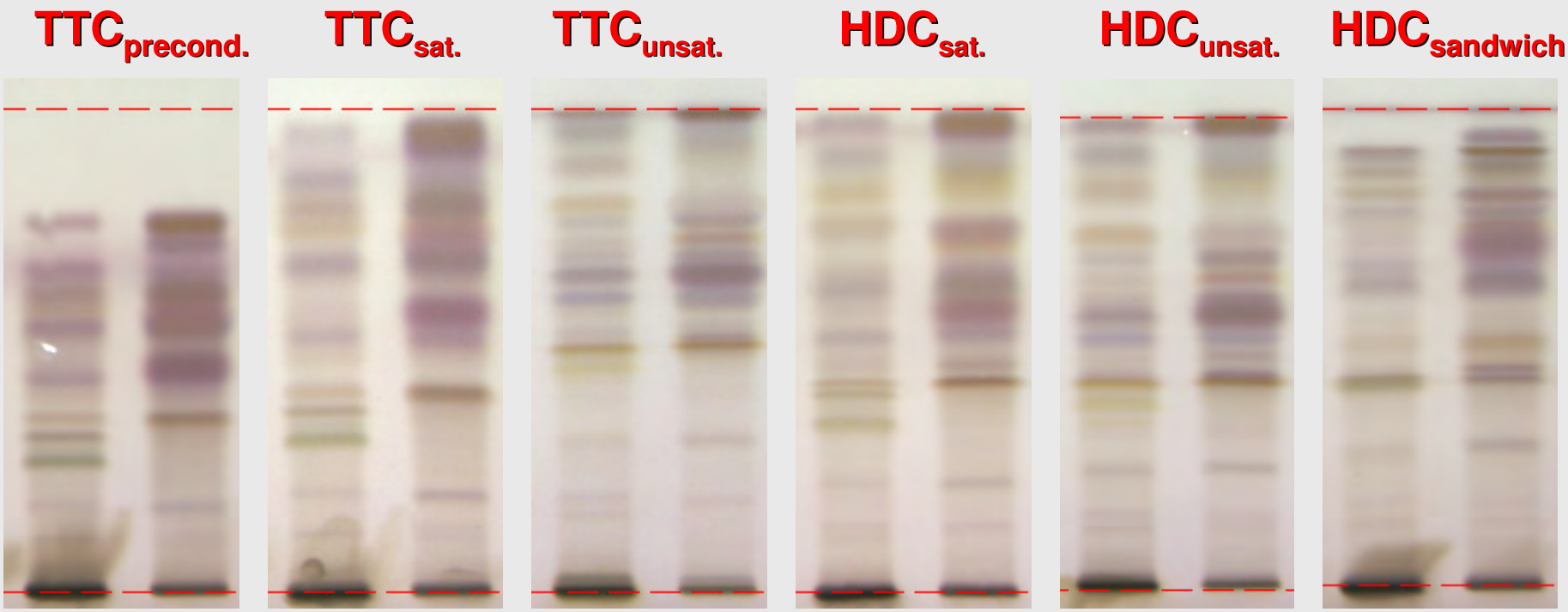
2: (Plate) Pre-conditioning

3: (Mobile phase) Evaporation

4: (Formation of) Secondary fronts



Effects of the chamber on the separation of *Schisandra*



HPTLC silica gel 60 F_{254}, toluene - ethyl acetate - acetic acid (70 : 33 : 3)

Left: *S. chinensis*, right: *S. sphenanthera*

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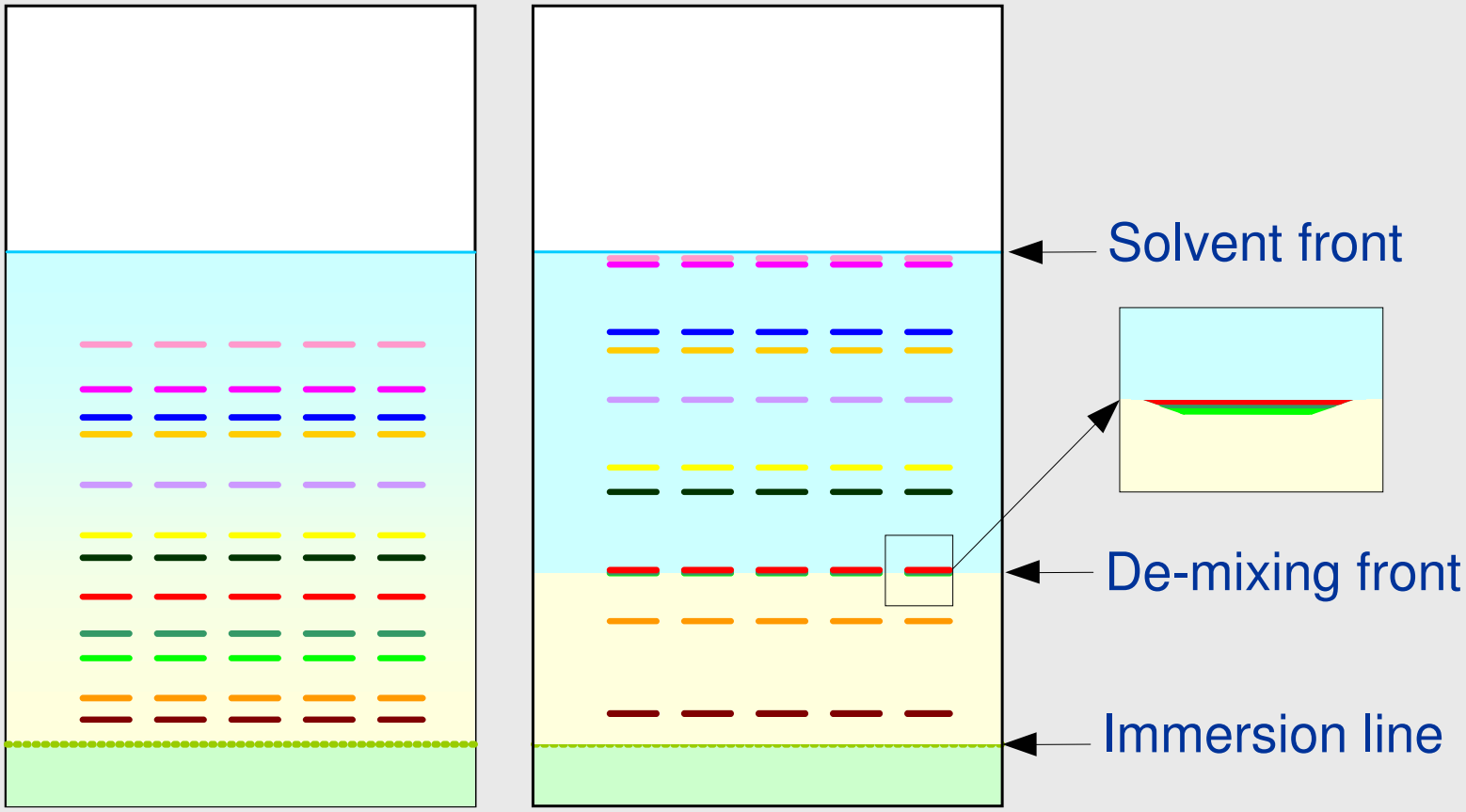
Secondary front

Saturated chamber

Sandwich



Virtual front

Sandwich

Saturated chamber

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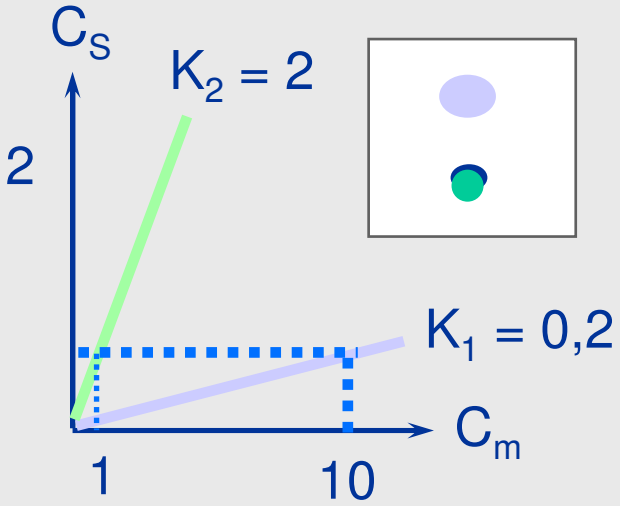
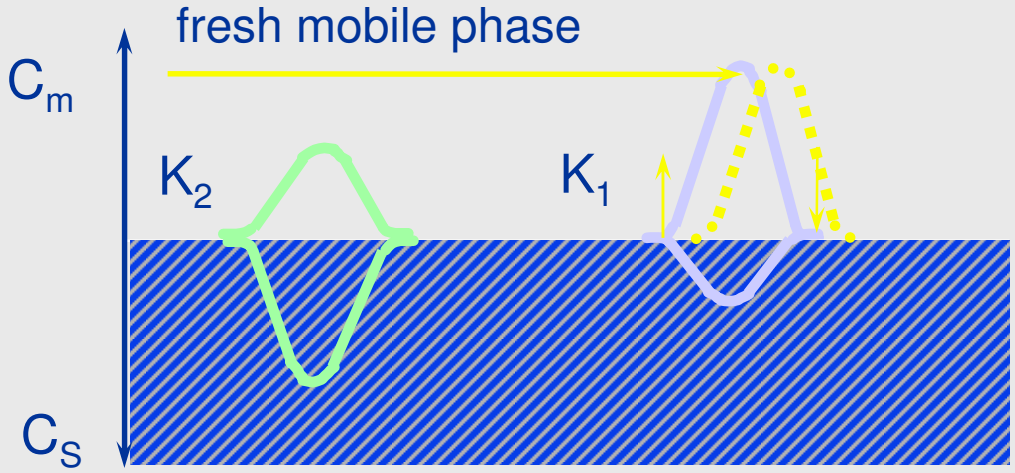
Partition-/adsorption isotherm





$$K = \frac{C_s}{C_m}$$

$$\text{selectivity} \quad a = \frac{K_1}{K_2}$$

K	partition coefficient
C_S	concentration in stationary phase
C_M	concentration in mobile phase

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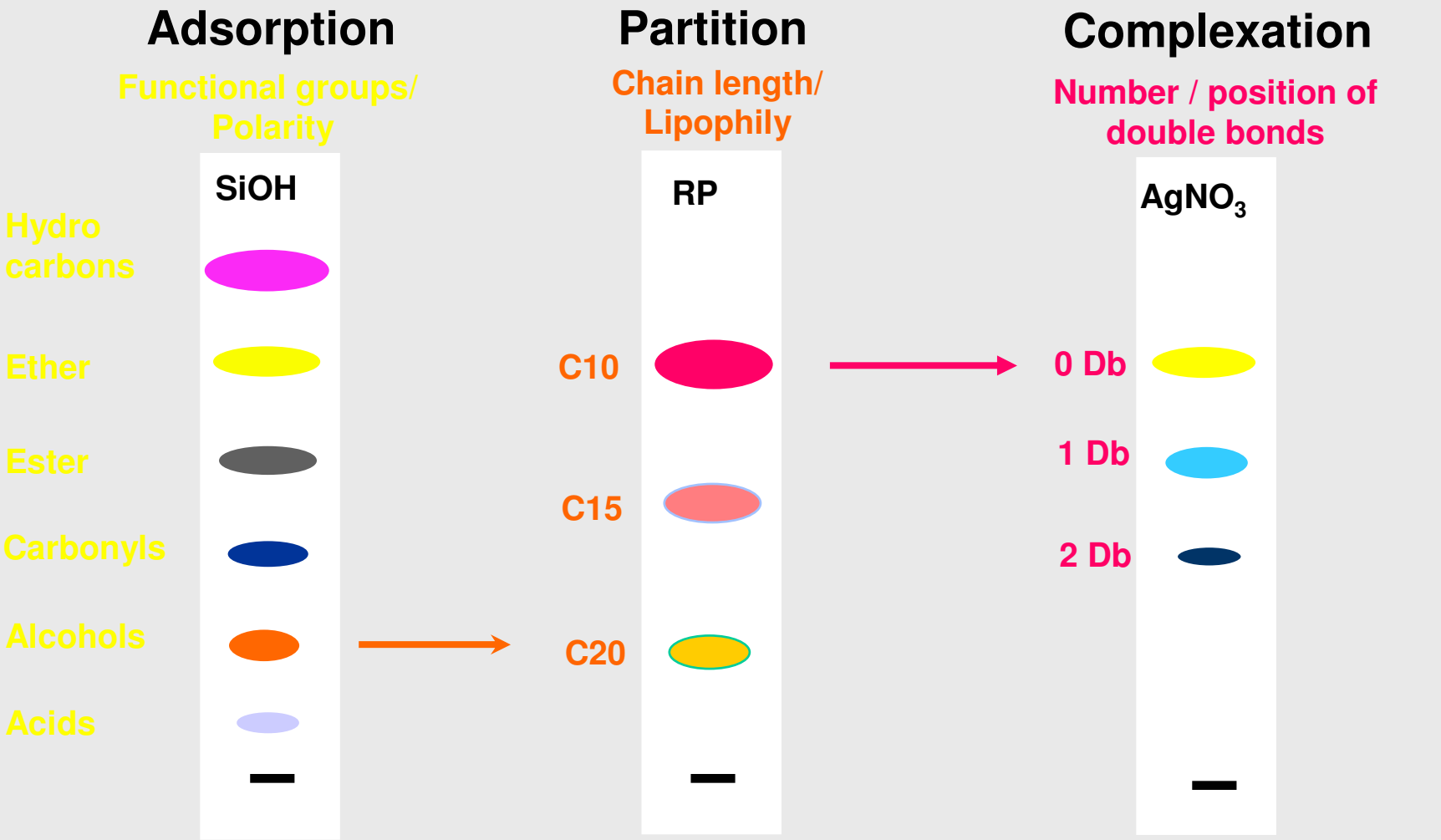
Separation mechanisms in TLC



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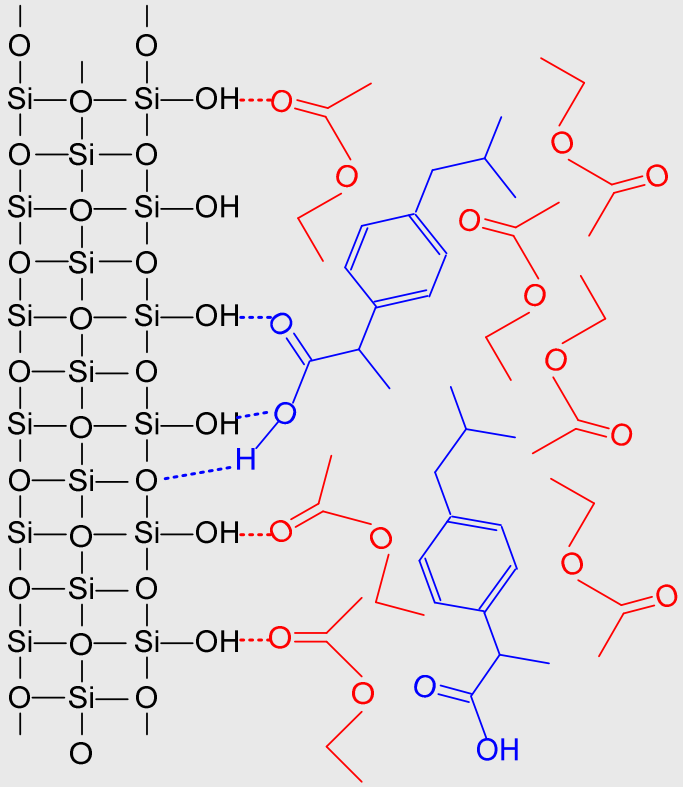
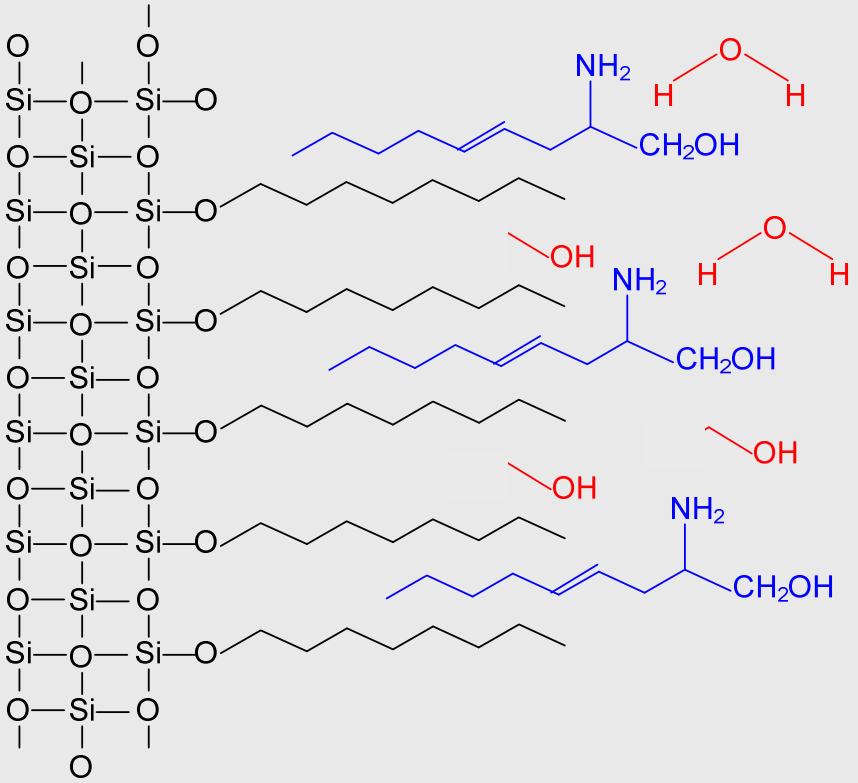
Separation mechanisms

Adsorption



Partition



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R_f -value/hR_f-range

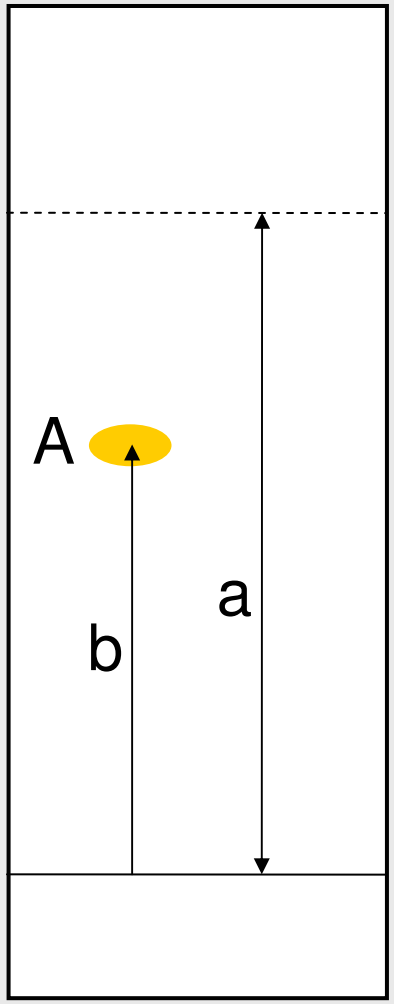
- Retardation factor or ratio to front
- Relative position of a zone on the TLC/HPTLC-plate

$$R_f = \frac{b}{a} < 1$$

a: migration distance of the mobile phase front

b: migration distance of the fraction

$$hR_f = R_f * 100$$



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Separation←→ resolution

$$R_s = 2\Delta z / w_{b1} + w_{b2}$$

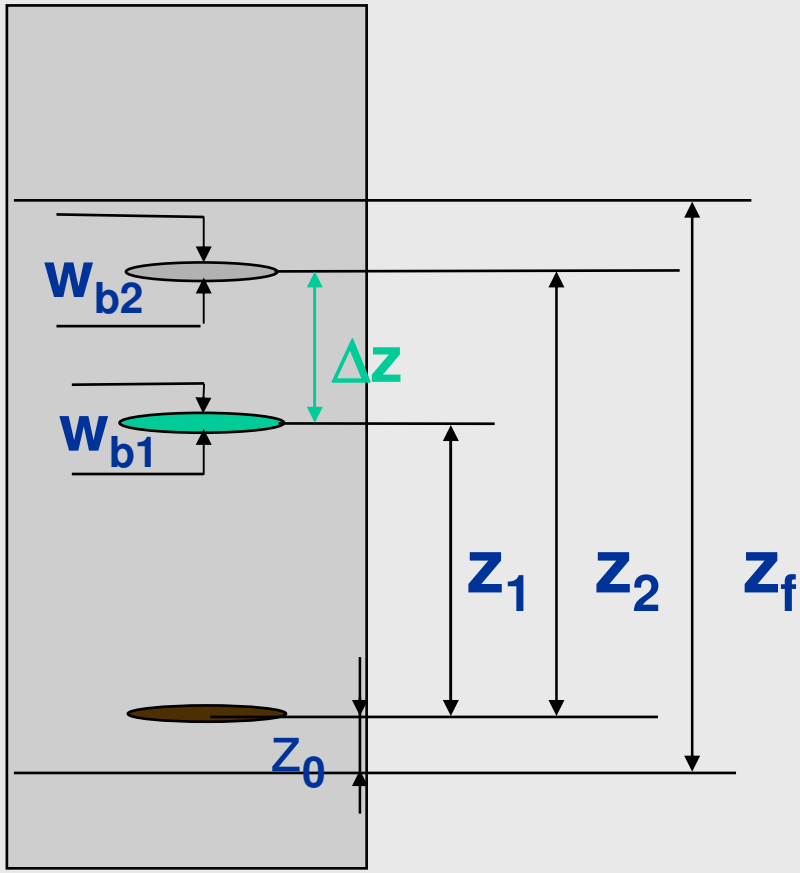
$$R_s = {}^1/_4(\alpha-1)(R_F N)^{1/2}(1-R_F)$$

a b c

$$\alpha = \frac{(R_{F2})[1-(R_{F1})]}{(R_{F1})[1-(R_{F2})]}$$

$$N = z_x / H \quad R_F$$

$$H = w_b^2 / 16 z_x$$



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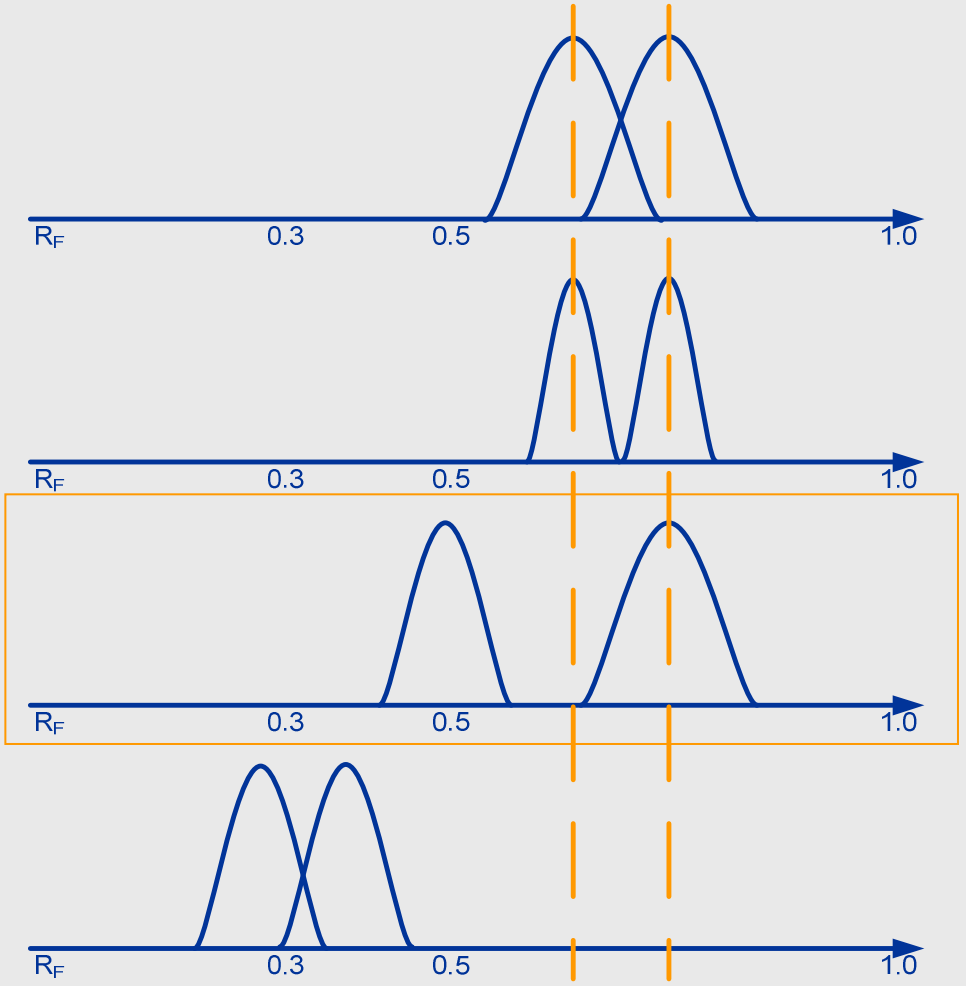
Effects of N, α, and Rf

- Selectivity



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Solvent strength and selectivity

Effect of selectivity at constant solvent strength

a) Hexane with 15% ethyl acetate
b) Hexane with 20% acetone
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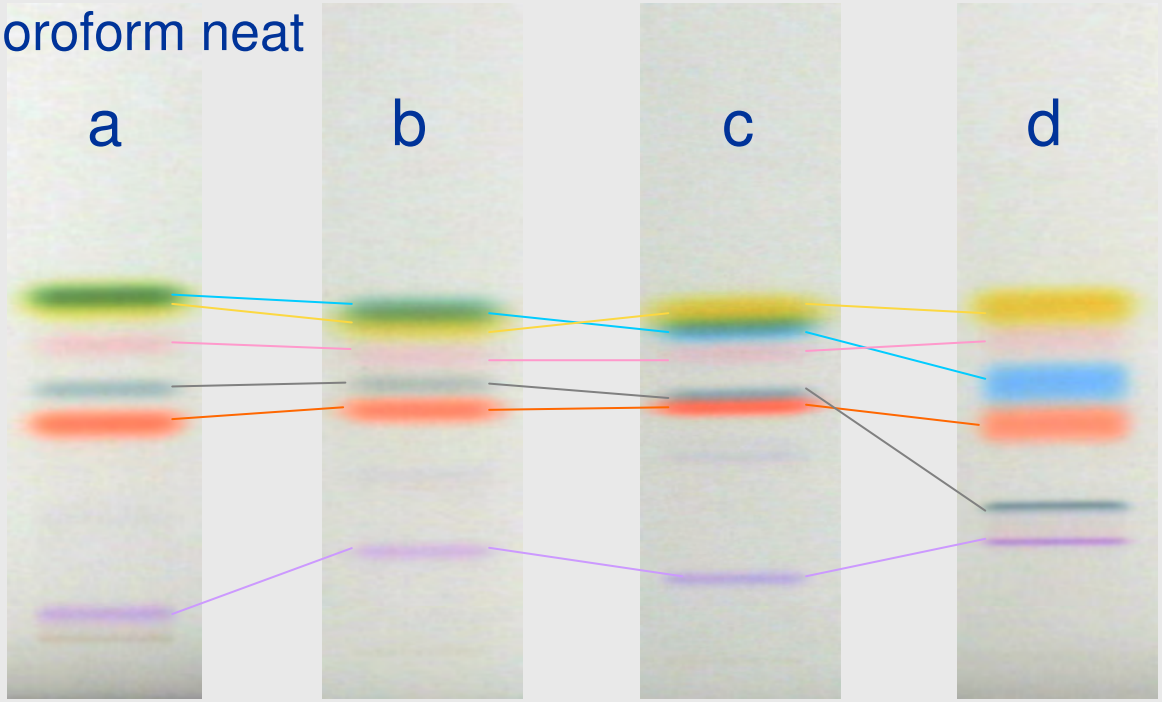
d) Chloroform neat



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The b-term: layer quality $(R_F N)^{1/2}$

- N is number of theoretical plates over entire separation distance
- Affects resolution only by square root
- Resolution is proportional to R_F
- Calls for high R_F

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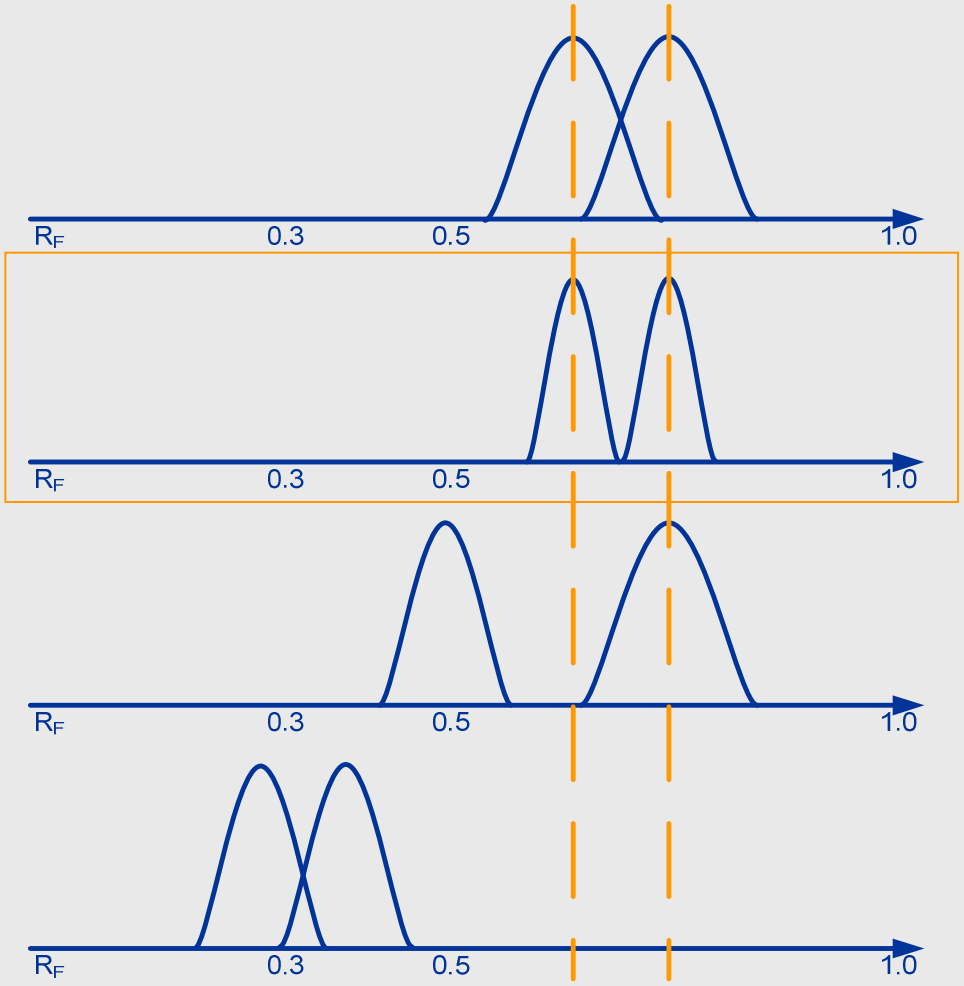
Effects of N, α, and Rf

- Increased N



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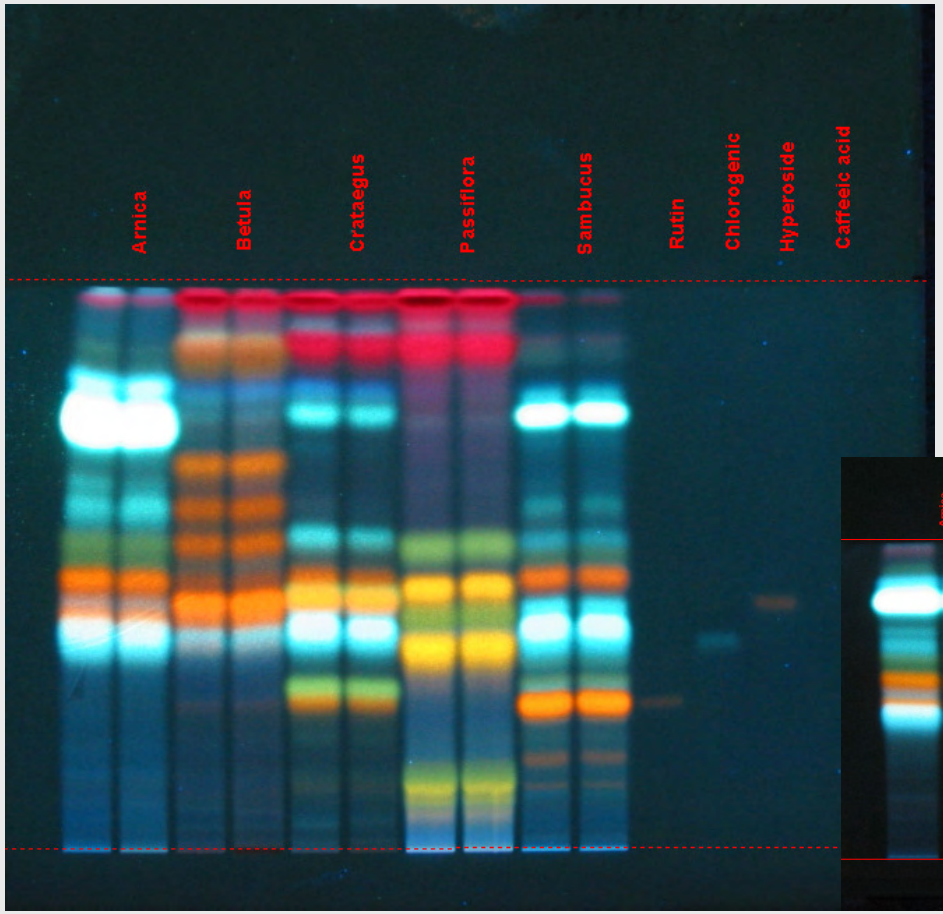
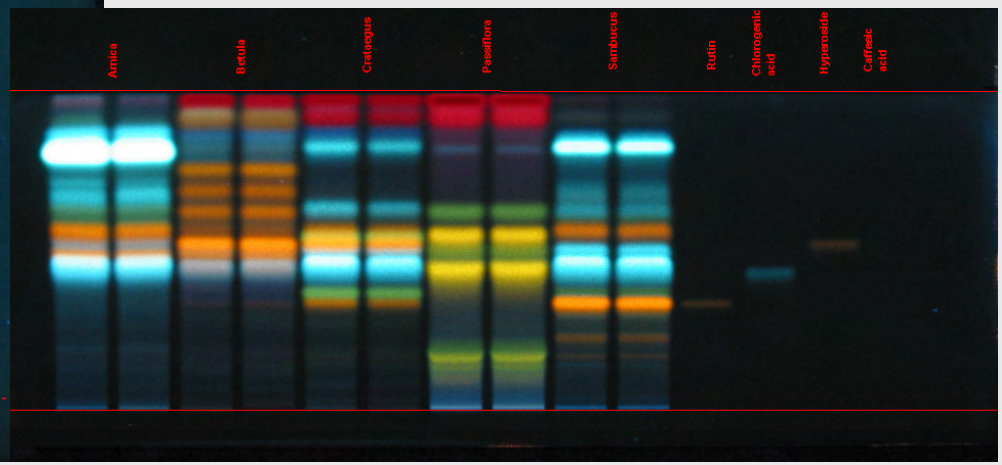
Comparison TLC-HPTLC

Flavonoids (Ph.Eur.5)



← TLC plate 20 x 20 cm

HPTLC plate 20 x 10 cm



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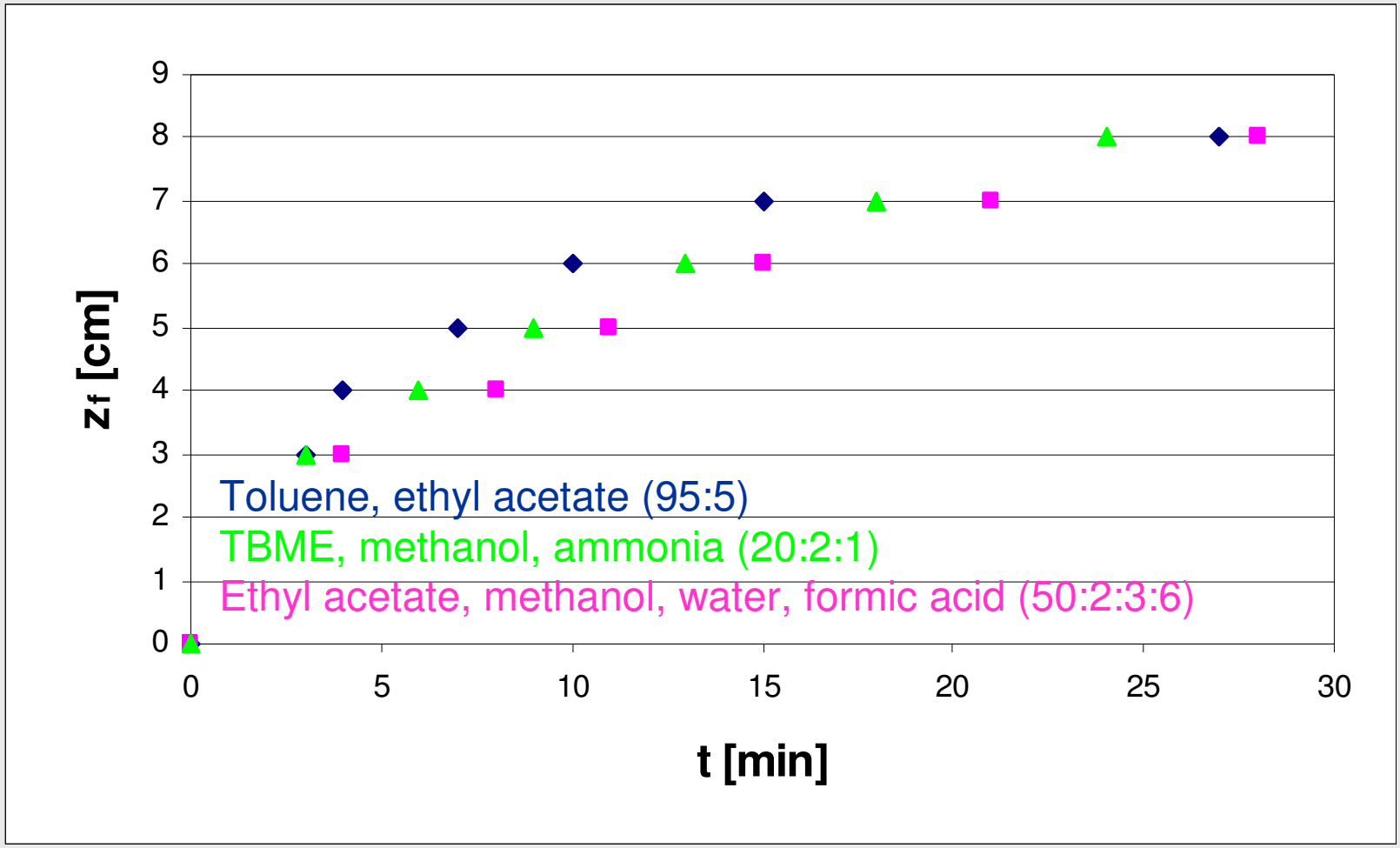
Mobile phase flow



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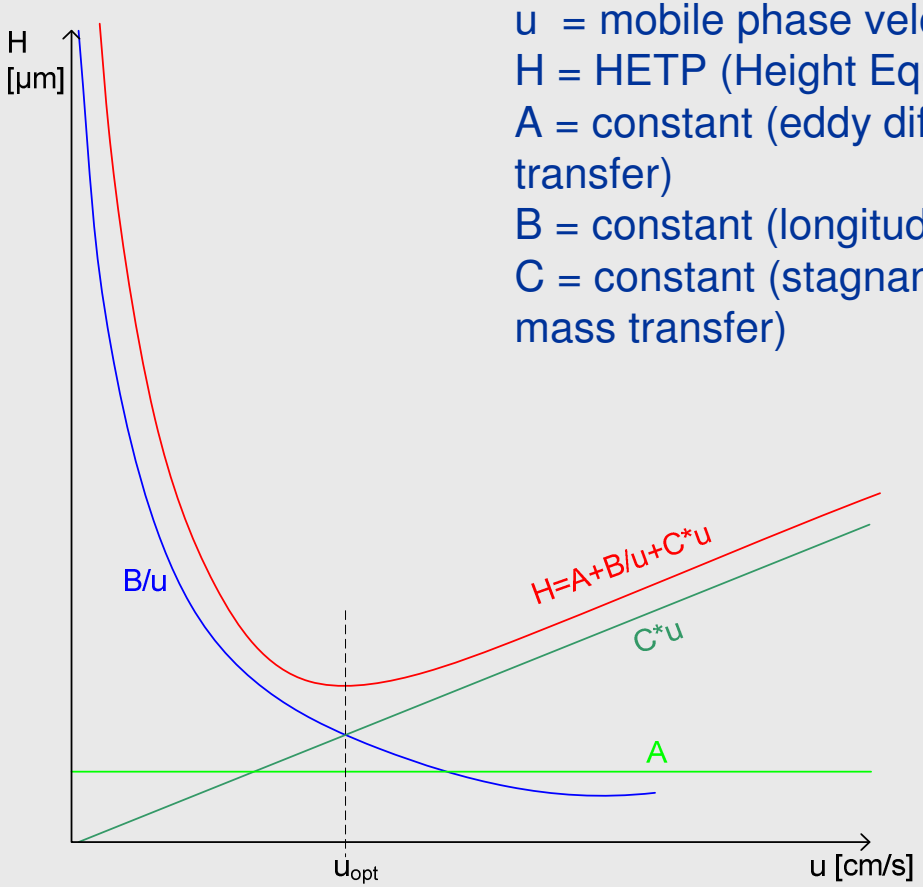
Van Deemter Equation



u = mobile phase velocity
H = HETP (Height Equivalent to a Theoretical Plate)
A = constant (eddy diffusion, mobile phase mass transfer)
B = constant (longitudinal diffusion)
C = constant (stagnant mobile phase, stationary phase mass transfer)

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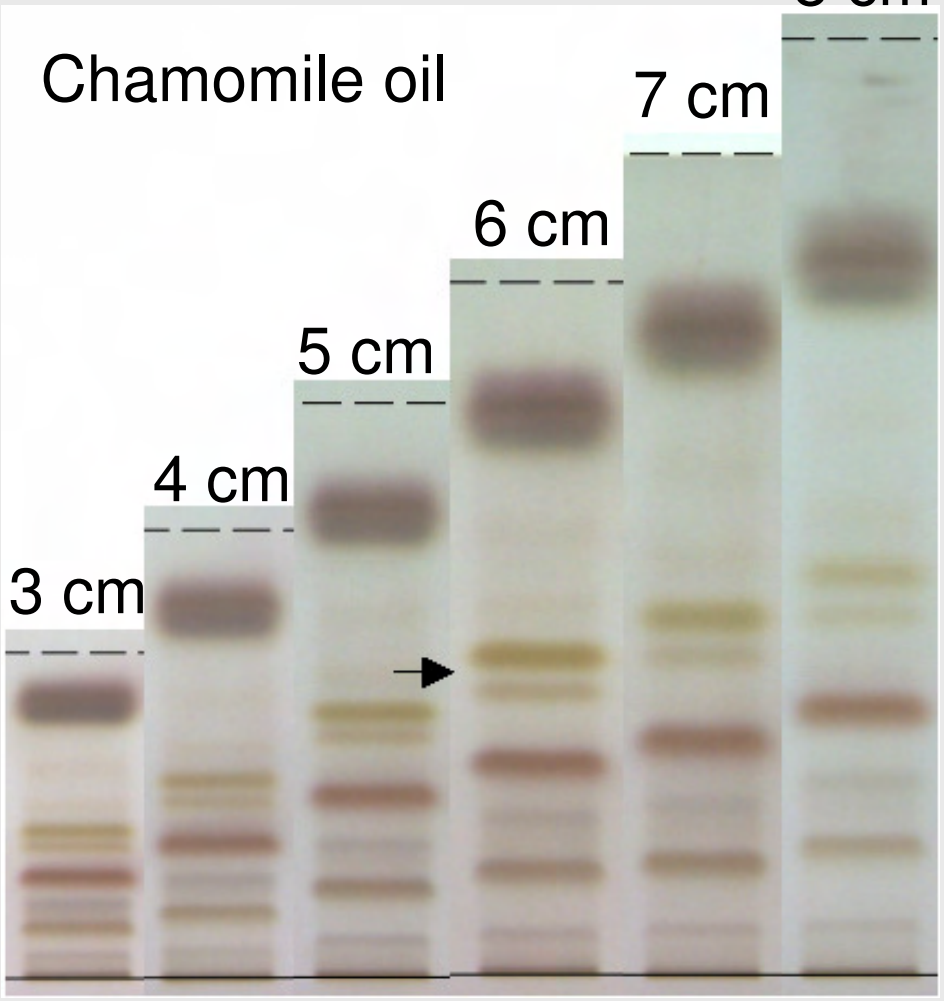
Developing distance



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Developing distance

- 5 - 7 cm for HPTLC (6 cm)
  - about 10 minutes
  - extension only if many components present
- 12 - 15 cm for TLC (12 cm)
  - about 30 minutes
  - extension does not improve result

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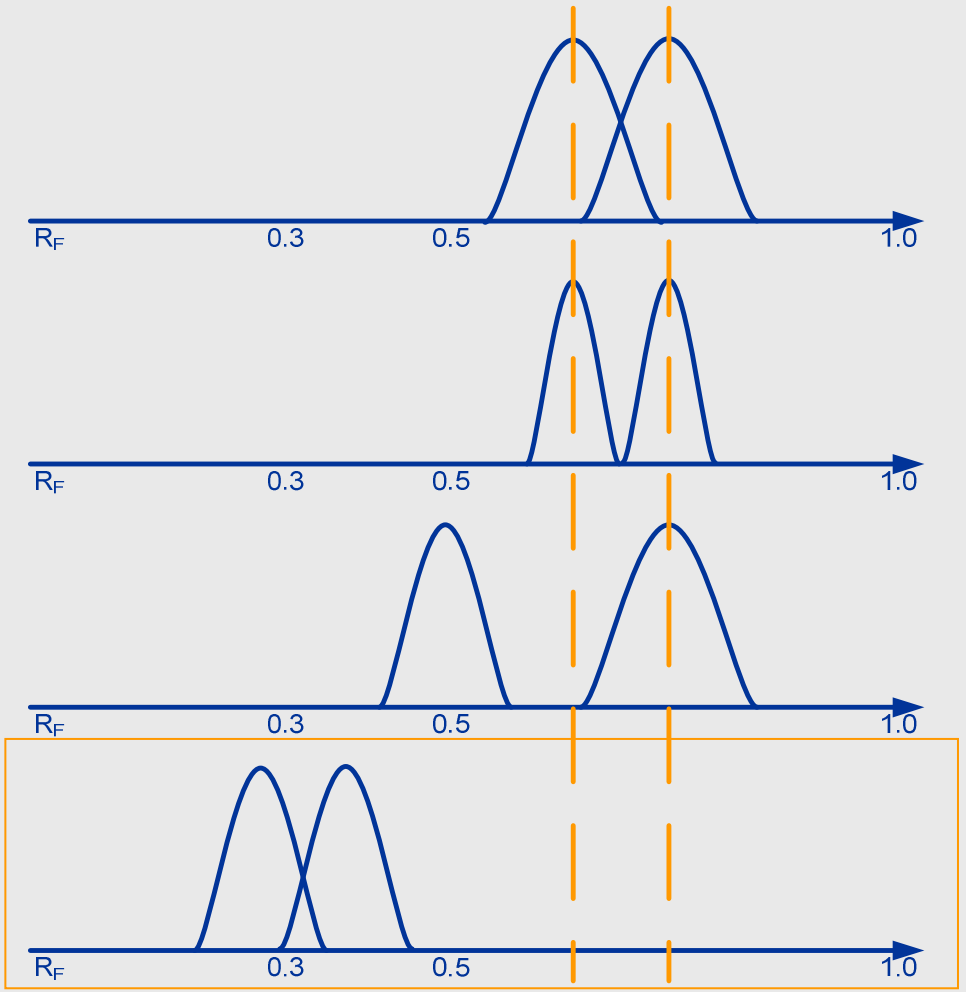
The c-term: **(1-R_F)**

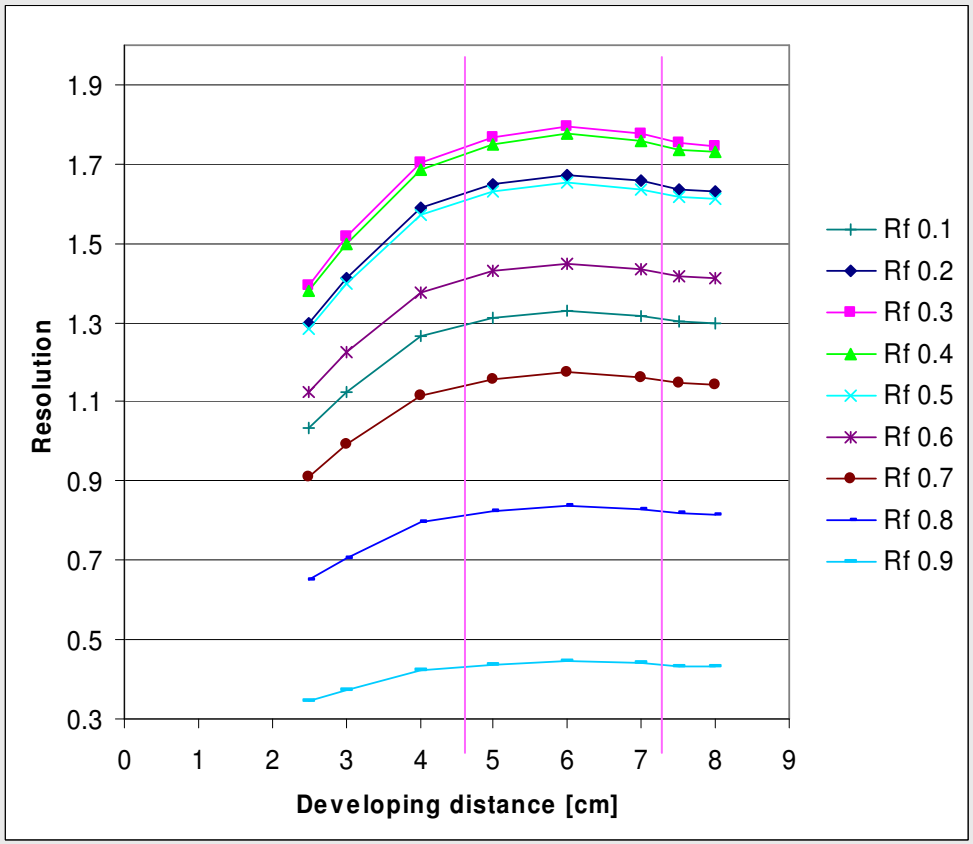
- Calls for low R_F
- Extreme R_F effect:
  - R_F =1 yields R_s=0
  - R_F =0 yields R_s=0
- b and c term:
  - R_F must be optimized
  - R_s maximum at R_F=0.3
- In N_s chamber R_F<0.75

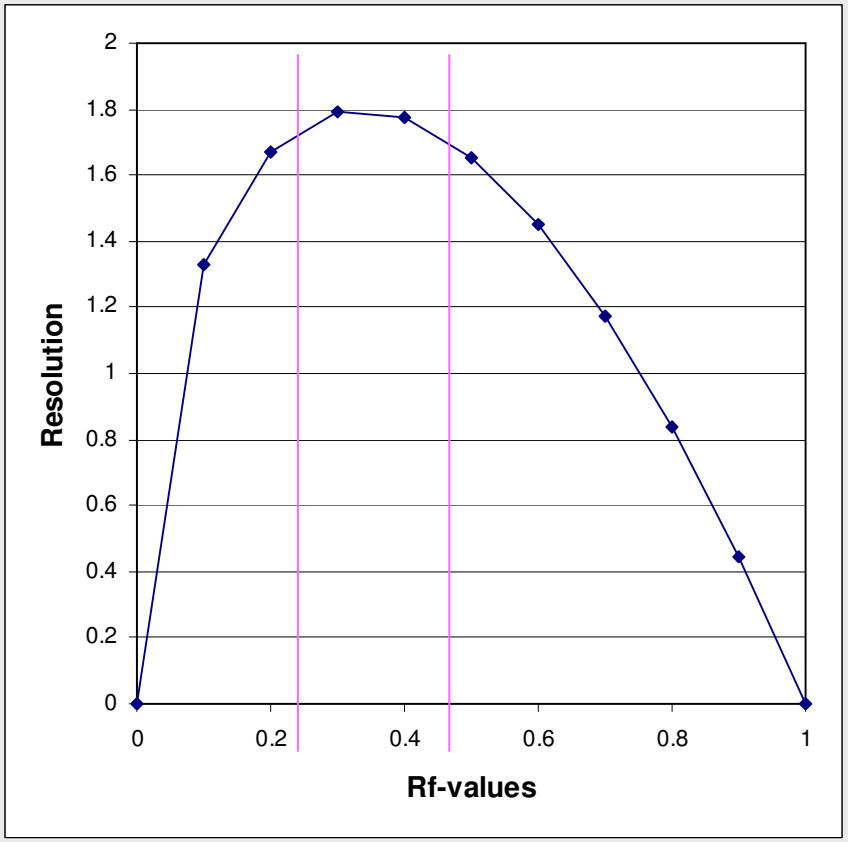
Effects of N, α, and Rf



- Position

Influence of the developing distance and R_f-values on the resolution





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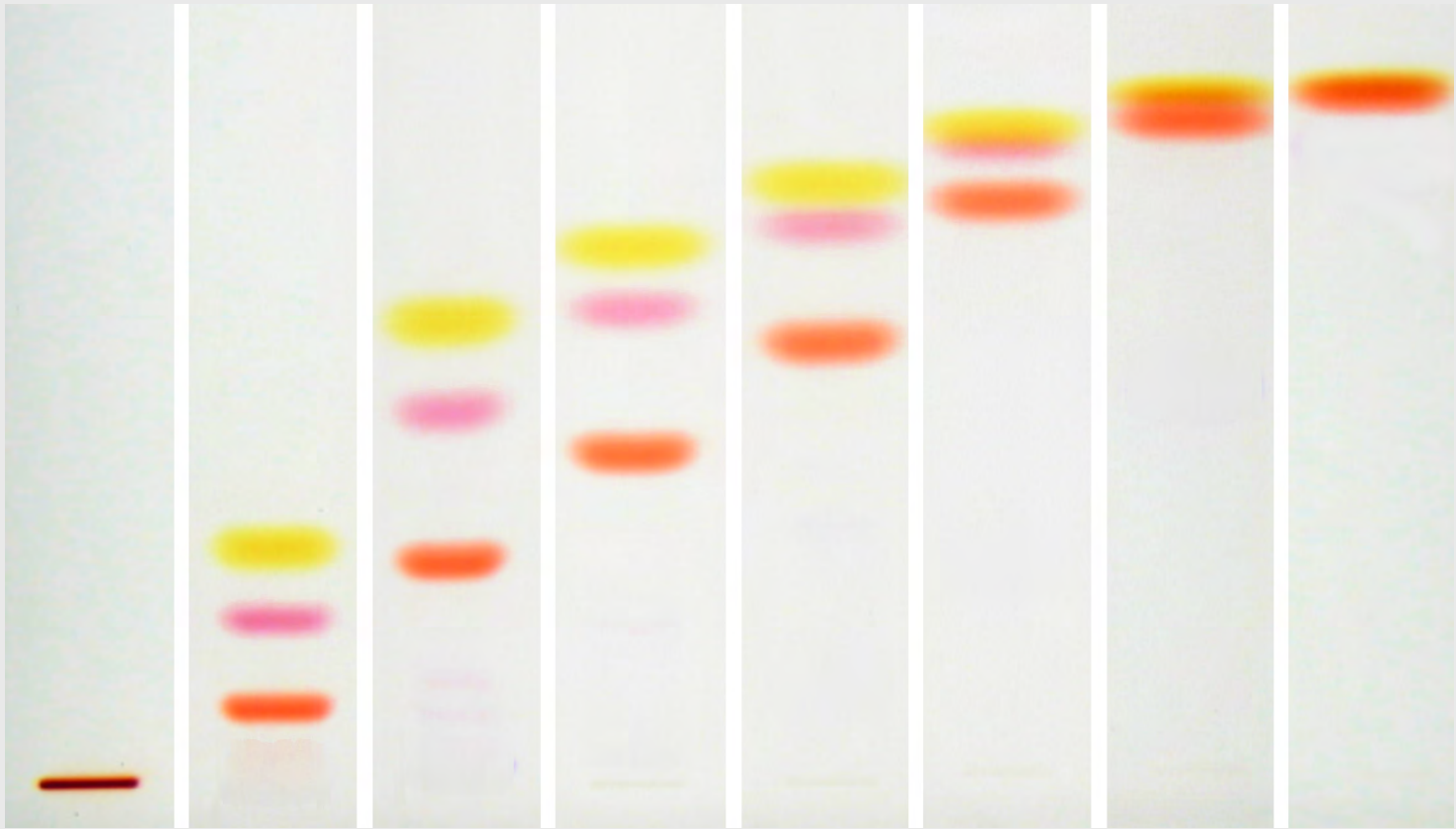
Solvent strength



Hexane	100	95	90	85	80	70	60	10
	0	5	10	15	20	30	40	90 Ethyl acetate

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The mobile phase

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The function of the mobile phase

- Dissolution of the sample
- Separation of sample and matrix
- Transport to optimal R_F-range
- Determination of selectivity of separation
- Affects plate height (HETP) through viscosity
- Not identical to developing solvent (!)

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Requirements for the developing solvent

- Simple composition
- Small polarity differences between components
- Non-toxic
- Defined “pure“ quality
- No reactivity with sample
- Low viscosity
- “Optimal“ volatility

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Classification of solvents

- Polarity
  - non polar — hexane
  - polar protic — methanol
  - polar aprotic — acetone
- Snyder: interaction with
  - H^+- donor — chloroform
  - H^+ - acceptor — 2-propanol
  - dipole — CH_2Cl_2
- Localized adsorption
  - weakly polar, non-localizing
    dichloroethane
  - polar, basic, localizing
    MTBE
  - polar, non-basic, localizing
    acetonitrile

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Solvent strength - ε^o

- Dimensionless number between -0.25 to 1.2
- Adsorption energy per area unit of solvent molecule on a given adsorbent
- Independent of adsorbent activity
- Pentane defined to be = 0.00

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Solvent strength (ε^o) on silica gel

Pentane / Hexane	0.00	Ethyl acetate	0.48
Cyclohexane	0.04	Acetone	0.50
CCl_4	0.11	Acetonitrile	0.60
Toluene	0.27	Dioxane	0.60
Isopropyl ether	0.28	Pyridine	0.70
Dichloromethane	0.30	Propanol	0.82
Chloroform	0.36	Methanol	0.95
Diethyl ether	0.43	Acetic acid	>>1

Geiss, Fundamentals of TLC, Huethig 1987

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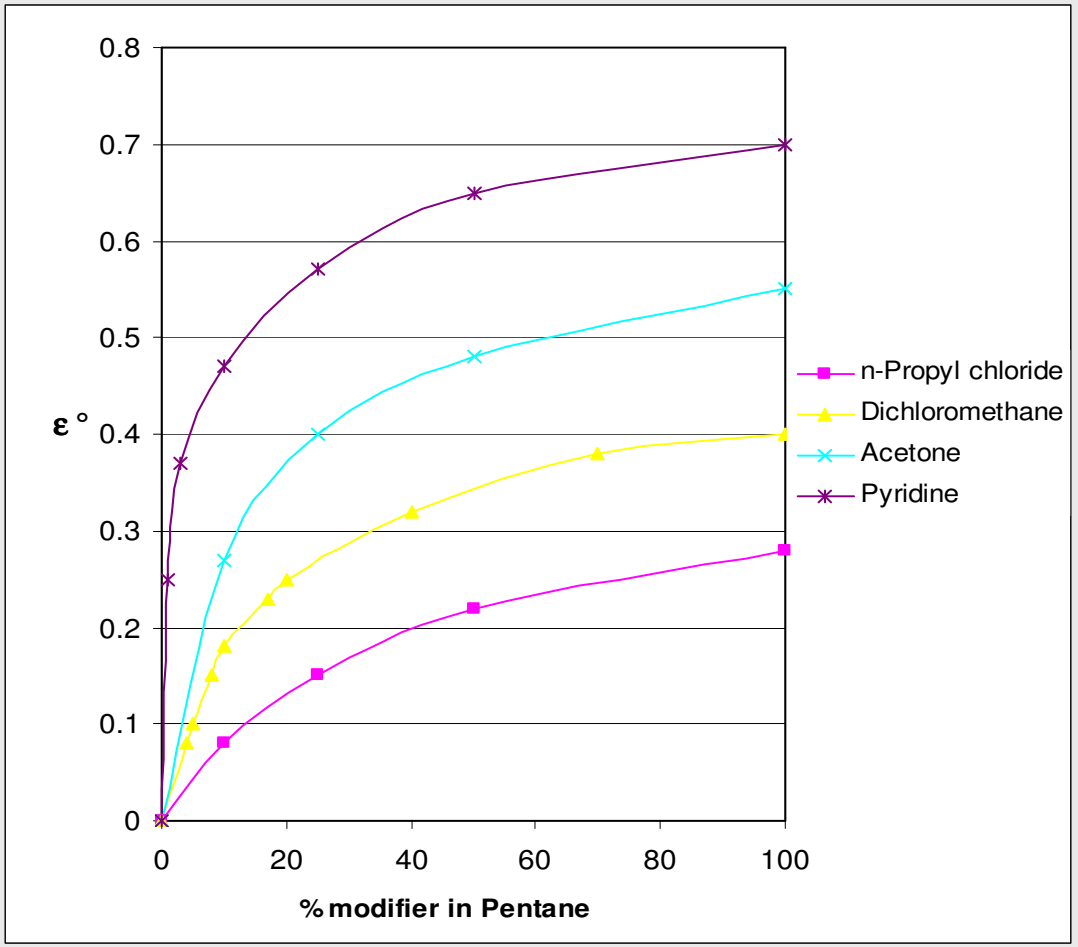
Solvent strength of binary mixtures

The solvent strength of mixtures is not additive!



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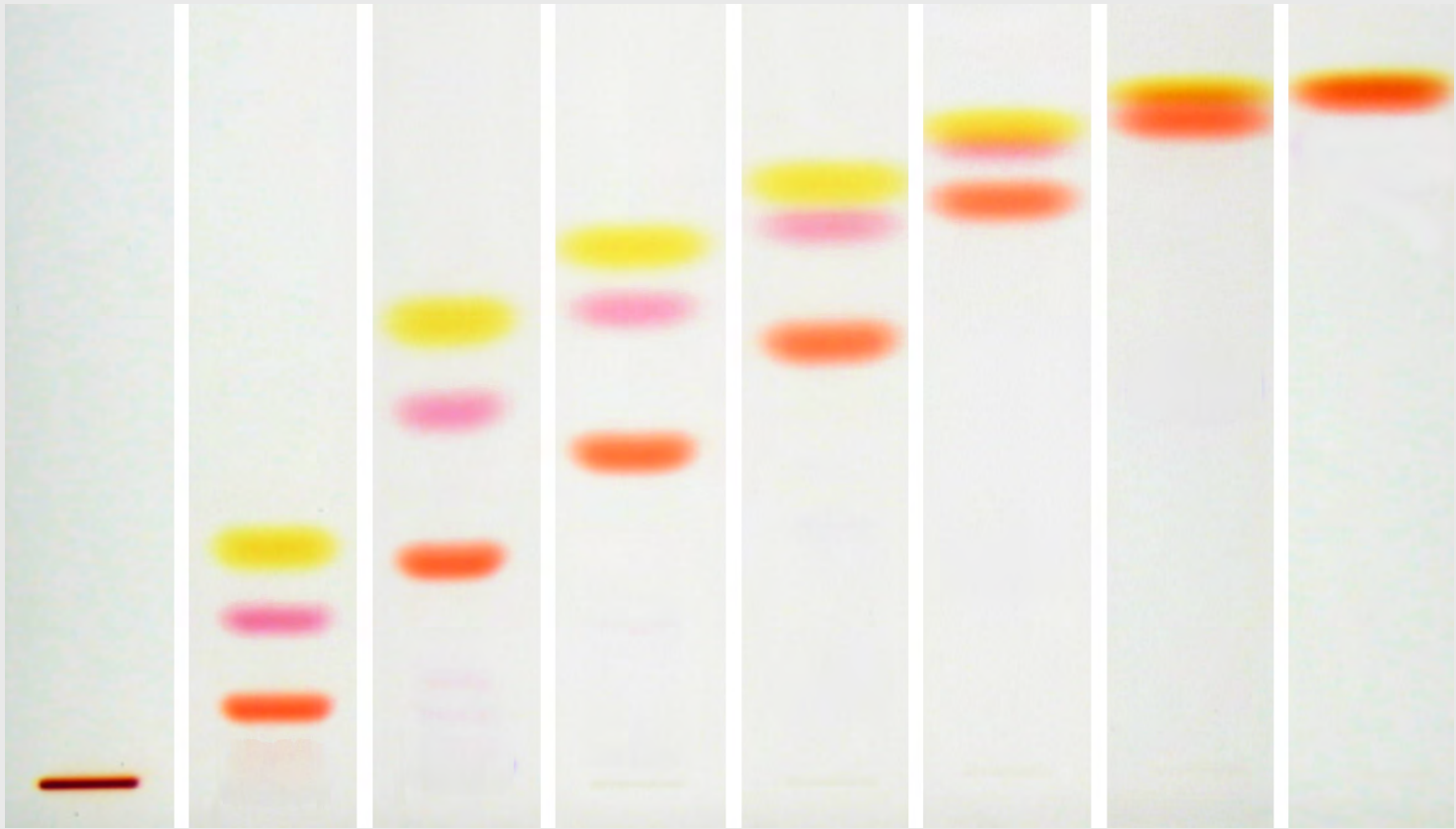
Solvent strength



Hexane	100	95	90	85	80	70	60	10
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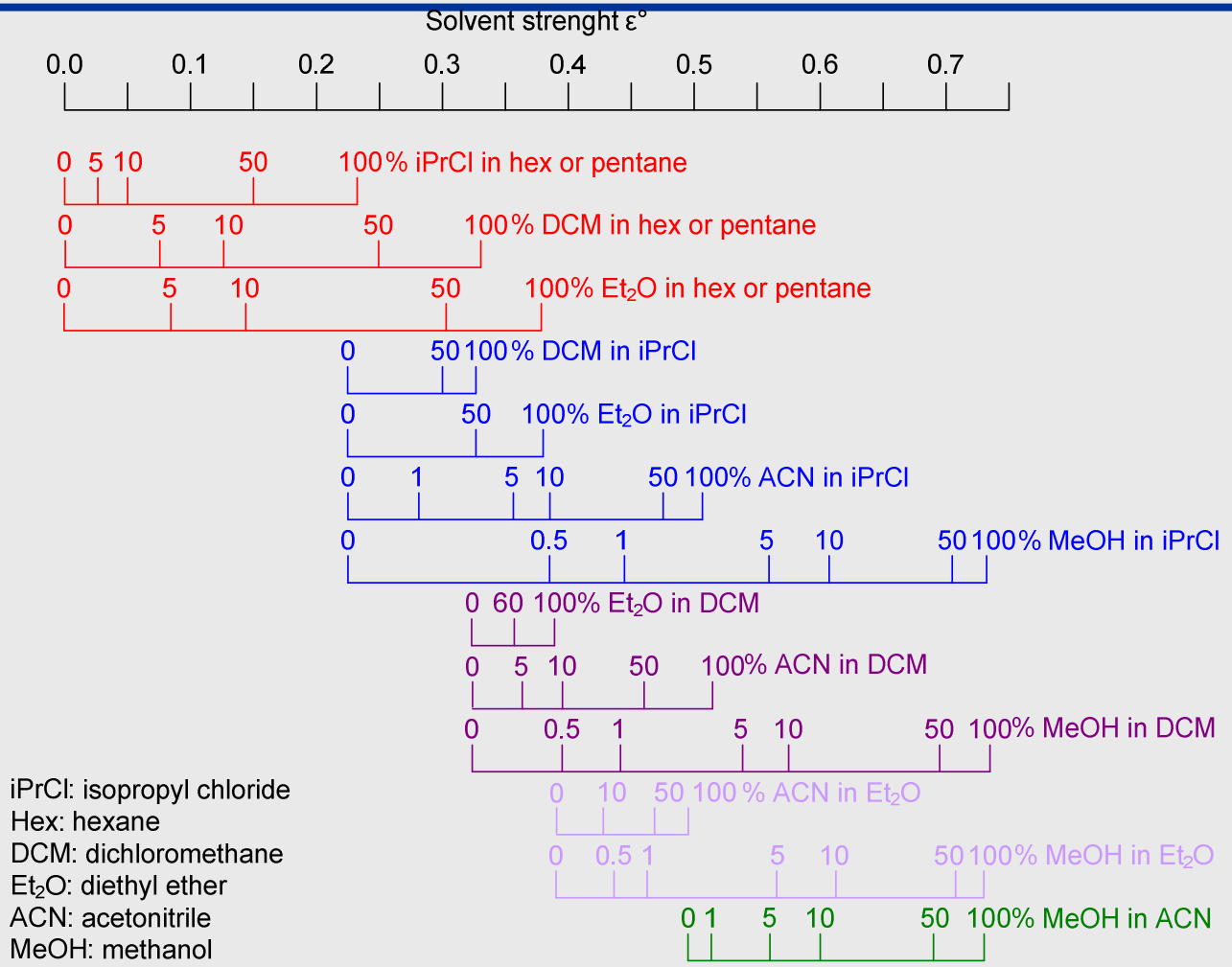
Solvent strength of mixtures



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Solvent strength and selectivity

Effect of selectivity at constant solvent strength

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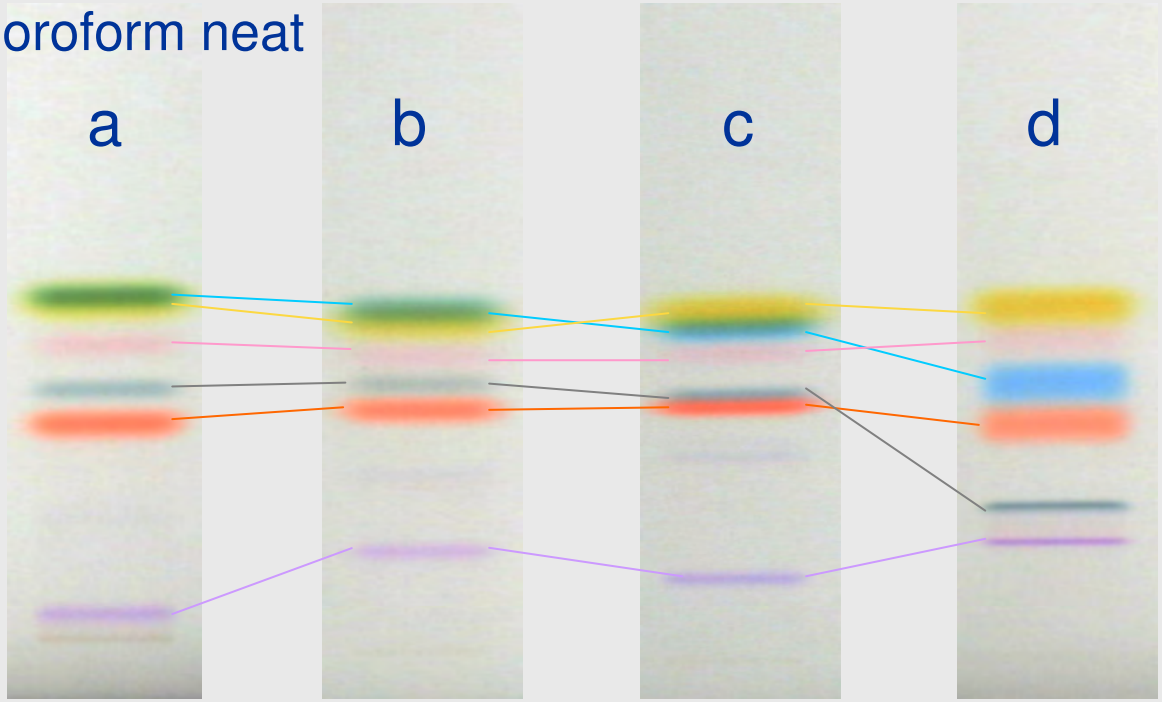

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Selectivity groups (Snyder)

I	Aliphatic ethers, trialkylamines, trialkylphosphates
II	Aliphatic alcohols
III	Pyridine, THF, DMSO, DMF, diethylene glycol
IV	Benzyl alcohol,ethylene glycol,acetic acid, formamide
V	Dichloromethane, 1,2-dichloroethane
VI	Ketones, esters, dioxane, nitriles
VII	Aromatic (halogenated) hydrocarbons,nitro comp.
VIII	Chloroform, water, fluoroalcohols, m- cresol

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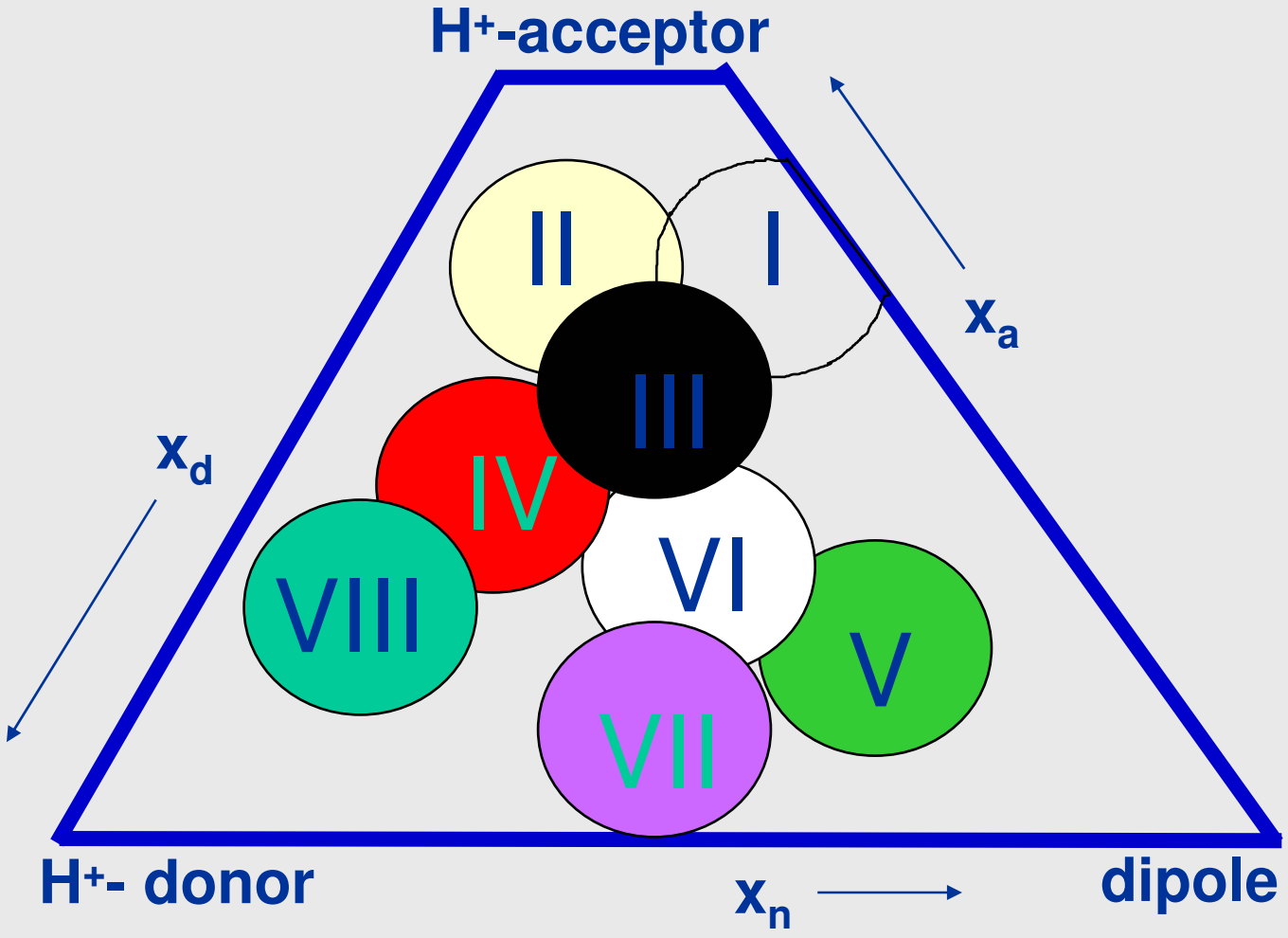
Selectivity triangle



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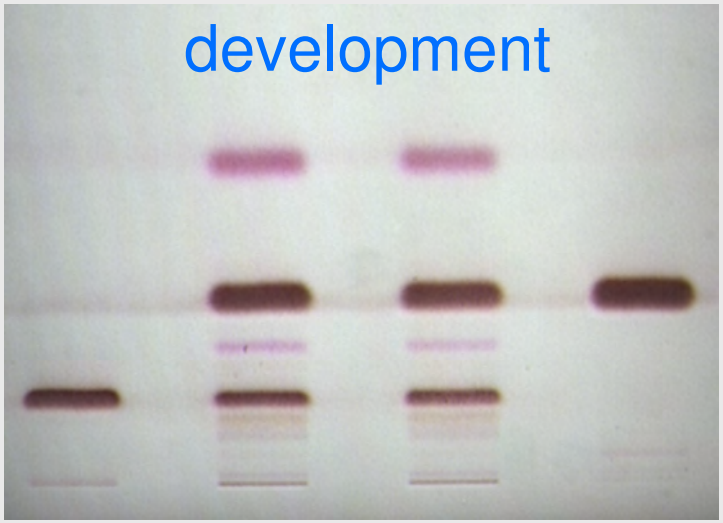
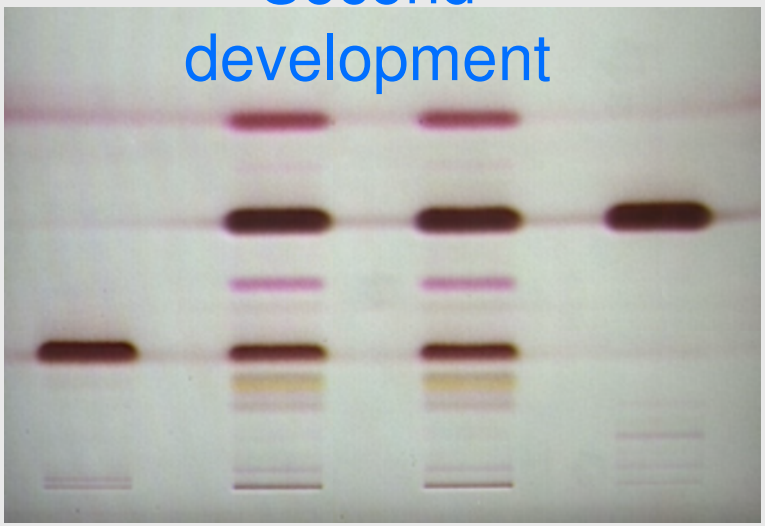
Multiple development (same mobile phase)

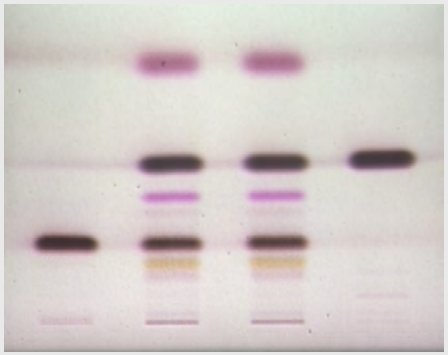
First development



TLC

Second development





Single development on a HPTLC plate

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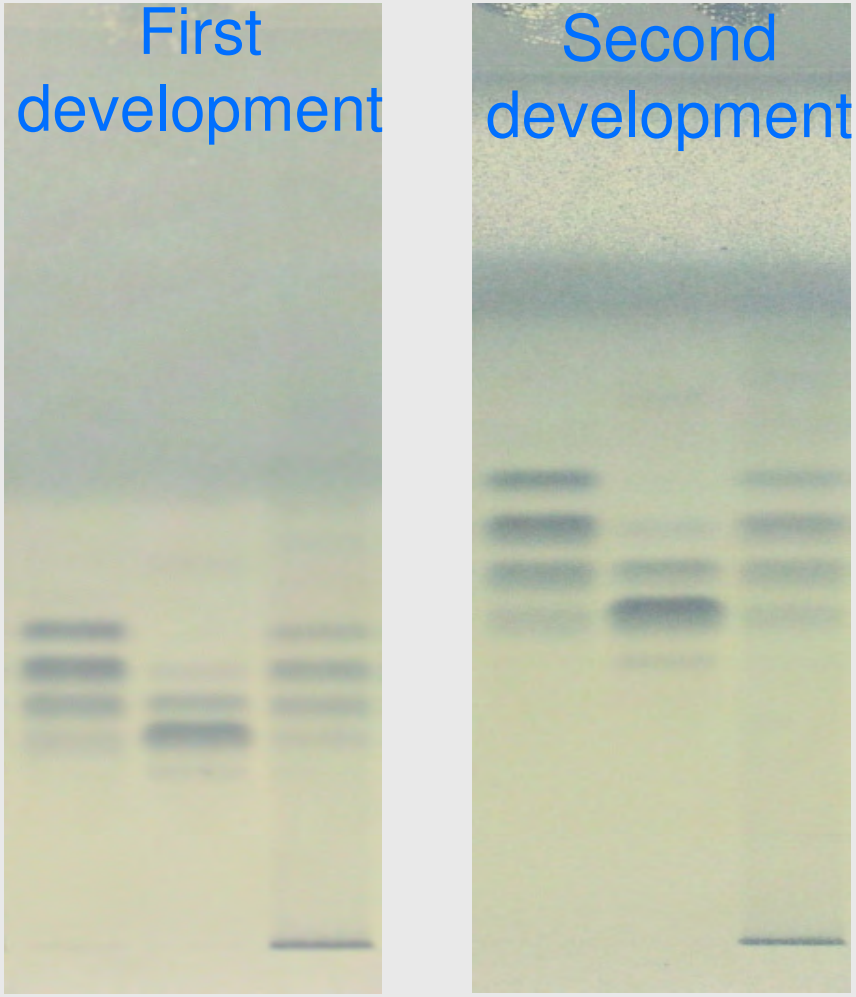
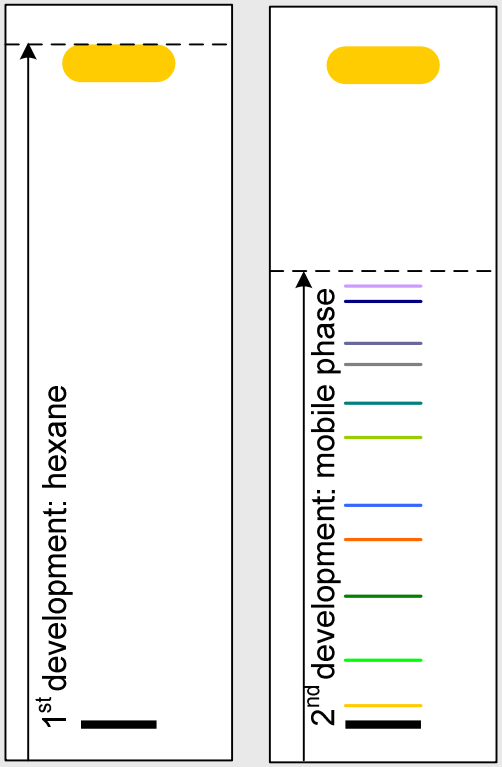
Multiple development (same mobile phase)

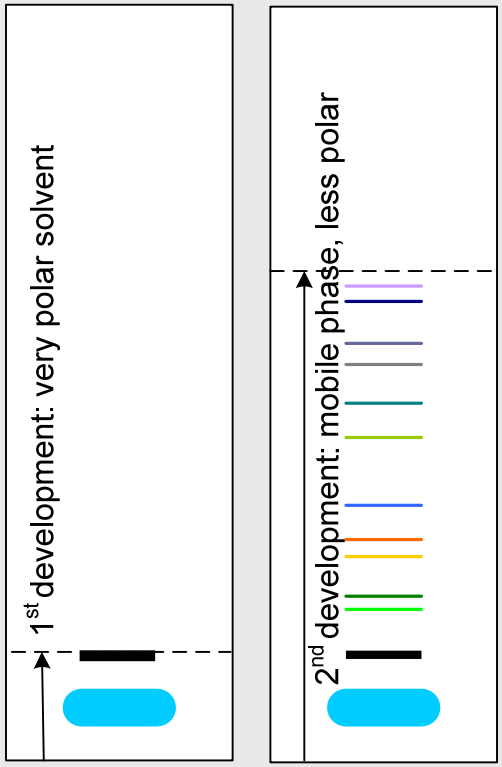


Multiple development (different mobile phase)

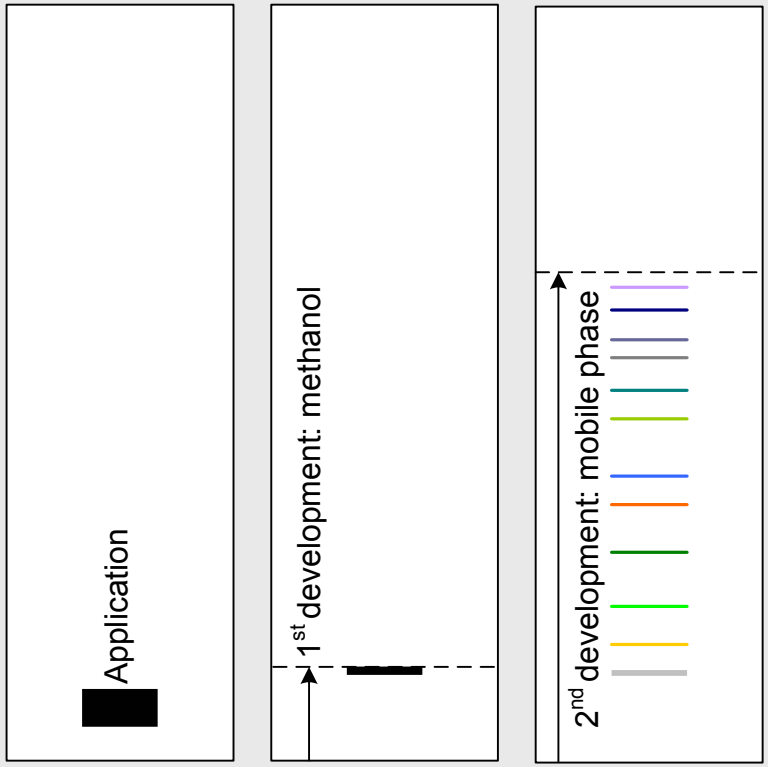
Removal of non-polar matrix



Removal of polar matrix



Focusing of zones



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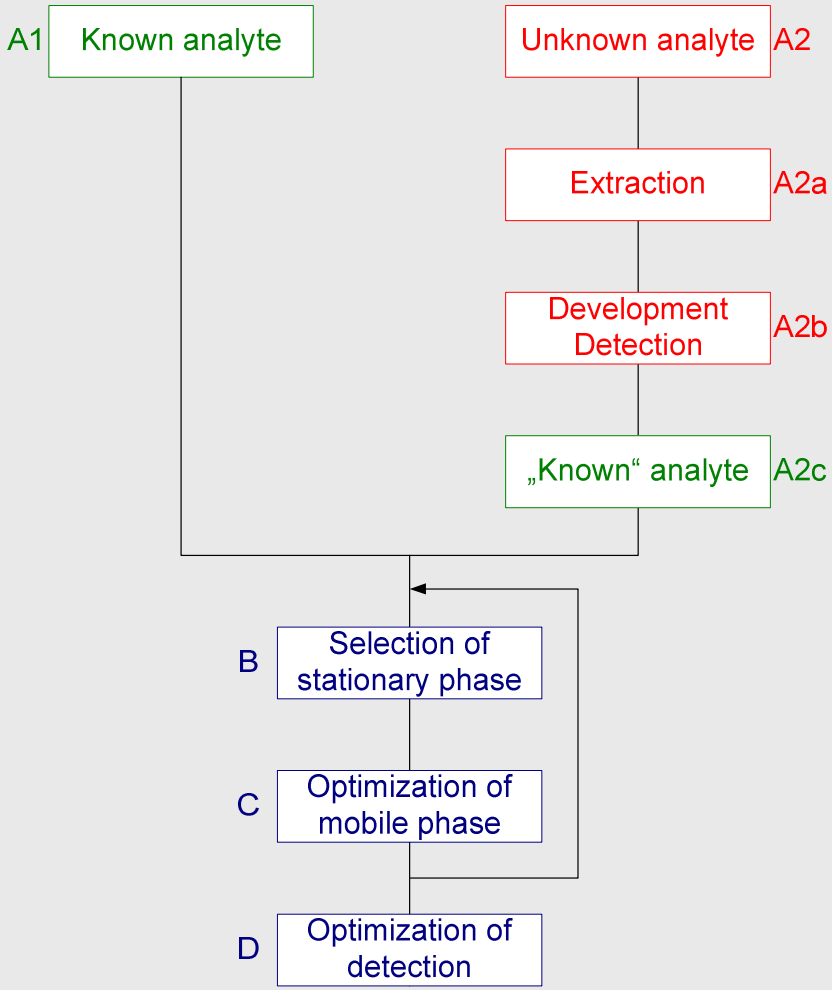
Method development



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Screening solvents for extraction

Neutral	Acidic	Basic
Heptane	Methanol - acetic acid (9:1)	Methanol - ammonia 25% (8:2)
Toluene	Water - acetic acid (9:1)	Water - ammonia 25% (8:2)
MTBE		
DCM		
Chloroform		
Acetone		
Ethanol		
Methanol		
Ethanol - water (7:3)		
Methanol - water (8:2)		
Water		

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Screening systems

1) Toluene, ethyl acetate (95:5) / sulfuric acid (anisaldehyde)

→ non-polar compounds and essential oils

2) Chloroform, methanol, water (70:30:4) / sulfuric acid (anisaldehyde)

→ saponins and lignans

3) Ethyl acetate, acetic acid, formic acid, water (100:11:11:27) / NP(PEG)

→ flavonoids

4) Acetonitrile, water, formic acid (30:8:2) / ninhydrin → amino acids

5) 1-Butanol, acetic acid, water (7:1:2) / sulfuric acid (anisaldehyde)

→polar compounds

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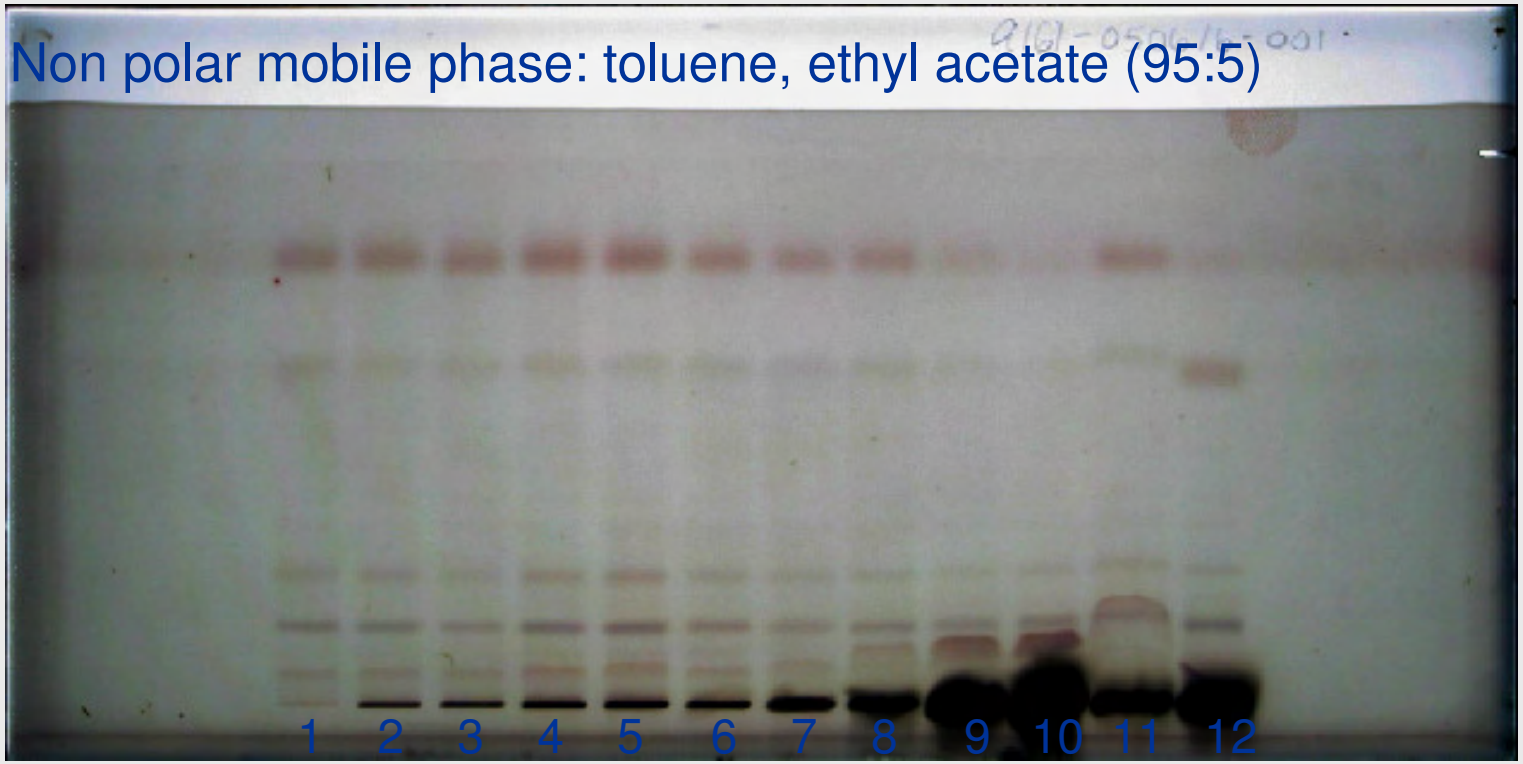
Extraction of an unknown sample



Extraction solvent: 1: heptane; 2: toluene; 3: MTBE; 4: DCM; 5: chloroform; 6: acetone; 7: ethanol; 8: methanol; 9: ethanol-water(7:3); 10: methanol-water (8:2); 11: methanol-acetic acid (9:1); 12: methanol-ammonia 25% (8:2).

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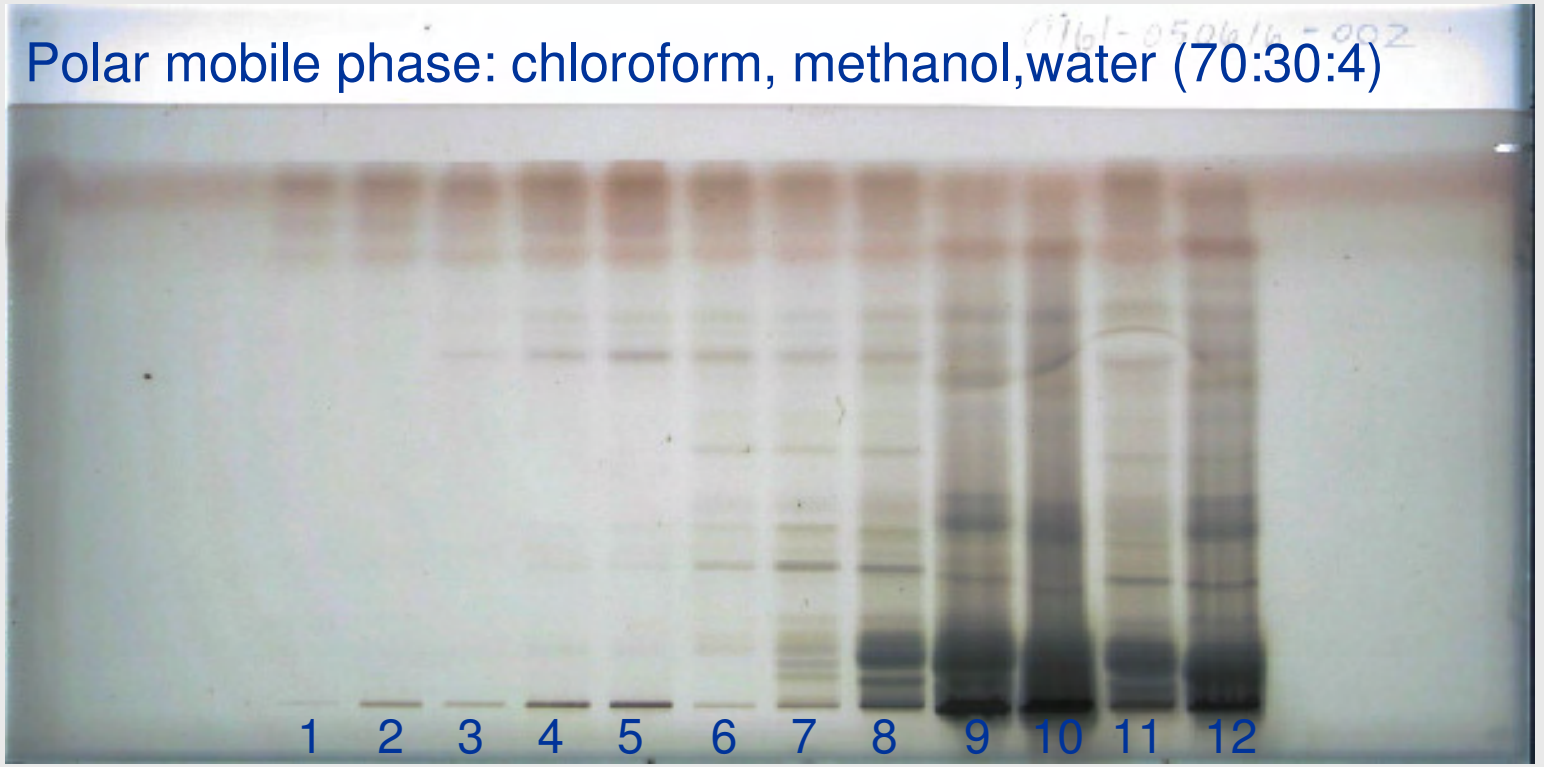
Extraction of an unknown sample



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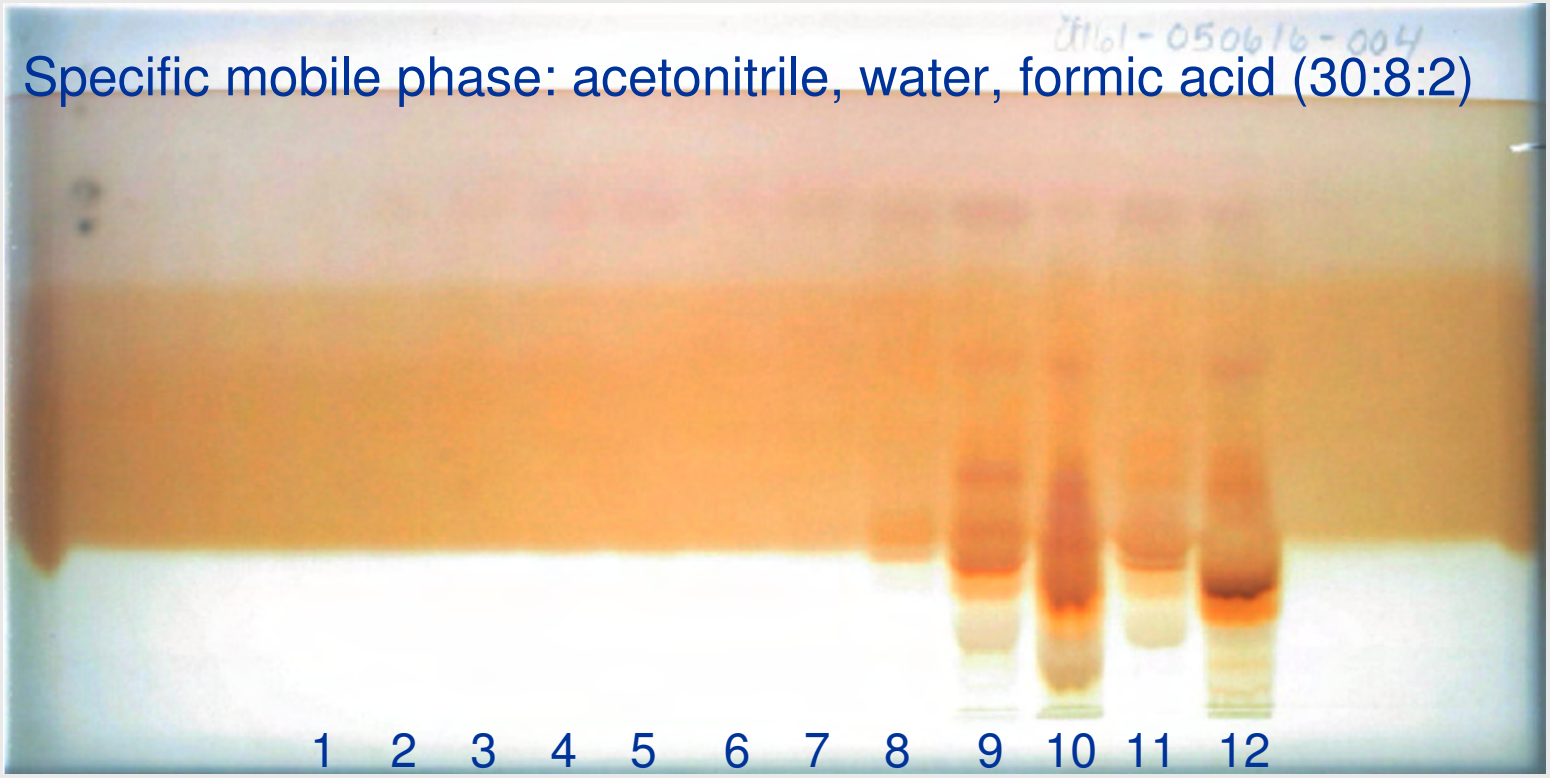
Extraction of an unknown sample



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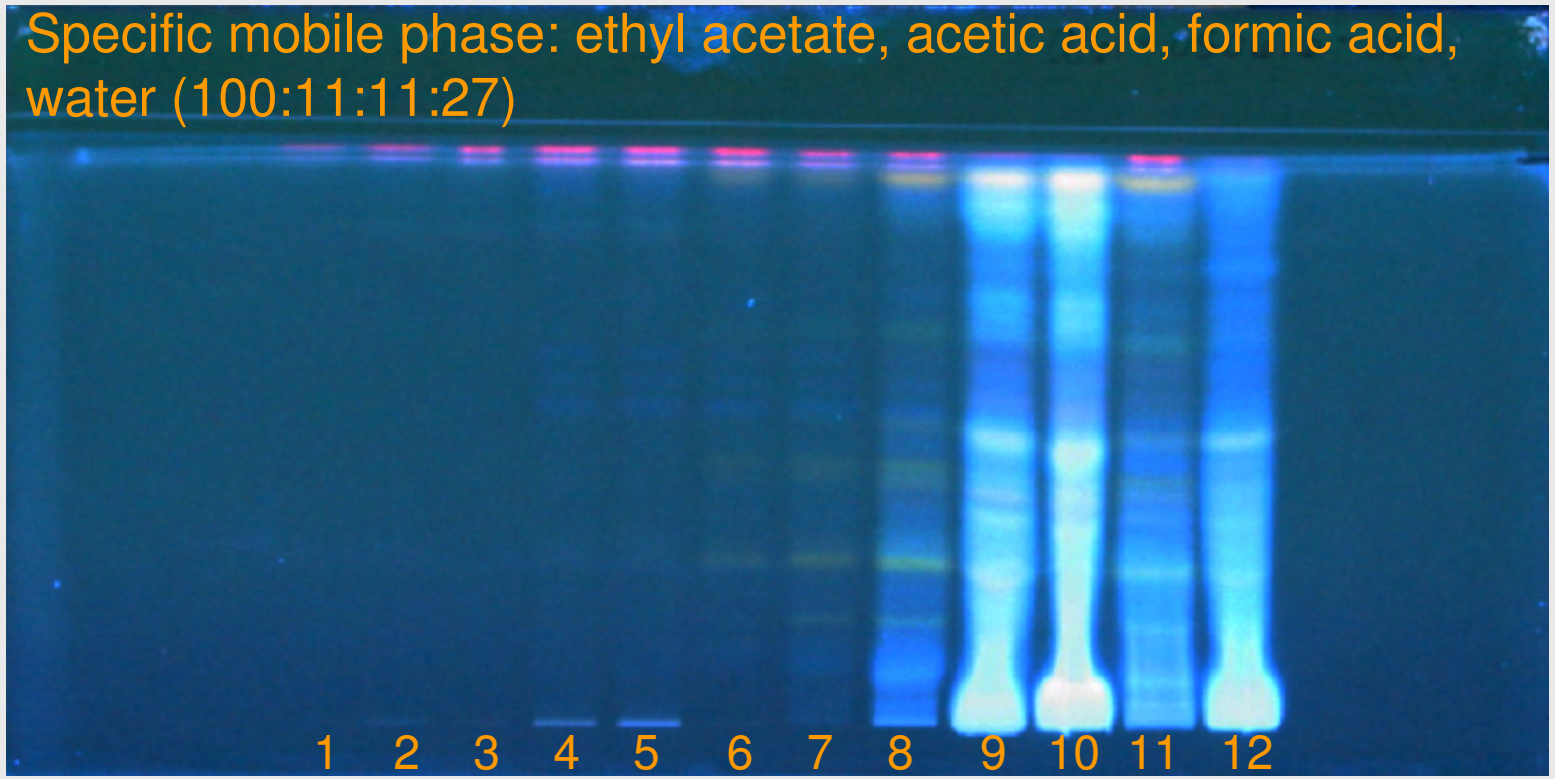
Extraction of an unknown sample



Extraction solvent: 1: heptane; 2: toluene; 3: MTBE; 4: DCM; 5: chloroform; 6: acetone; 7: ethanol; 8: methanol; 9: ethanol-water(7:3); 10: methanol-water (8:2); 11: methanol-acetic acid (9:1); 12: methanol-ammonia 25% (8:2).

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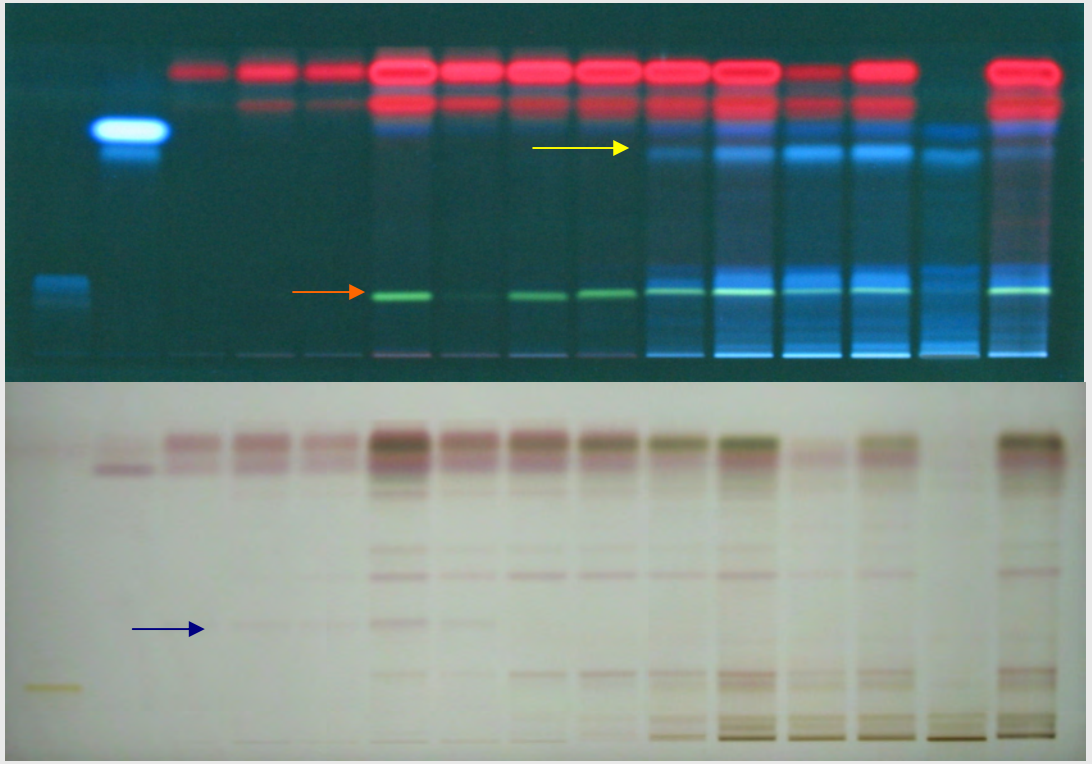
Extraction: Stinging Nettle



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

1, 2: standards; 3-14 extracts of Nettle obtained with: 3:hexane, 4: toluene, 5: MTBE, 6: DCM, 7: ethyl acetate, 8: isopropanol, 9: ethanol, 10: methanol, 11: methanol-water (8:2), 12: ethanol-water (7:3), 13: ethanol-water (1:1), 14: water; 15 extract of Nettle obtained by reflux with methanol)

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Practical approaches

- Standard systems
- Spot test
- 4-solvent-approach
- Prisma-model
- CAMAG - Optimization scheme

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Standard systems (adsorption)

- Chloroform - methanol
- Chloroform - acetone
- Dichloromethane - methanol
- Ether - toluene
- Ether - hexane - acetic acid
- Ethyl acetate - methanol
- Ethyl acetate - toluene

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Standard systems (partition)

- Chloroform - methanol - water
- Chloroform - methanol - water - ammonia
- Chloroform - methanol - water - acetic acid
- Butanol - acetic acid - water
- Butanol - pyridine - water
- Ethyl acetate - formic acid - water

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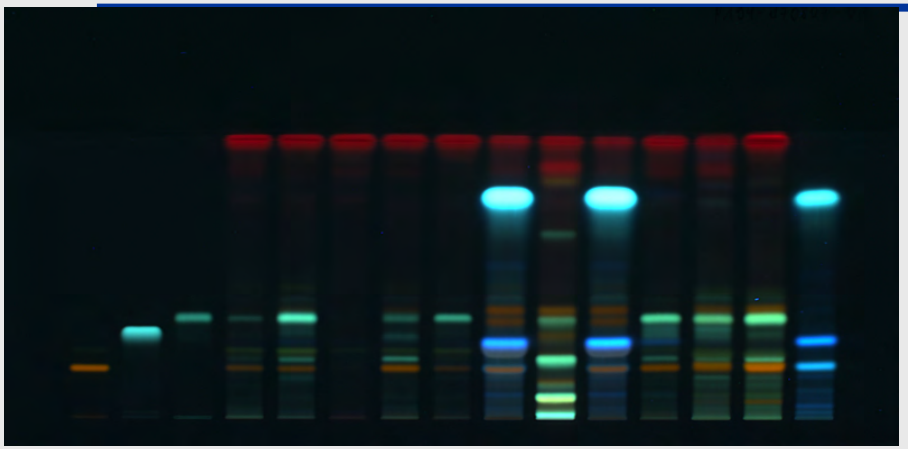
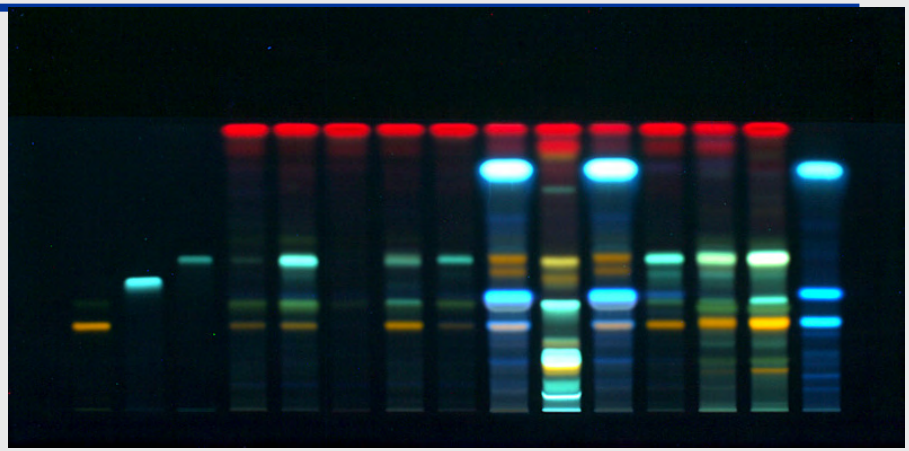
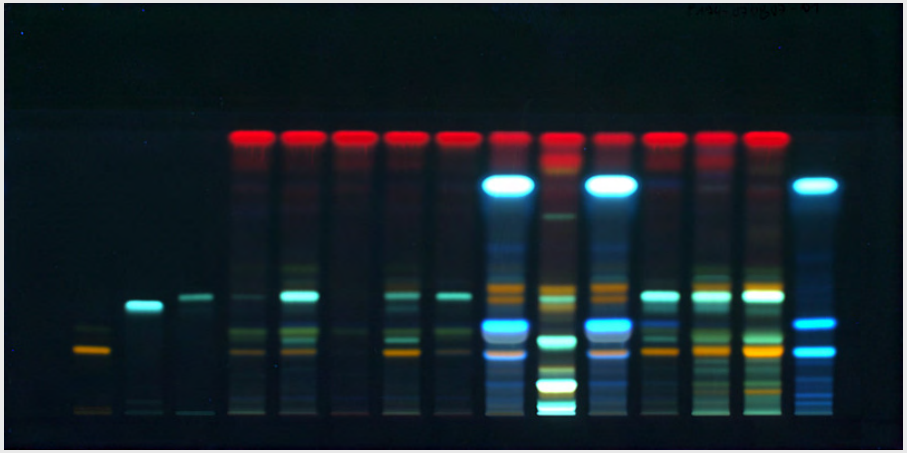
Standard systems: example flavonoids



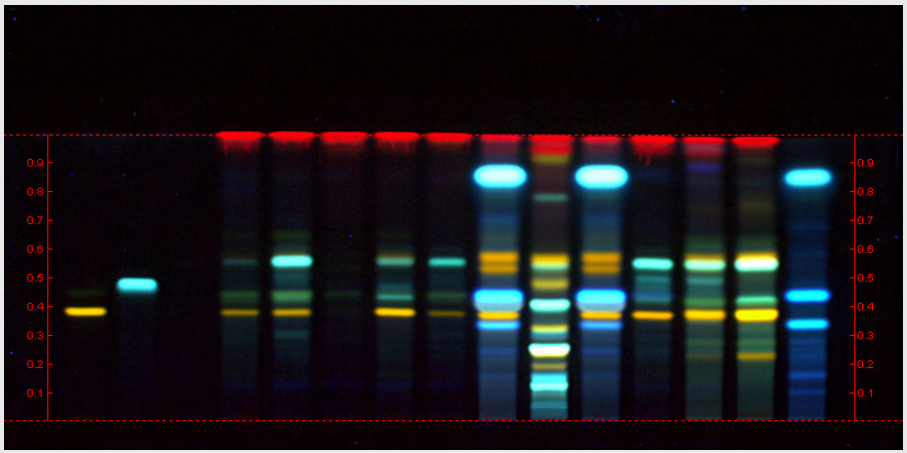
ethyl acetate, formic acid, acetic acid, water (15/1/1/2)



ethyl acetate, formic acid, water, MEK (50/10/10/30)



ethyl acetate, formic acid, water (80/10/10)



ethyl acetate, formic acid, acetic acid, water (100/11/11/26)

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Spot test: trial and error

- Dissolve sample in least polar solvent
- Apply several spots (autosampler)
- Apply ~ 20 µL of various solvents onto center of spots
- Evaluate circular chromatograms
- Try solvent mixtures

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Spot test: advantages and disadvantages

- Rapid and fully automated
- Number and type of solvents is flexible
- Information about
  - solvent strength
  - suitable selectivity

- Miniaturized ring chromatogram
- Unsaturated, open system
- Not systematic

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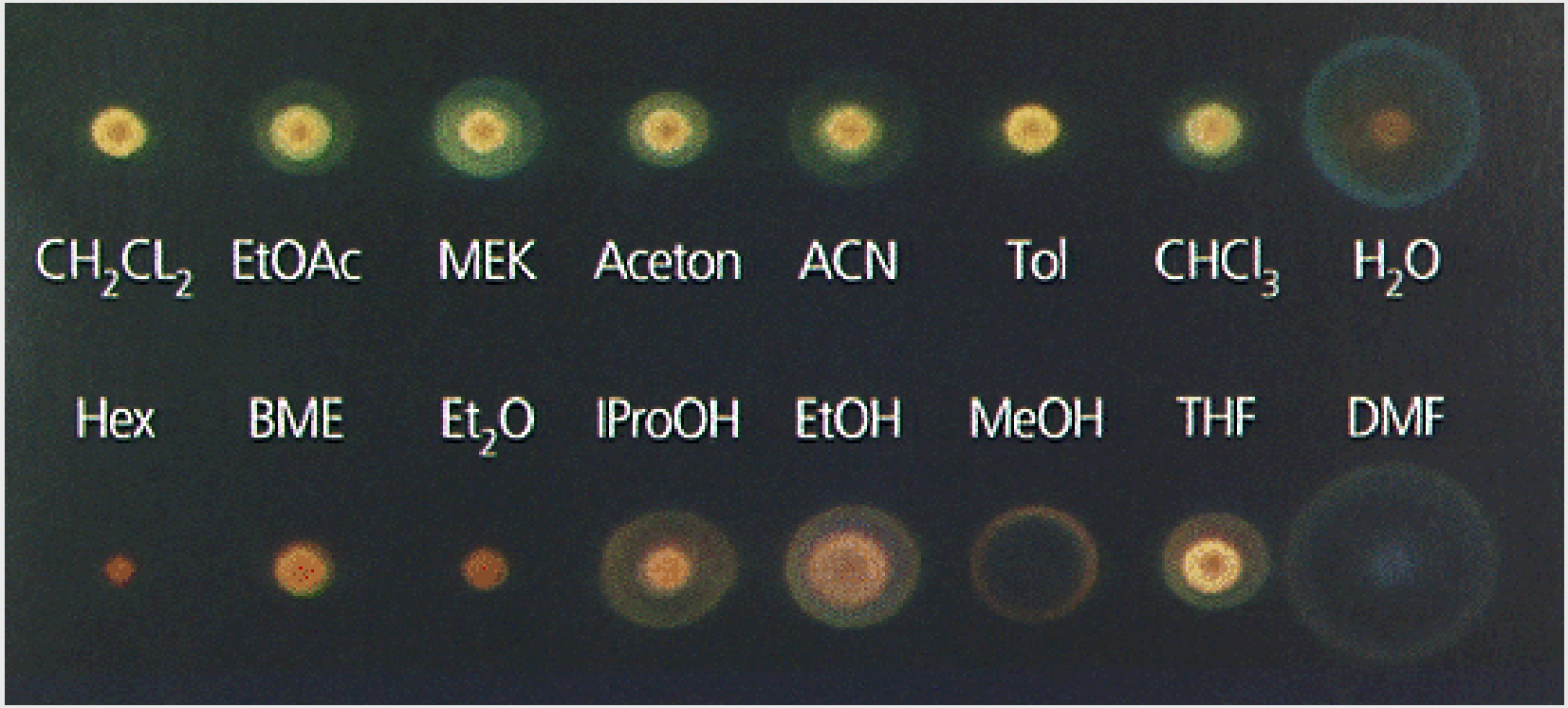
Spot test: example



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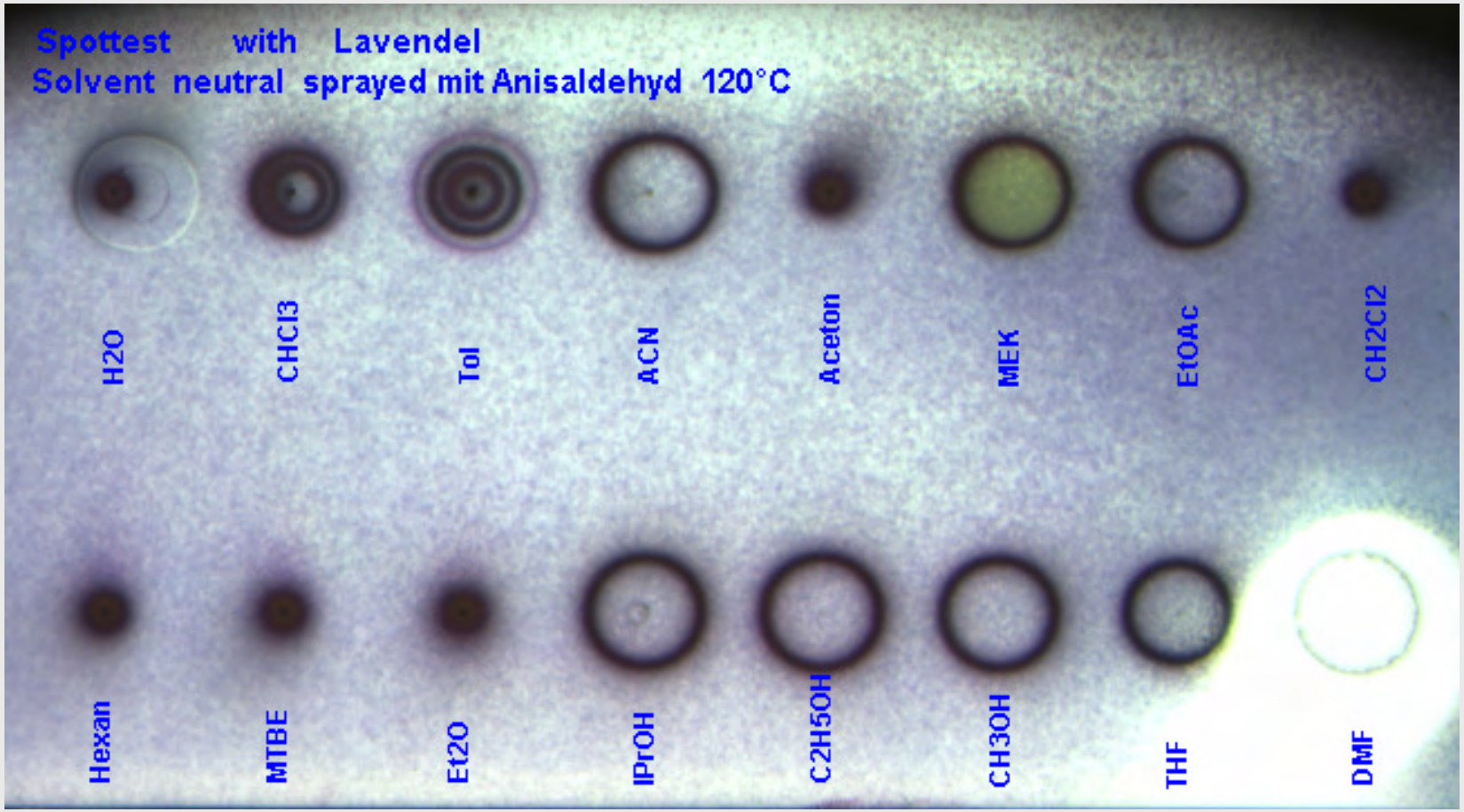
Spot test: example Lavender oil



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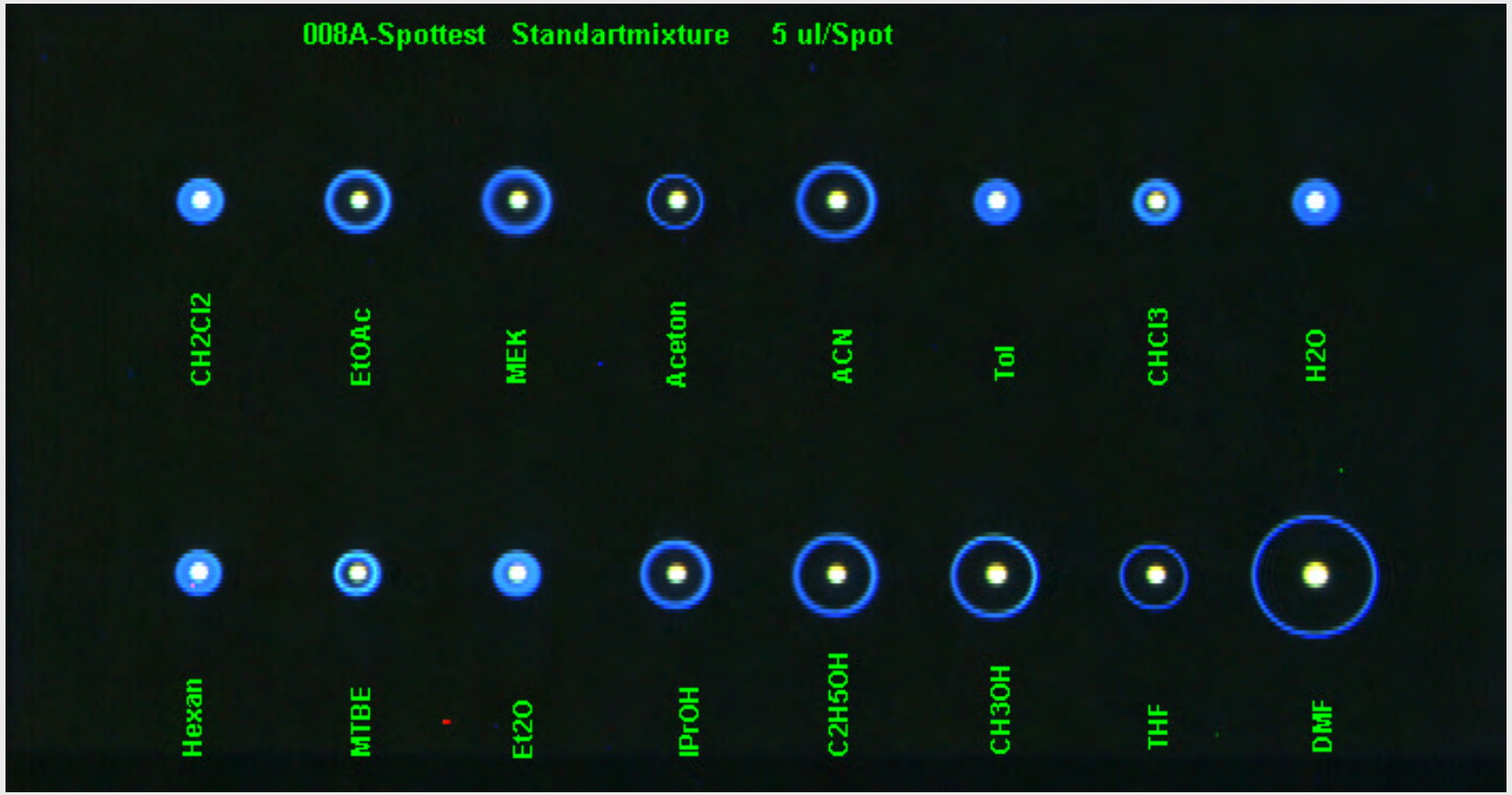
Spot test: example *Hydrastis 1*



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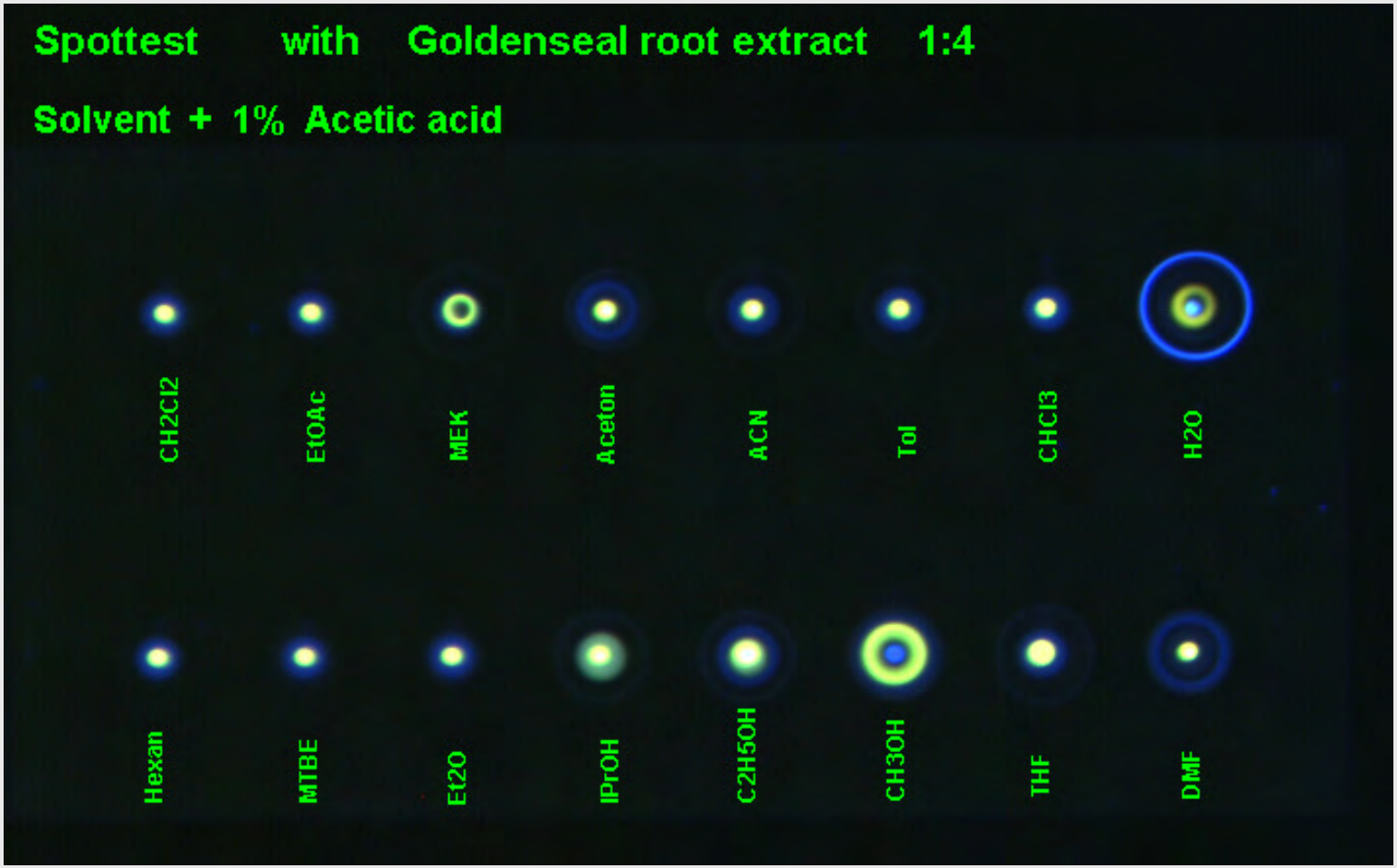
Spot test: example *Hydrastis* 2



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4 solvent approach: step 1

Heptane /	DCE /	ACN /	MTBE
neutral	non-localizing	localizing dipolar	localizing basic

Find solvent strength

ε^o	DCE/Hept	ε^o	ACN/Hept	MeOH/DCE
0.00	0	0.35	8	
0.05	3.5	0.40	24	
0.10	10	0.45	52	
0.15	18	0.50	88	
0.20	32	0.60	100	
0.25	58	0.70		28
0.30	100	0.80		52

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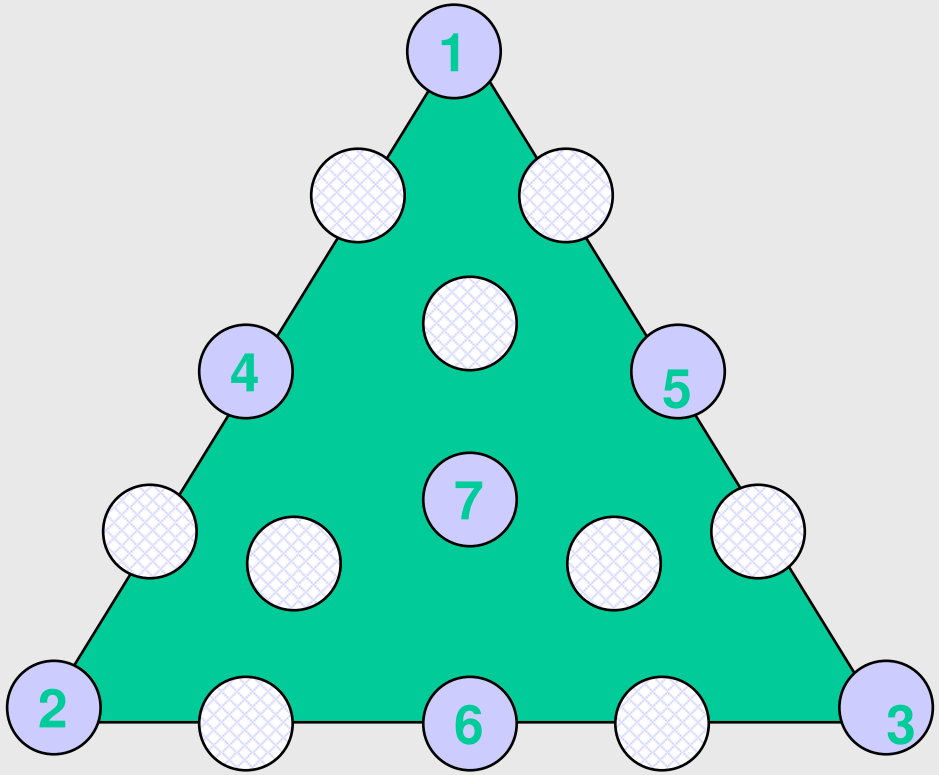
4 solvent approach: step 2

At suitable solvent strength change selectivity

1) DCE/Heptane
2) MTBE/Heptane
3) ACN/Heptane
4) 1+2 (1:1)
5) 1+3 (1:1)
6) 2+3 (1:1)
7) 1+2+3 (1:1:1)
... (x:y:z)



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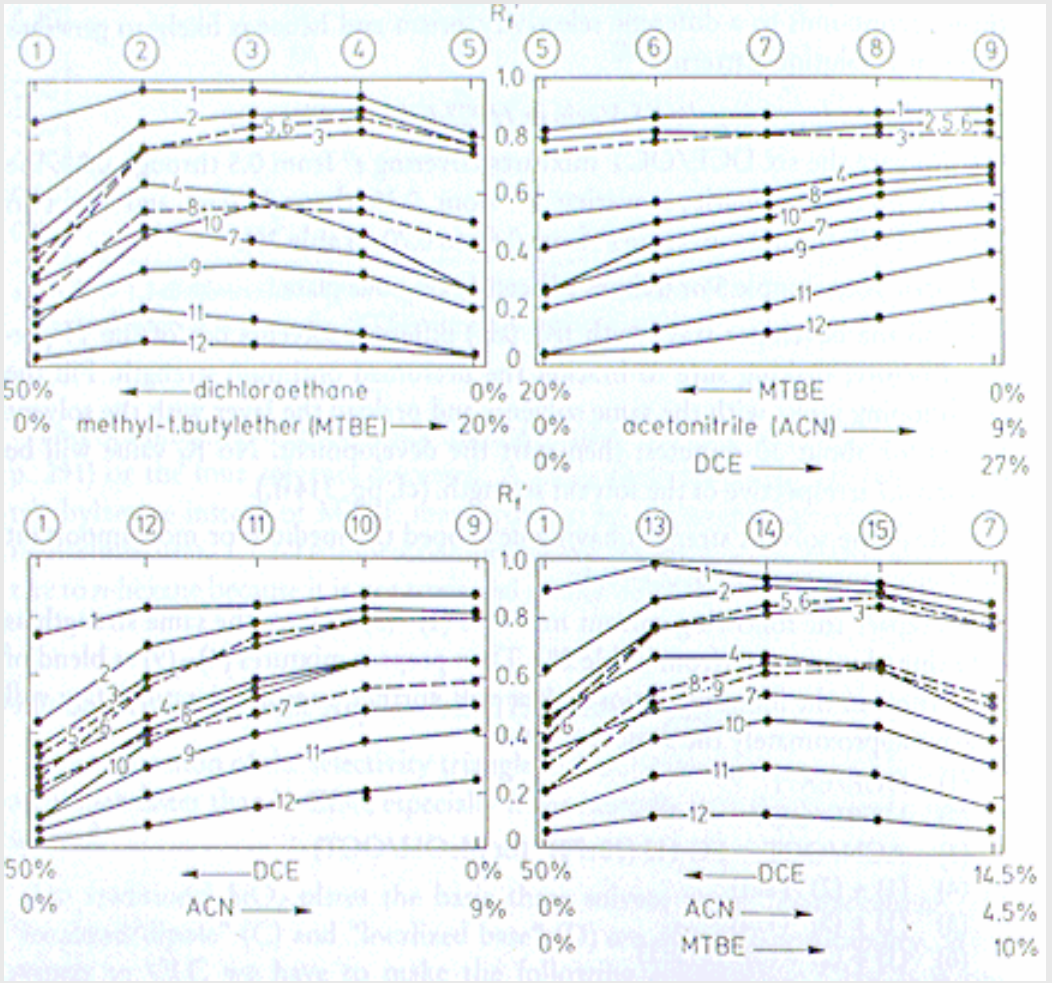
4 solvent approach: example



4 solvent approach: advantages /disadvantages

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- Well understood
- Easy and straight forward to perform
- Good agreement with theory

- Elaborate preparation
- Not suitable for polar compounds

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The Prisma model: step 1

Select 8 - 10 neat solvents

diisopropyl ether	I	2.4	dichloromethane	V	3.1
MTBE	I	2.7	dichloroethane	V	3.5
diethyl ether	I	2.8	ethyl acetate	VI	4.4
2-propanol	II	3.9	dioxane	VI	4.8
ethanol	II	4.3	acetone	VI	5.1
methanol	II	5.1	acetonitrile	VI	5.8
THF	III	4.0	toluene	VII	2.4
pyridine	III	5.3	benzene	VII	2.7
acetic acid	IV	6.0	chloroform	VIII	4.1
formamide	IV	9.6	water	VIII	10.2

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The Prisma model: step 2

- Adjust solvent strength to yield R_F between 0.2 and 0.8 (0.3 !)
- Strong solvents are “diluted” with hexane
- Weak solvents are made stronger with: water, acetic acid, diethylamine **(small amounts)**

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The Prisma model: step 3

- Select the three “best” solvents
- Equalize solvent strength
- Calculations are based on P’ (see table)
- Dilution to the strength of the weakest solvent with hexane

$$P' = \sum Pi\ \Psi$$

Ψ= volume increment of components

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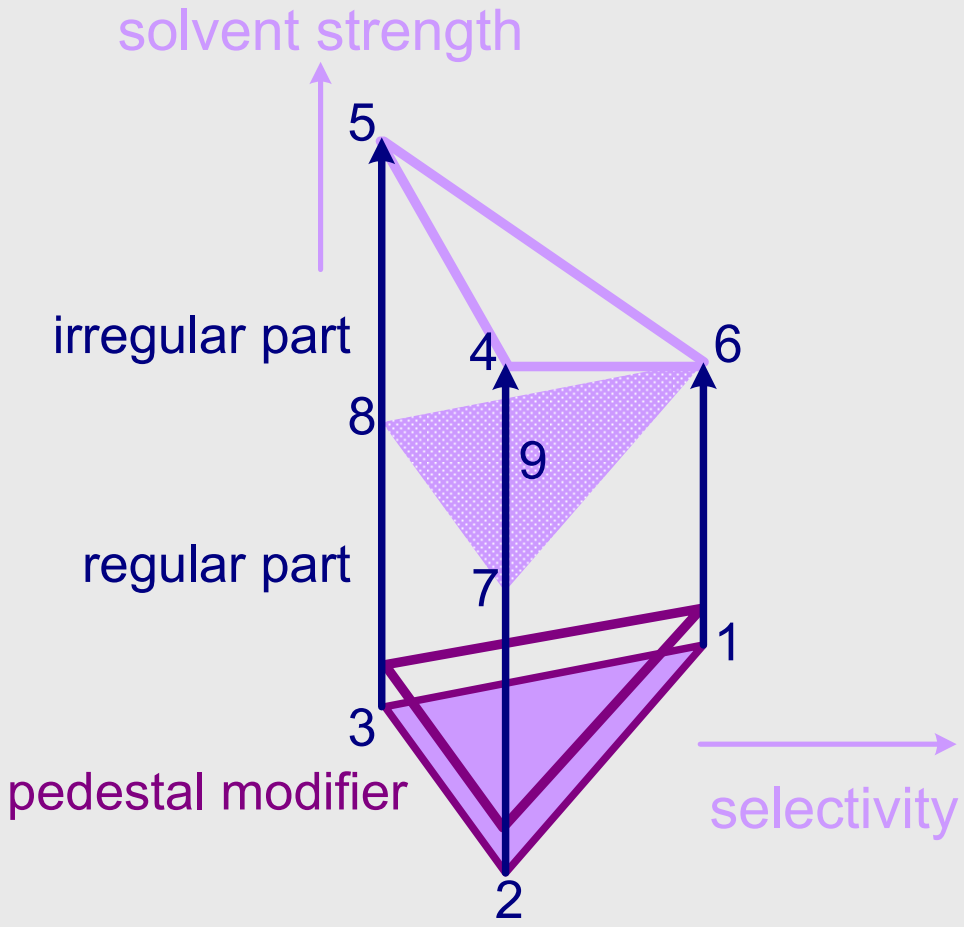
The Prisma model: step 3



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The Prisma model: step 4

- Change selectivity
- Mix components of equal strength

example:

MTBE (2.7), DCM (3.1), chloroform (4.1)

MTBE:	neat	
DCM:	diluted:	2.7/3.1= 87% (13% hexane)
Chloroform:	diluted:	2.7/4.1= 66% (34% hexane)

1:1:1 mix (P'=2,7): 33.3% MTBE + 29% DCM + 22% C + 15.,7% hexane

The Prisma model: advantages / disadvantages

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- Easy to use
- Rapid results
- Universally applicable

- Difficulties with polar samples
- Calculations can only give hints
- Effects of the gas phase are not included

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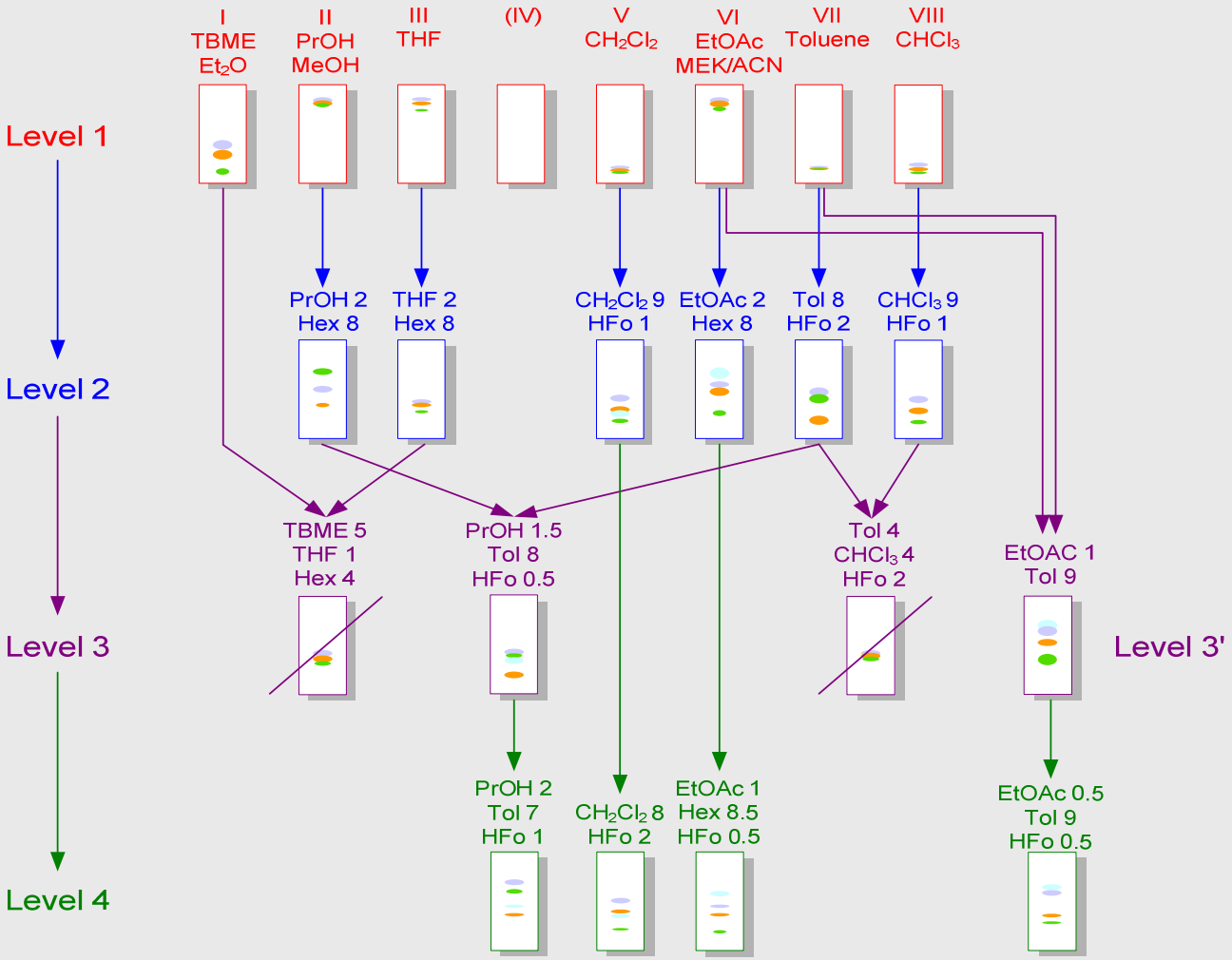
The CAMAG - optimization scheme



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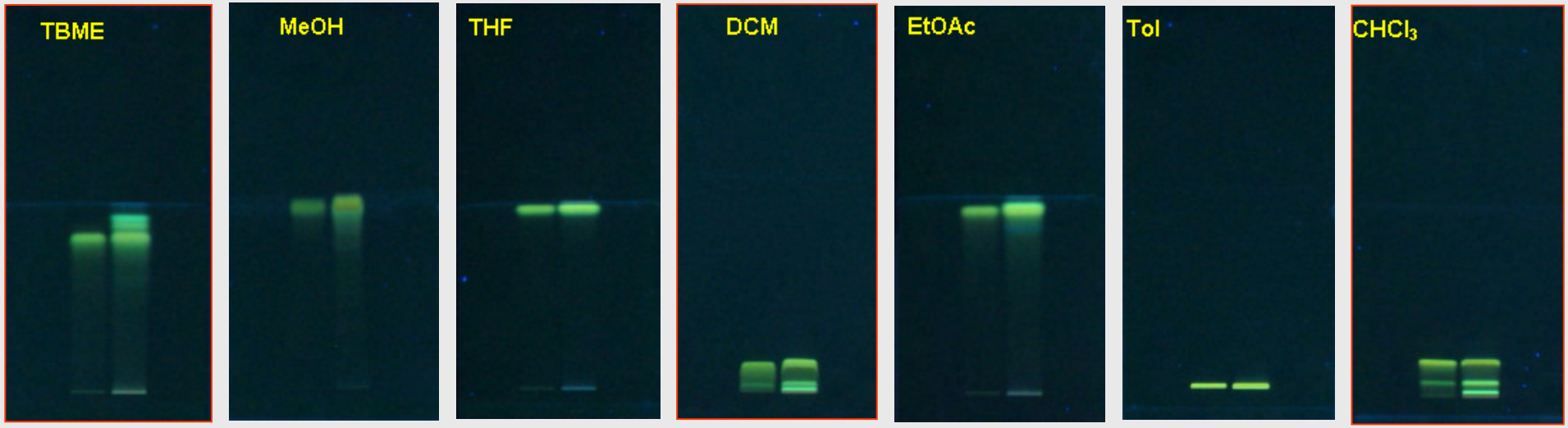
CAMAG - scheme: Example level 1



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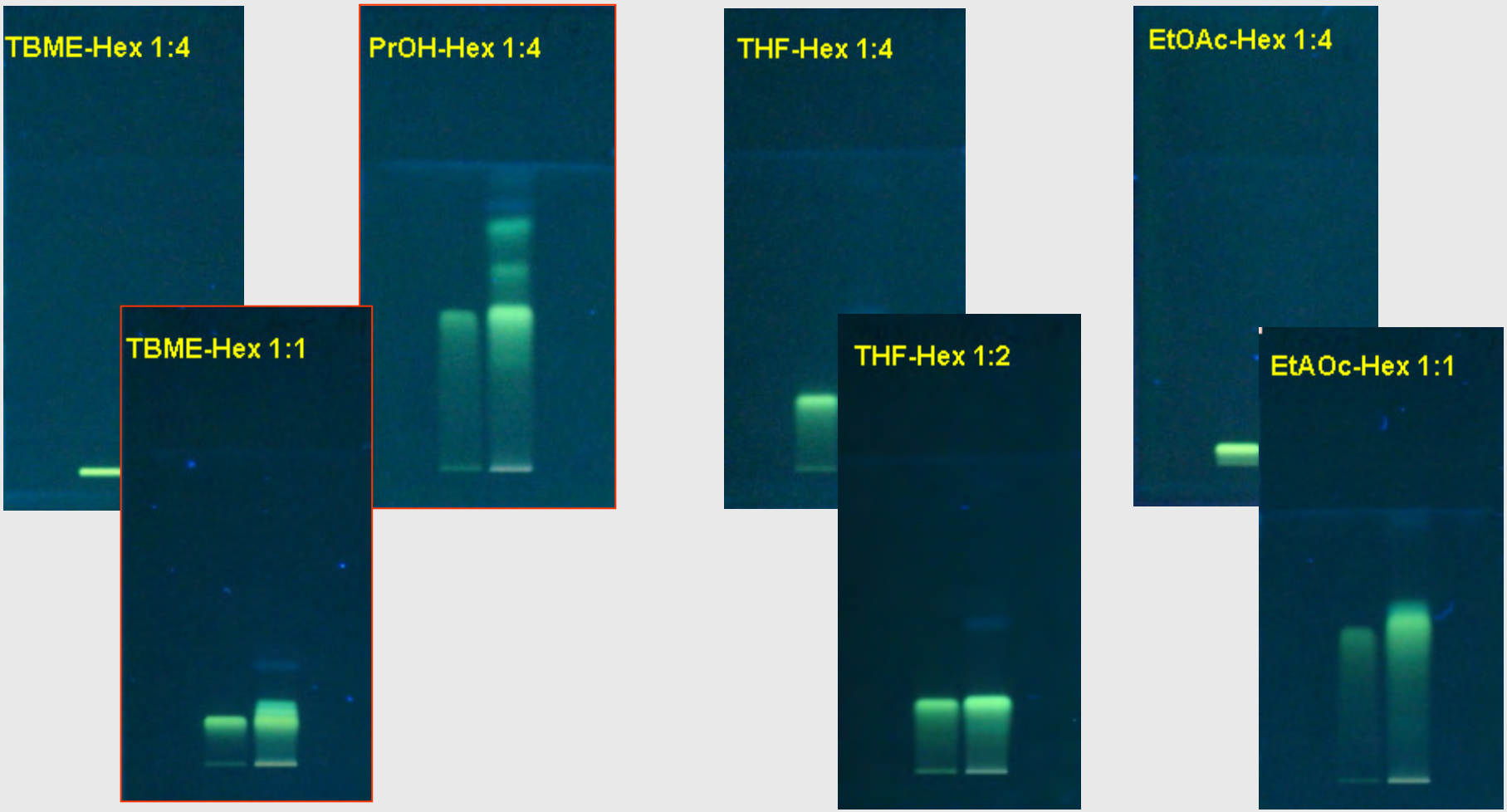
CAMAG - scheme: Example level 2



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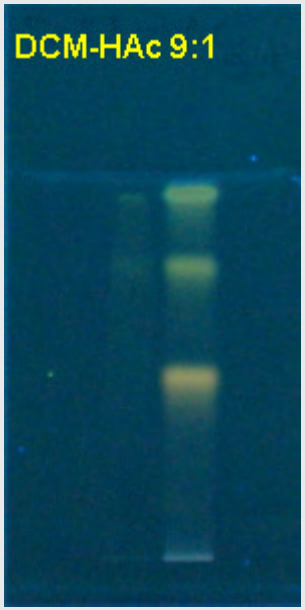
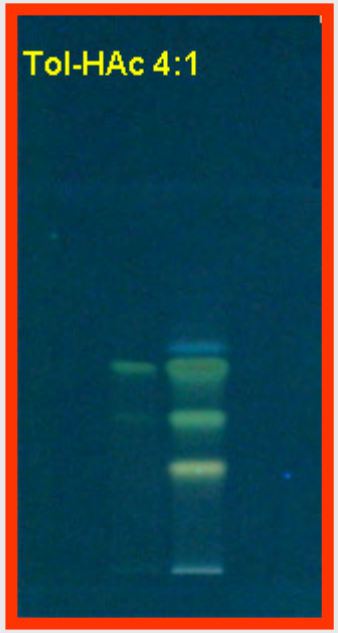
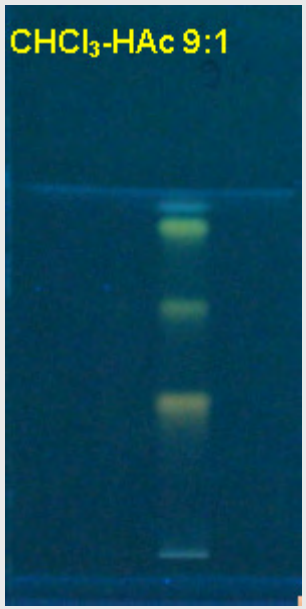
CAMAG - scheme: Example level 2







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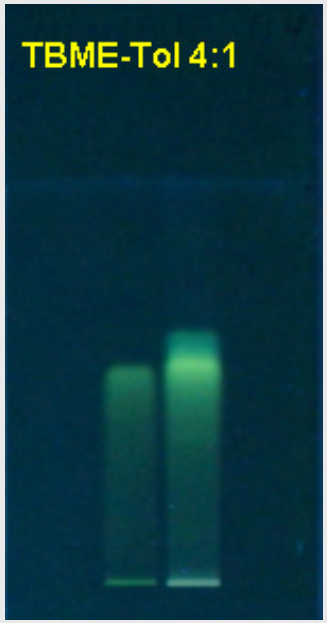
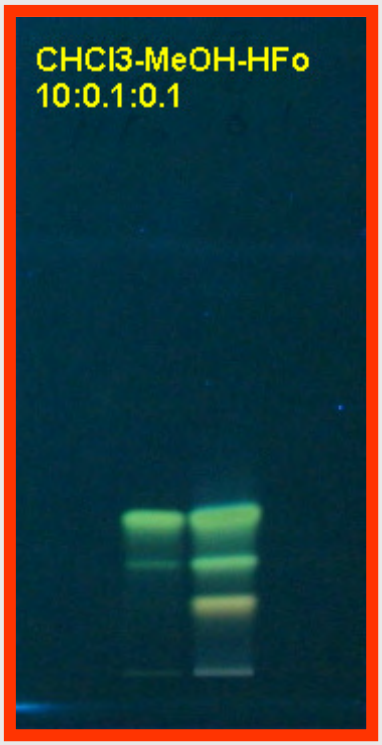
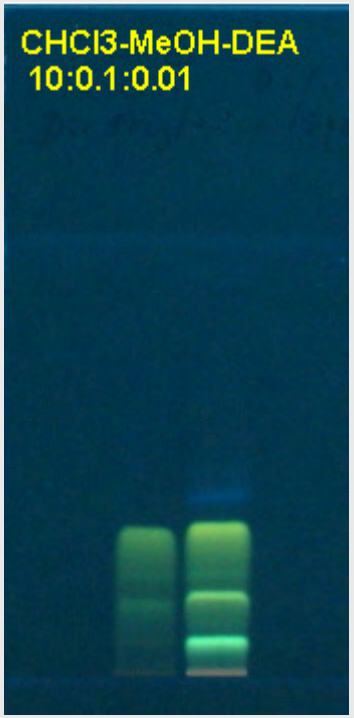
CAMAG - scheme: Example level 3



CAMAG - scheme: Example level 4





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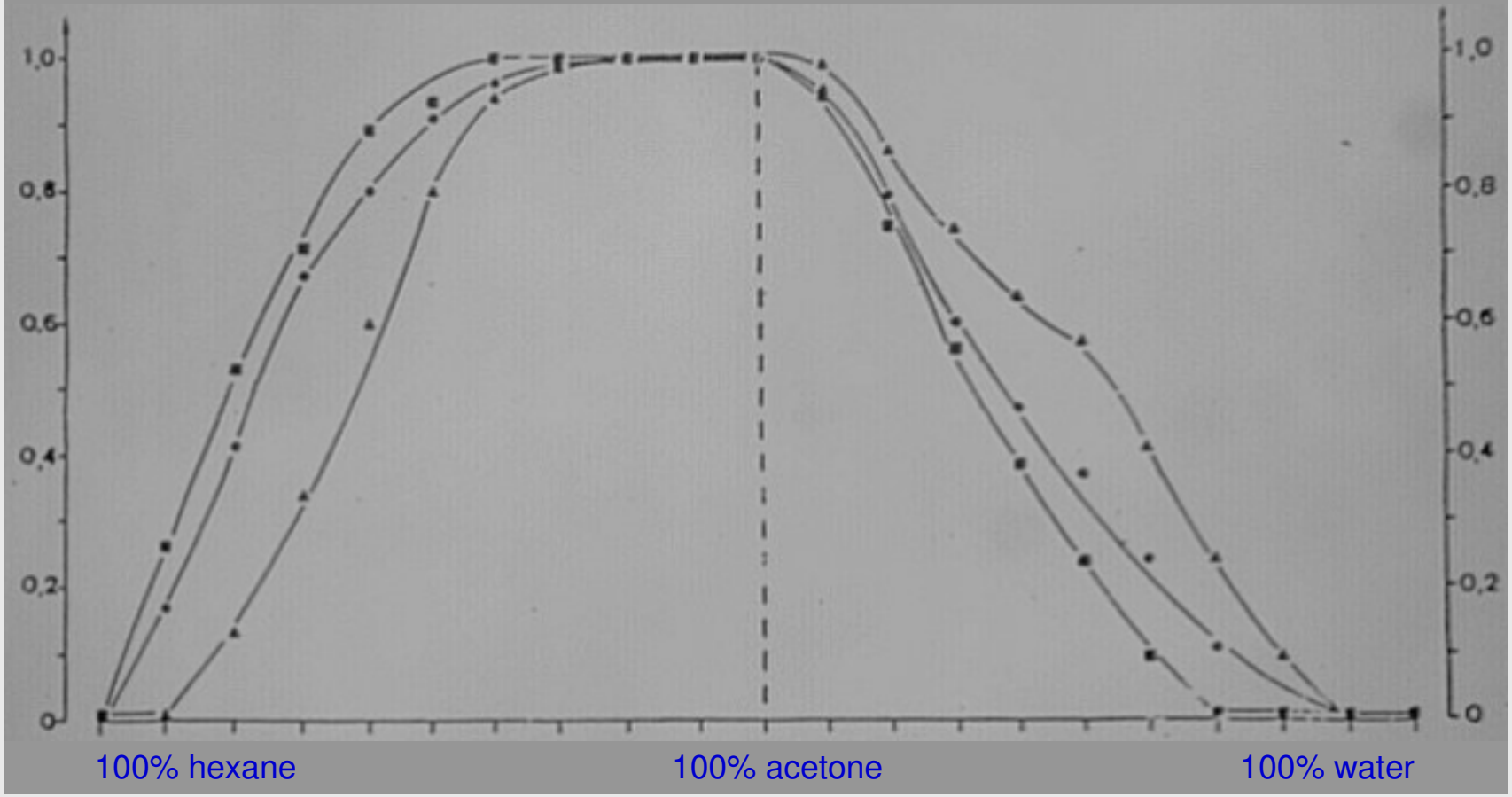
Switching the mode of separation

Pregnelolone, testosterone, estriol, detection $MnCl_2/H_2SO_4$, UV 366, normal chamber



Comparison of bonded phases
Mode switching

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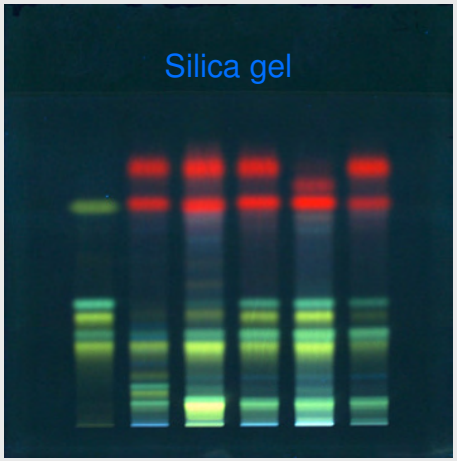
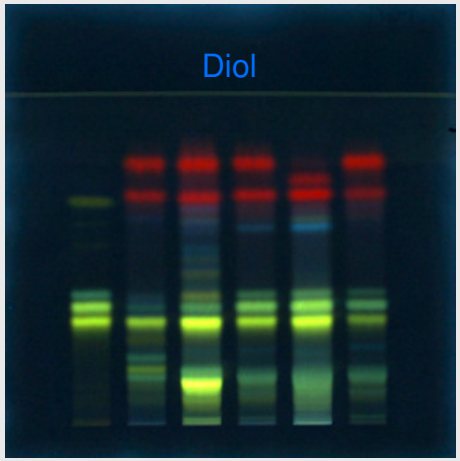
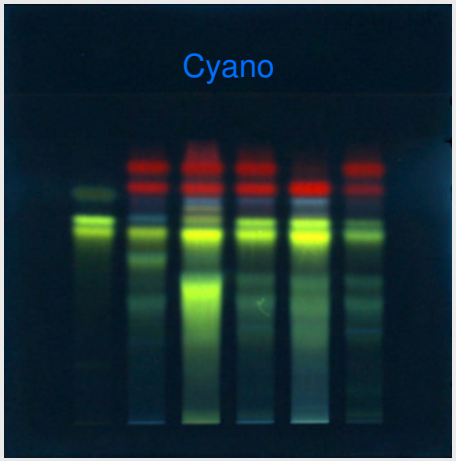
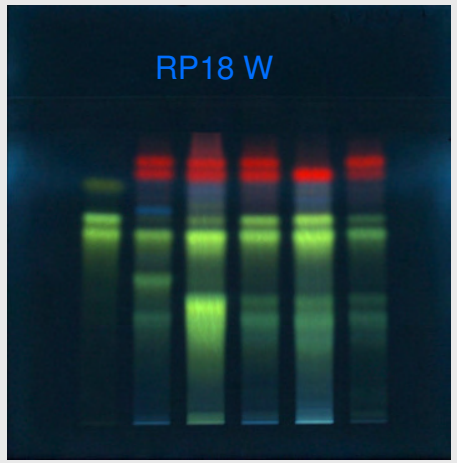
Normal phase: THF, toluene, formic acid, water (24:12:3:1.5)

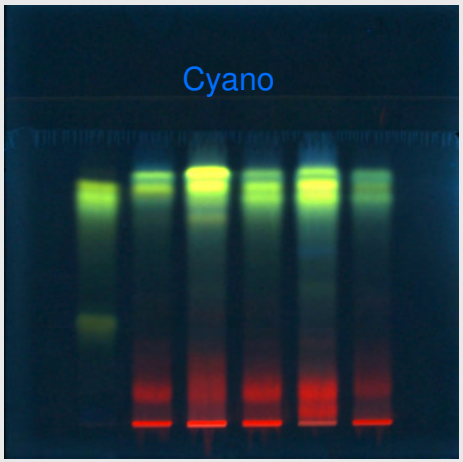


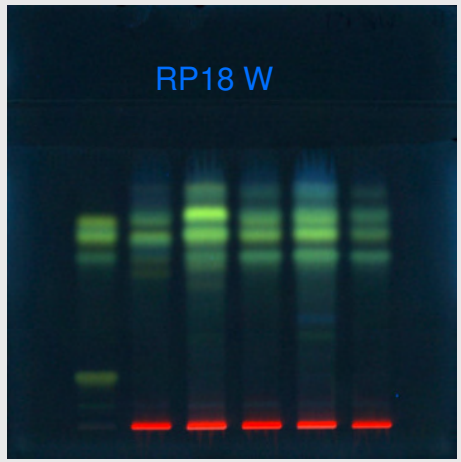






Reversed phase: methanol, formic acid, water (5.5:1:4.5)





Vitexin, orientin, isovitexin, isoorientin, chrysin, and 5 Passion Flower samples

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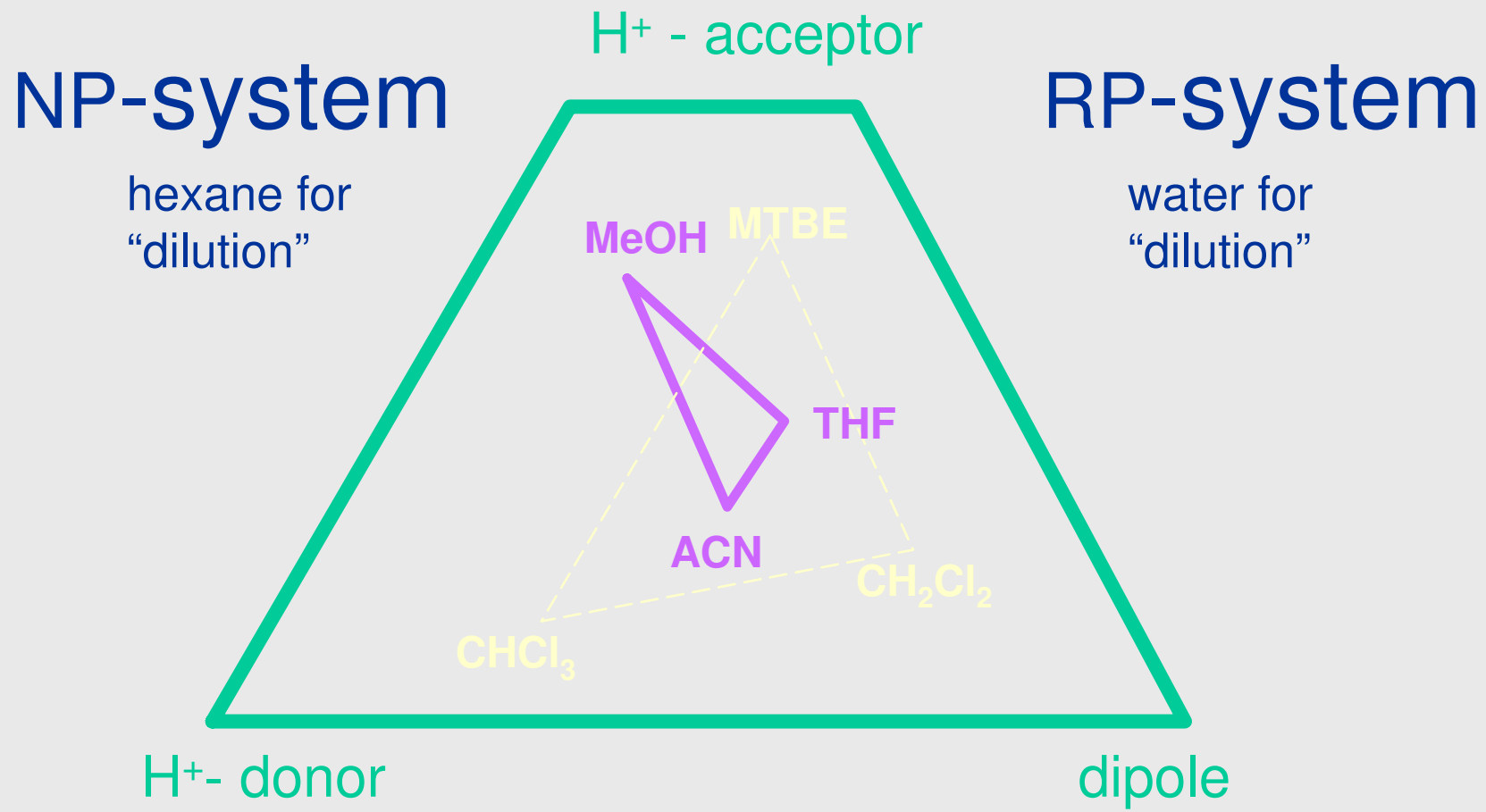
Optimization of RP-systems



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Solvent strength and selectivity

Solvent	Solvent Strength	Selectivity
water	0.0	VIII
methanol	2.6	II
acetonitrile	3.2	VI
acetone	3.4	VI
dioxane	3.5	VI
ethanol	3.9	II
isopropanol	4.2	II
THF	4.5	III

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Other aspects

- Stability during chromatography
- Effects of humidity

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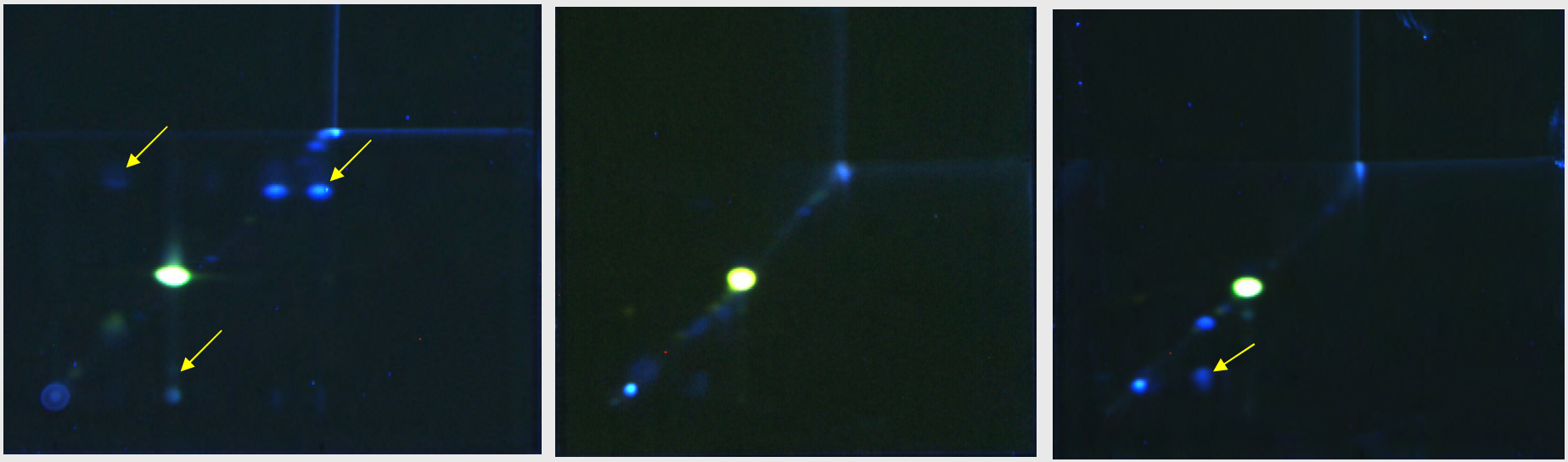
Stability in the chromatographic system



2D chromatography of Goldenseal

Chinese Ph. CAMAG USP

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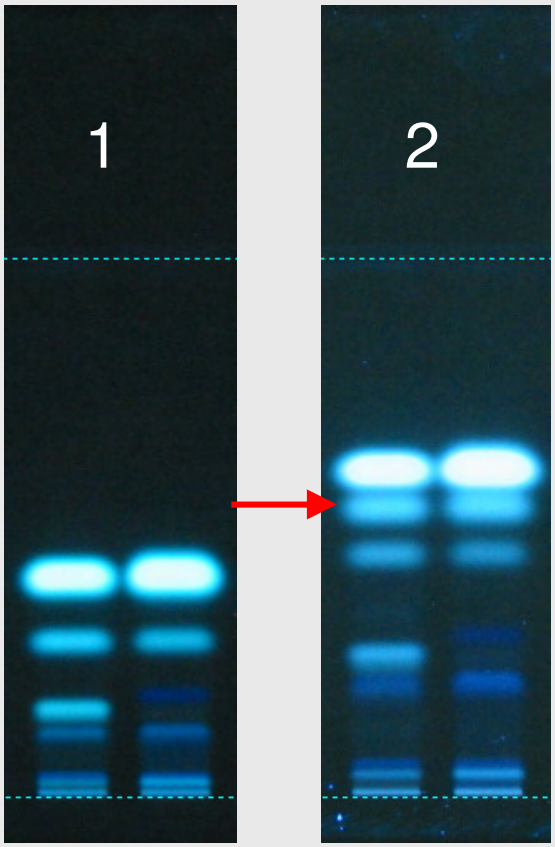
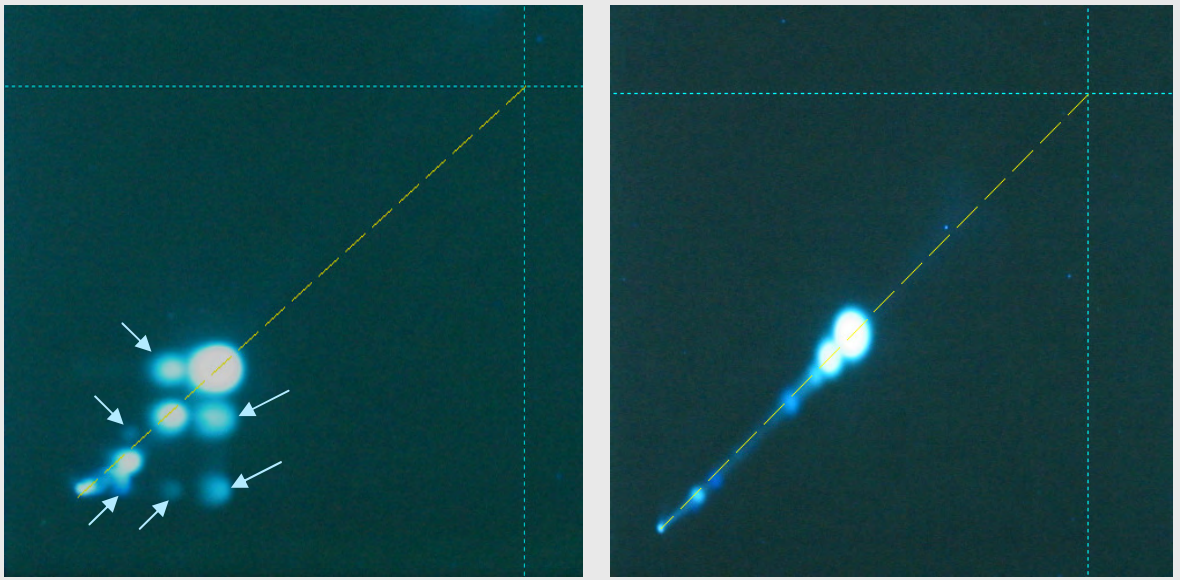
Stability in the chromatographic system





2D Chromatogram *Angelica sinensis*.

Left: formation of artifacts (arrows),
Right: the analyte it stable

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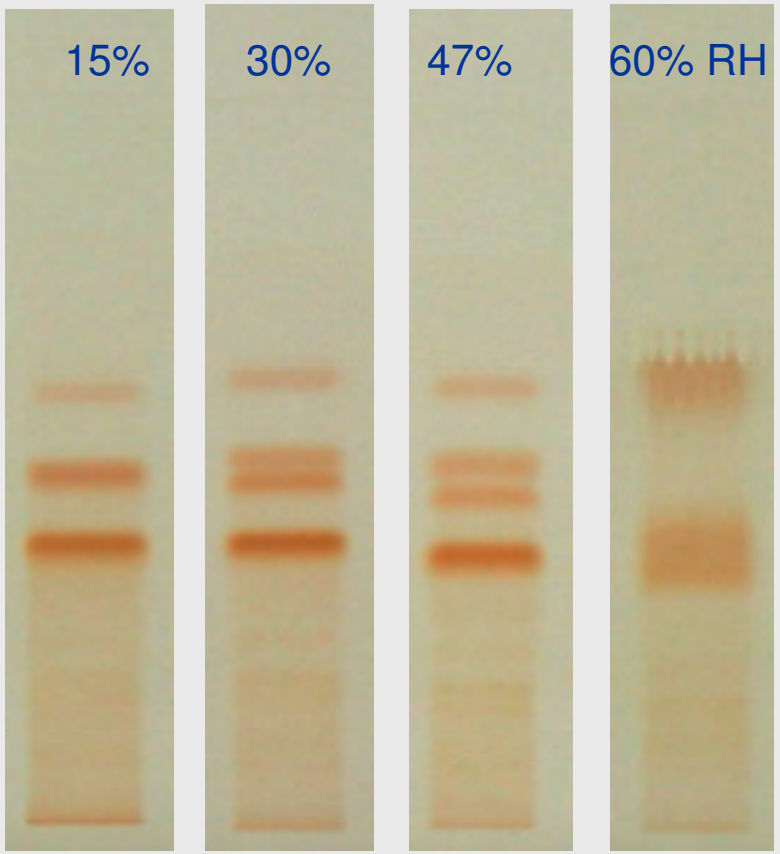
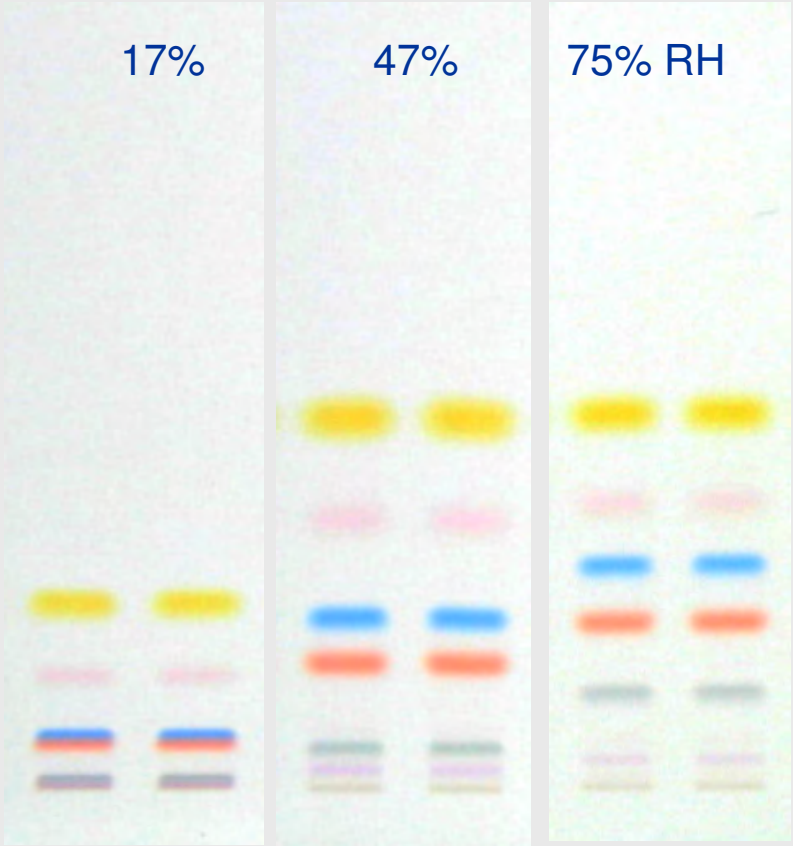
Humidity/Activity

Green tea: Polyphenols



Toluene, acetone, formic acid (4.5:4.5:1)

Test dye mixture



Toluene neat

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Contact information

Lab@camag.com

eike.reich@camag.com

www.camag-laboratory.com

What's new?

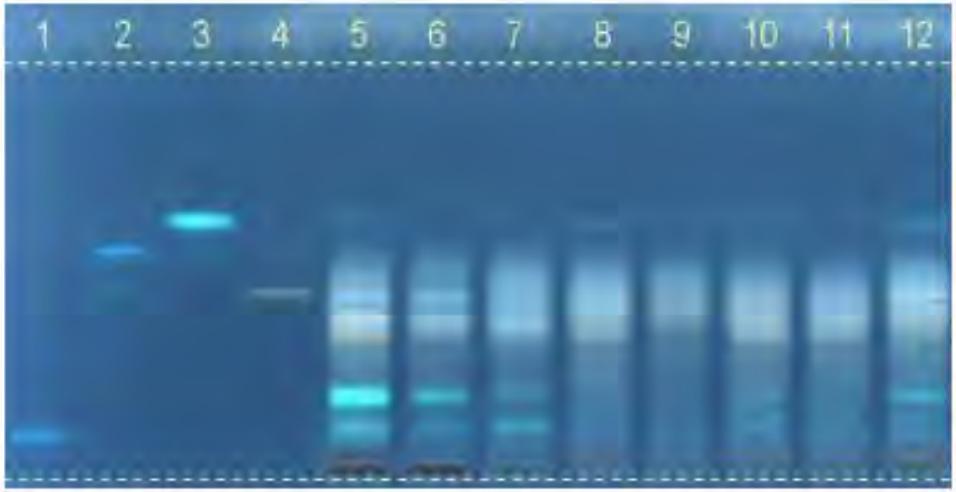
New Publication
Separation of phospholipids by HPTLC - an investigation of important parameters >>

New book
"Thin Layer Chromatography in Phytochemistry" features a chapter written by scientists from CAMAG Lab >>

Conferences, seminars and workshops
CAMAG collaborates with the United States Pharmacopeia USP >>

The International Symposium for High Performance Thin-Layer Chromatography took place in Helsinki, Finland, 16 - 18 June 2008 >>

HPTLC of Black Cohosh



New TLC VISUALIZER:
The powerful evaluation, visualization and archiving system >>

CBS 100 about «Bio-activity based analysis of irradiated sunscreens using HPTLC and in situ detection with Vibrio fischeri» >>

CAMAG Laboratory

a center for applied HPTLC

- Broad range of services
- Dedicated to research
- Source of information

Services

Method development and validation

- Feasibility studies
- Contract analyses
- Training

Publications

- HPTLC for the analysis of medicinal plants, by E. Reich / A. Schibli
- HPTLC identification of Hoodia gordonii
- Validation of HPTLC identification methods for botanicals