Workshop
Planar Chromatography
Part II

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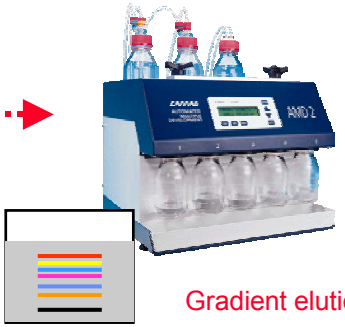
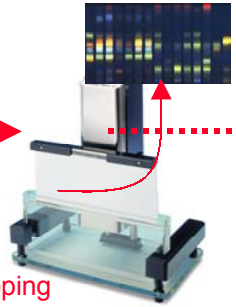
University of Hohenheim
Stuttgart, Germany

Planar Chromatography

Fully automatic application

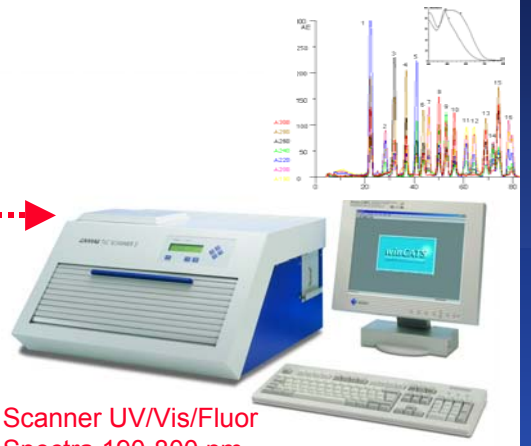


Gradient elution



Dipping

Spraying



Scanner UV/Vis/Fluor
Spectra 190-800 nm

Half automatic application

Fully automatic chromatography

Horizontal development

Heating

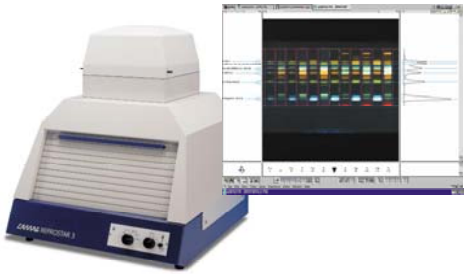
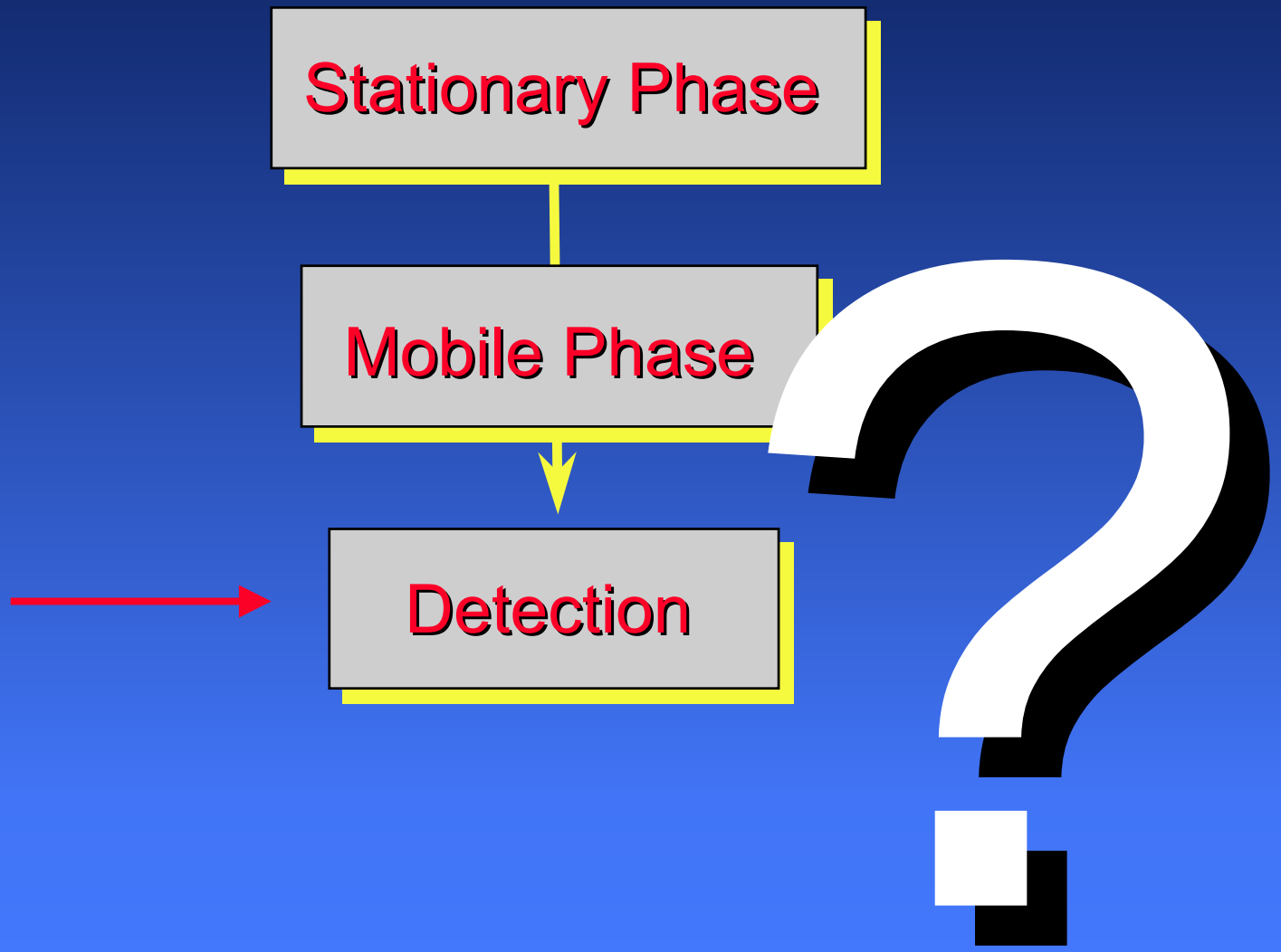


Image documentation
Video densitometry

Application Chromatography Derivatization Evaluation

yesterday today

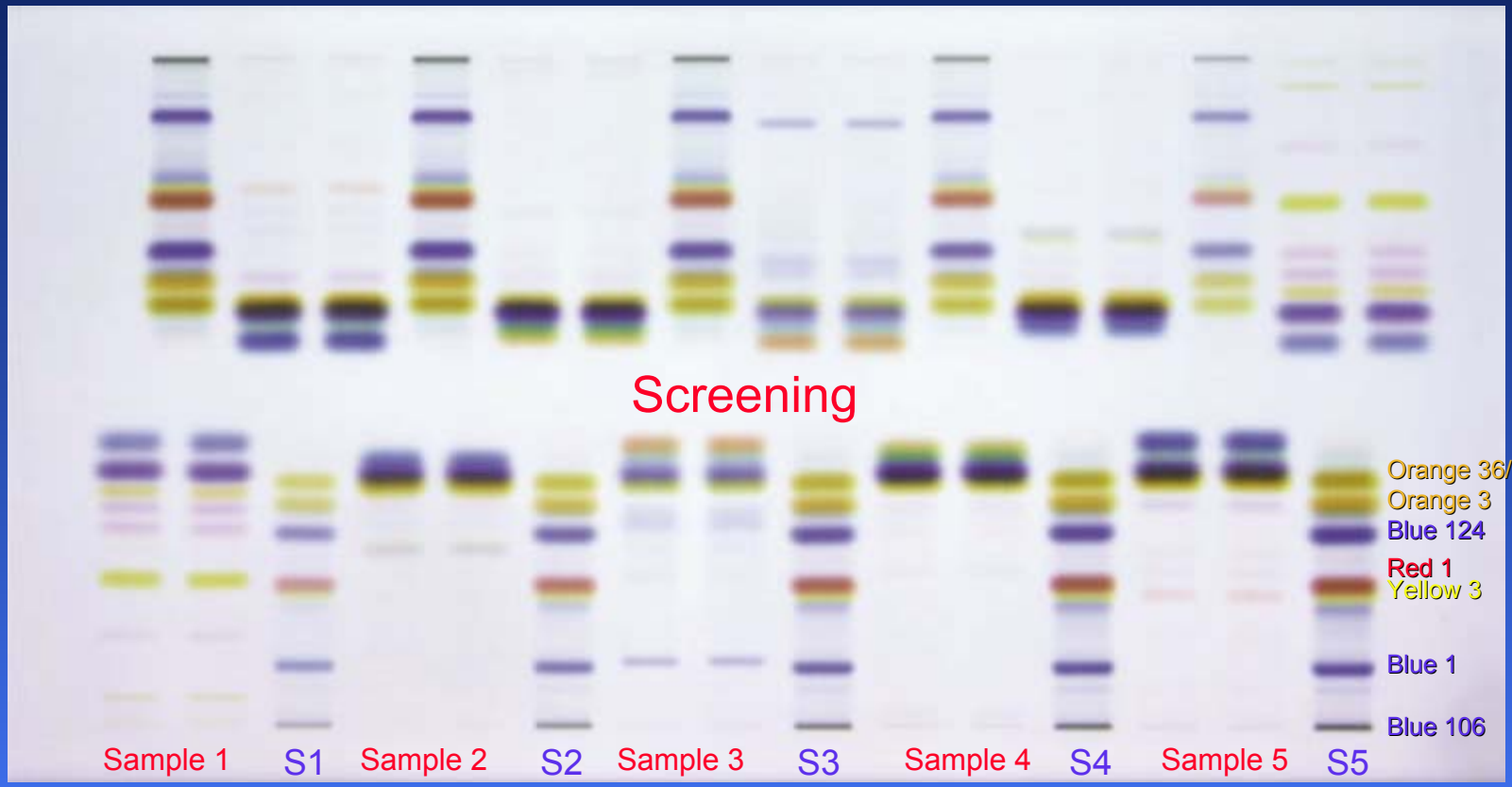
Which TLC system?



Overview of detection

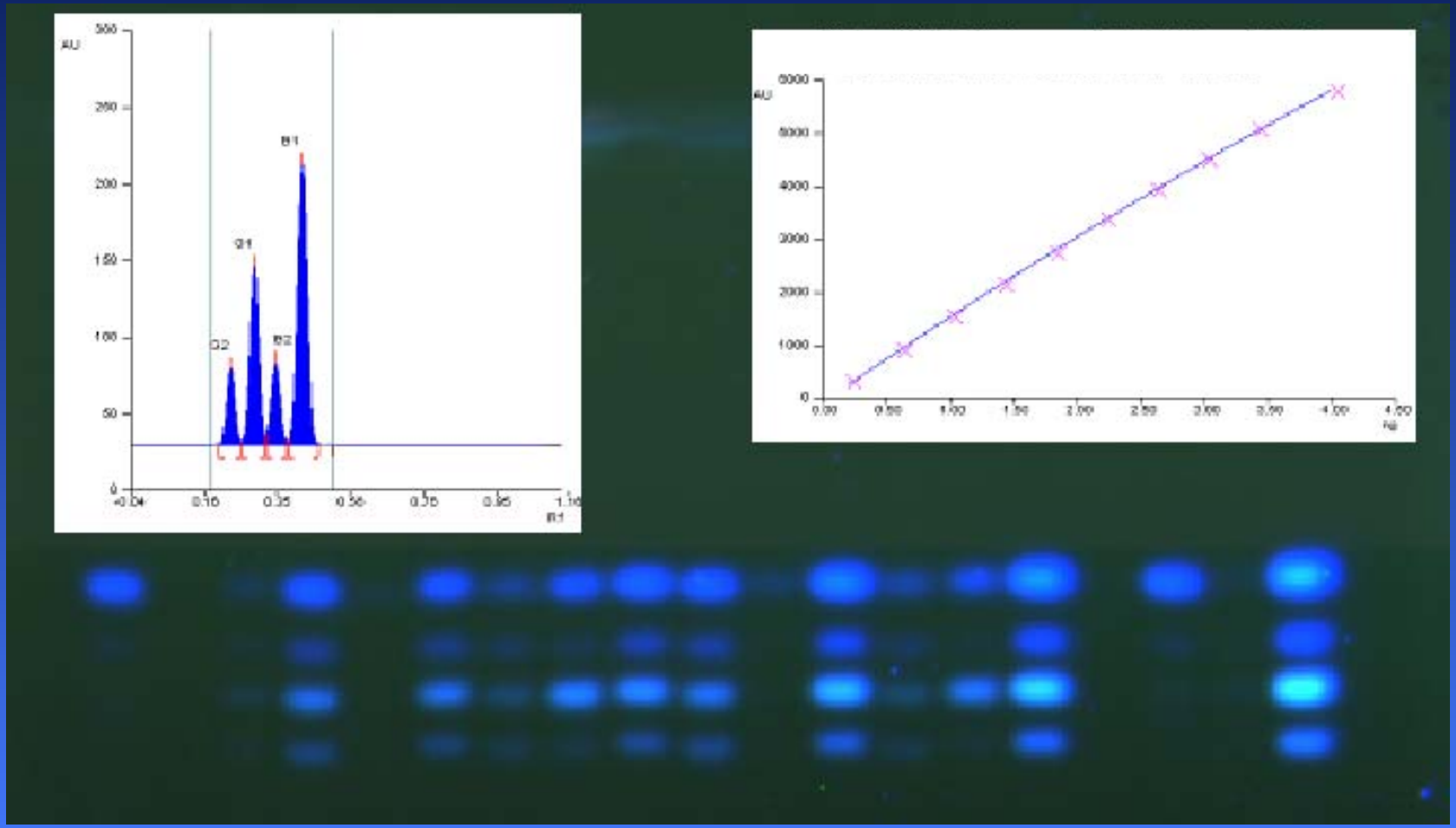
- ✓ Commonly used detection methods
- ✓ Pre- and postchromatographic derivatization
- ✓ Universal and group characterizing reagents
- ✓ Derivatization - sensitivity of reagents
- ✓ Derivatization techniques
- ✓ Microchemical and microbiological detection
- ✓ Absorbance scan
- ✓ Fluorescence scan
- ✓ Multi-wavelength scan
- ✓ Multiple detection
- ✓ Spectra recording and library search

Allergenic disperse dyes in textiles



A. Bonhoff et al., STR Testing & Inspection AG, Steinach, Switzerland, optimized at CAMAG Lab, see CBS 82

Aflatoxins in foodstuffs



CAMAG Laboratory Application Note 12.4

Commonly used in situ detections

Physical detection

- Optomechanical scan
- Video densitometry
- Further techniques

- ✓ Absorbance UV/VIS
- ✓ „Fluorescence quenching“
- ✓ Fluorescence

- Autoradiography etc.

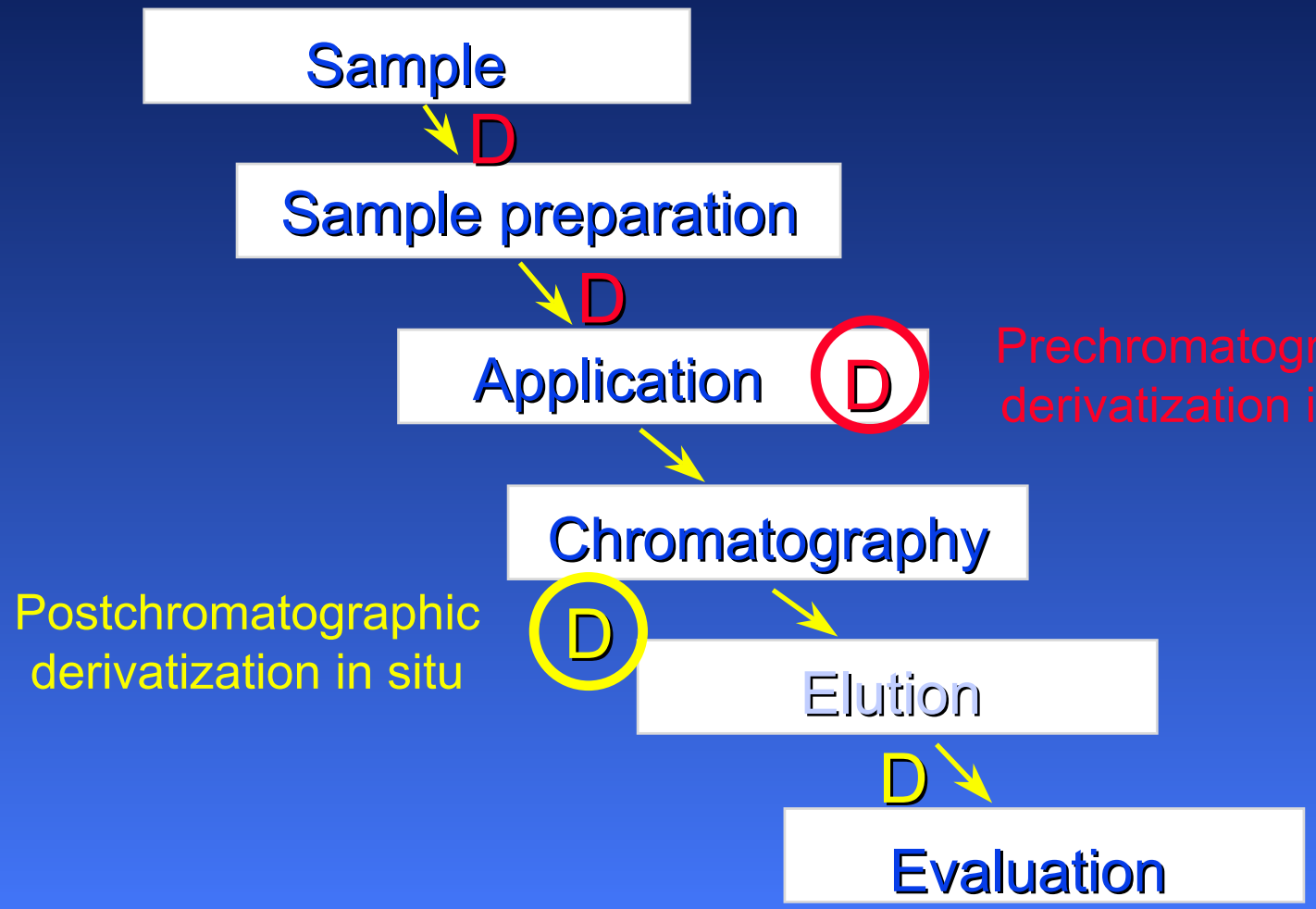
Microchemical detection

- Prechromatographic derivatization
- Postchromatographic derivatization
- Universal reagents
- Group characterizing reagents

Microbiological & biochemical detection

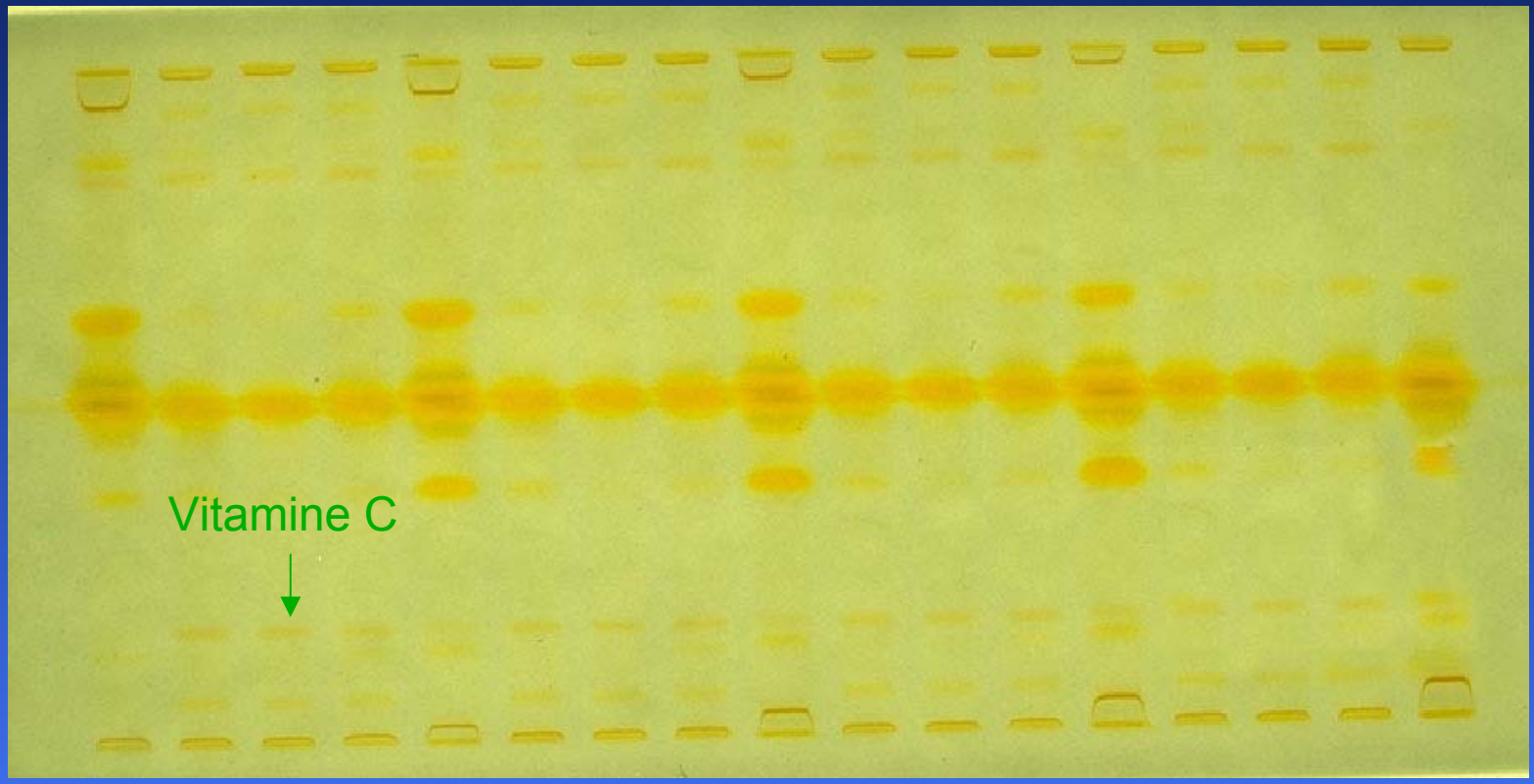
- Bioautography
- Enzyme-substrate-reaction

Derivatization



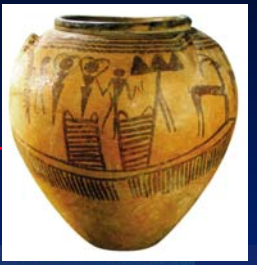
Note: By derivatization in situ all samples are derivatized simultaneously!

Prechrom. derivatization during sample preparation

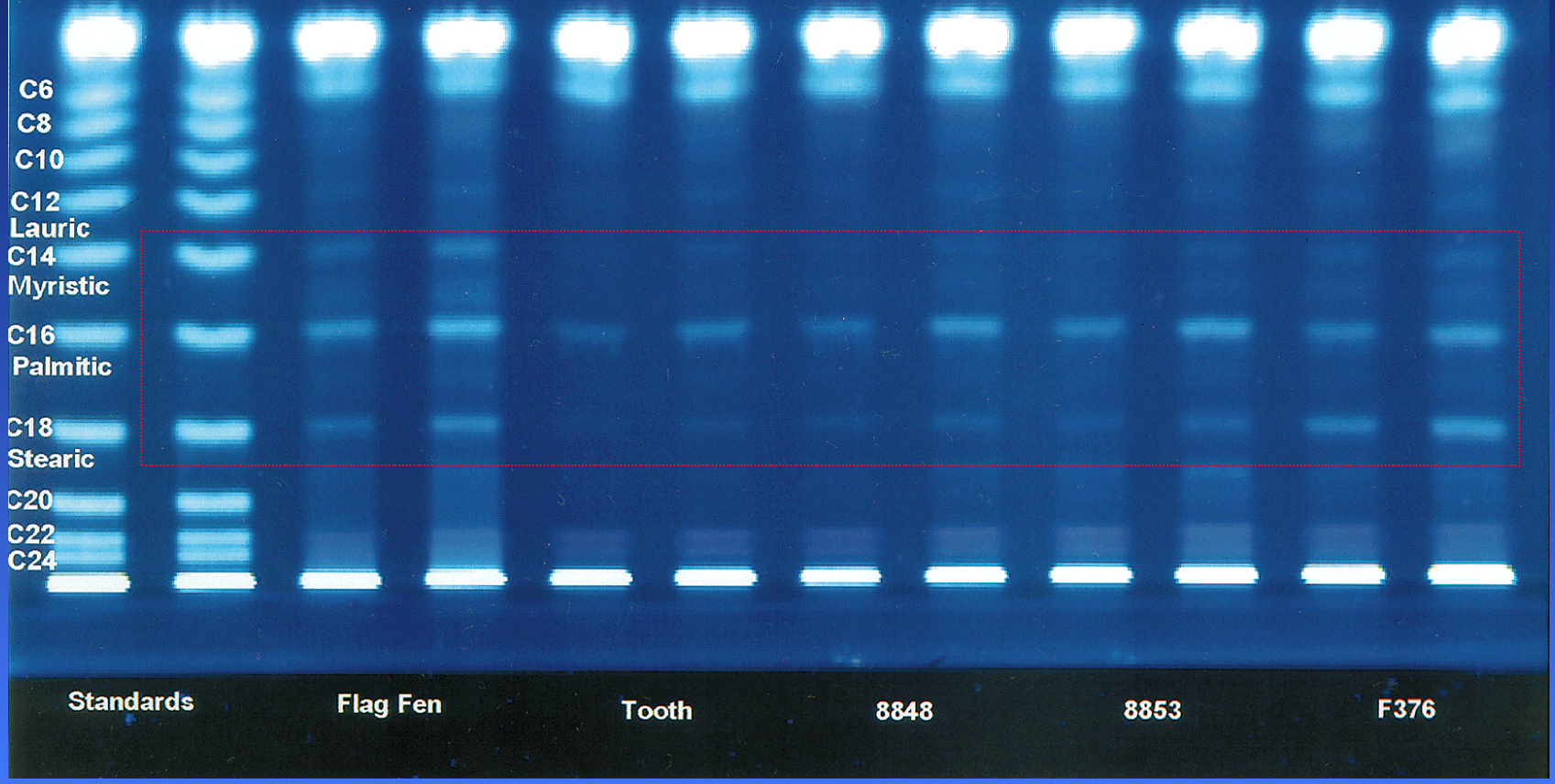


Derivatization with 2,4-Dinitrophenylhydrazin, see CBS 66

Prechromatographic derivatization in situ



What ate our forefathers? Fatty acids in archaelogical artifacts



P. Jones, Time Team & CAMAG Team at Food Science Research Laboratory,
University of Bournemouth, GB, see CBS 85

Prechromatographic derivatization

Advantages

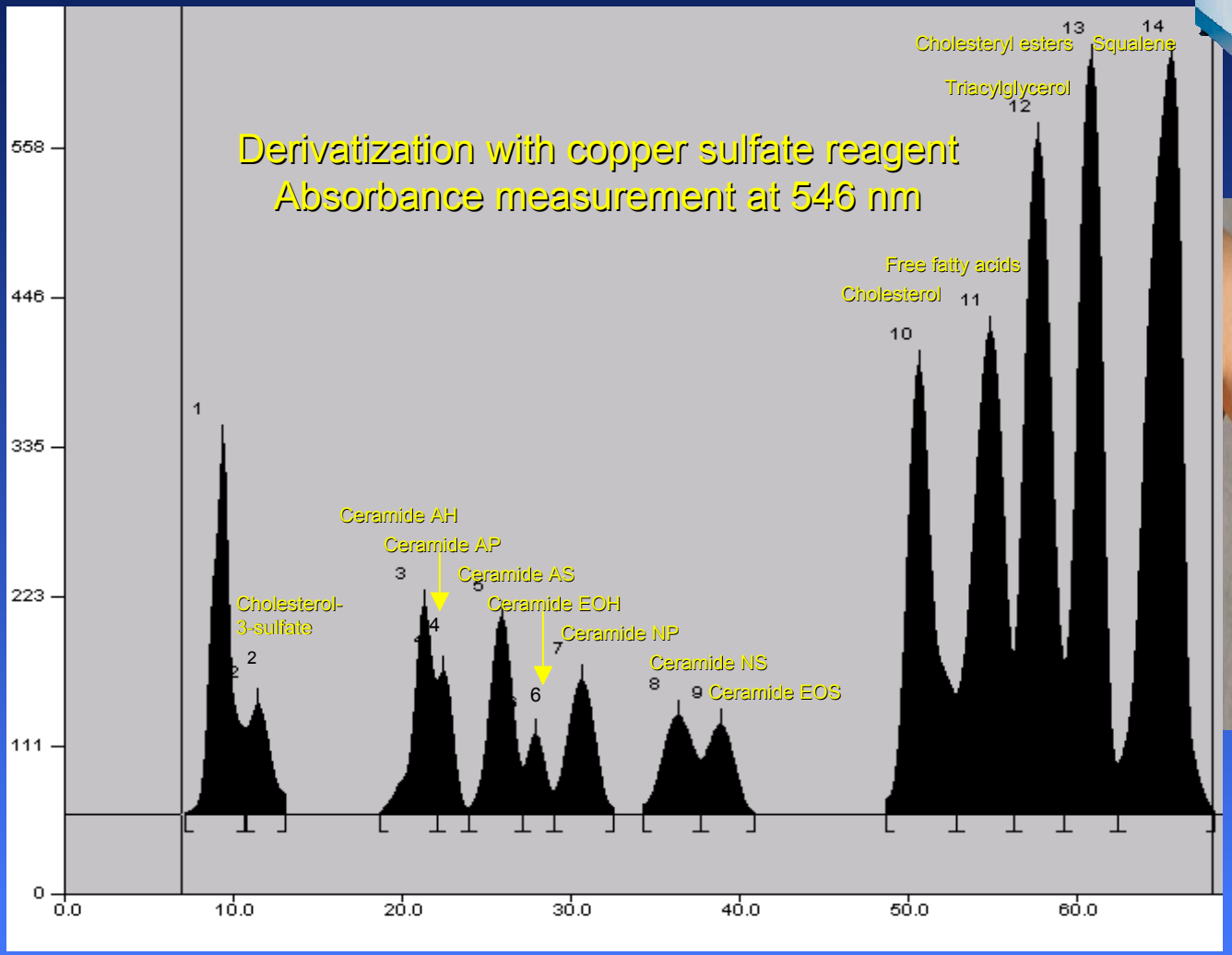
- Enabling the chromatographic separation at all
- Increase in stability of substances to be separated
- Decrease of reactivity of substances with stationary phase, decrease of strong polarities
- Transfer to not volatile derivatives
- No increase of background by reagents in excess because these can still be removed
- Derivatization in vessel: improved extraction efficacy by changing substance properties

Prechromatographic derivatization

Disadvantages

- Reagent influences other sample and matrix constituents
- Chromatographic properties of different sample constituents can be equalized by formation of great molecule groups
- Varying sample and matrix composition influences quantitative derivatization

Stratum corneum lipids



K. Raith et al., University of Halle, see CBS 90

Postchromatographic derivatization in situ

Advantages

- No influence on separation
- Optimal reaction kinetics at different substance concentrations (reaction of substances, not solutions)
- Reaction under identical conditions
- Additional confirmation of results

Disadvantages

- Interference by absorption or fluorescence of reagents in excess
- Technical difficulties

Microchemical derivatization

Examples for universal reagents

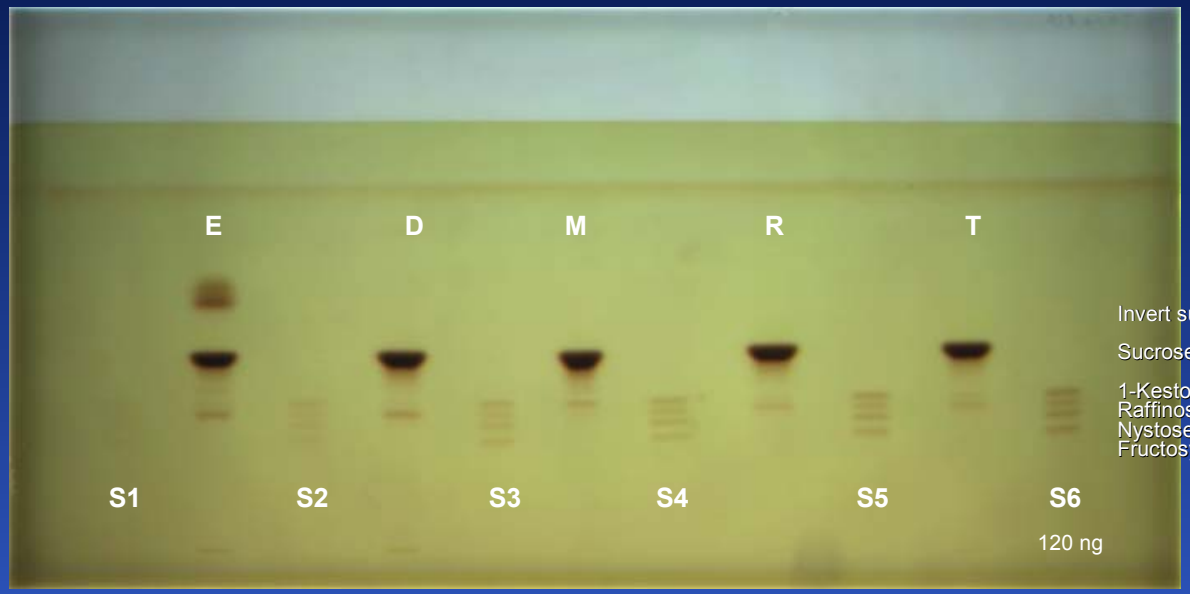
- Carbonizing with acids
- Aldehyde/acid reactions
- Molybdato phosphoric acid
- Iodine or bromine vapors

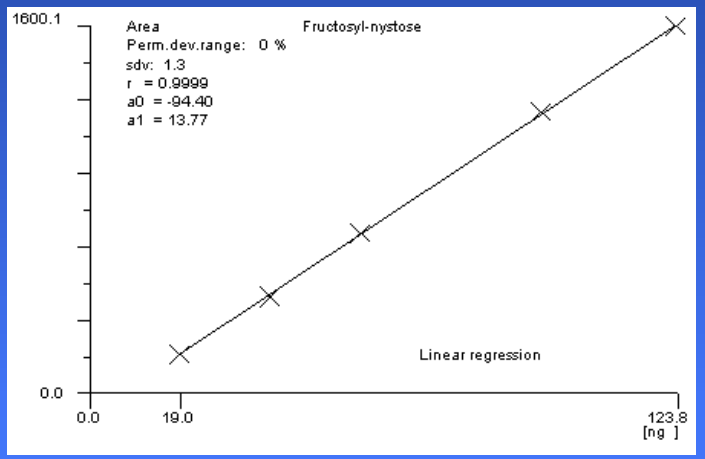
Examples for group characterizing reagents

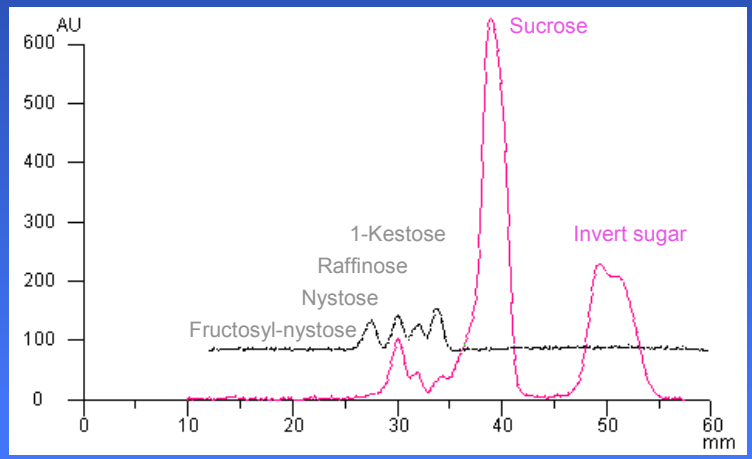
- Ninhydrin
- Diazotization
- Hydrazine derivatives
- Dansylation

Jork, H., Funk, W., Fischer, W., Wimmer, H.: Thin-Layer Chromatography, volume 1a and 1b, VCH, Weinheim, 1989 and 1993.

Oligosaccharids in beet molasses

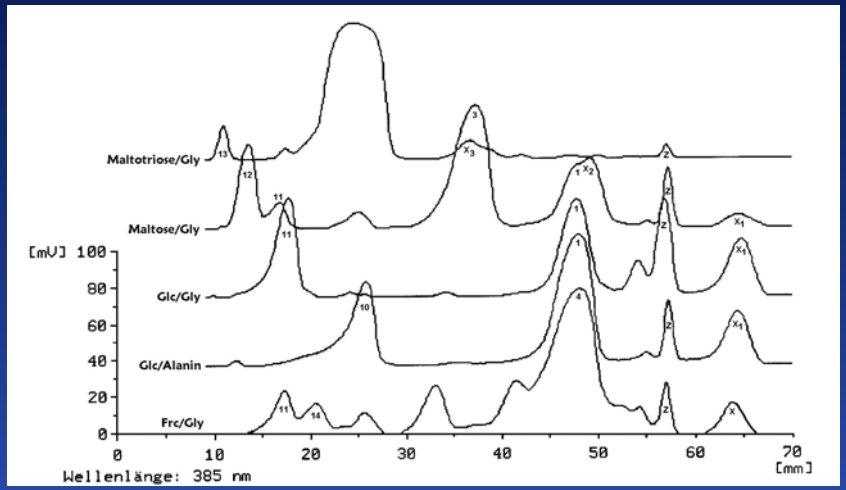






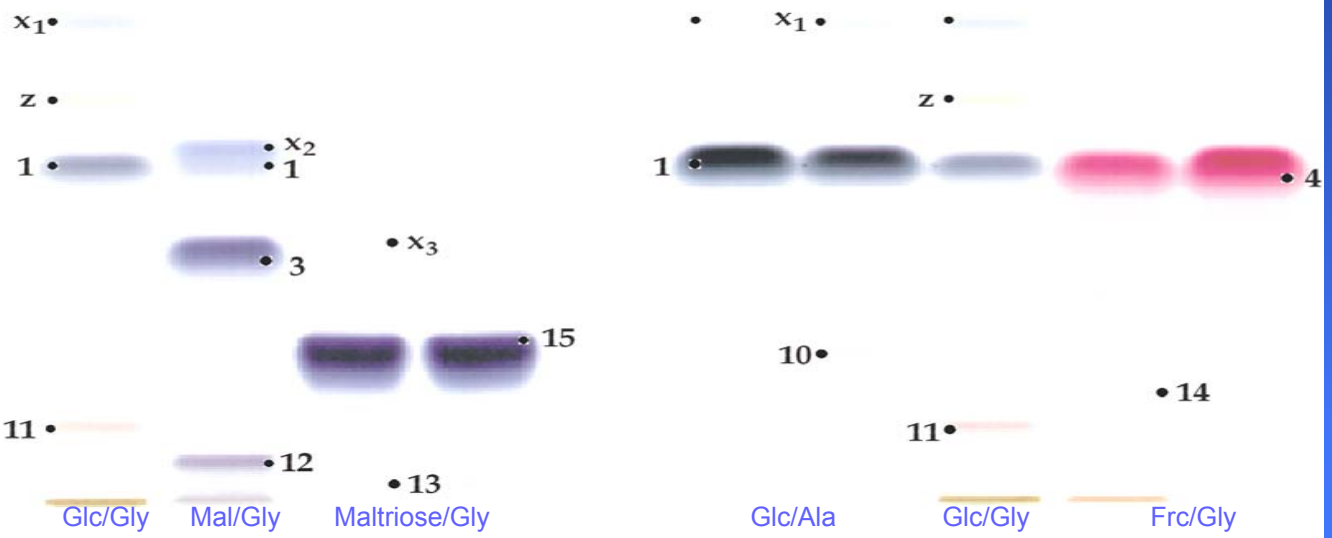
G. Lodi et al., University of Ferrara, Italy, see CBS 84

Carbohydrates in model systems



1 = D-Glucose
3 = Maltose
4 = Fructose
10, 11, 12, 13 = Amadori compounds
14 = Heyns compounds
15 = Maltooligosaccharides

Monitoring of Maillard reaction



L. Kroh et al., Inst. for Food Chemistry, TU Berlin, see CBS 82

Derivatization - sensitivity of reagents

1 µg sugar each detected by

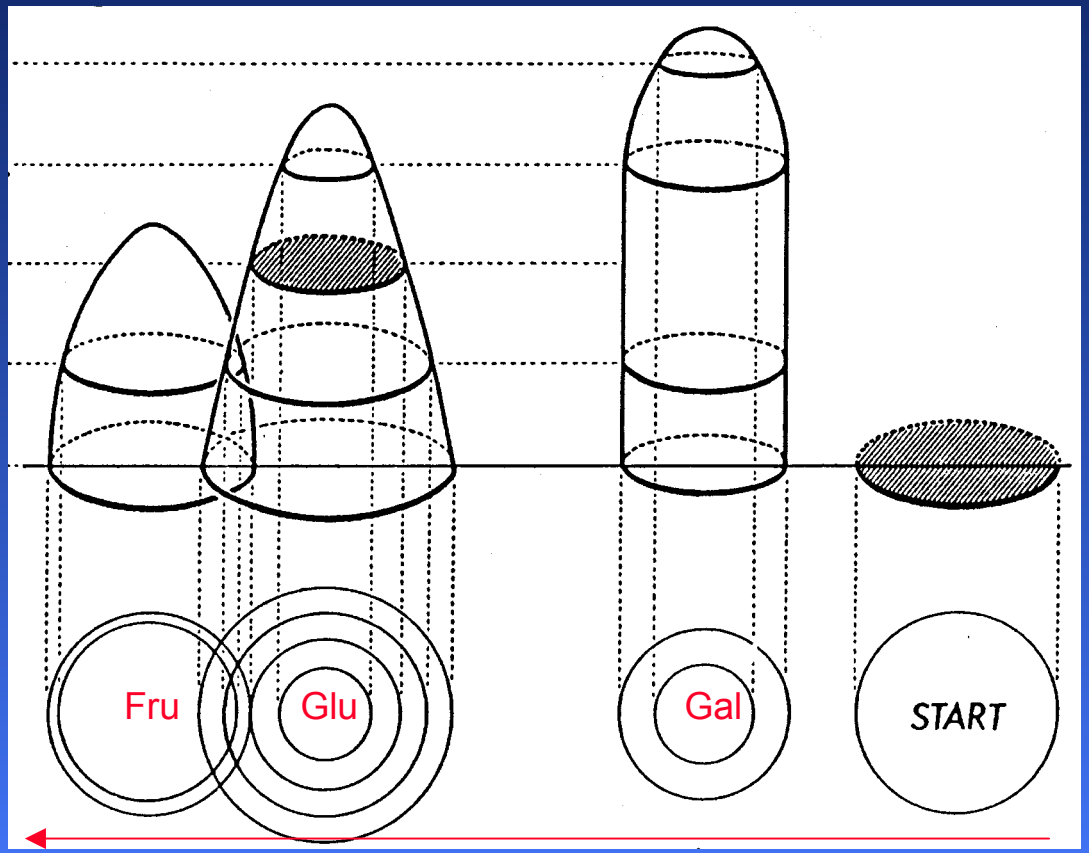
Iodine vapor

Anisaldehyde reagent

GOD reagent

Anthrone reagent

Aniline phthalate reagent

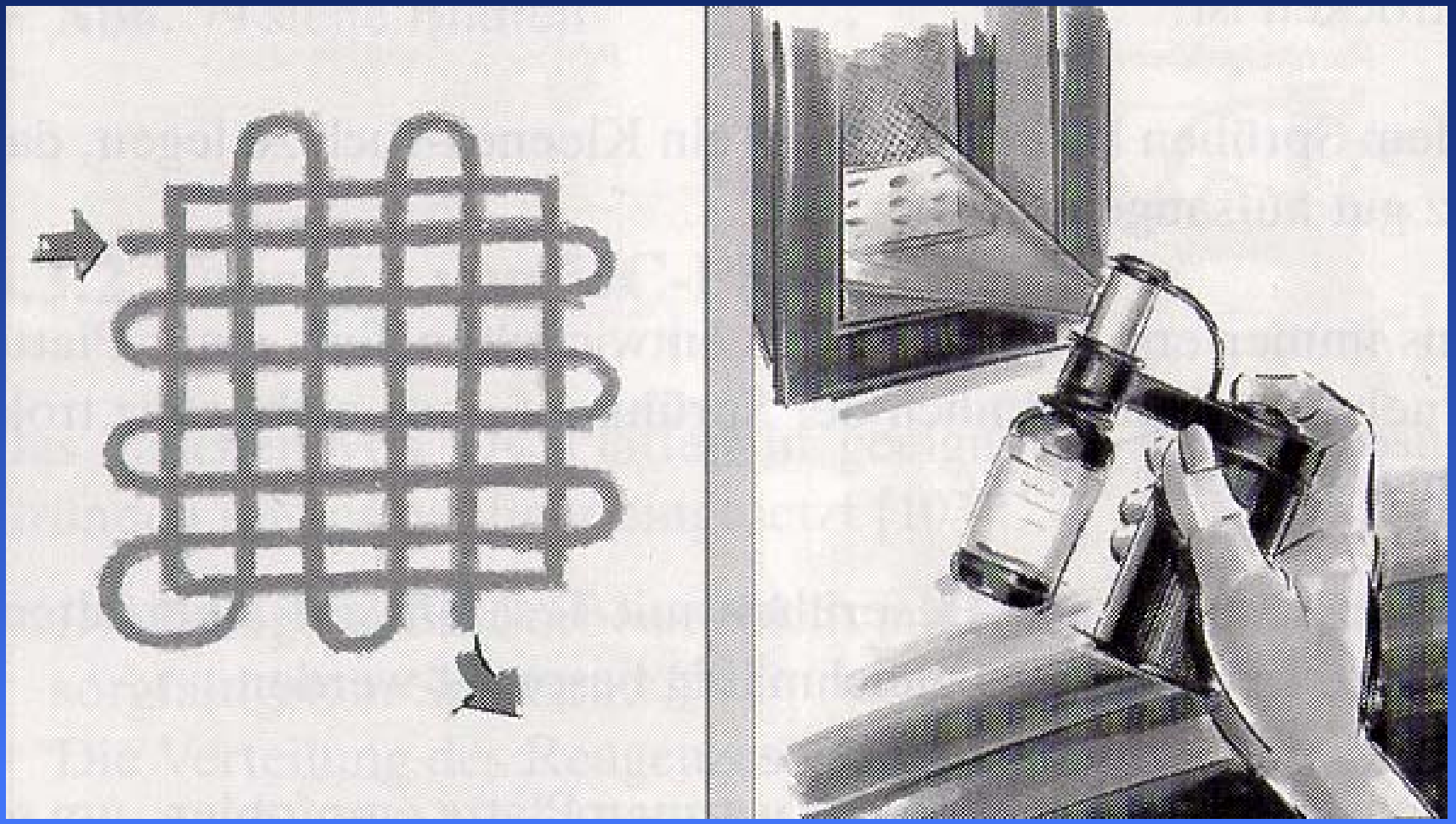


Jork, H., Funk, W., Fischer, W., Wimmer, H.: Thin-Layer Chromatography, volume 1a and b, VCH Weinheim 1990 and 1994.

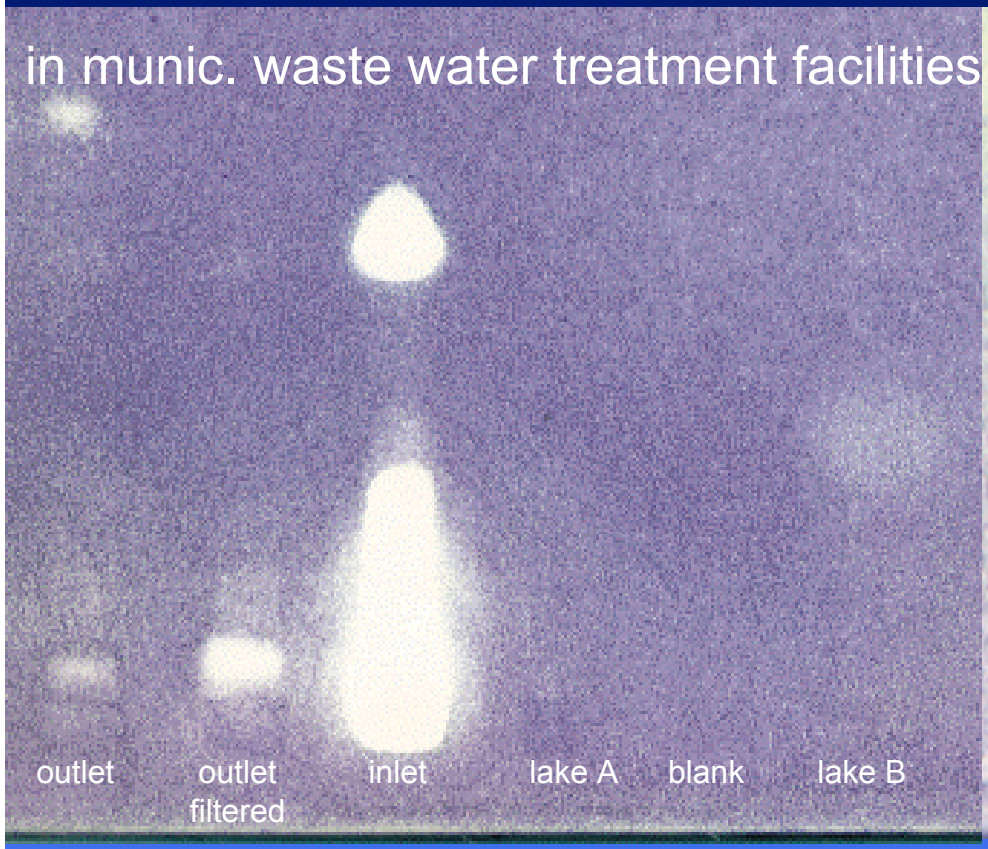
Derivatization techniques

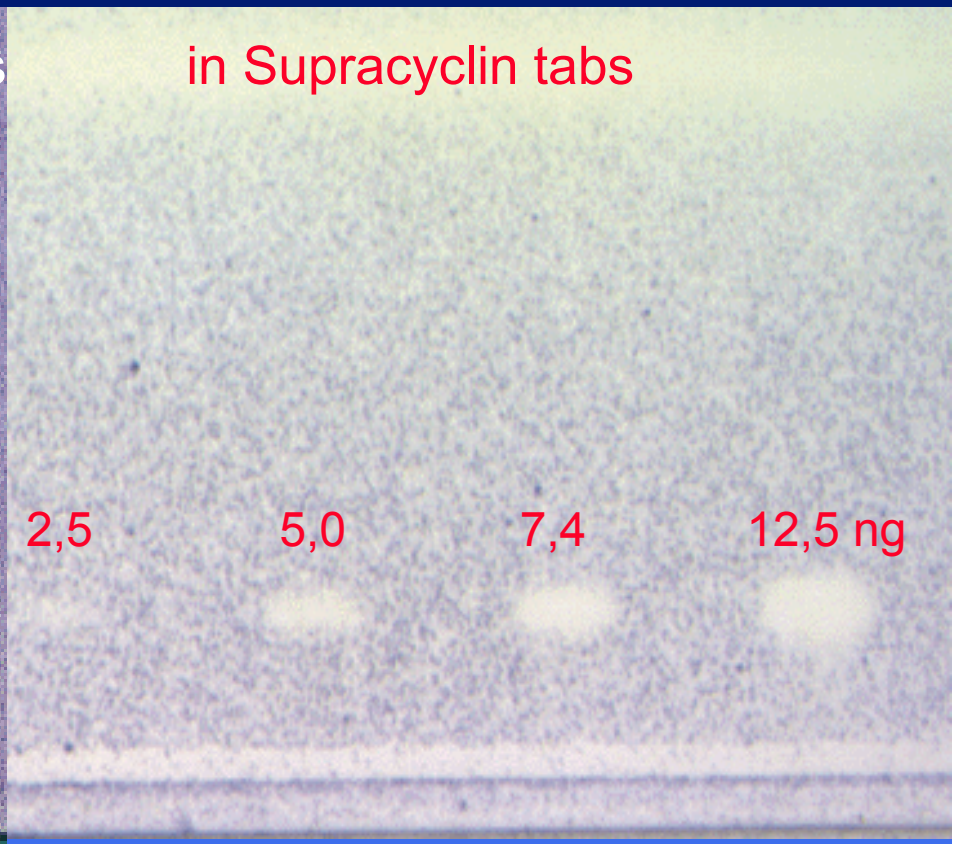
Derivatization by	Reproducibility (see CBS 72)	⟷	Without derivatization
• Dipping Device - the best way to do it	3 %		2%
• Spraying	5%		
• Evaporation			
• Mobile phase			
• Incorporation into layer			

Spraying scheme



Microbiological detection of antibiotics

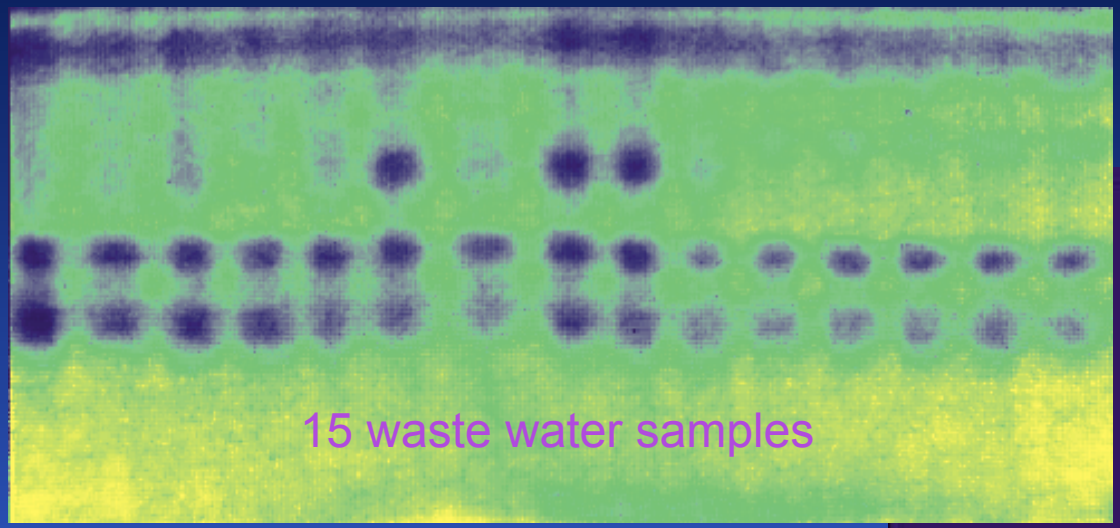


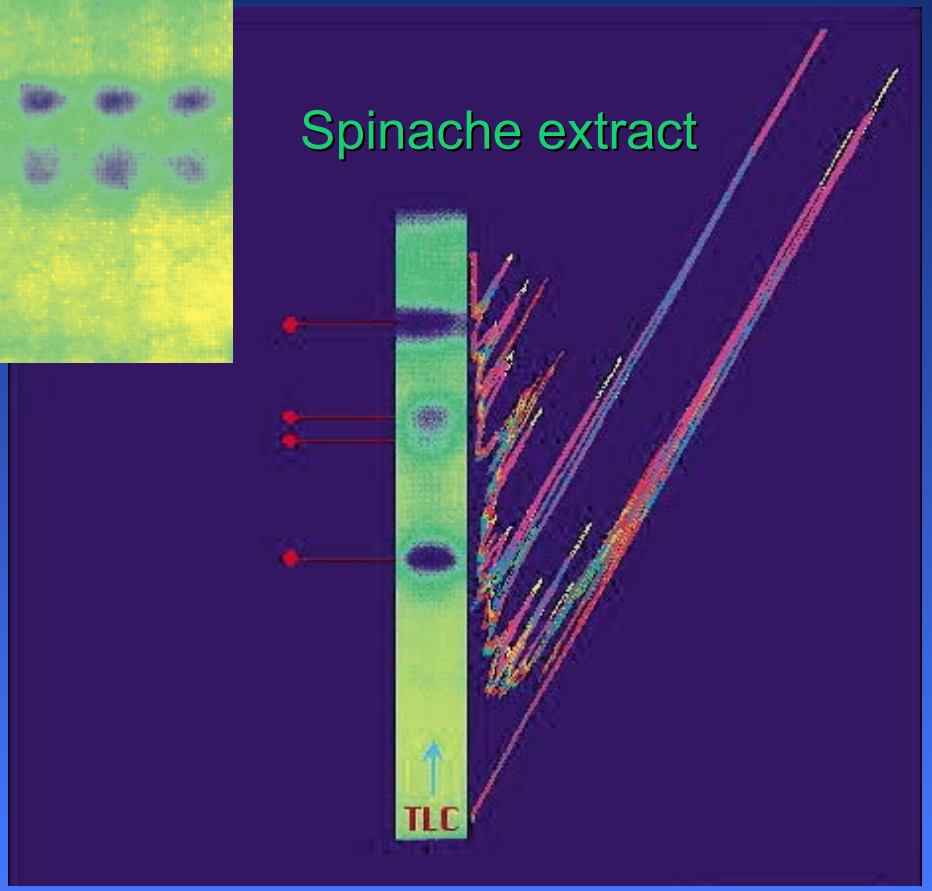


C. Weins, Staatl. Inst. für Gesundheit und Umwelt, Saarbrücken

Merck Bioautographic Test Kit „Chrom Biodip®“ , see CBS 85

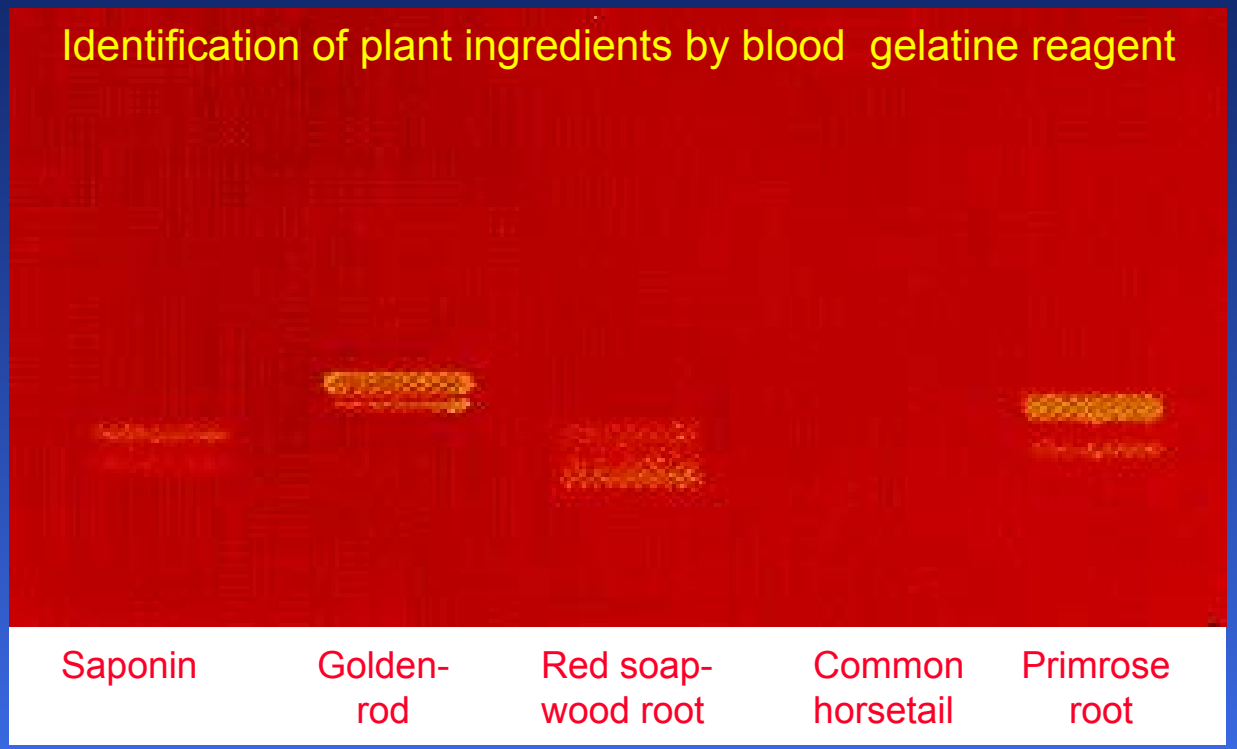
Microbiological detection of toxic compounds





W. Kreiss et al., Bayer AG,
Chroma Dex Test Kit “BiolumineX“, see CBS 88

Microbiological detection of saponins

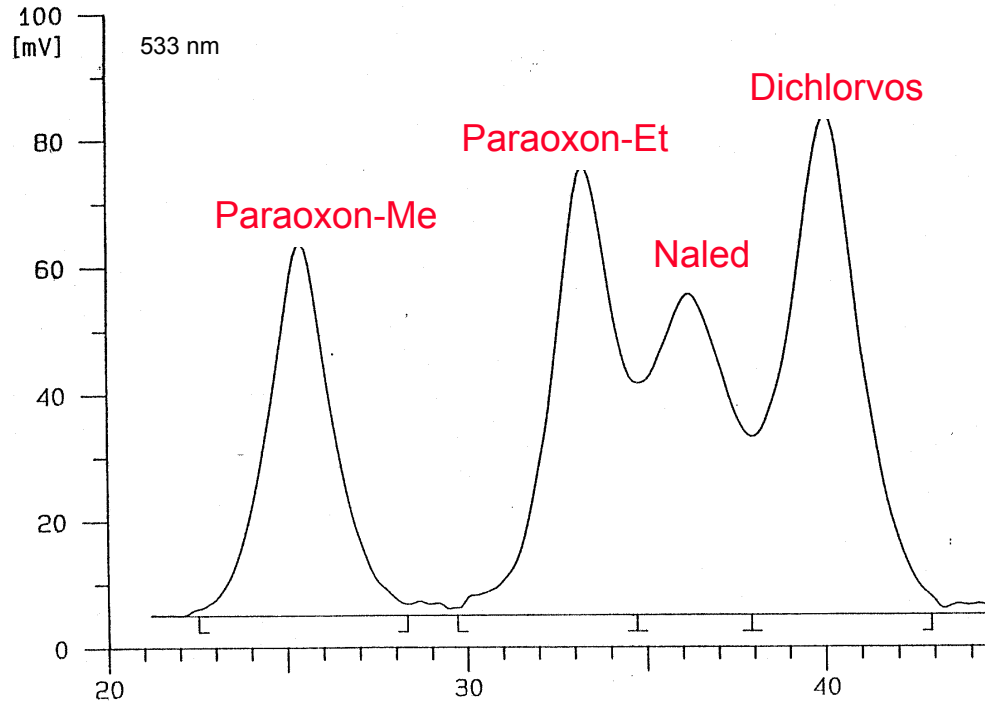


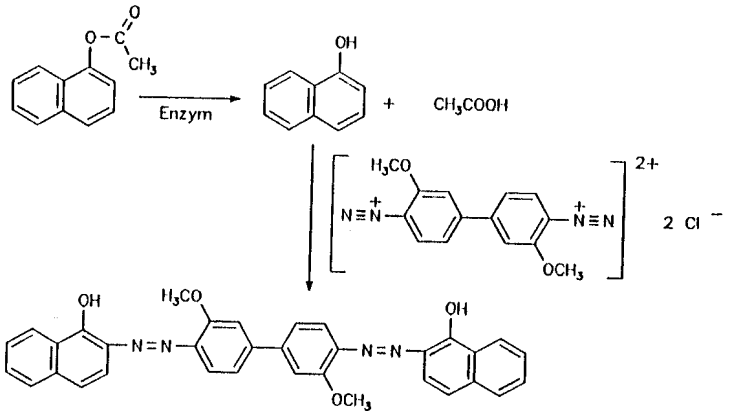
Hahn-Deinstrop, E.: Applied Thin-Layer Chromatography. Best practice and avoidance of mistakes, 2000, Wiley-VCH, Weinheim, ISBN 3527-298398.

Biochemical detection

Enzymes		used for...
Peroxidase	→	quinones
Urease, amylase	→	heavy metals, metal containing fungicides
Trypsin, chymotrypsin, cholinesterase	→	organo phosphates, carbamates, pentachlorphenol

Biochemical detection of cholinesterase inhibitors

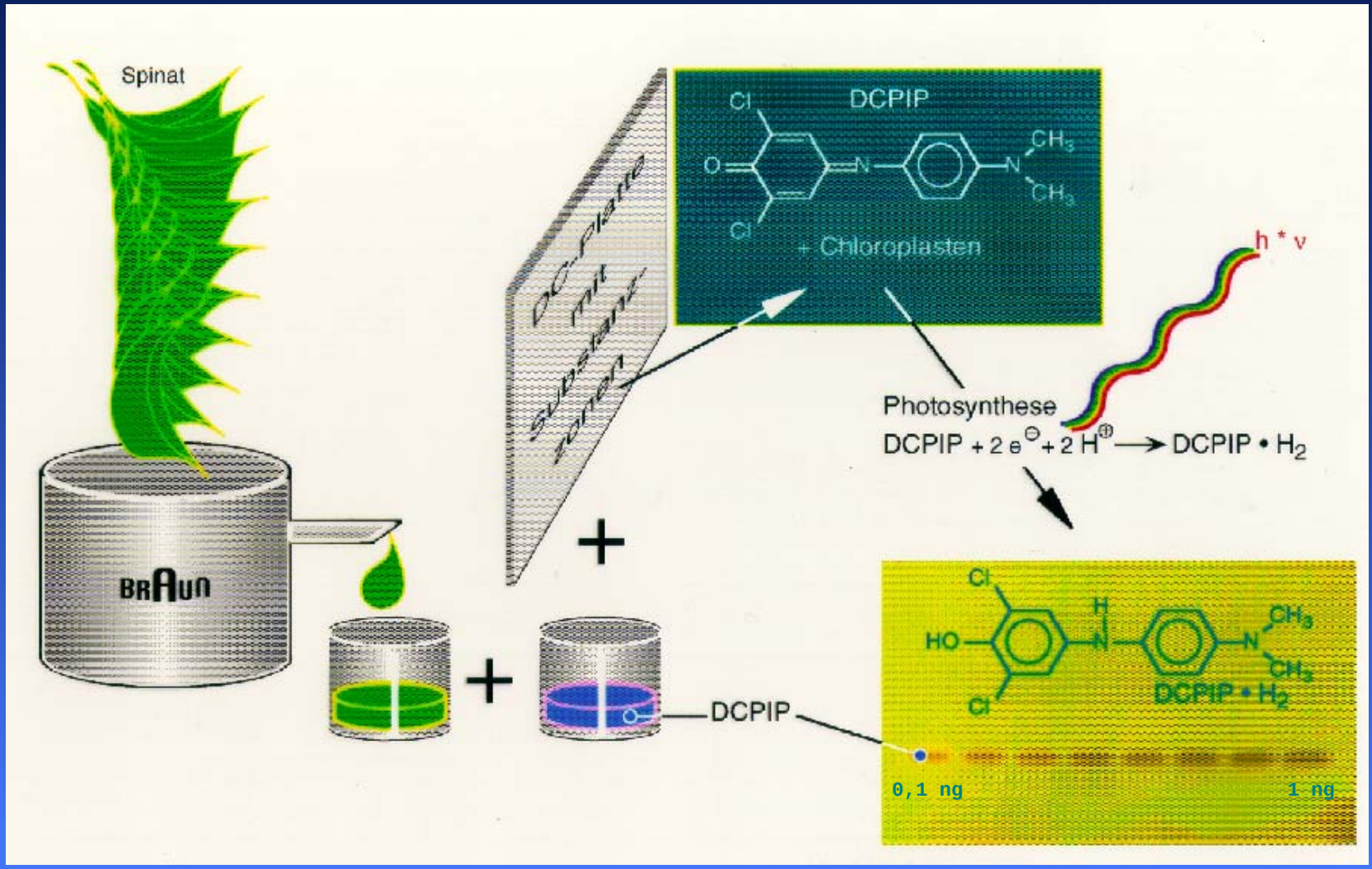




1. Dipping in a solution of cholinesterase
2. Incubation at 37 °C for 30 min
3. Dipping in a solution of
   1-naphthyl acetate and fast blue salt B

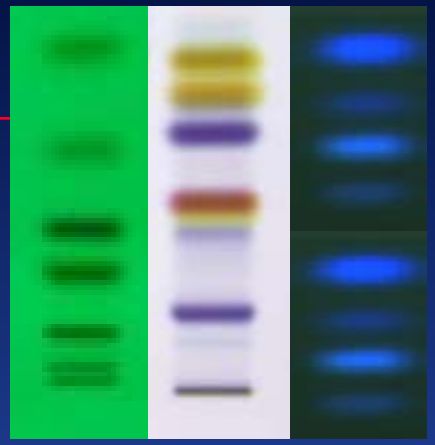
C. Weins, Staatl. Inst. für Gesundheit und Umwelt, Saarbrücken

Biochemical detection of photosynthesis inhibitors



klaus.burger@mail.isis.de

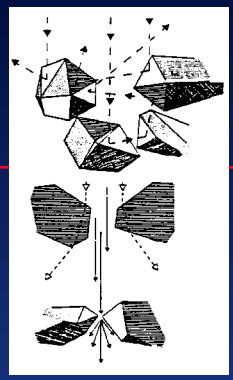
Scanning



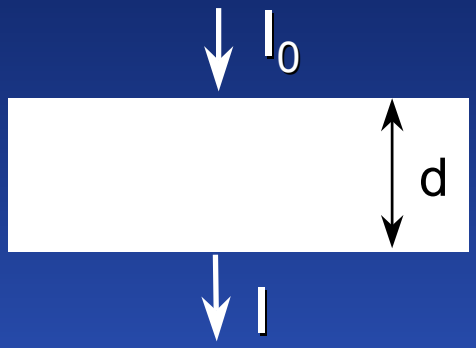
→ **UV, VIS, Fluorescence**

- Absorbance scan
- Fluorescence scan
- Multi-wavelength scan
- Multiple detection
- Spectra recording and library search

Absorbance



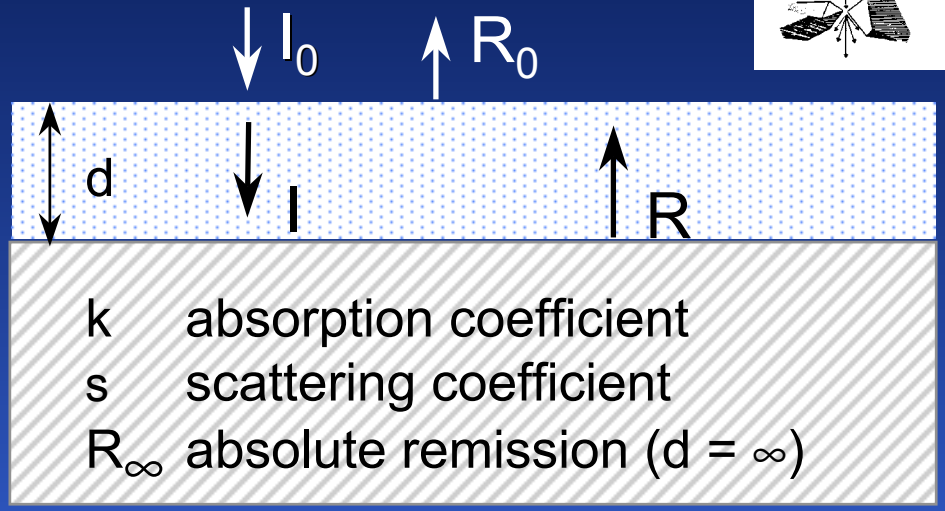
in solution (transmission)



$$E = \varepsilon * c * d$$

$$E \sim c$$

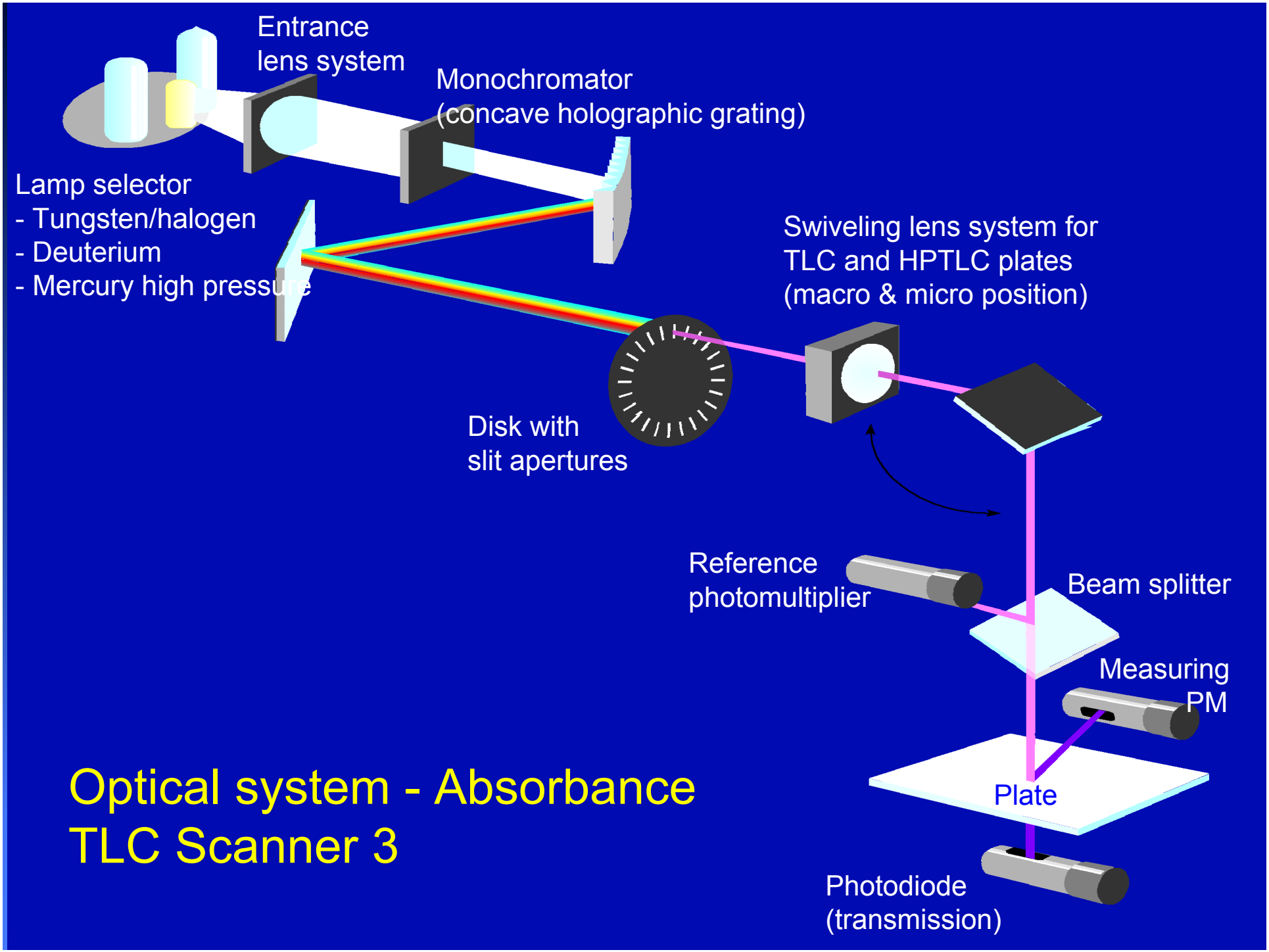
on the layer (remission)



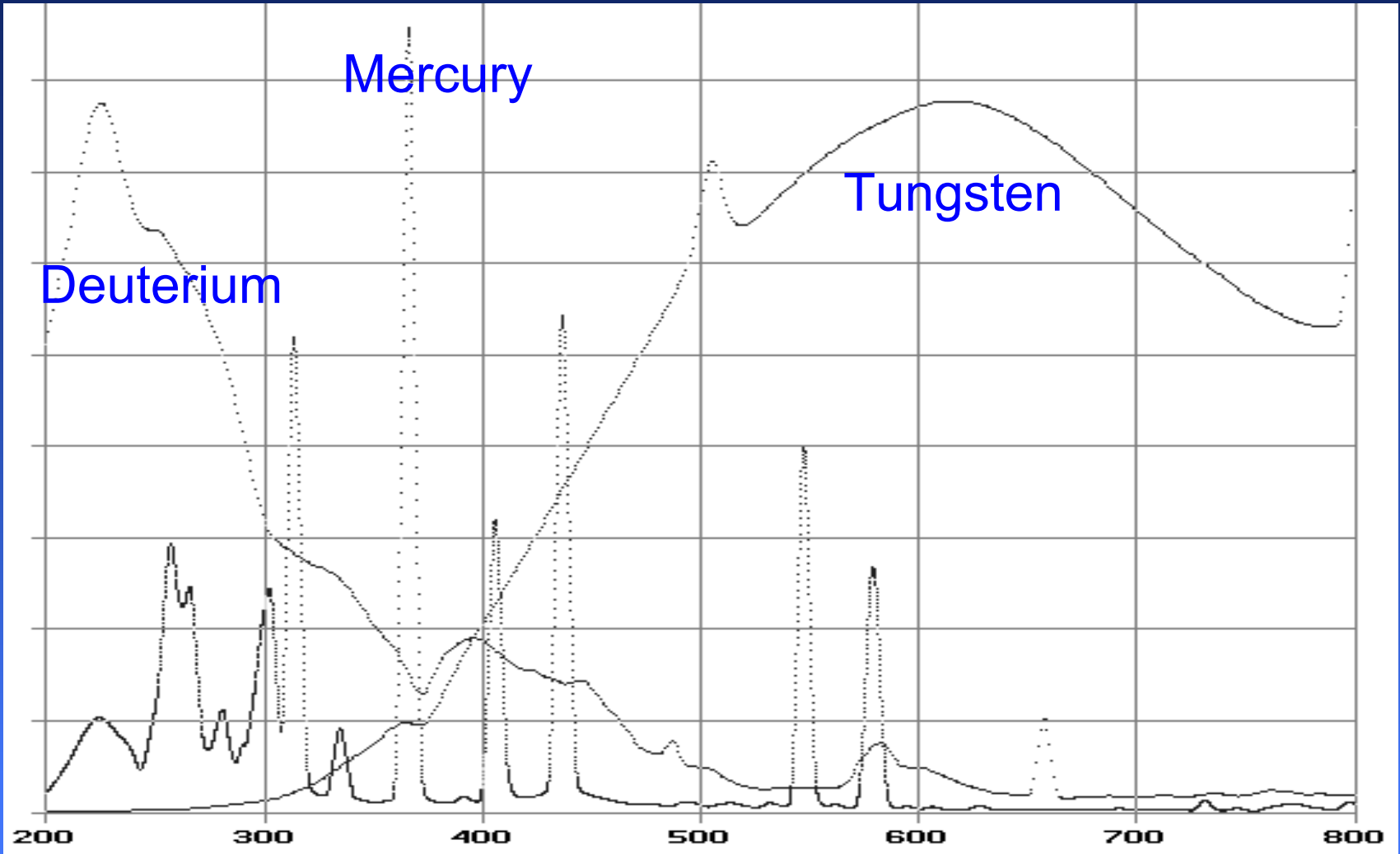
Kubelka Munk function

$$F(R_\infty) = \frac{k}{s} = \frac{(1 - R_\infty)^2}{2 R_\infty}$$

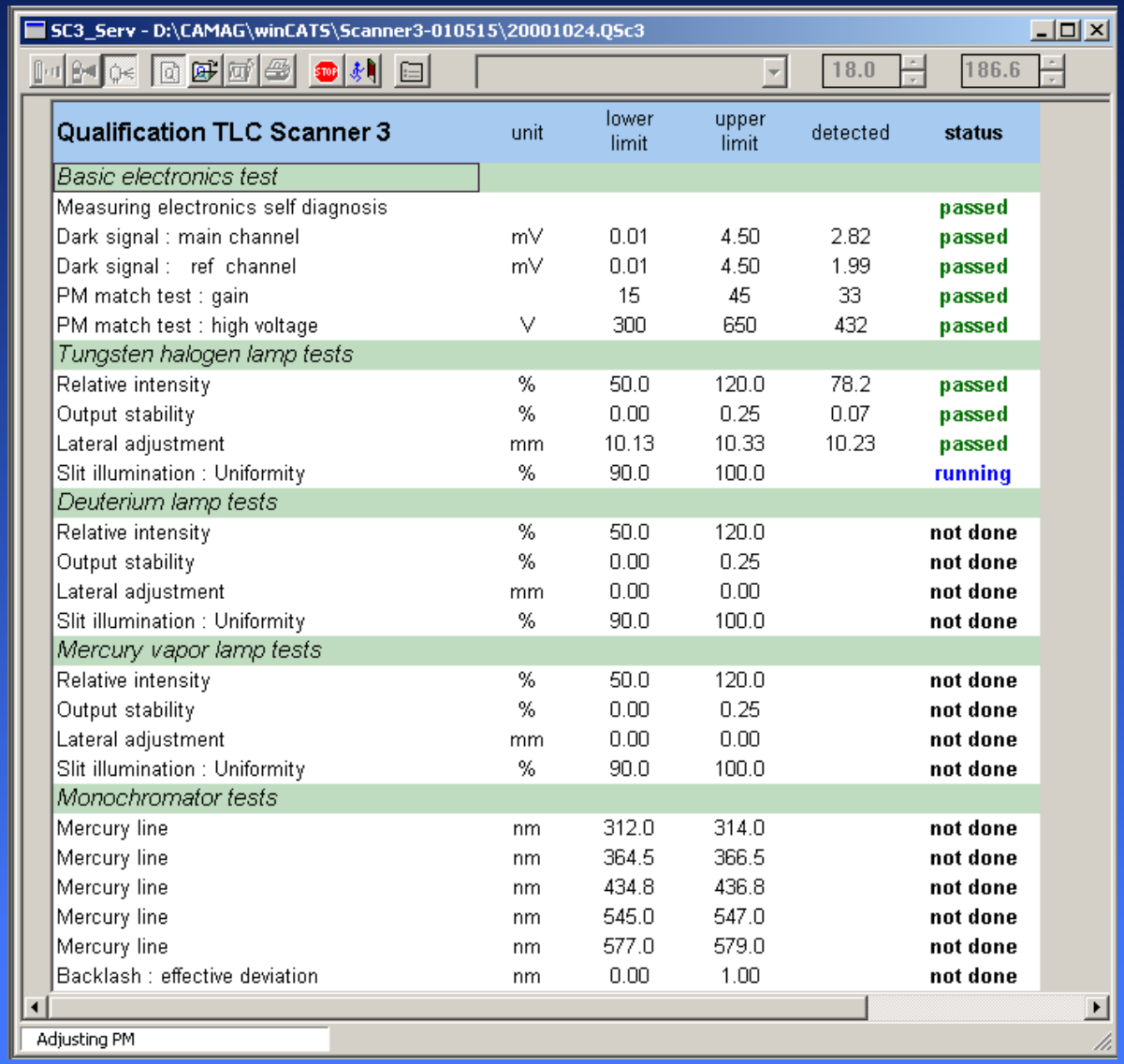
Preconditions:
- $d = \infty$
- $R_0 = 0$
- no direct reflexion
- dp < 1 mm


Entrance
lens system
Monochromator
(concave holographic grating)
Lamp selector
- Tungsten/halogen
- Deuterium
- Mercury high pressure
Swiveling lens system for
TLC and HPTLC plates
(macro & micro position)
Disk with
slit apertures
Reference
photomultiplier
Beam splitter
Measuring
PM
Plate
Photodiode
(transmission)
Optical system - Absorbance
TLC Scanner 3

Emission spectra of the lamps



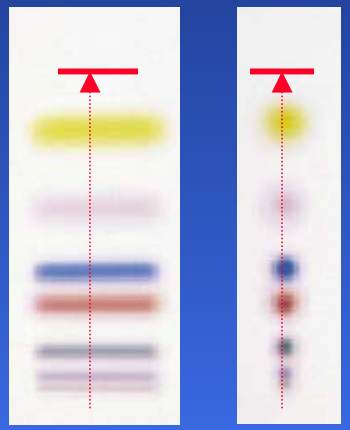
Scanner validation



Qualification TLC Scanner 3	unit	lower limit	upper limit	detected	status
Basic electronics test					
Measuring electronics self diagnosis					passed
Dark signal : main channel	mV	0.01	4.50	2.82	passed
Dark signal : ref channel	mV	0.01	4.50	1.99	passed
PM match test : gain		15	45	33	passed
PM match test : high voltage	V	300	650	432	passed
Tungsten halogen lamp tests					
Relative intensity	%	50.0	120.0	78.2	passed
Output stability	%	0.00	0.25	0.07	passed
Lateral adjustment	mm	10.13	10.33	10.23	passed
Slit illumination : Uniformity	%	90.0	100.0		running
Deuterium lamp tests					
Relative intensity	%	50.0	120.0		not done
Output stability	%	0.00	0.25		not done
Lateral adjustment	mm	0.00	0.00		not done
Slit illumination : Uniformity	%	90.0	100.0		not done
Mercury vapor lamp tests					
Relative intensity	%	50.0	120.0		not done
Output stability	%	0.00	0.25		not done
Lateral adjustment	mm	0.00	0.00		not done
Slit illumination : Uniformity	%	90.0	100.0		not done
Monochromator tests					
Mercury line	nm	312.0	314.0		not done
Mercury line	nm	364.5	366.5		not done
Mercury line	nm	434.8	436.8		not done
Mercury line	nm	545.0	547.0		not done
Mercury line	nm	577.0	579.0		not done
Backlash : effective deviation	nm	0.00	1.00		not done

Slit scan

- Resolution increases with
  - number of datapoints (smaller micro steps)
  - decreasing width of slit - but less intense signal
- Slit dimension
  - Slit length ⟷
    - bands 50 - 70 %
    - spots 120 %
  - Slit width ↕



the higher, the better light intensity, but the worse resolution

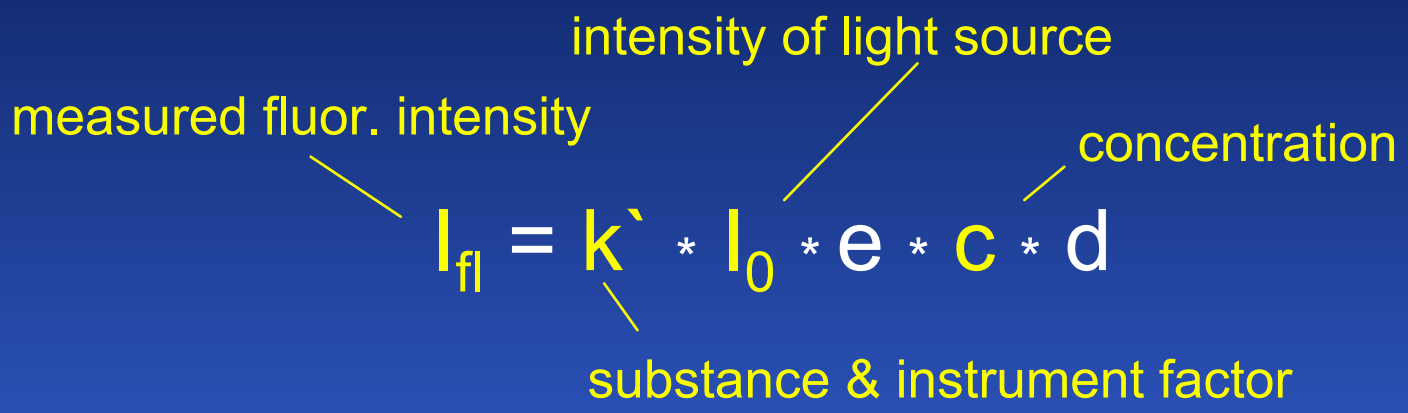
Fluorescence

- Substances with fixed molecular structure
- Fluorophore

- ✓ Aromatic systems
- ✓ Compounds with conjugated double bonds
- ✓ Carbonyls
- ✓ Condensated heterocycles

Fluorescence

Linear correlation between fluorescence intensity and concentration:

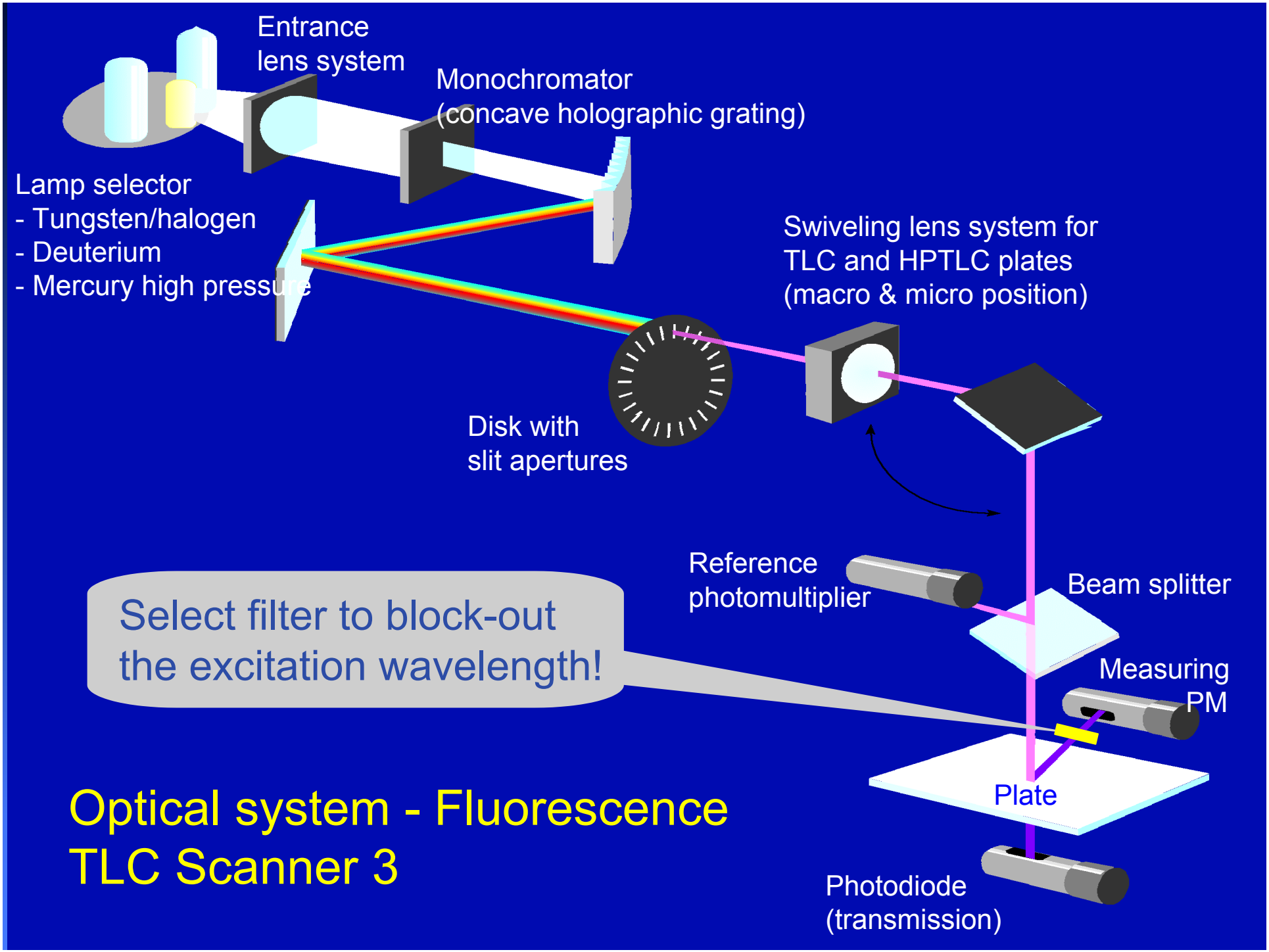


Preconditions

- strict monochromatism of the exciting light
- low concentration level

Advantages

- high sensitivity (pg-range)
- high selectivity
- wide linear concentration range


Entrance
lens system
Monochromator
(concave holographic grating)
Lamp selector
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- Deuterium
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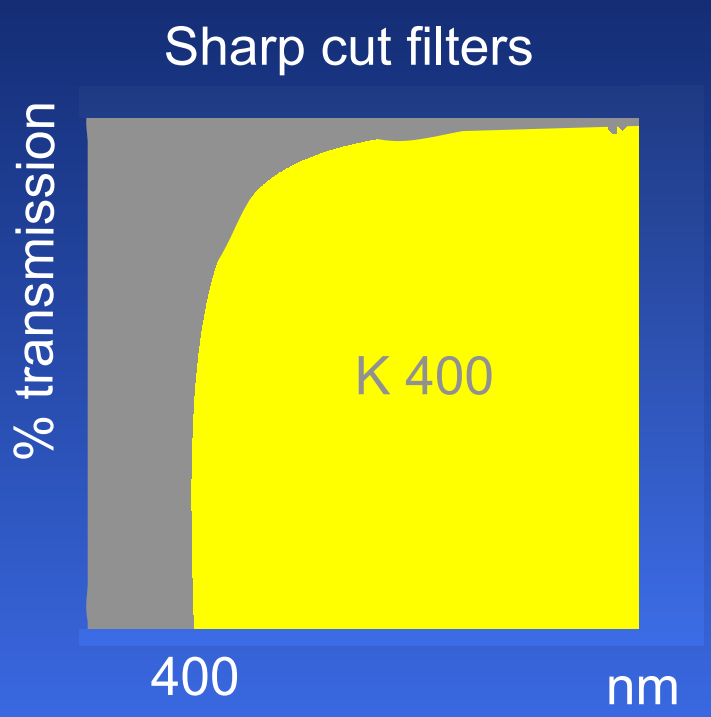
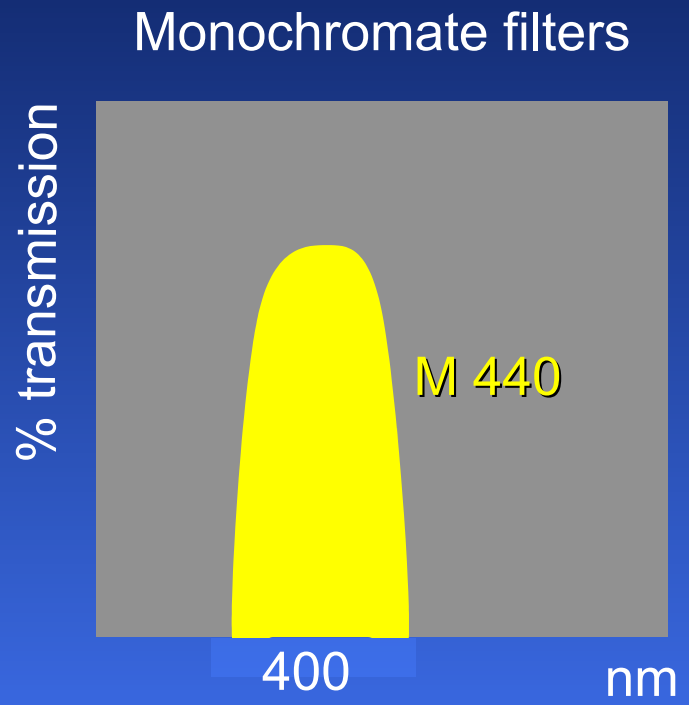
Swiveling lens system for
TLC and HPTLC plates
(macro & micro position)
Disk with
slit apertures
Reference
photomultiplier
Beam splitter
Measuring
PM
Plate
Photodiode
(transmission)
Select filter to block-out
the excitation wavelength!
Optical system - Fluorescence
TLC Scanner 3

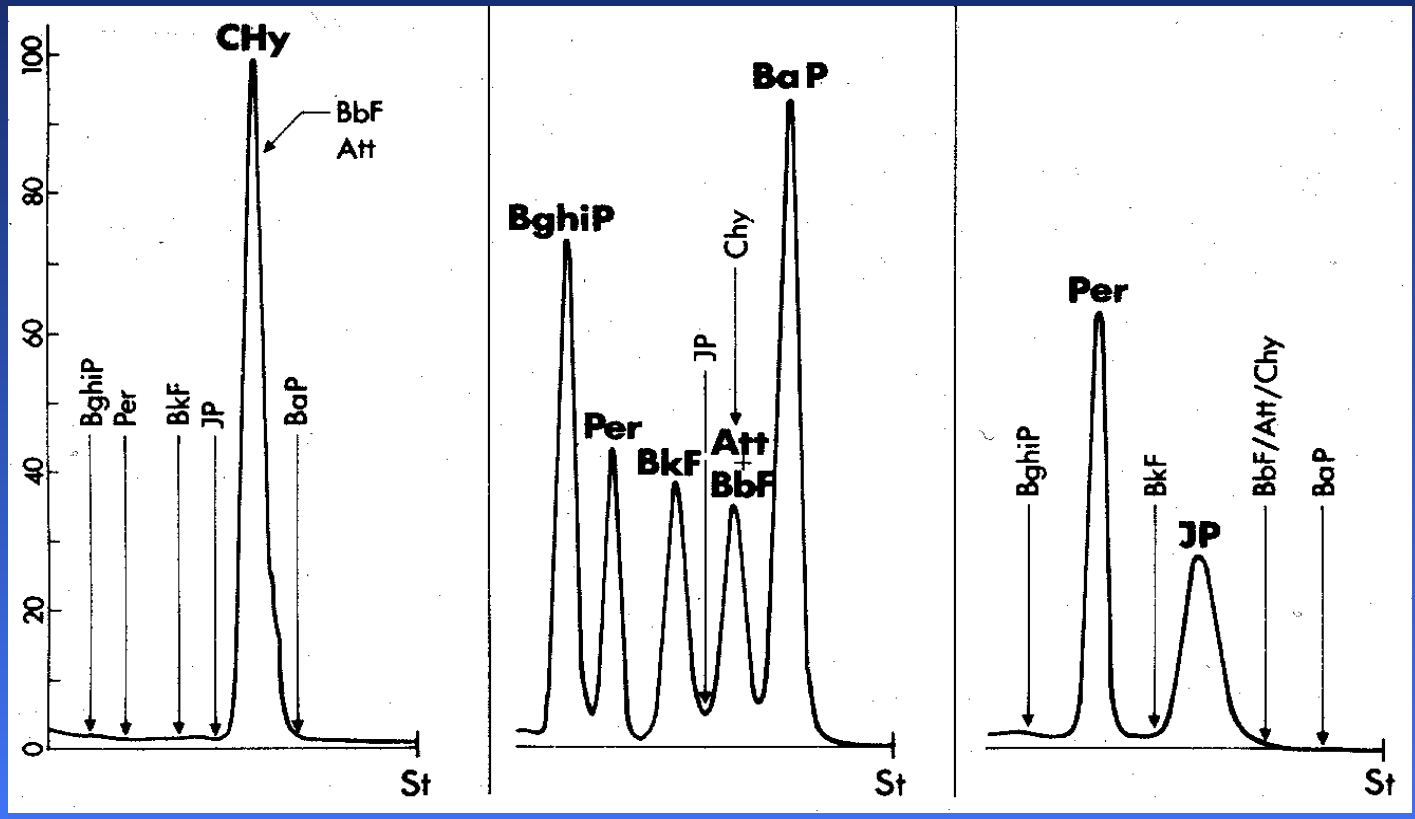
Types of fluorescence filter





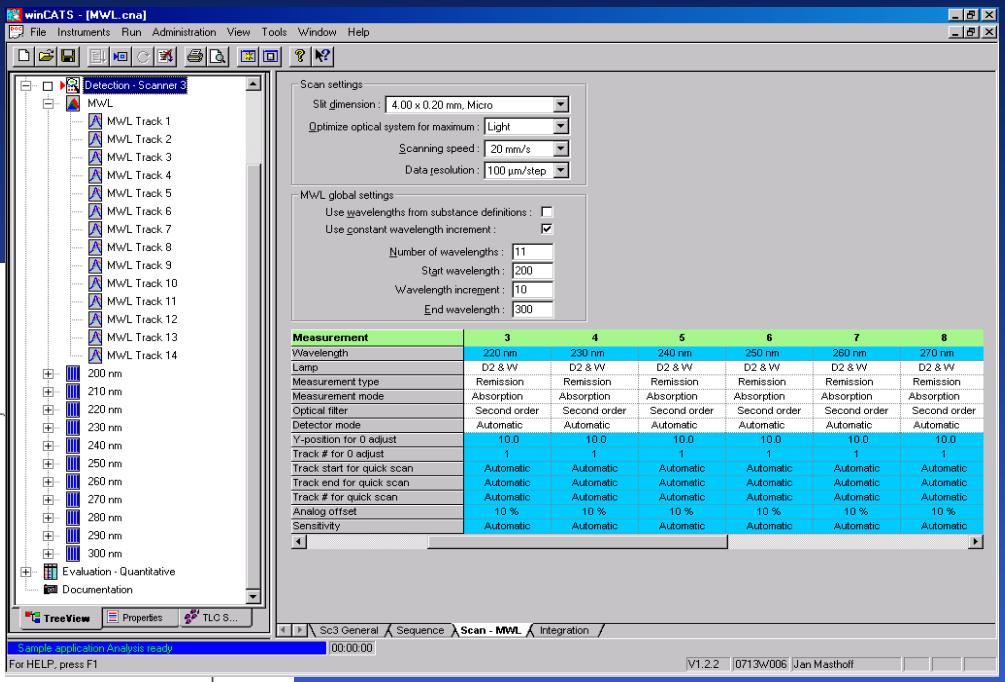
Selectivity by ideal excitation and filter combination

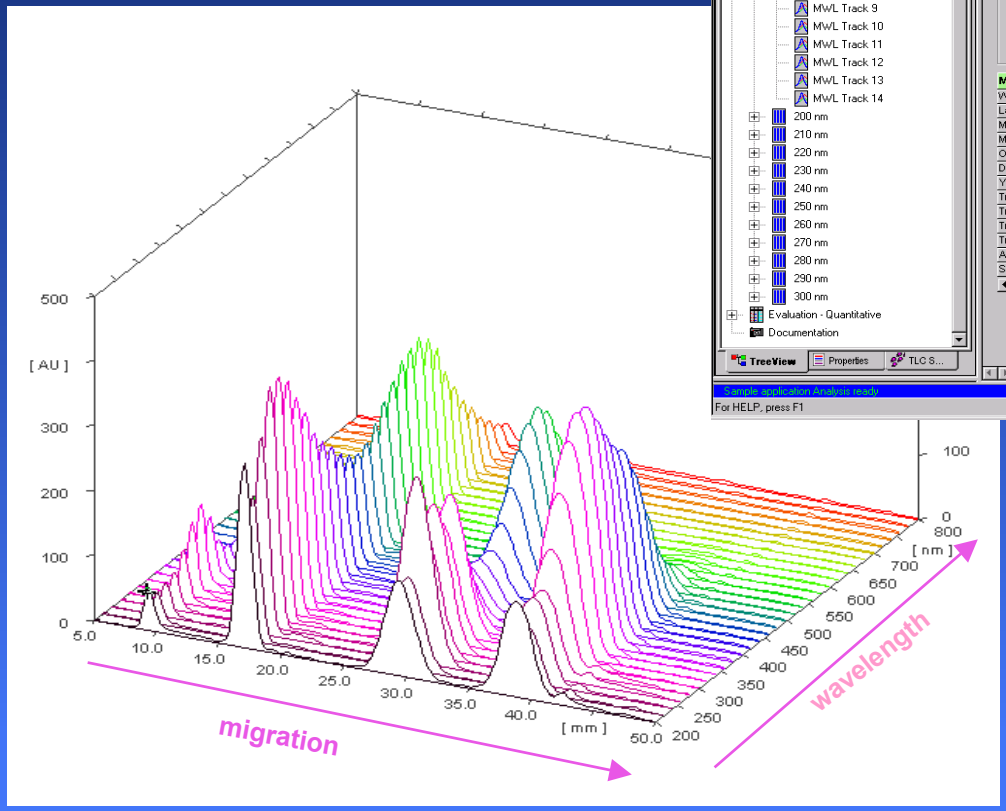
266/M365 nm 365/M436 nm 436/M578 nm



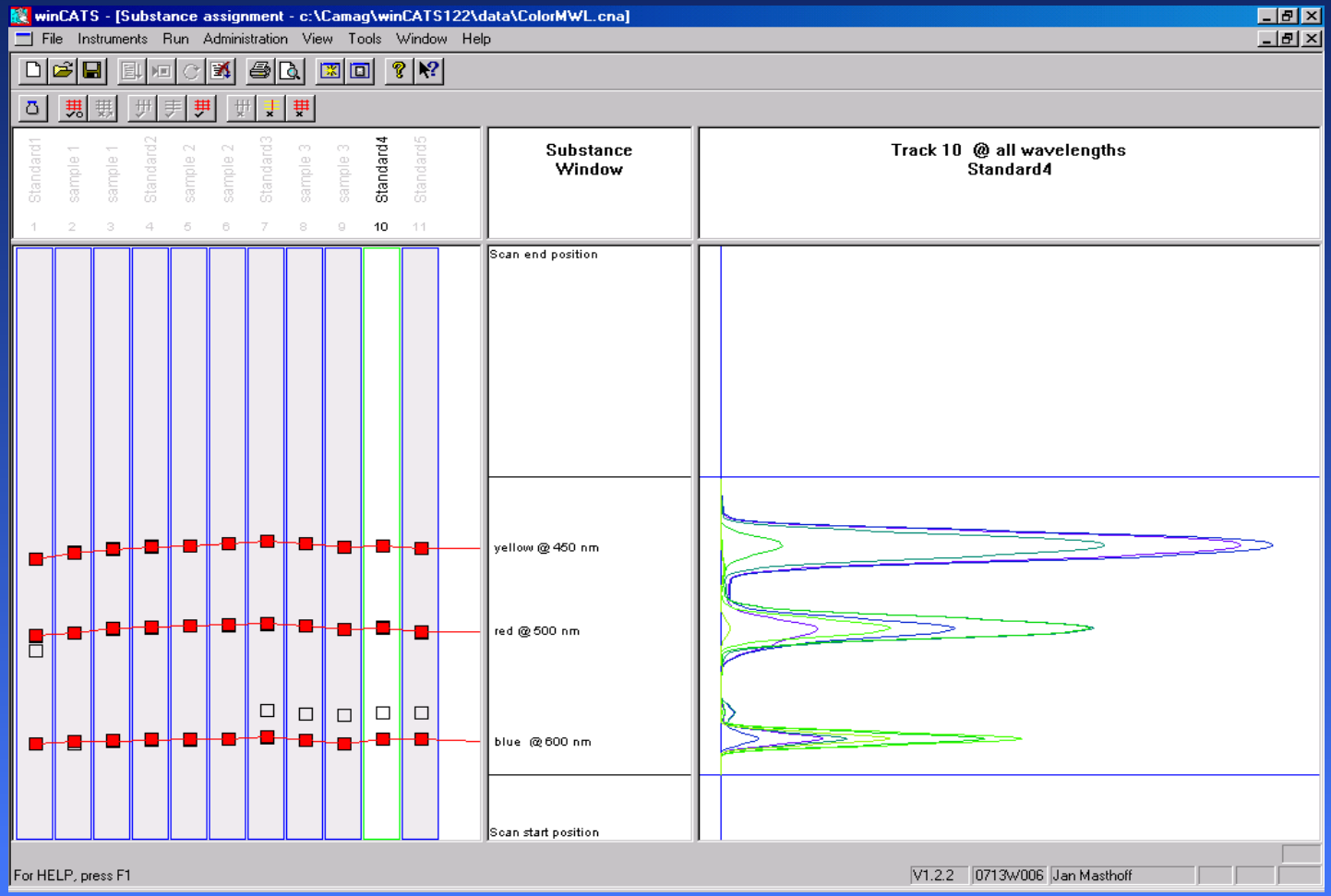
Jork, H., Funk, W., Fischer, W., Wimmer, H.: Thin-Layer Chromatography, volume 1a and b, VCH Weinheim 1990 and 1994.

Multi-wavelength scan

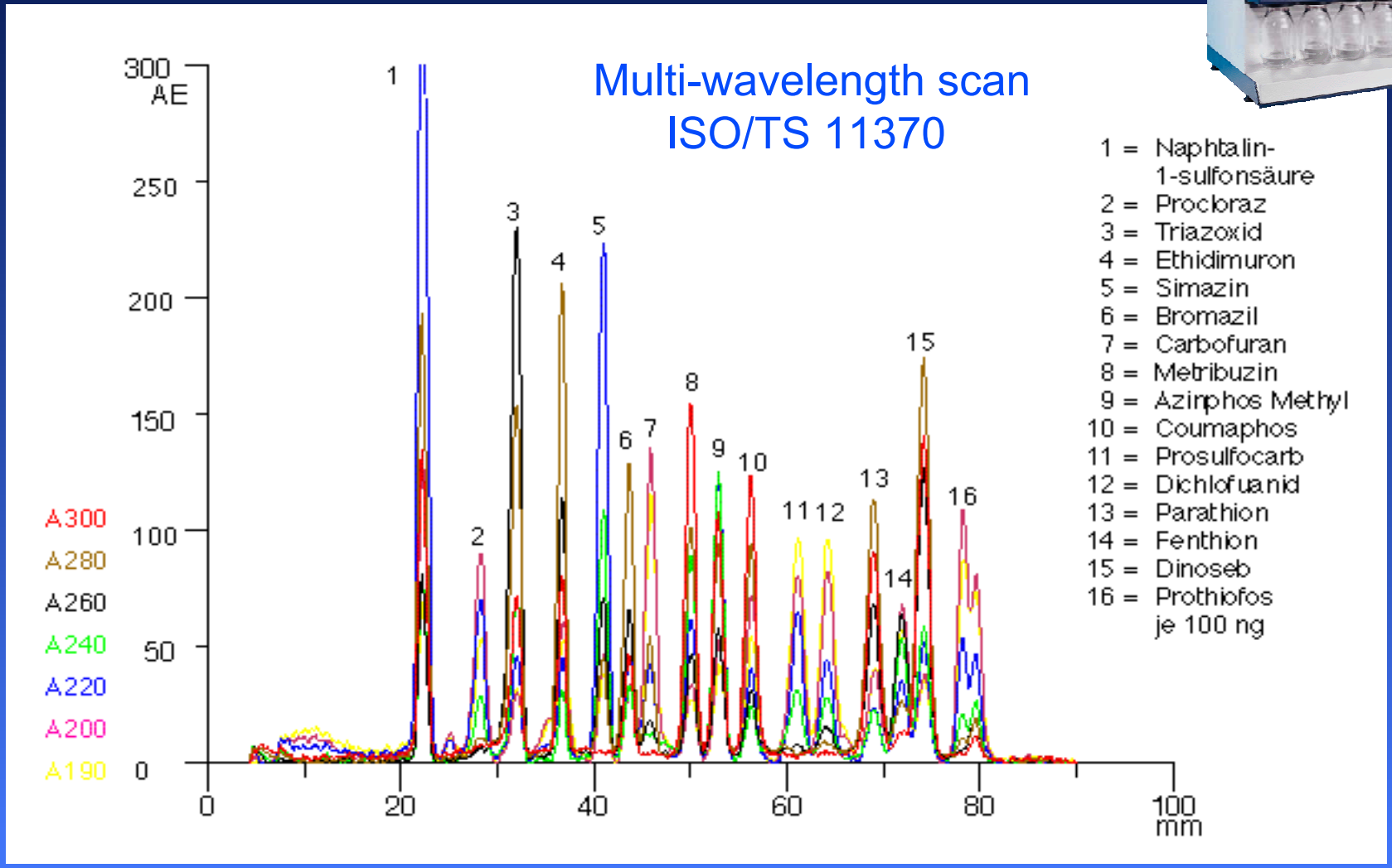




Multi-wavelength scan

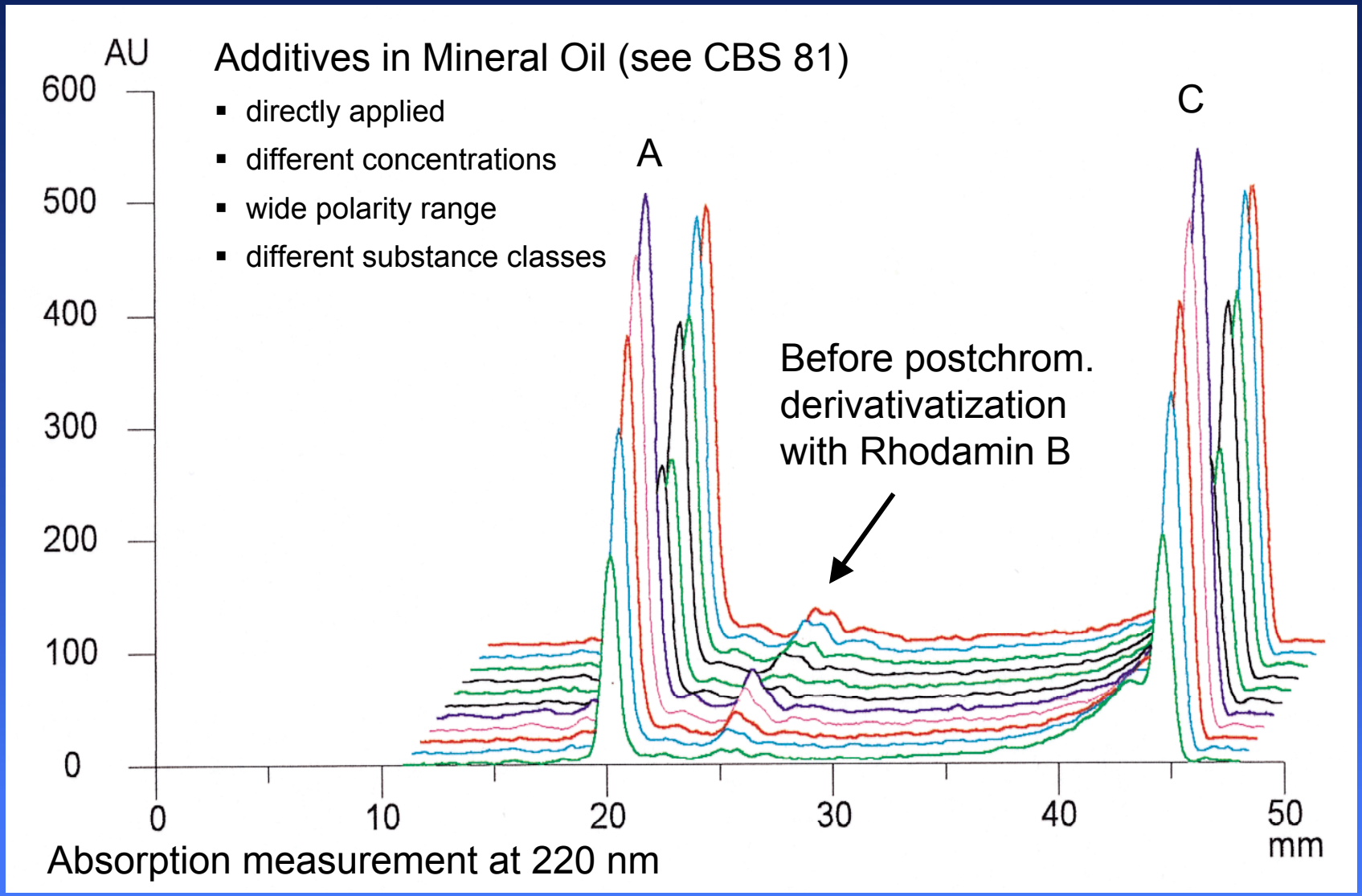


Pesticides in drinking and surface water

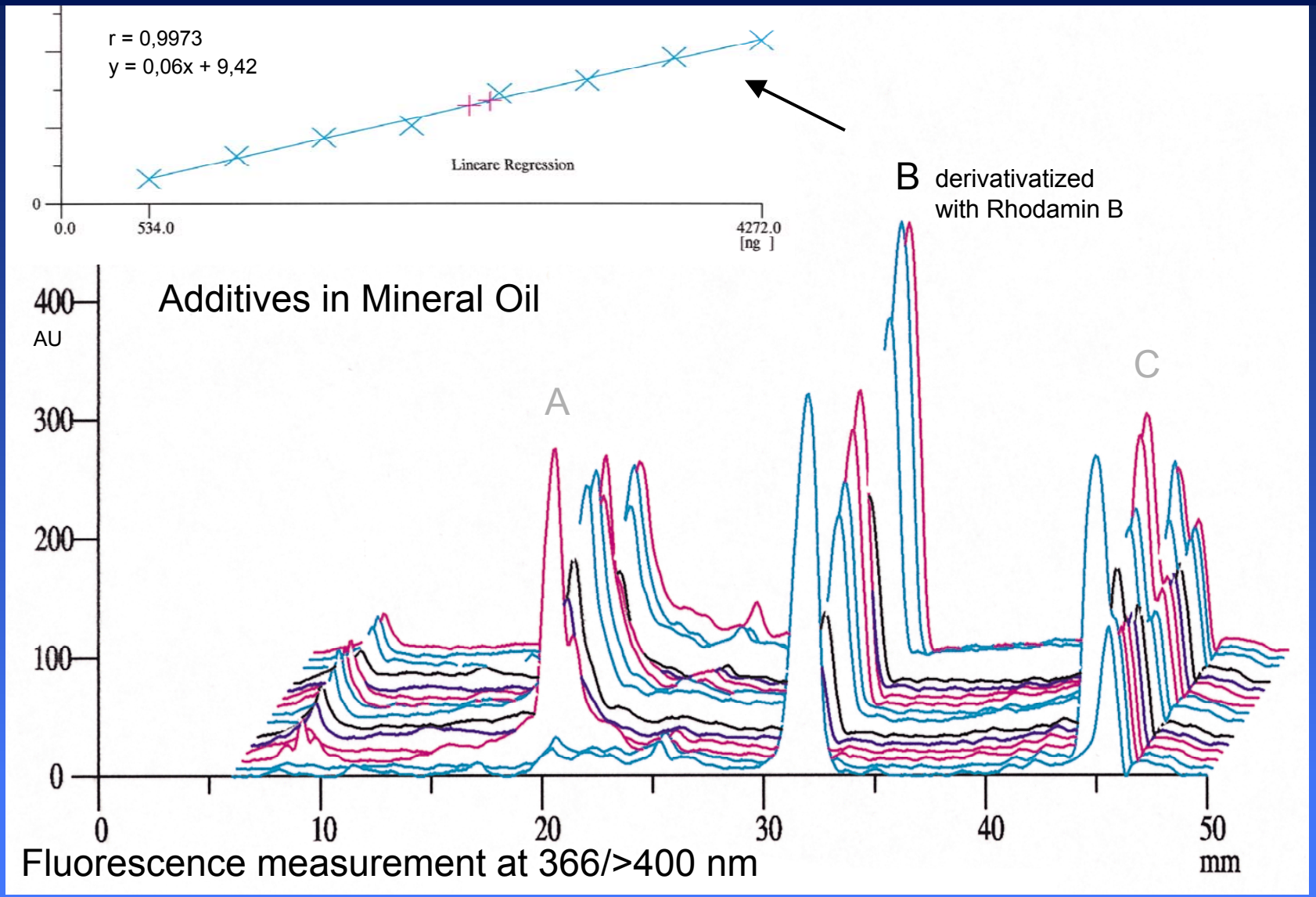


klaus.burger@mail.isis.de

Multiple detection



Multiple detection



Spectra recording

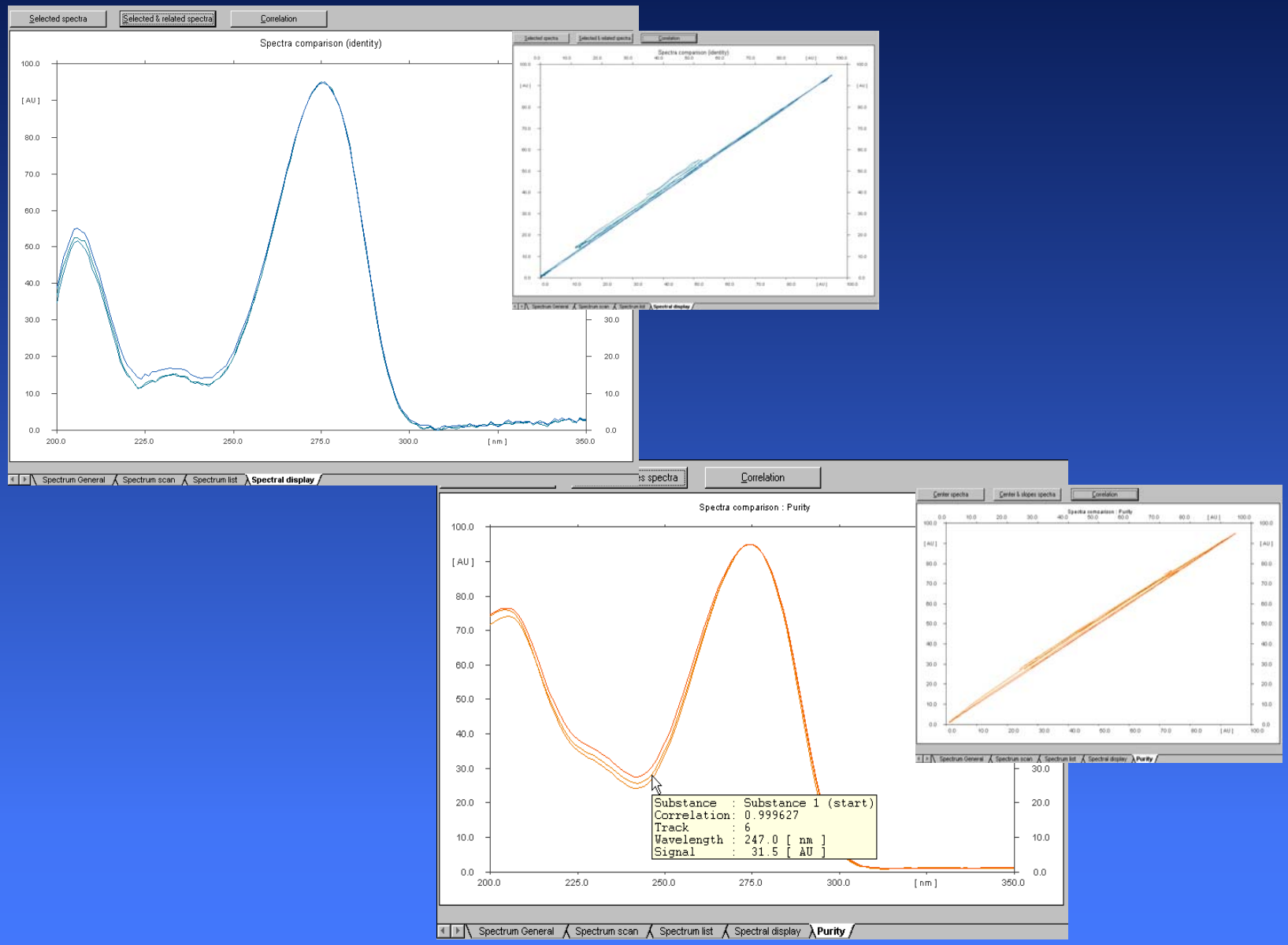
$$\lambda_{\text{Sample corr.}} = \lambda_{\text{Sample}} - \lambda_{\text{Lamp}} - \lambda_{\text{Background}}$$

Difference to spectra in solution

- no solvent
- adsorbed on the layer

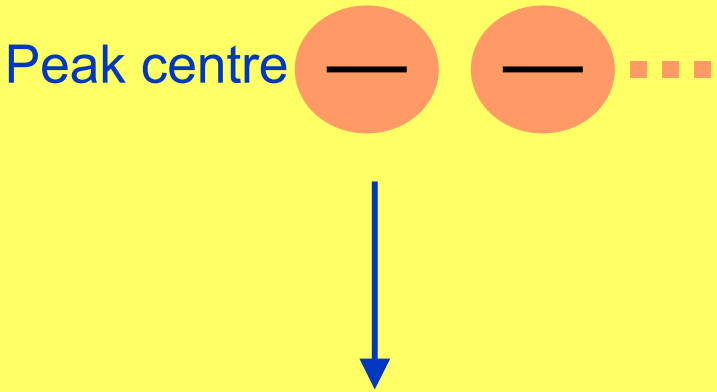
Note: Compare spectra at similar concentrations!

Spectra recording



Spectra calculation

Identity



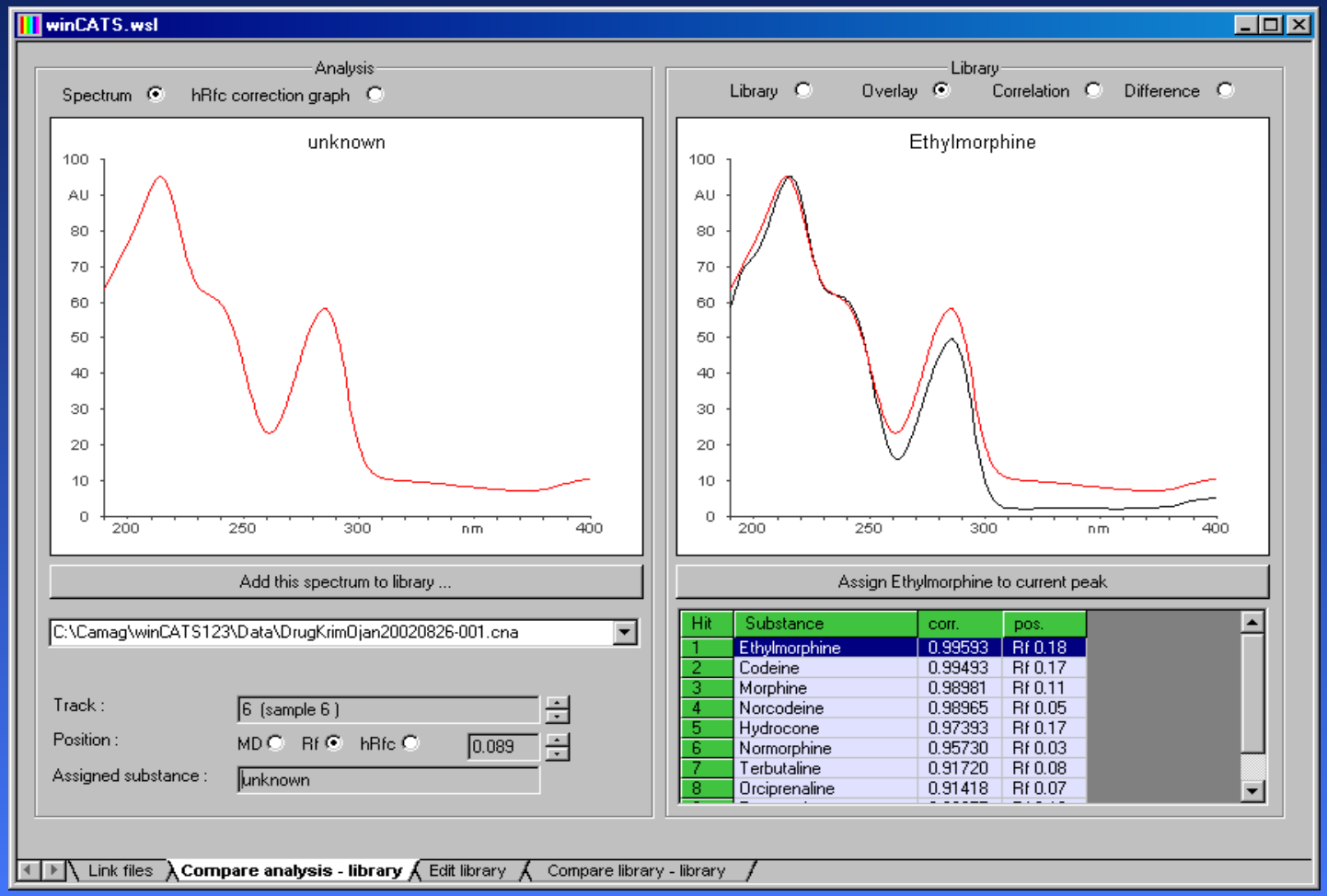
Comparison of correlations of
2 standards adjoining
&
analysis - adjoining standard

Purity

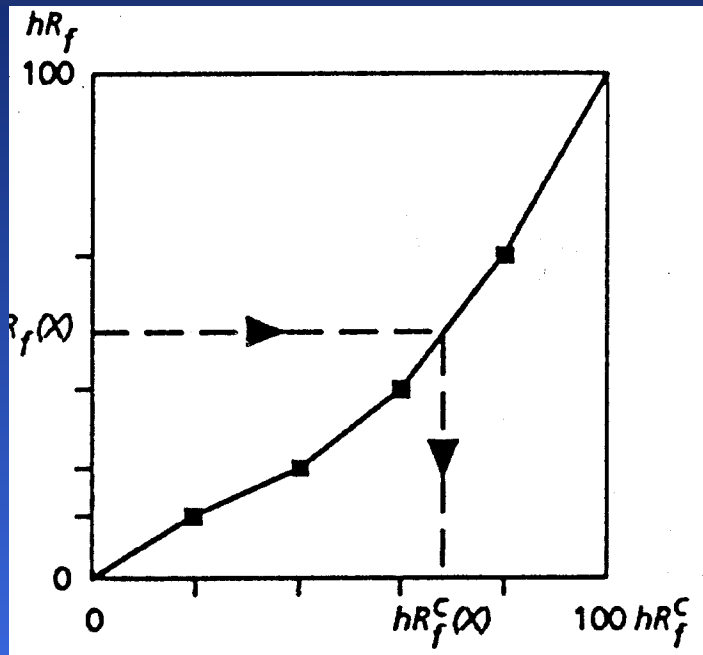
Peak start
Peak centre
Peak end

Comparison of correlations of
peak start - peak centre
&
peak centre - peak end

Spectra library



Calculation of hR_F^C



$$R_f^C(X) = R_f^C(A) + \frac{\Delta^C}{\Delta}\ [hR_f(X) - hR_f(A)],$$

$$\Delta^C = hR_f^C(B) - hR_f^C(A) \text{ and}$$

$$\Delta = hR_f(B) - hR_f(A)$$

Overview of quantification

- ✓ Calibration
- ✓ Dual wavelength scan
- ✓ Track optimization
- ✓ Videoscanning or conventional scanners
- ✓ Validation
- ✓ GLP

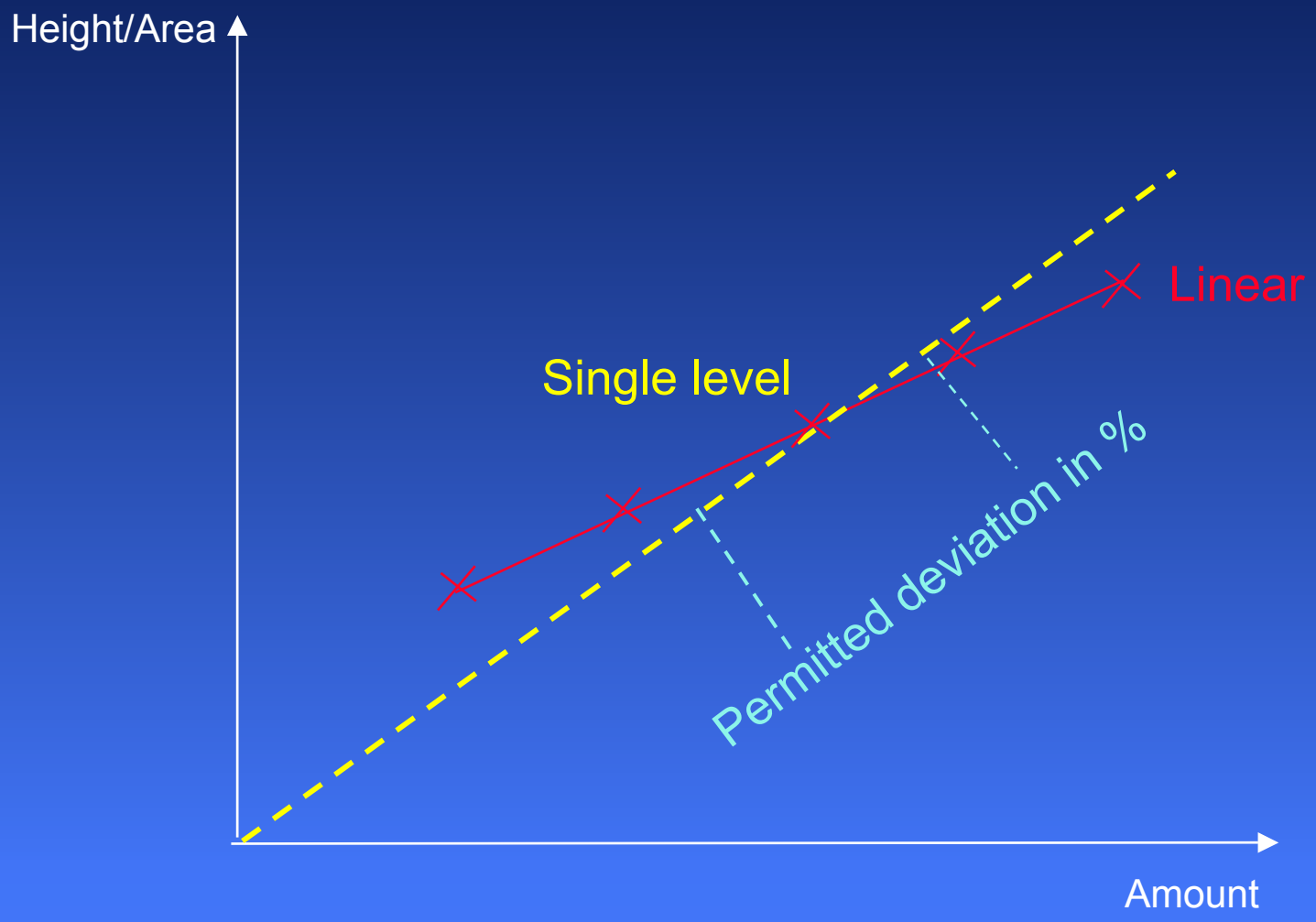
Calibration

- start with limit of quantification LOQ
- use external standard

1. Single standard calibration

- only 1 standard level
- = linear function through standard level and origin
- Precondition: first use linear regression to determine the permitted deviation in %
- analyte concentration around the standard level
- more tracks of analytes on one plate!

Single standard calibration



Calibration

Multi level calibration

- permitted deviation 0 %
  (within the calibration function)
- at least 5 standard levels (DIN 38 402 part 52)

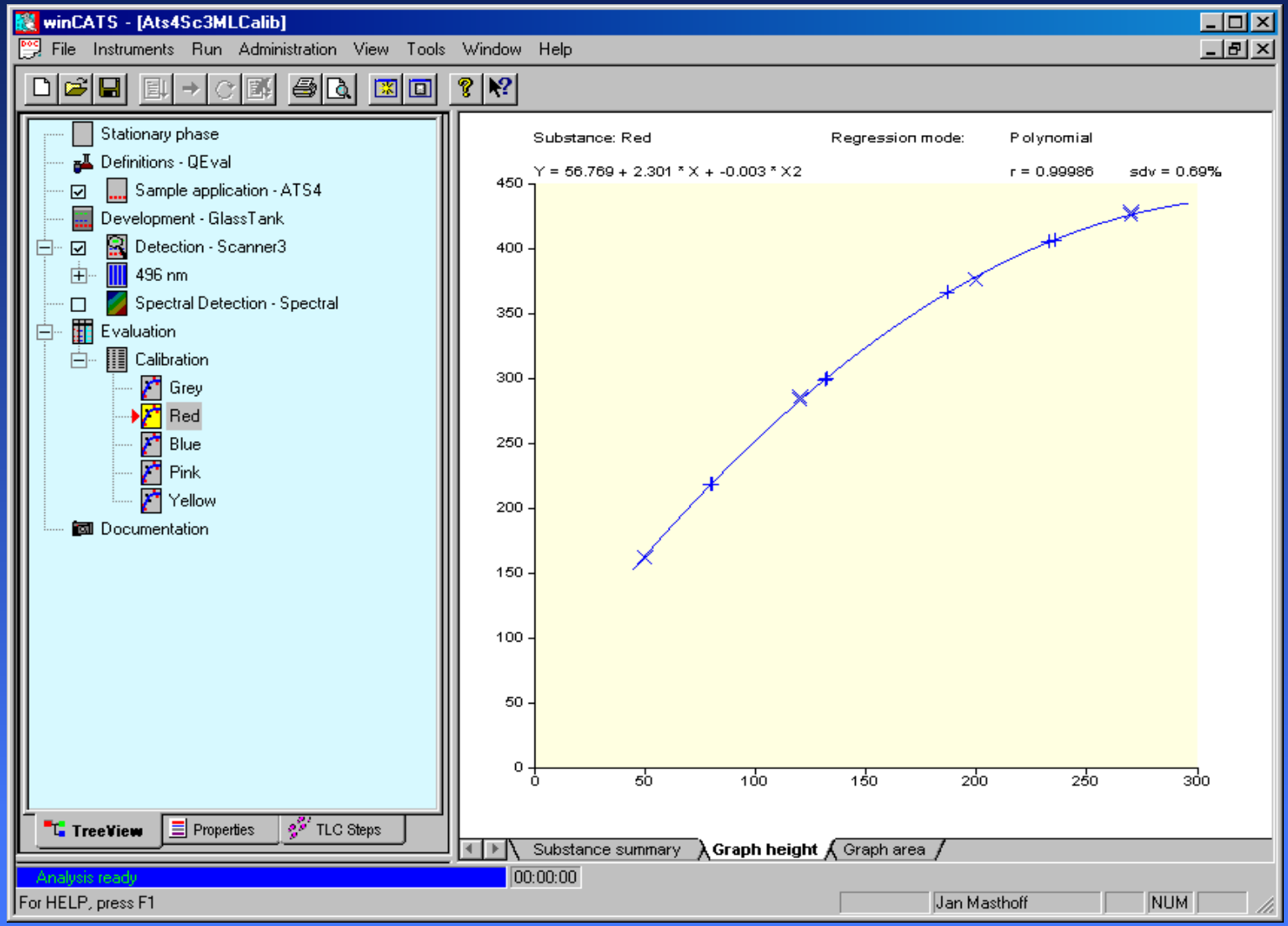
2. Linear calibration function $y = ax + b$

- narrow concentration range (1:10)
- generally by fluorescence measurement

3. Polynomial function $y = ax^2 + bx + c$

- wide concentration range (1:100)

Polynomial regression



Calibration

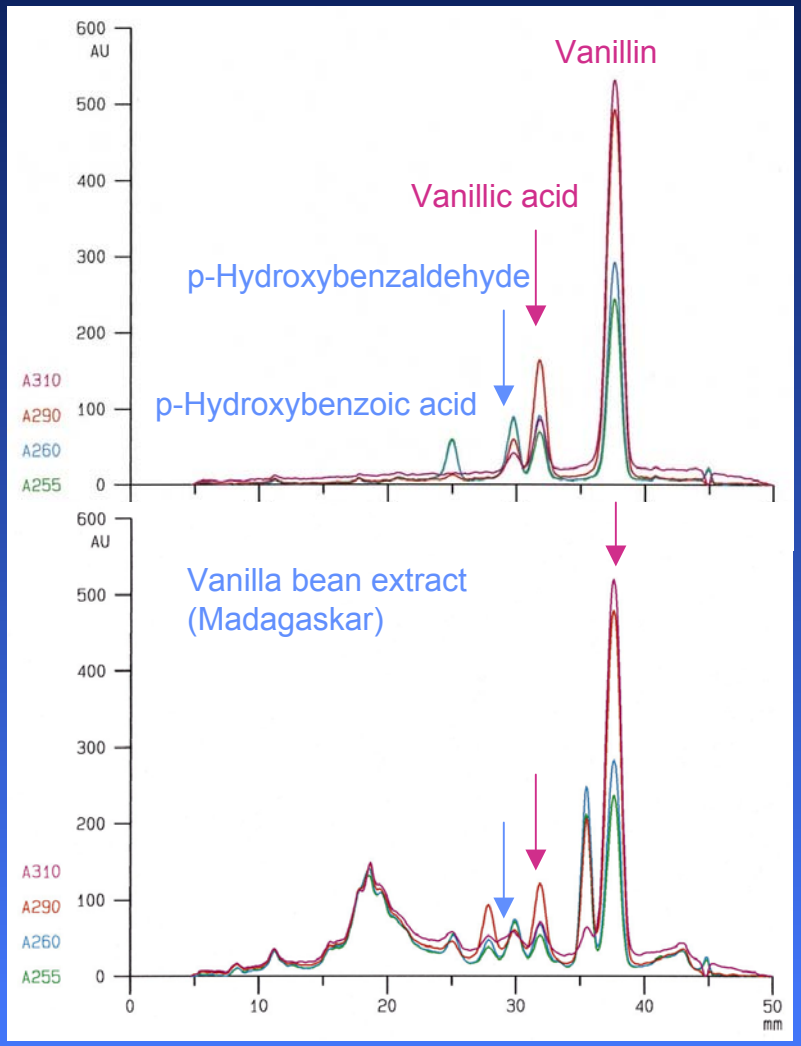
4. Michaelis Menten 1 $y = (a_1 * x)/(a_2+x)$

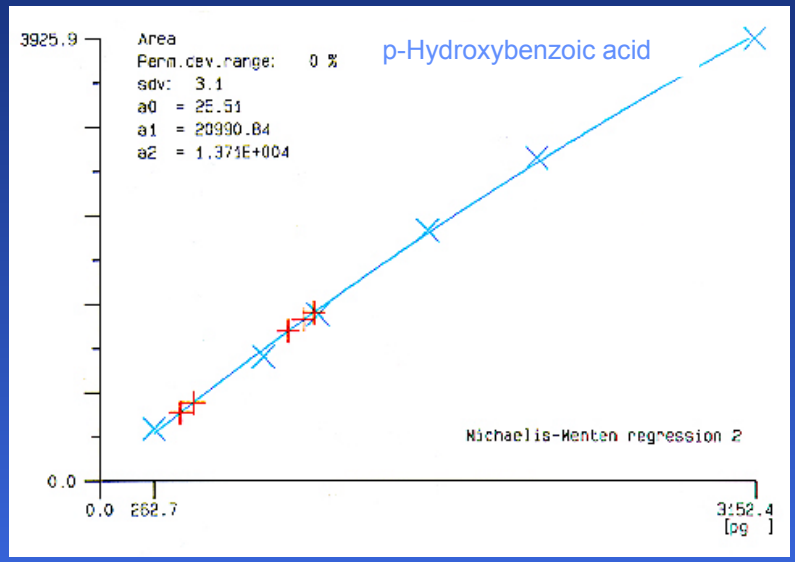
- saturation curve
- through origin
- wide concentration range (1:100)
- high concentrations

5. Michaelis Menten 2 $y = a_0 + (a_1 * x)/(a_2+x)$

- like MM 1, but
- not through origin

Vanilla bean extracts





S. Lavoine et al., Studio de Creation de Parfumerie, Mourgins cedex and Biolandes, Labrit, France, see CBS 81

Modes of dualwavelength scan

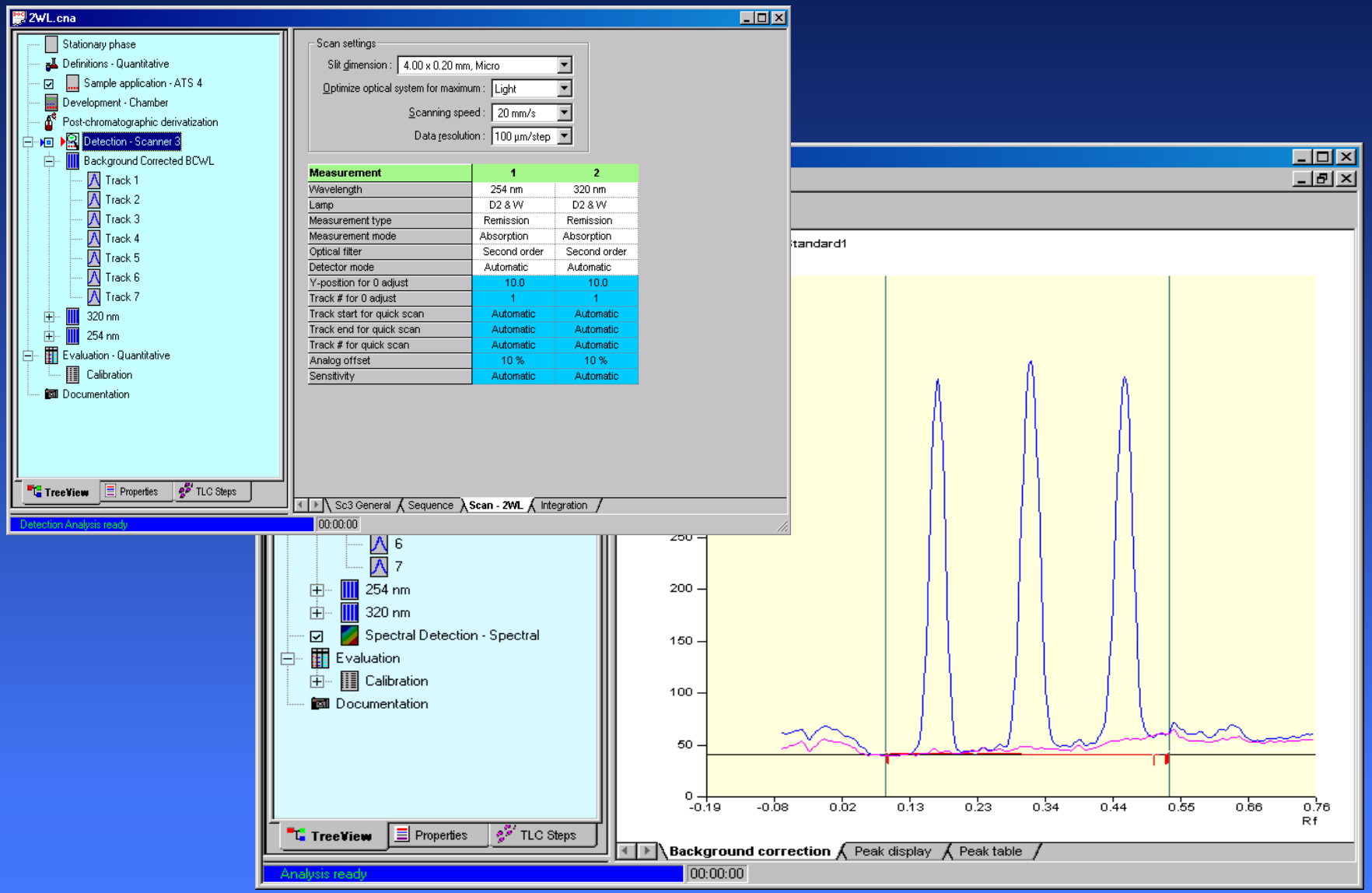
2 Wavelengths on the same track

- successively via 1 monochromator
- wavelength of maximum absorbance minus wavelength of minimum absorbance

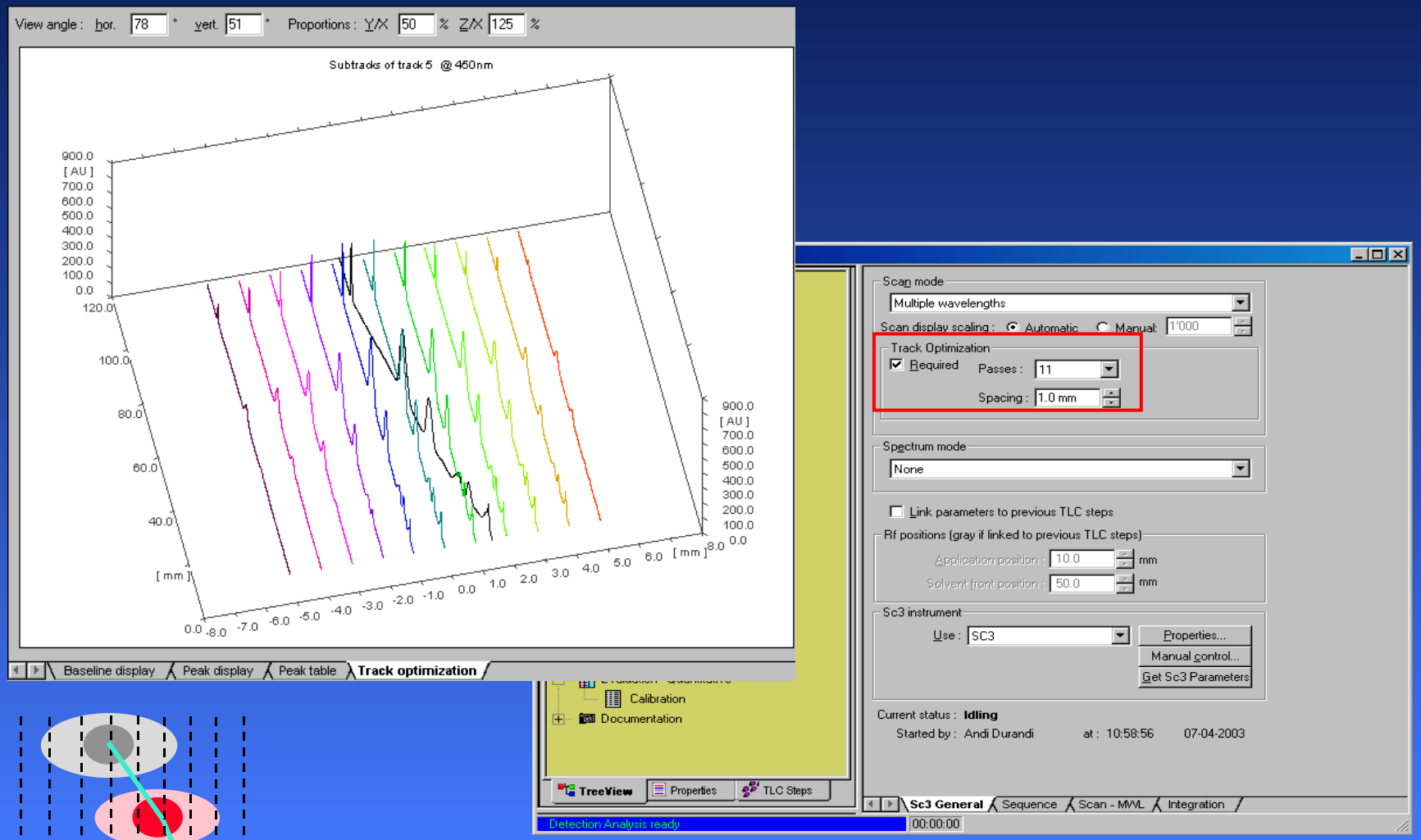
Scan of 2 tracks with the same wavelength

- sample track minus blank track (between two sample tracks)

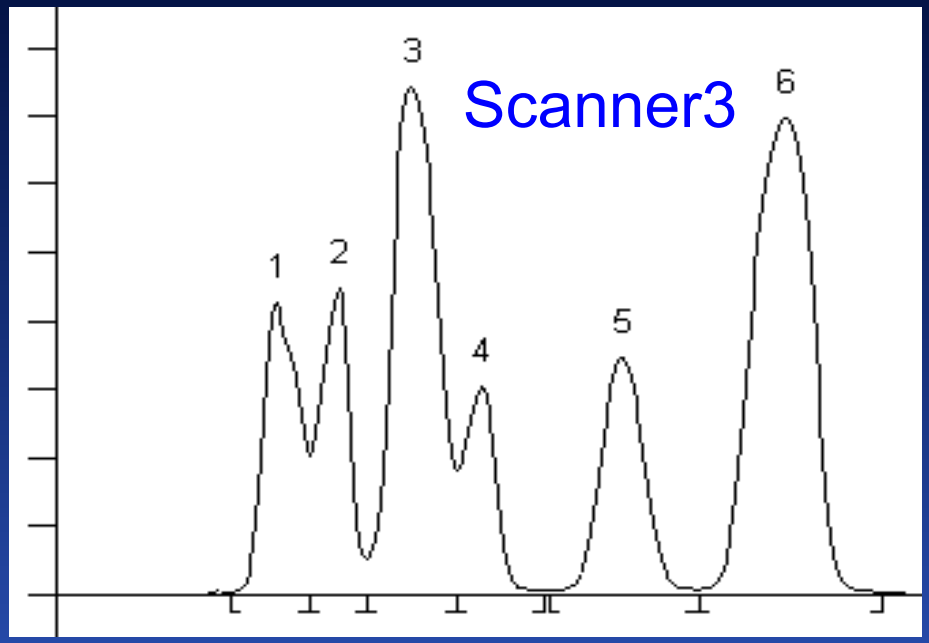
Dualwavelength scan

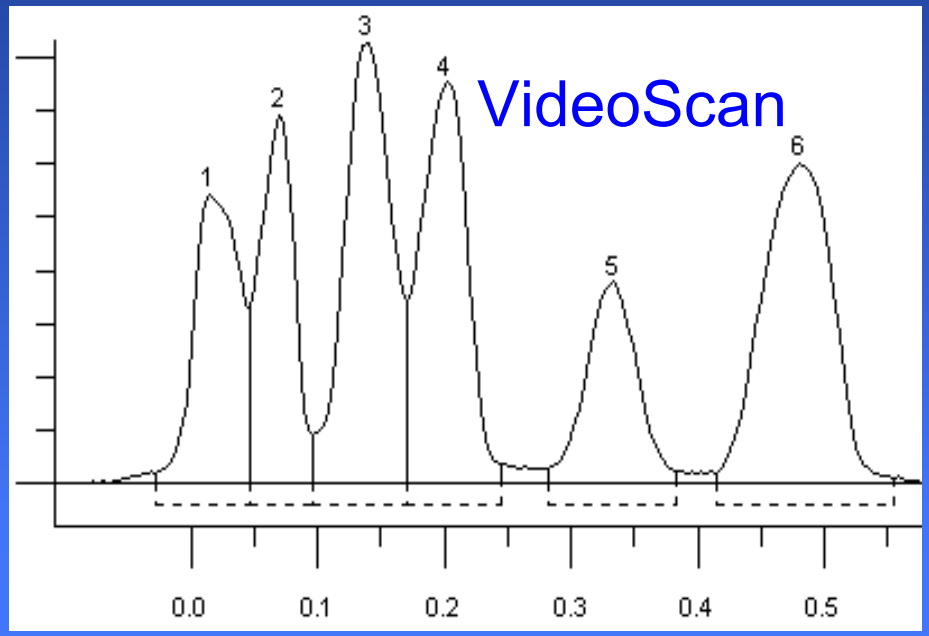


Track optimization



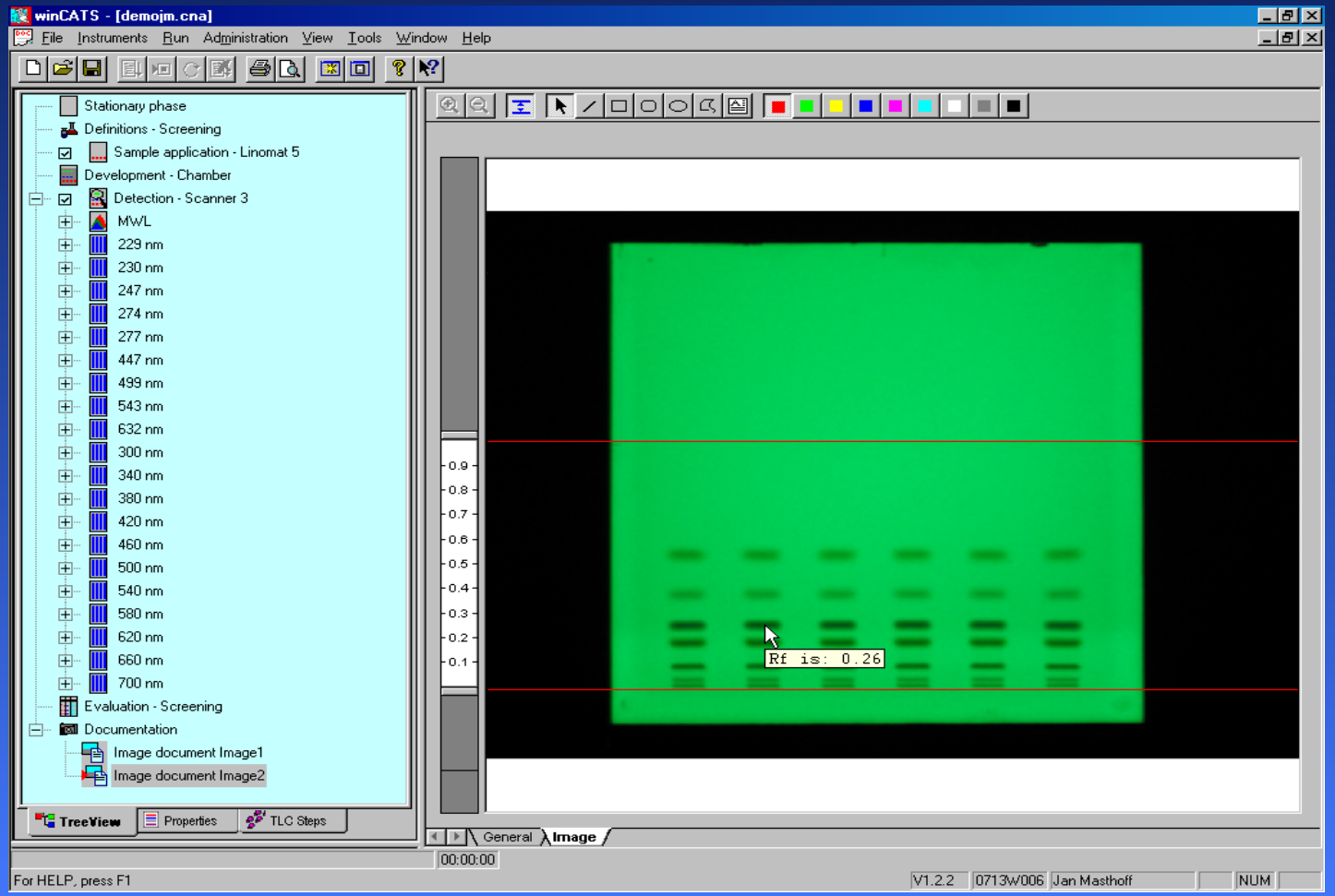
Note: Only for spotwise application





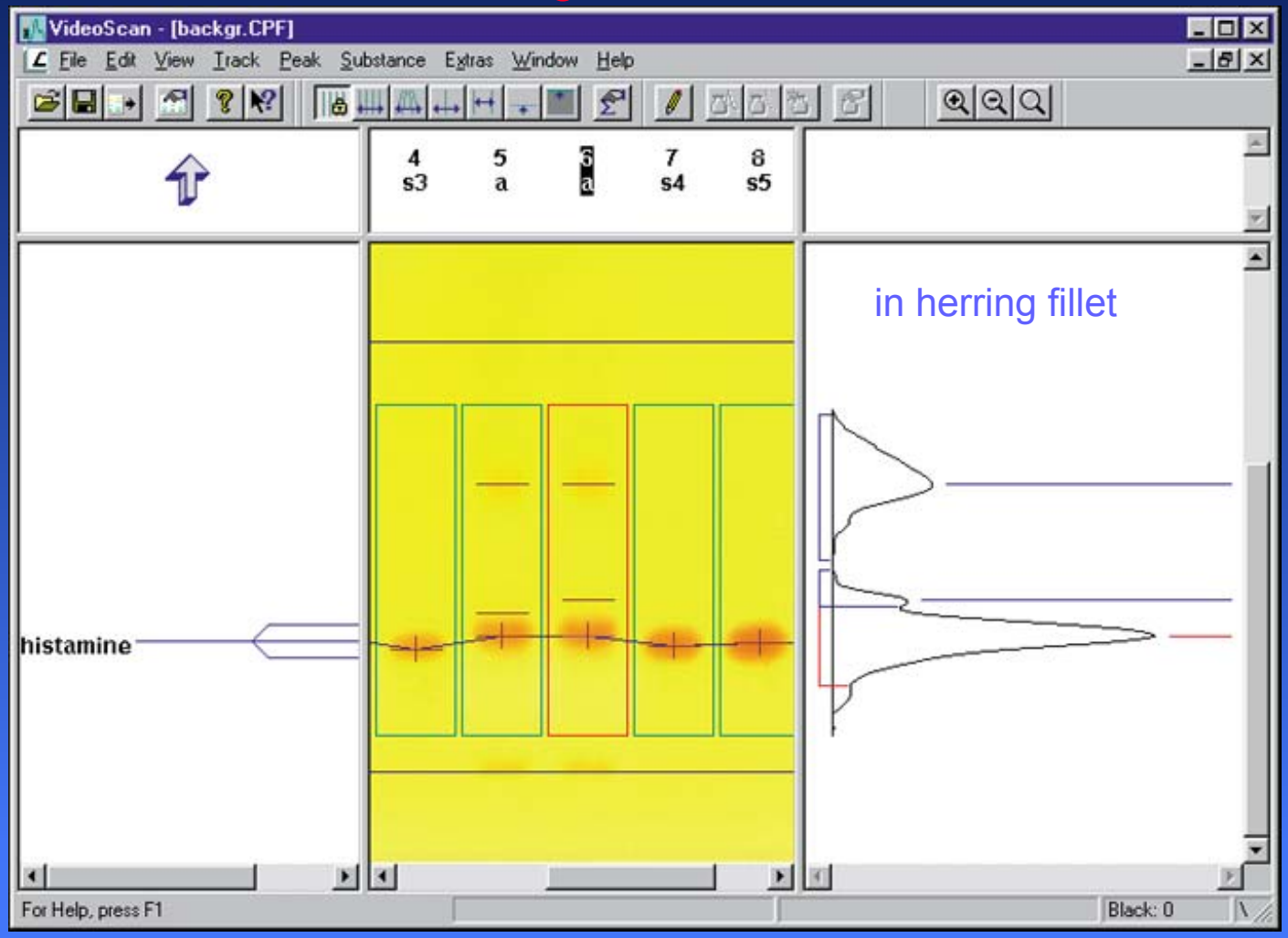
Videoscanning or conventional scanners?

Image documentation



Histamine in fish and fish products

All at one glance and click!



M. Stoyke, IVPT, Berlin, see CBS 83

Videoscanning or conventional scanners?

VideoScan

- very fast
- less costs
- image documentation and its quantification
- but...

TLC Scanner3

- optimal reproducibility
- the whole UV-range
- spectral selectivity
- spectra recording

Validation

= official qualification of an analytical method showing that it fulfills the intended purpose

Prerequisites

- ✓ characterized, homogeneous sample
- ✓ exact formal analytical method
- ✓ characterized reference substances (stability tested, of known purity and origin)
- ✓ validated instruments (IQ, OQ, PQ)
- ✓ statements concerning tolerated values & deviations, limit values, purpose of the procedure

Parameters of validation

1. Specifity

   Differentiate specifically the impurities, degradation products, byproducts etc. from the analyte

   Note: R_F value, chromatogram & spectra comparison, blank sample, standard addition, ruggedness tests

2. Selectivity

   Differentiate selectively the impurities, degradation products, byproducts etc. from the analyte

   Note: R_F value, resolution, peak asymmetry, spectra purity

3. ”Linearity” or better analysis function

   Functional correlation between measured value and concentration

   Note: Sdv, linearity test acc. to Mandel

Resolution according to Snyder

$$R_S = \frac{1}{4}\left(\frac{K_1}{K_2} - 1\right) * \sqrt{\overline{R_F} * N} * (1 - \overline{R_F})$$

α β γ

$$N = \frac{z_F}{\overline{H}}$$

α	Selectivity term
β	Layer quality
γ	Solvent influence
$\overline{R_F}$	Mean R_F value of the substances to be separated
N	Number of theoretical plates of a given plate (mean)
$\overline{z_F}$	Migration distance
$\overline{H}$	Height Equivalent to a Theoretical Plate, HETP (mean)

Selectivity of great influence: $R_s \sim \alpha$

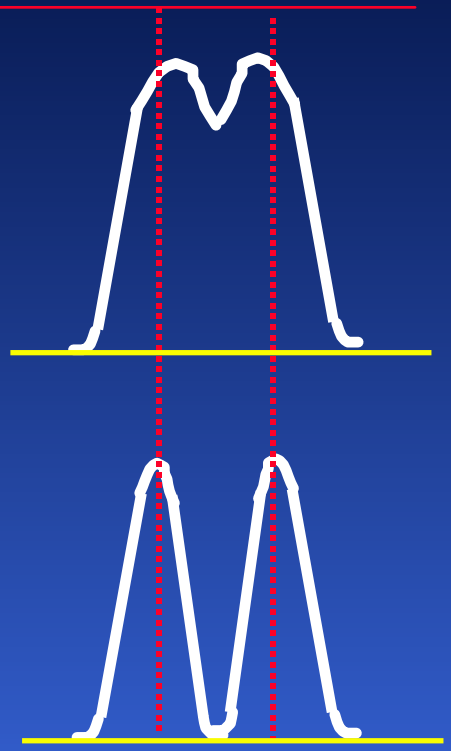
Migration distance of slight influence: $R_s \sim \sqrt{N_i}$

Resolution

Difference of migration distances

$$R_S = \frac{2\,(z_{F_1} - z_{F_2})}{w_1 + w_2}$$

Sum of the peak widths on the basis/2



z_{F_1}, z_{F_2} Migration distance

w_1, w_2 Peak width on the basis

- ✓ Different K-values, i.e. different slopes of the isotherms, selectivity
- ✓ Work in the linear range of the isotherms (decrease concentration)
- ✓ Optimal migration distance - just as high as necessary to avoid increased diffusion

Parameters of validation

4. Sensitivity

Change of concentration per change of signal,
reliability of the results

Note: Slope of the function

5. Working range

Calibration range in which quantitative statements
are allowed

Note: Starting at the limit of quantification, variances-F-test

6. Limit of quantification (LOQ)

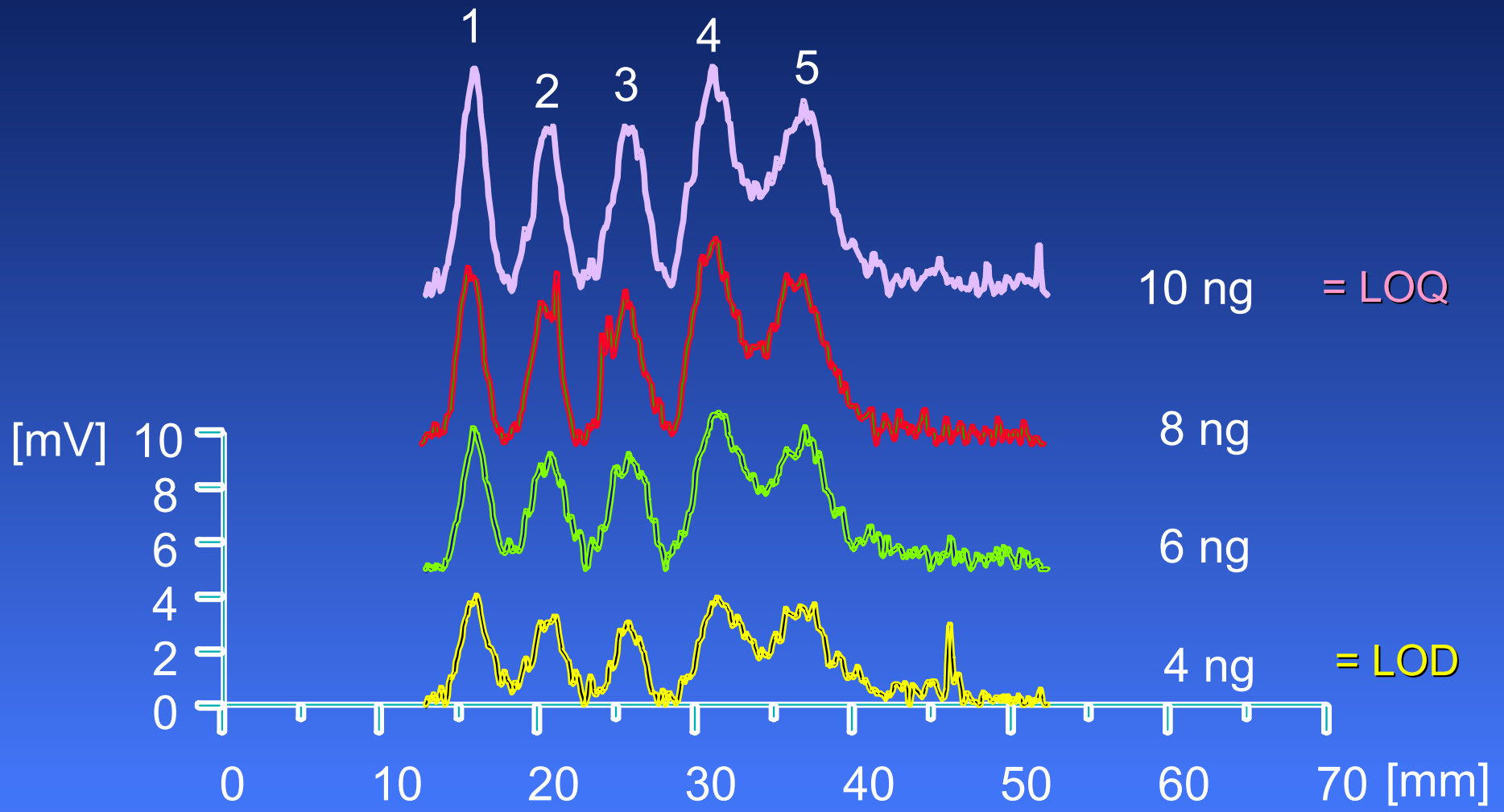
Concentration, at which a substance can be quantitatively evaluated and is statistically significant different from zero

= 2-3 fold detection limit (LOD)

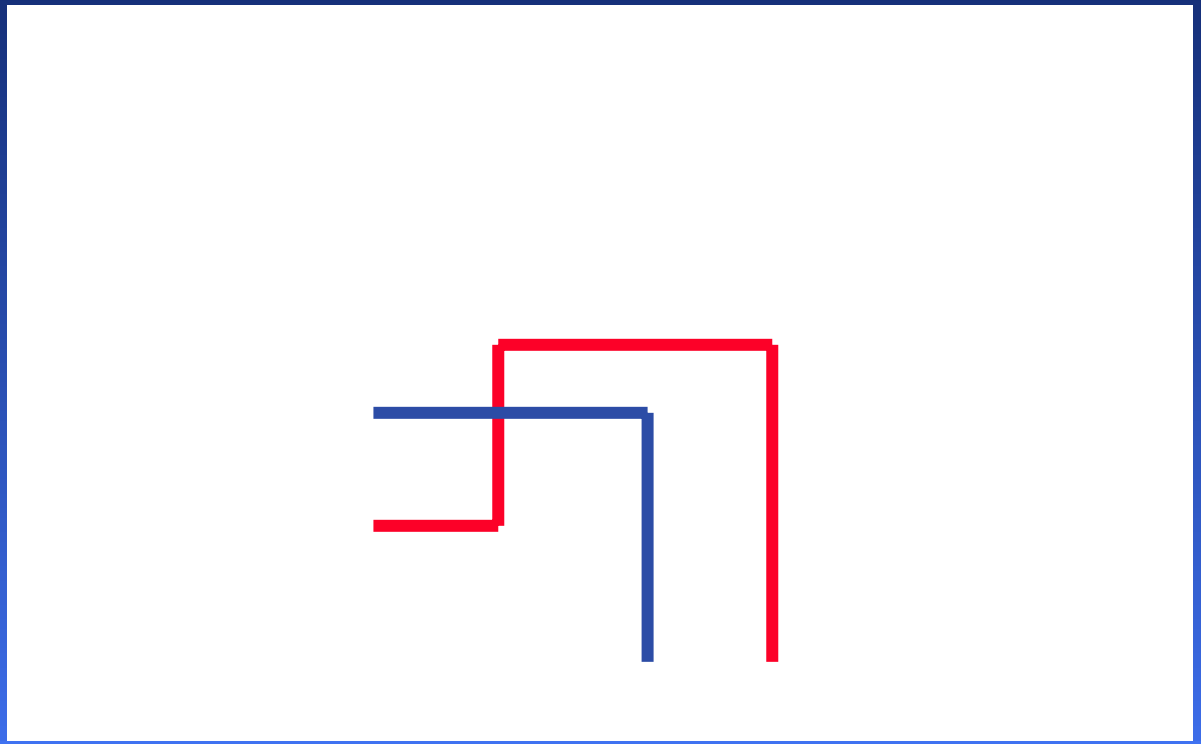
= concentration, at which a substance can be detected

= 3 fold noise signal (= variation of the baseline)

Limit of detection and quantification



Limit of detection and quantification



Parameters of validation

7. Trueness ("Accuracy" = systematic & random errors)

True result of an analysis

Note: Systematic errors... recovery rate, recovery function with and without matrix (difference in slope and intercept with the y-axis, variances-F-test)

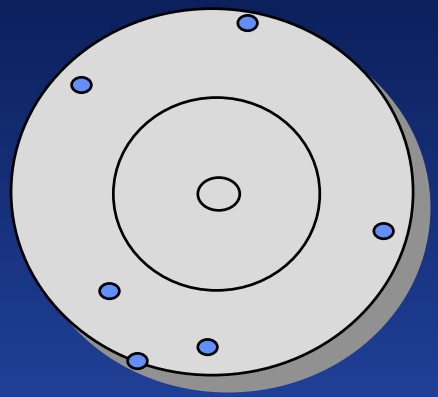
8. Precision

Deviation of the results

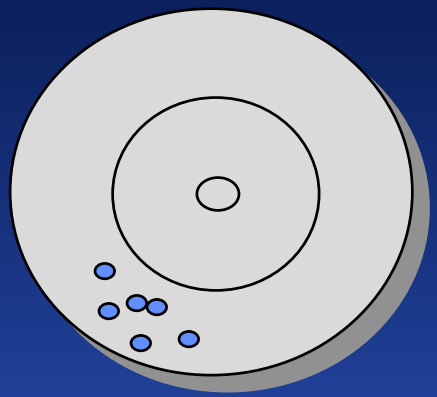
Note: Random errors.... sdv, coefficient of variation, confidence interval

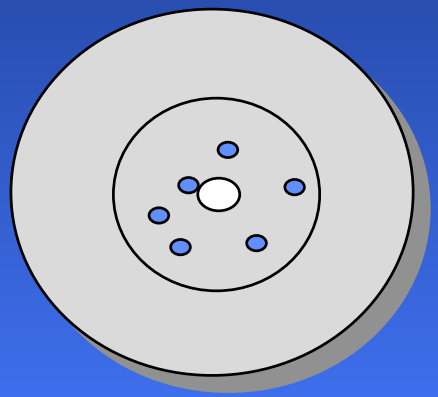
- repeatability (same day/person/instruments)
- intermediate precision or reproducibility (different days/persons/instruments)

Precision & trueness poor!



Precision good, but trueness poor!

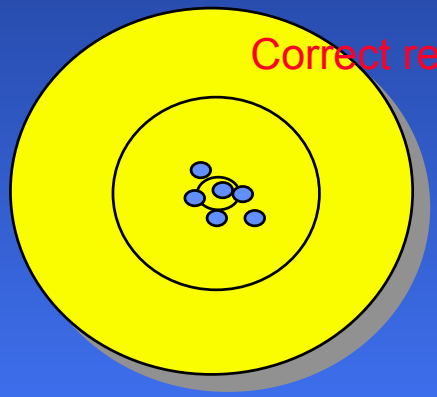




Precision poor, but trueness better!

That´s it!

Correct result!



Precision & trueness good!

Errors

Systematic errors

- Trueness, constant and/or varying
- Characterization by interception with y-axis

Random errors

- Reproducibility, precision
- Characterization by standard deviation

$$s_x = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n}} \rightarrow s = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n - 1}}$$

Sources of random errors

Total error or reproducibility: < 2-3 %

$$x_T^2 = x_A^2 + x_{PO}^2 + x_C^2 + x_{PL}^2 + x_M^2$$

x_T	total error	x_C	chromatography error
x_A	application error	x_{PL}	plate error
x_{PO}	positioning error	x_M	measurement error

- ✓ Measure n-tracks with the same amount of substance

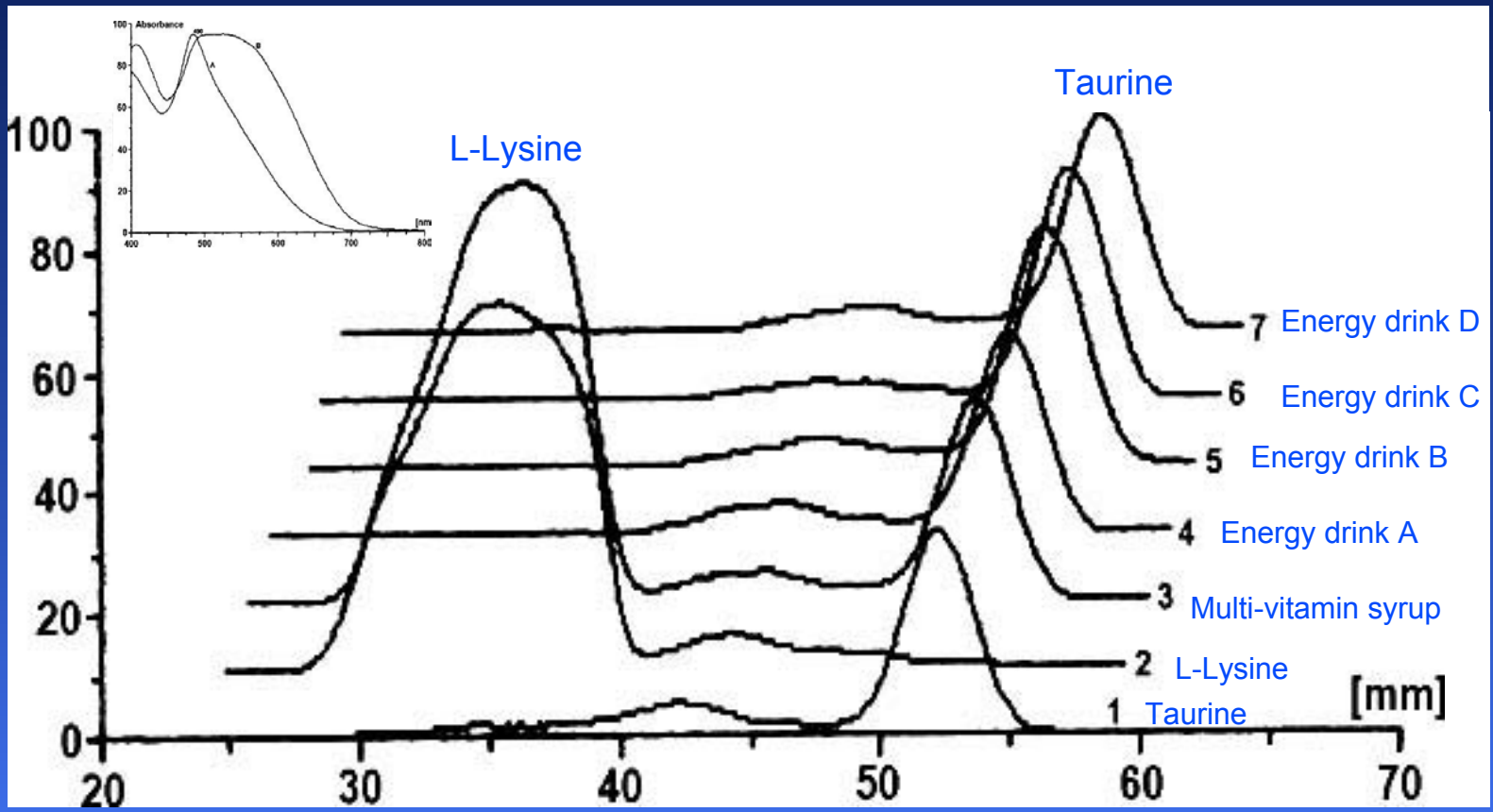
Measurement error: < 0,2 %

- ✓ Measure a given chromatogram track n-times (track distance: 0)

Sources of systematic errors

- insufficient purity of standards
- bad recovery rate of samples containing matrix
- scan perpendicularly to chromatography
- evaluation acc. to 100 % method
- force a calibration function to a linear one
- evaluation outside the working range
- sample and standard react differently with derivatization reagent
- instability of zones during scanning
- different shape of sample and standard zone caused by matrix

Amino acids in drinks



Intermediate precision < 1,6 %
Recovery rate 99,4 and 100 %

G. Indrayanto et al., University of Airlangga, Indonesia, see CBS 90

Facultative parameters

9. Ruggedness (stress test)

Usage of the method under varying conditions,

stability test of the whole procedure

Note: Sdv, coefficient of variation, confidence interval, resolution,

R_F value, selectivity, sensitivity

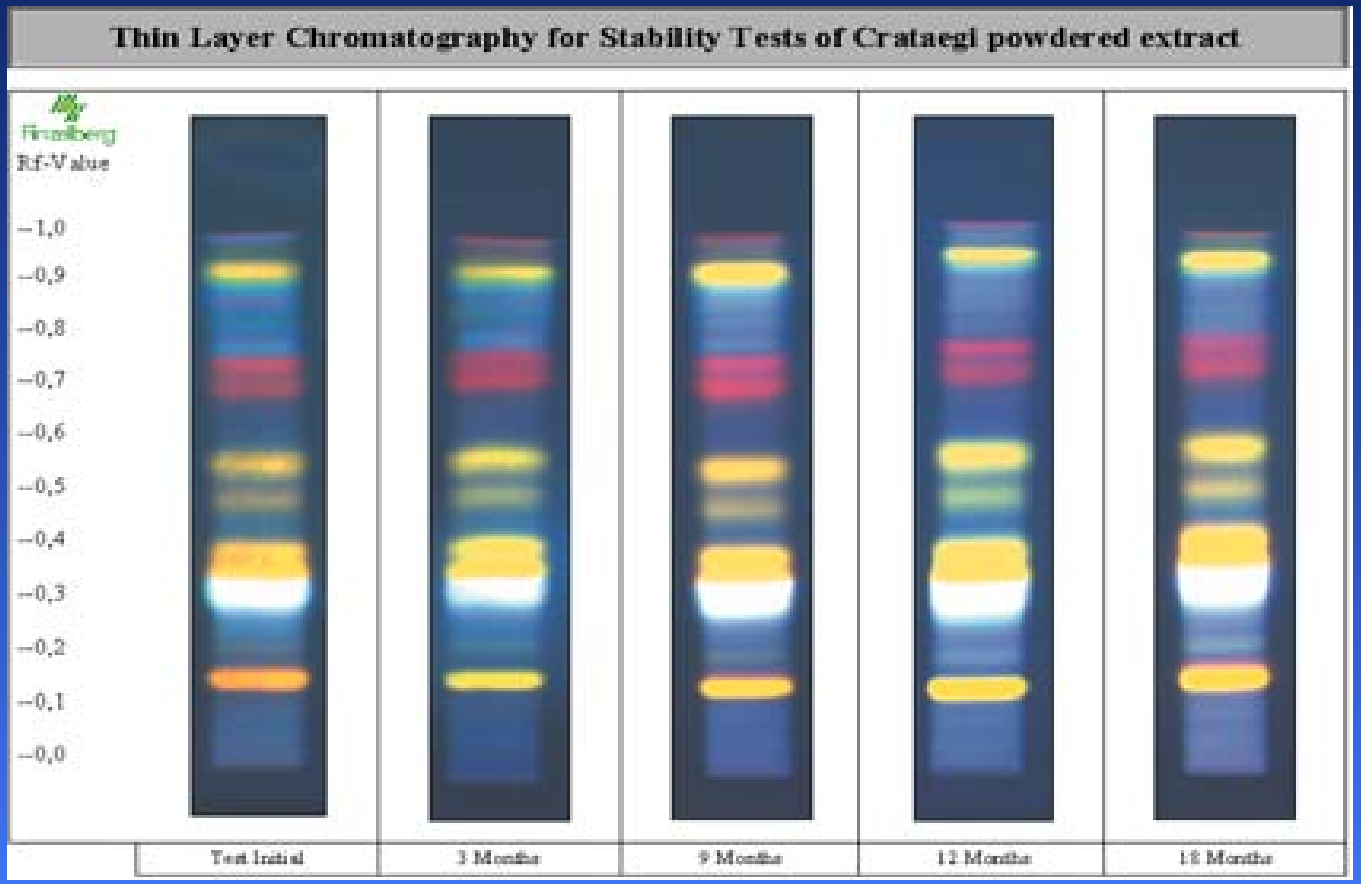
10. Control charts (mean value, recovery)

Check by control standard over a long period,

confirms trueness and reliability of a procedure

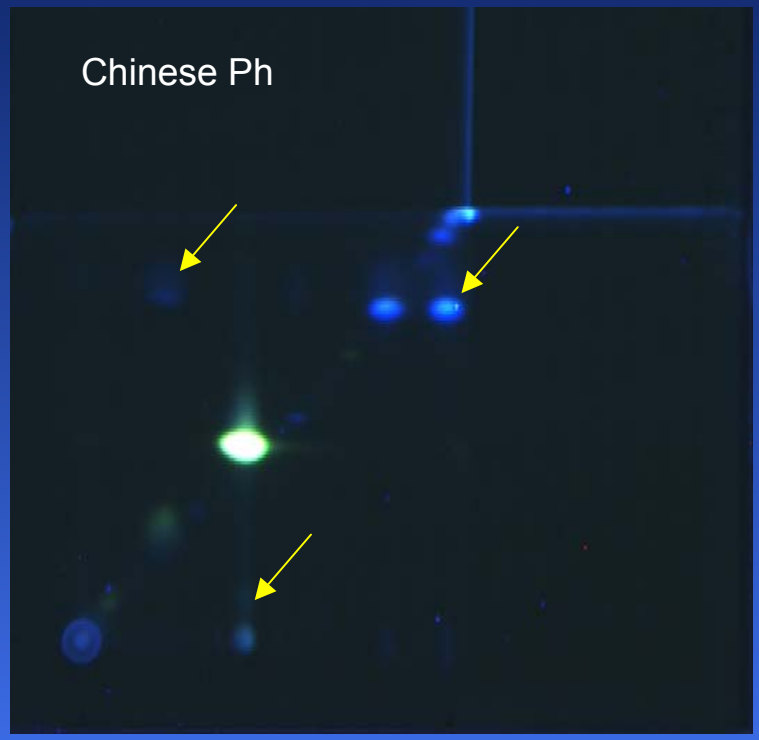
Note: Warning limit (2s) and control limit (3s)

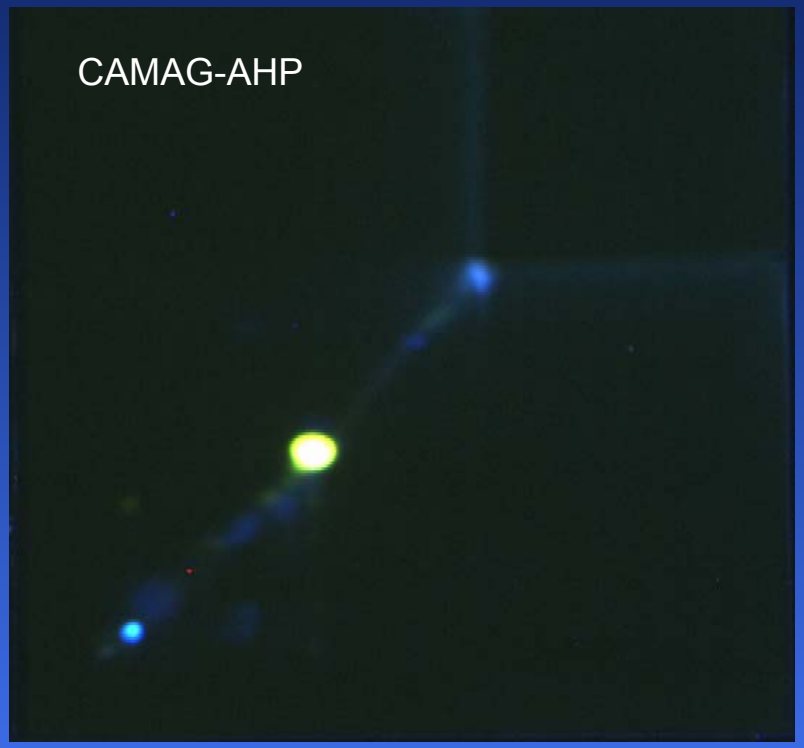
Stability test



K. Thiekötter, Finzelberg GmbH & Co. KG, see CBS 85

Stability test of golden seal

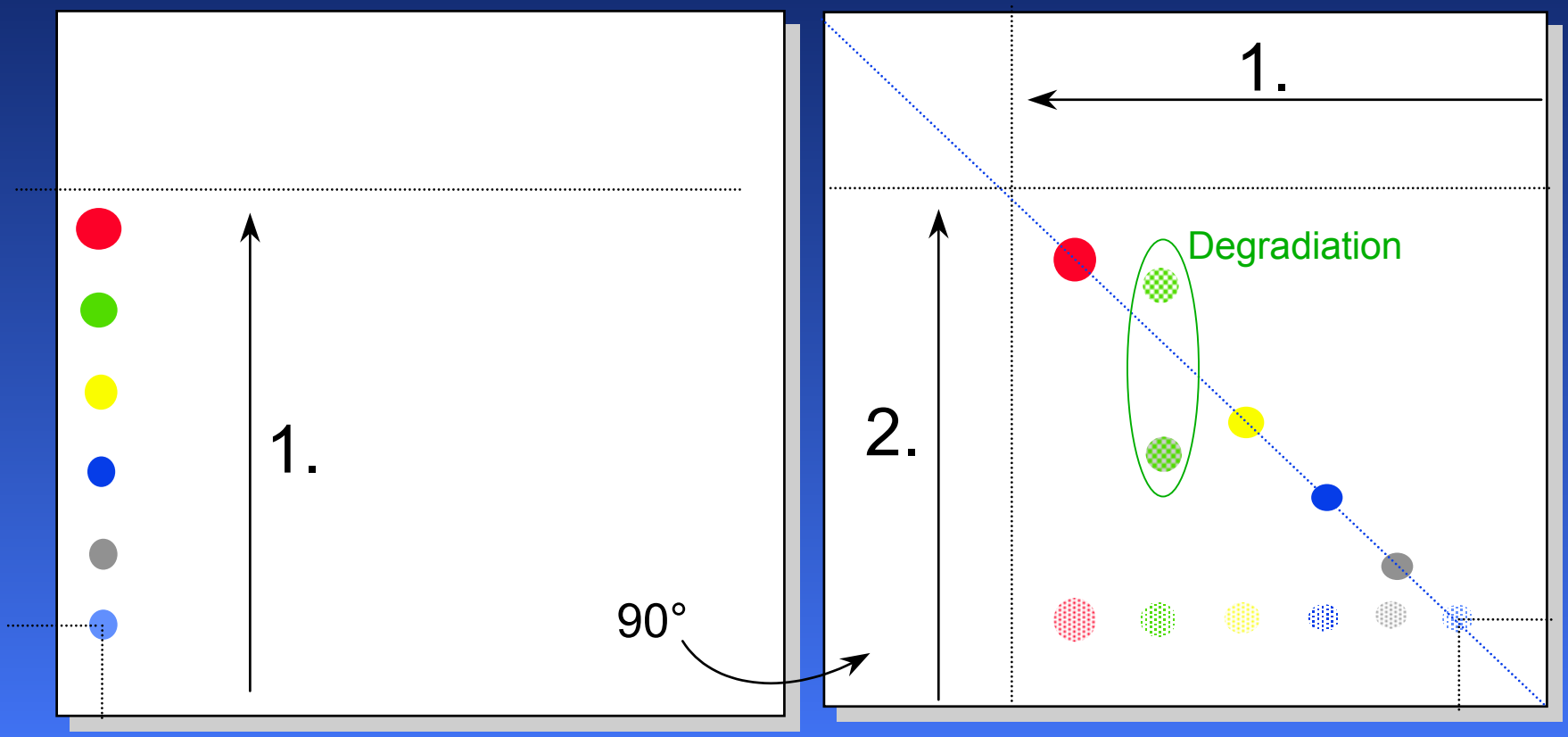




2-D separation of alkaloids, CAMAG Laboratory, Muttenz

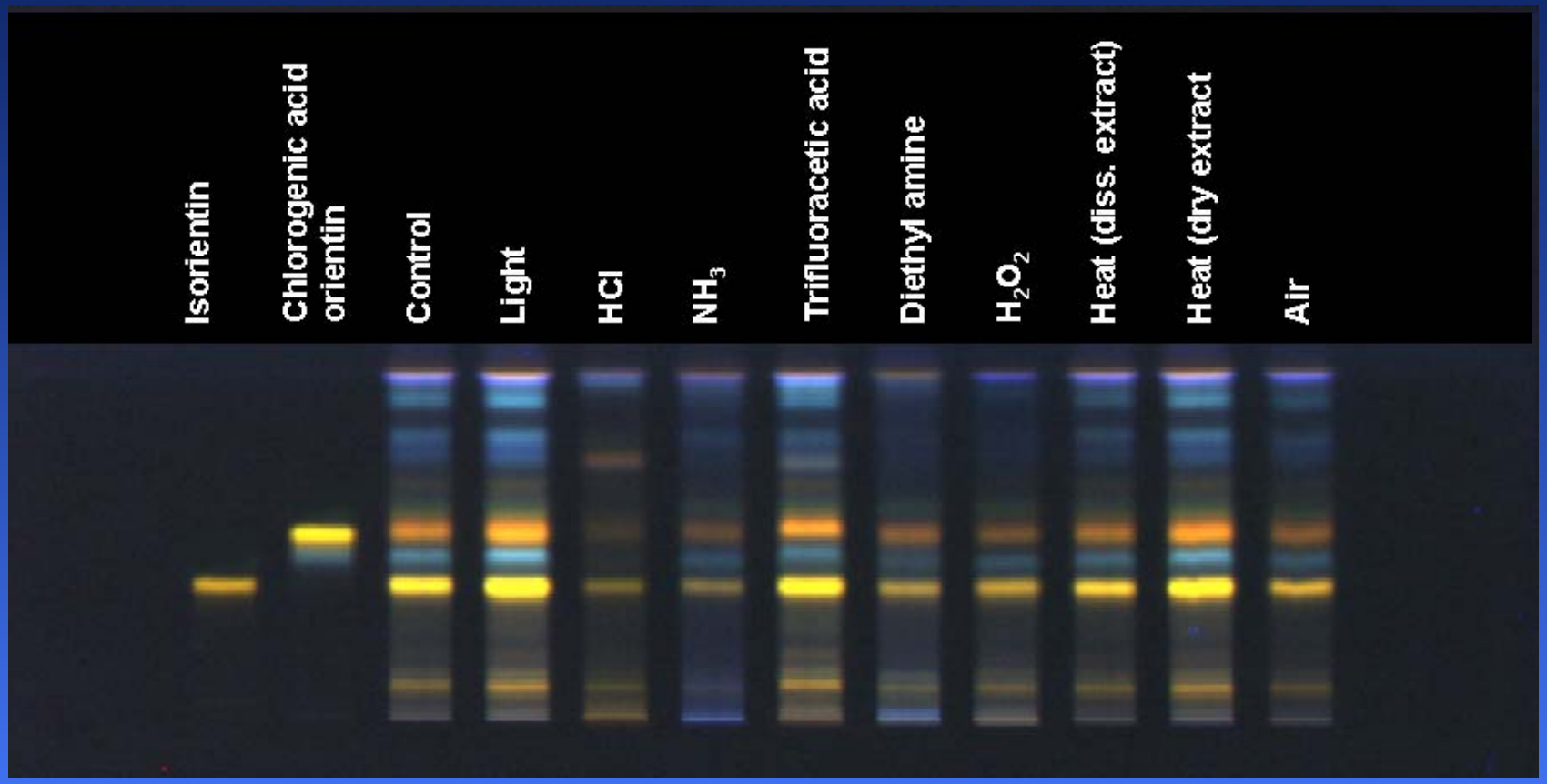
Stress test

2-D separation with intermediate reaction



... with the same solvent, plate turned by 90°

Stress test of Chaste Tree extracts

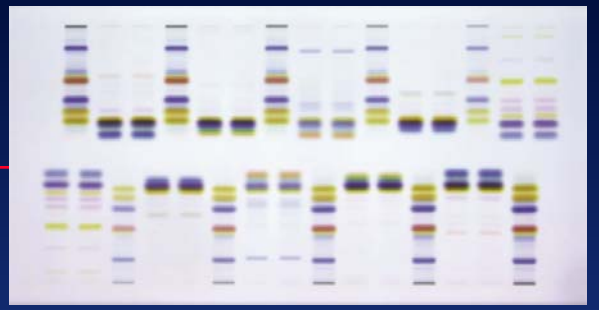


Diploma thesis of Franziska Wahli, Inst. of Pharm. Biology, University of Basel, 2002, at CAMAG Lab, see CBS 91

What is GLP/GMP?

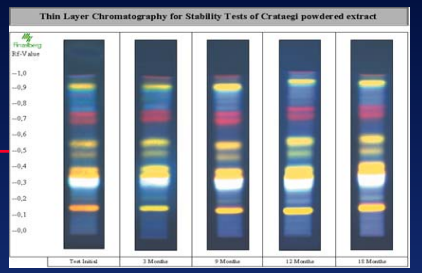
- ✓ Sanitary regulations, safety measure ...
- ✓ Continued, further education
- ✓ Test plans
- ✓ Standard operating procedures (SOP)
- ✓ Safety of data/archives
- ✓ Data integrity: validated instruments (IQ, OQ, PQ) and methods
- ✓ Report (duplication)
- ✓ Certified reference substances
- ✓ User validation (pass word) – 21 cfr 11

Why HPTLC?



- ✓ All information at first glance
- ✓ High matrix tolerance
- ✓ Less effort for sample preparation
- ✓ Flexible detection and identification
- ✓ Rapid, sensitive and cost-effective
- ✓ Separation under identical conditions

Disadvantages – not at all!



- ✓ (Room with air-conditioning system)
- ✓ (Reproducibility ⟷ thorough knowledge of factors of influence necessary!)
- ✓ (No black box, not fully automated, but info stored similar to a compact disk enables flexibility & creativeness!)
- ✓ (Open system (... additional vapor phase, multi-component mobile phase, activity of the sorbent) increases possibilities for a good separation!)