# HPTLC

## For the analysis of botanical materials and medicinal plants

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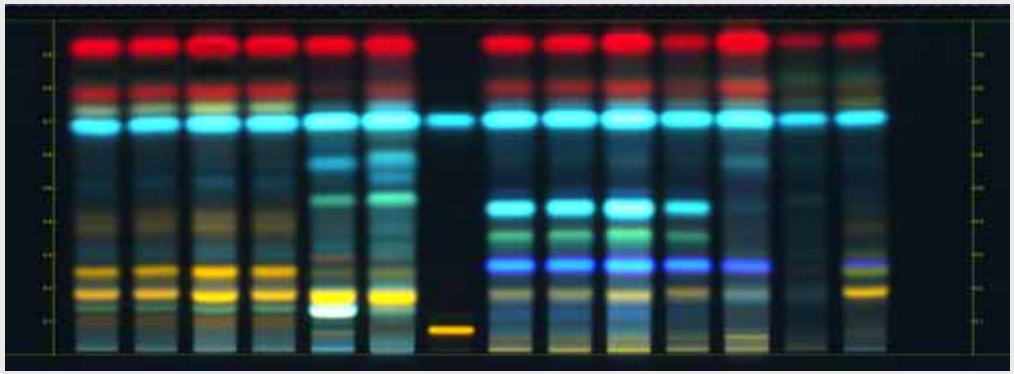

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# What will be discussed?

1. What exactly is HPTLC?
2. The elements of a standardized HPTLC methodology
3. Changing TLC methods into HPTLC methods
4. Development and validation of ID Methods
5. How new developments in HPTLC may affect the analysis of plants

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# Introduction

- Thin-layer chromatography (TLC) always was and still remains an important tool for the analysis of plants.
- Today there are two principal applications in this context: research and quality control. Both benefit from the advantages of the planar off-line principle and also in particular from low cost, simplicity, and flexibility.
- For decades TLC is integral part of monographs for medicinal plants in all pharmacopoeias and the primary method of identification.
- Growing expectation regarding performance charateristics have brought TLC methods to the limits.
- Since the turn of the century pharmacopoeias recognize the technical progress in instrumentation and improvements offered by high performance plates.
- Most recently HPTLC is being discussed as alternative to classical TLC.

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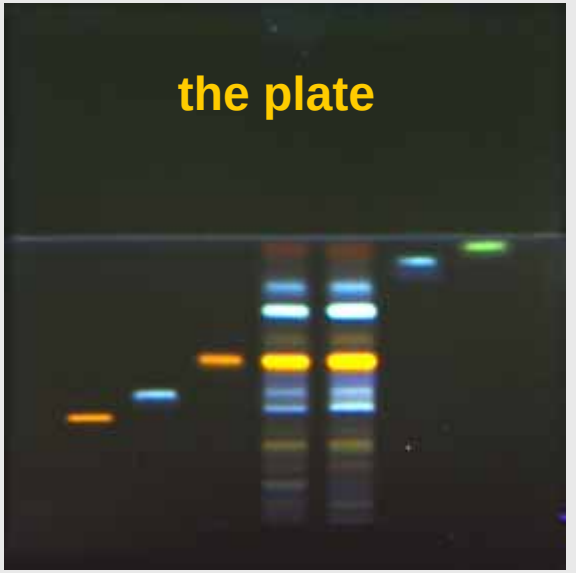
# The planar off-line principle

**application**

**development**

**detection and quantitative evaluation**

**documentation**




***FLEXIBILITY***

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# But what about reproducibility?

## The central problem:

- When two (or more) labs do the „same“ or think that they are doing the same, the results are not necessarily equal.
- Reason is the general method description in the Pharmacopoeias (EP 2.2.27, USP <201>, <621>, PhPRC ap. VI) which define „suitable” equipment and give ranges instead of values.
- JPXV 2.03 is still centered around self made 20x20 TLC plates !
- A table (EP) or a result description (USP, JP) can only define the most important aspects of a TLC chromatogram. That leaves room for interpretation. An atlas (PhPRC) provides clear guidance.
- Example: how can a color be described correctly?

**Most text books are even worse …**

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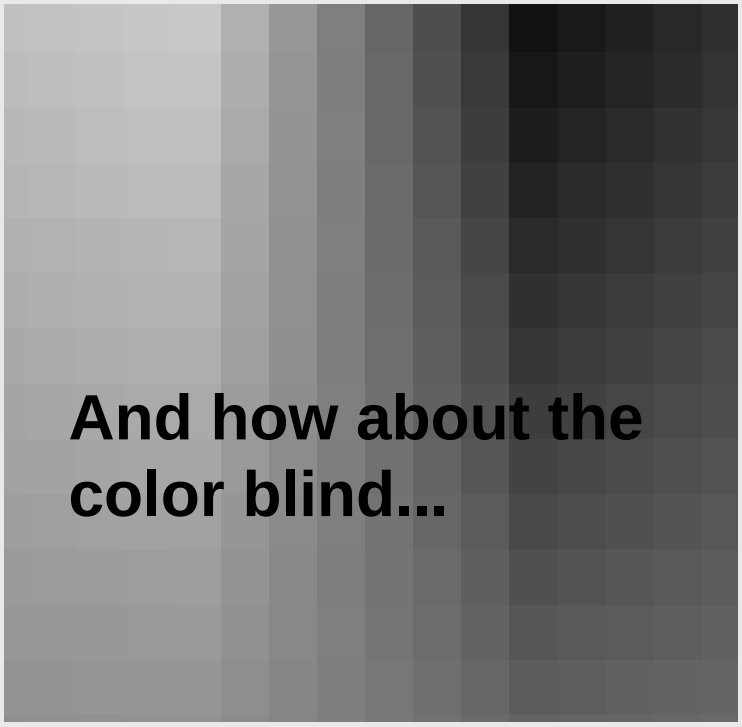
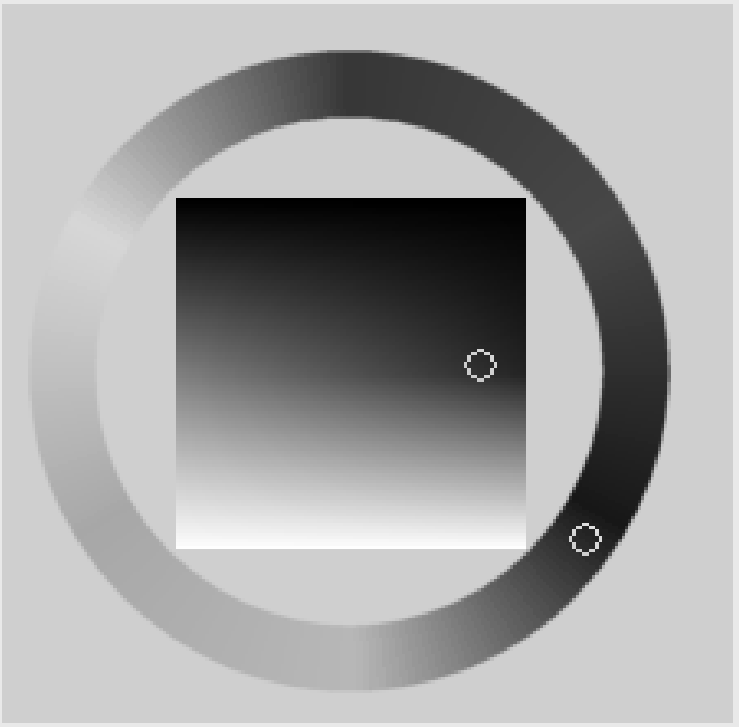
# What is blue?



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# PhEur: Possible choices in methodology

- TLC layer
- Manual application
- Transparent container (Pickle jar?)
- UV-Lampe (λ?)
- Manual spraying / immersion

- HPTLC layer
- Automatic application
- Automatic Developing Chamber
- Scanner
- Automatic immersion / spraying

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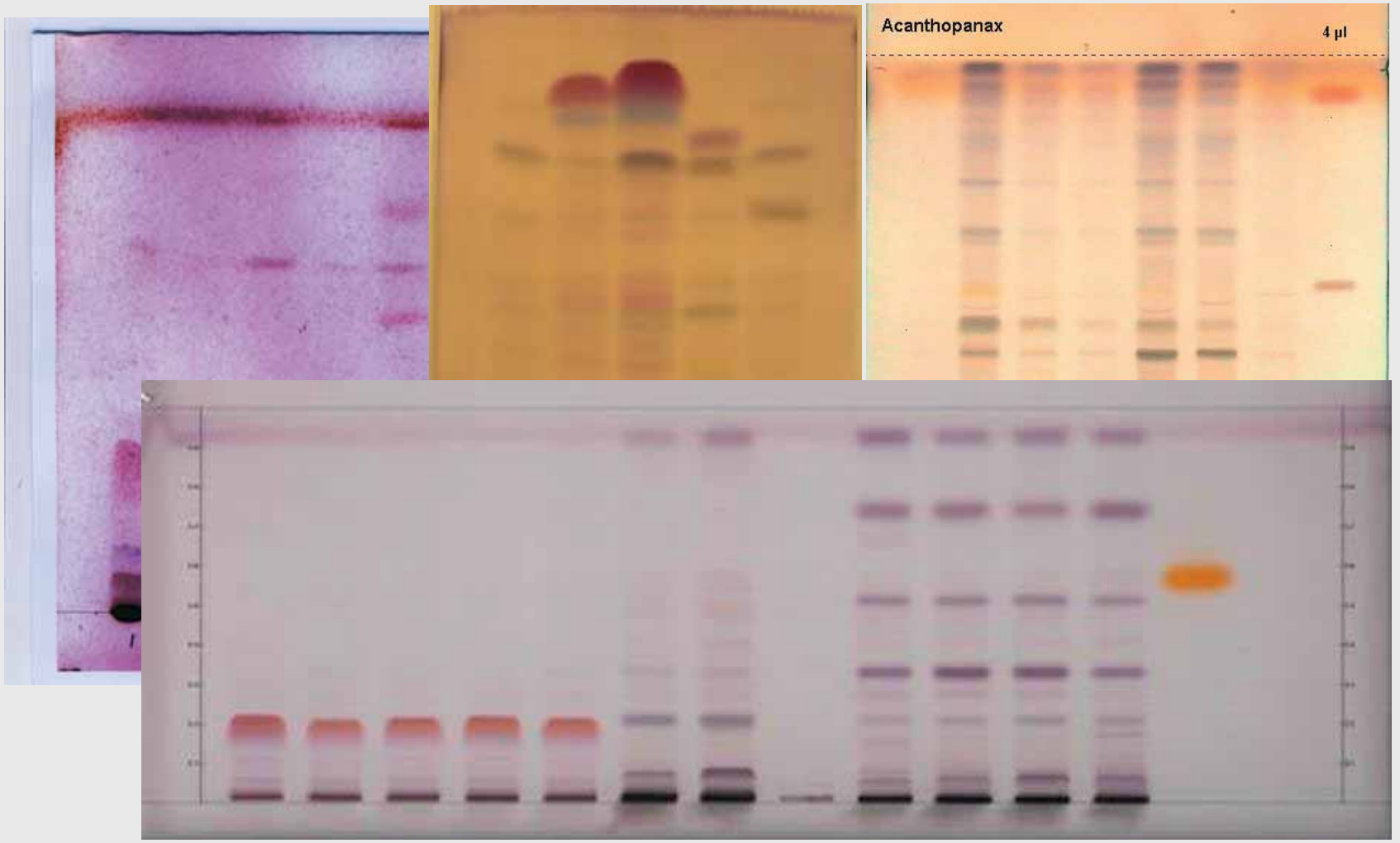
# Identification of *Acanthopanax*



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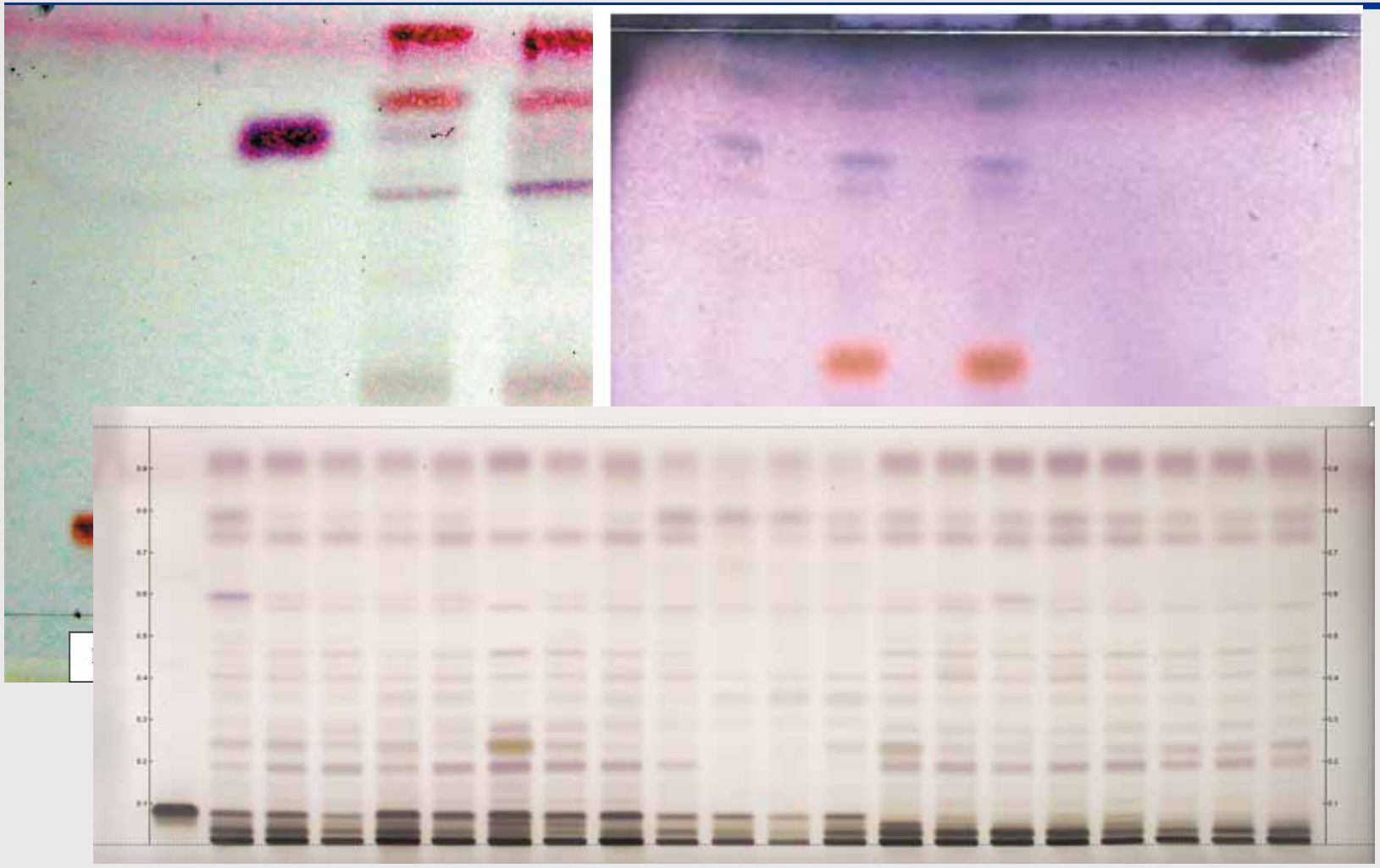
# Identification of Peonies



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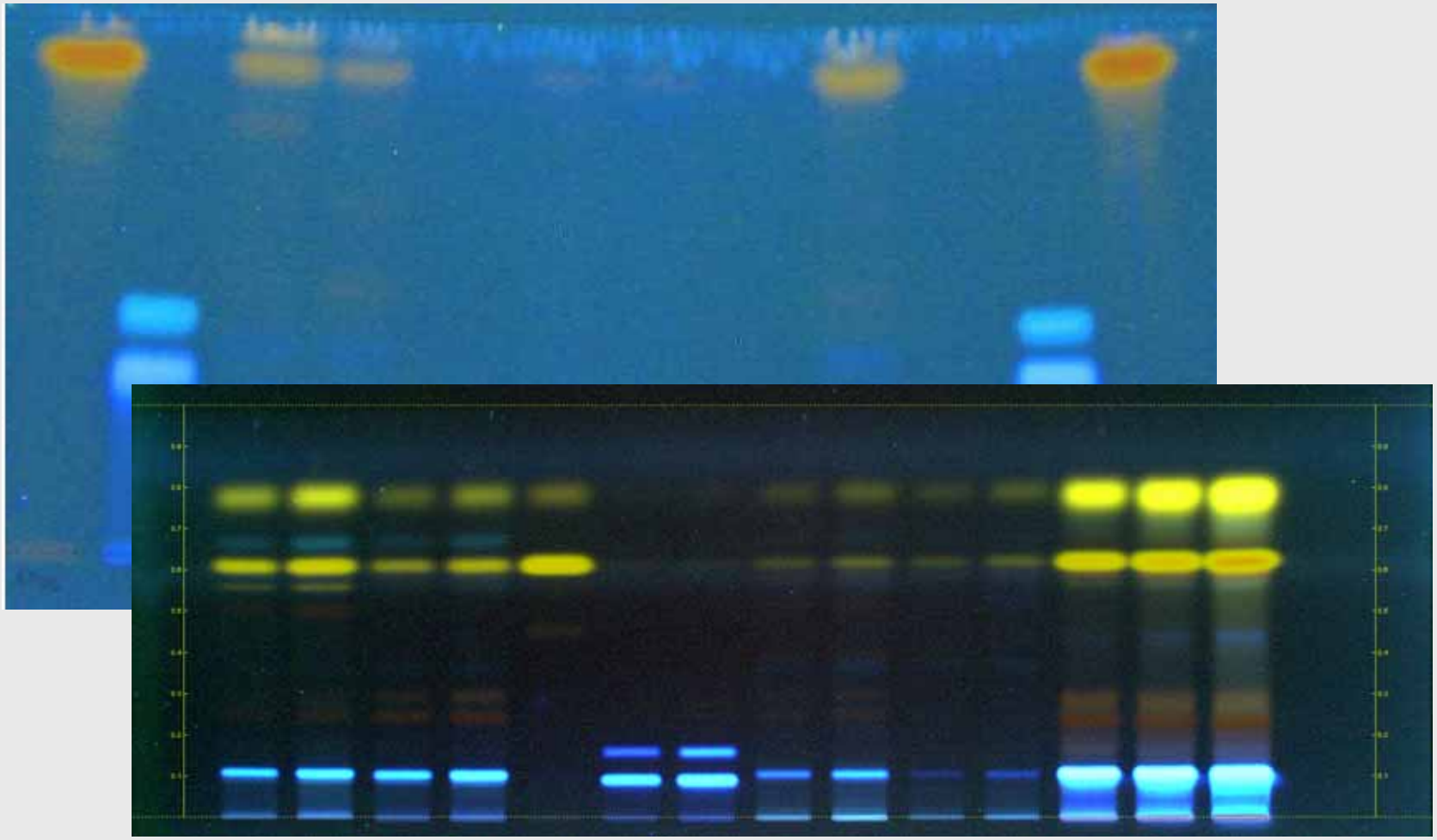
# Identification of Fleece flower



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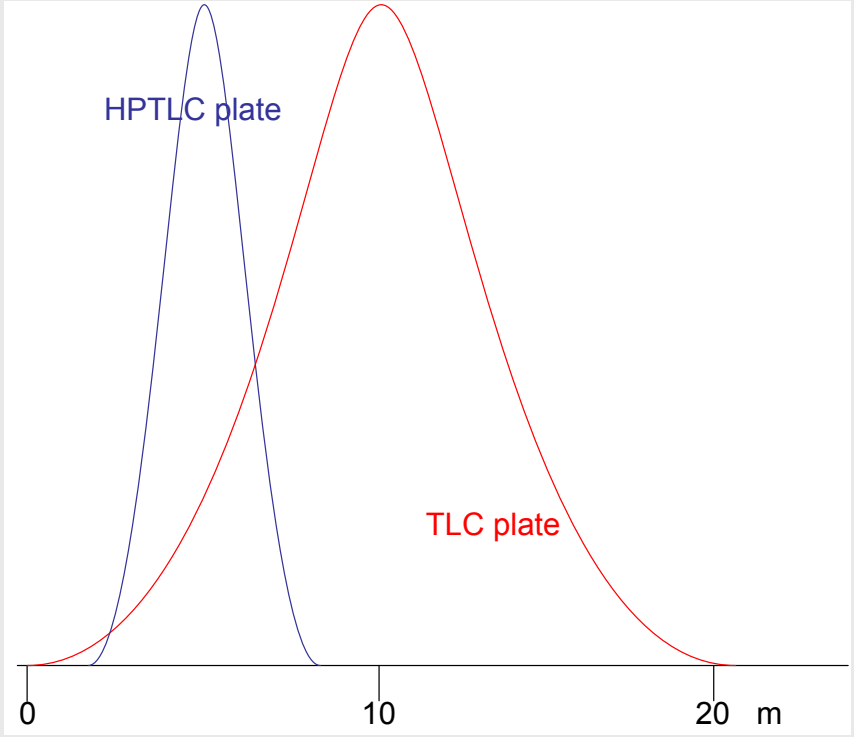
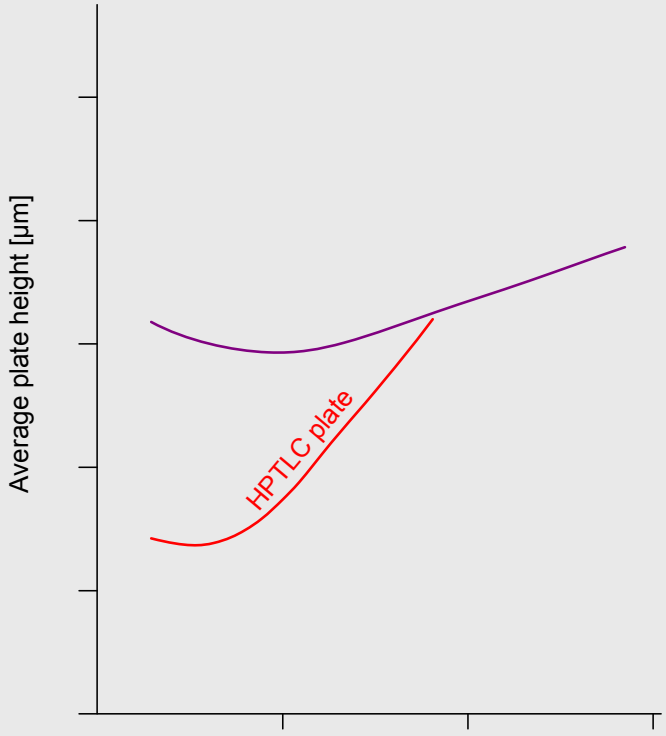
# Goal:
# International standardization of HPTLC

- What
  - HPTLC definition
  - Methodology
  - Equipment
- Why
  - Reproducibility of results
  - Validity of official methods
  - Quality assurance in a globalized world
  - Quality of published research
- How
  - International collaboration
  - Top down
  - Publication

# TLC or HPTLC

- Pharmacopoeias see difference primarily in the plate yet assume similar results



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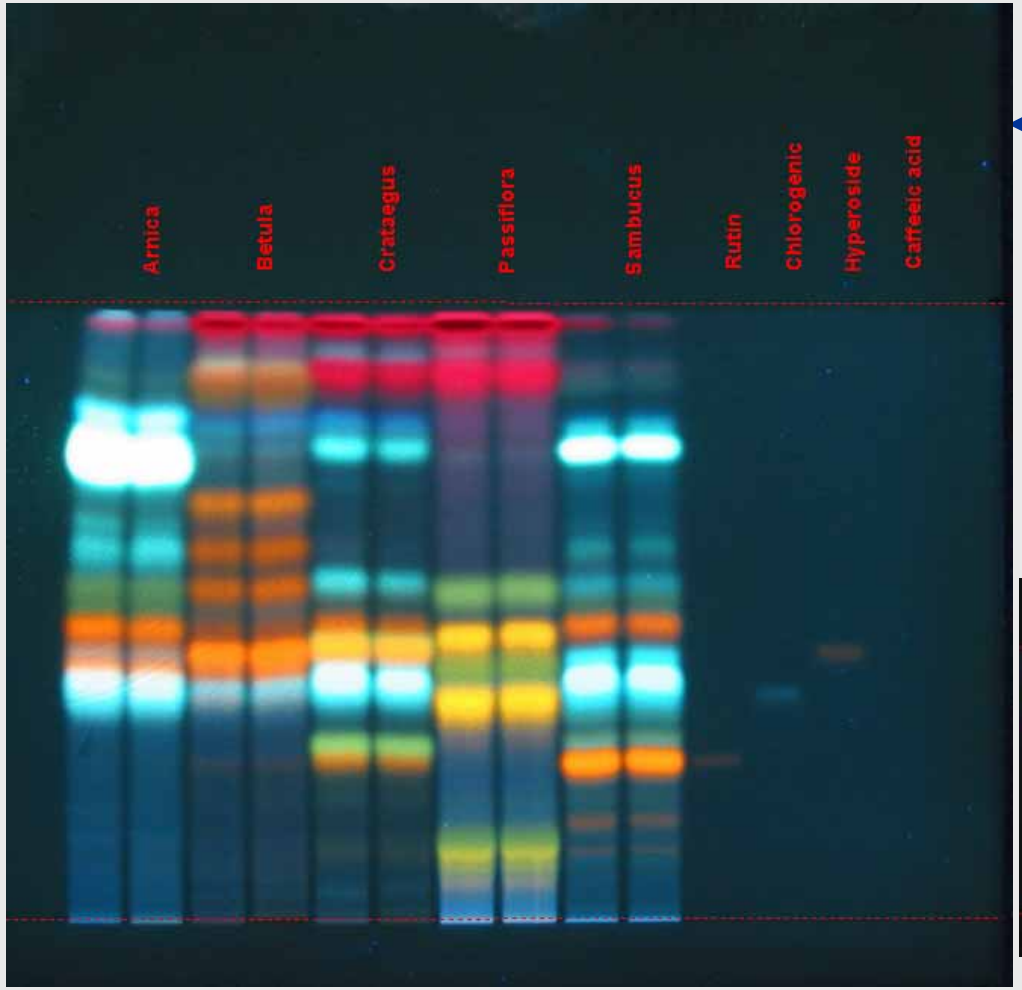
# Comparison TLC-HPTLC of flavonoids

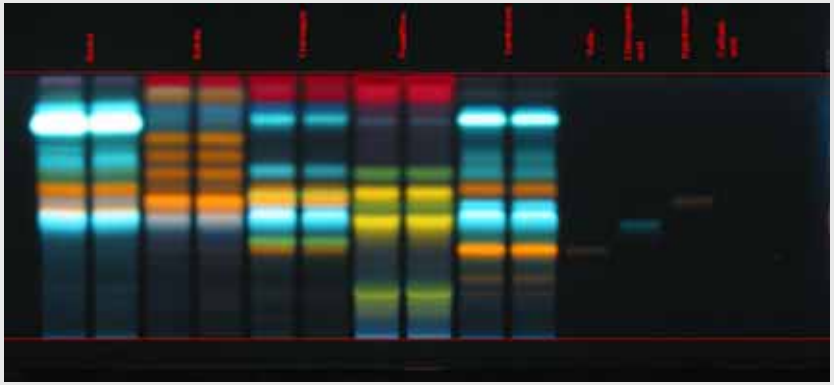



← TLC plate 20 x 20 cm

(135 mm)

HPTLC plate 20 x 10 cm

(60 mm) ↓



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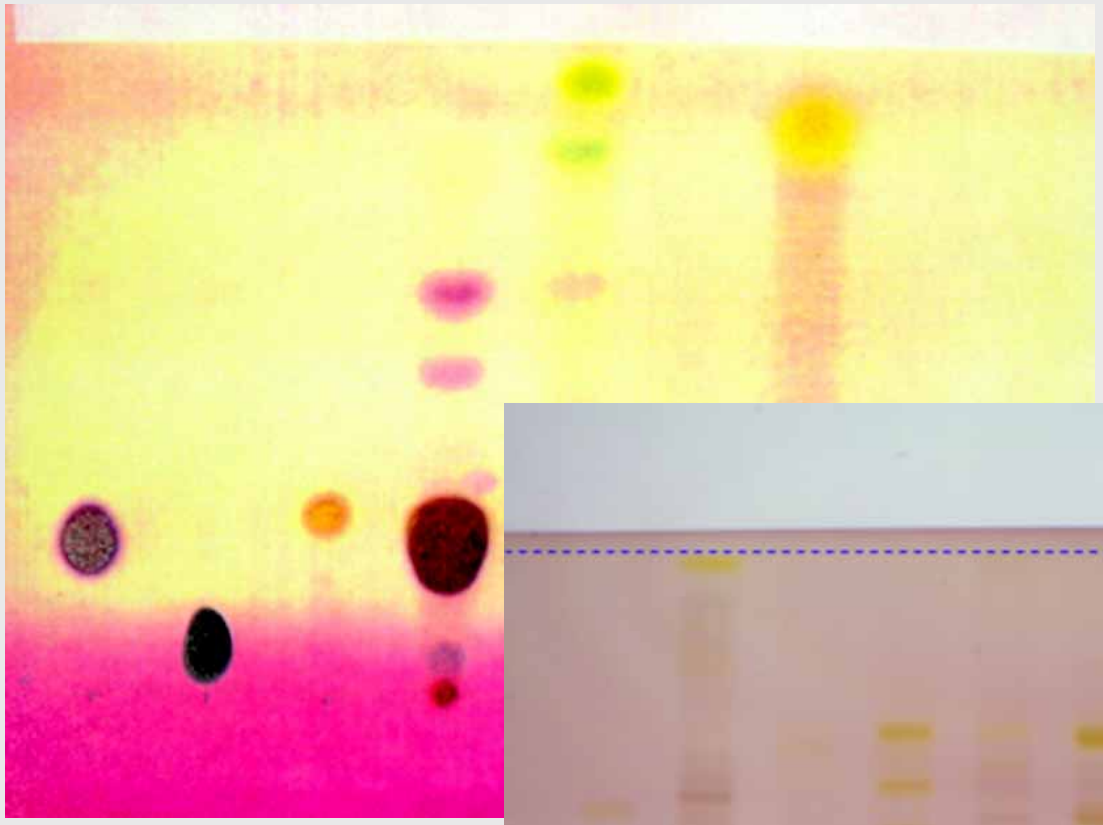
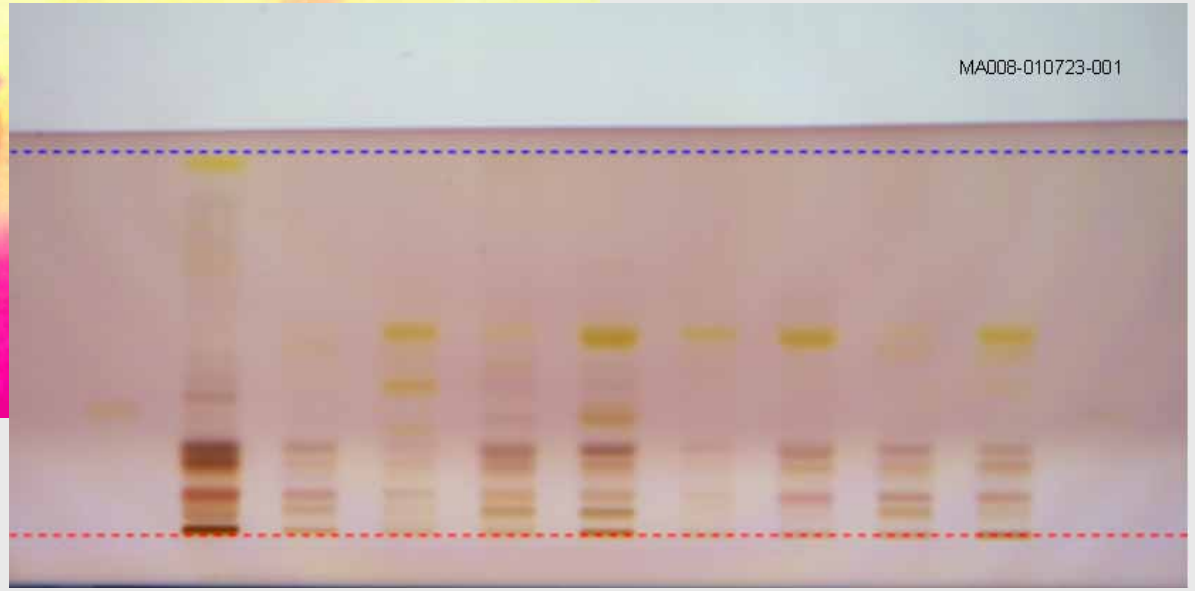
# TLC or HPTLC?





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# What is TLC?

- Chromatography for the poor (cheap)
- Simple manual chromatography for everyone (students?)
- Rapid
- Flexible
- Reference and test solution side by side
- “Just” qualitative, preliminary estimation at best

- Unpredictable
- Unreliable

→ Manual technique, simple instruments, TLC plates

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# What is HPTLC?

## **High Performance** Thin-Layer Chromatography

### TLC for the 21st century

- Instrumental TLC
  - Application
  - Development
  - Documentation
  - Densitometry
- Truly „plug and play“
- Fully cGMP compliant

### A new concept

- Suitable instruments
- Scientific basis
- Standardized methodology
- Validated methods

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# “HPTLC” on TLC plates?

- What is the point?
  - Saturation 1h vs. 20 min
  - Twice (10x) the solvent volume
  - 3x the developing time (15 vs. 6 cm)
  - Same cost per plate (20x20 vs. 10x10 cm)

  yet
  - Less resolution
  - No control of the development process

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# Standardization of methodology

- Plate setup and handling
- Sample application (as band)
- Chamber geometry and saturation
- Humidity control
- Developing distance
- Derivatization procedure
- Documentation (electronic images)
- Evaluation

SOP

A Standardized Approach to Modern High-Performance Thin-Layer Chromatography (HPTLC). Reich, E., Schibli, A., (2004) J. Planar Chromatogr. 17, 438-443

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# SOP for HPTLC

- Should be the basis for all work (in participating labs)
- Applies to all methods
- All deviations need to be recorded
- Our SOP is in full compliance with PhEur, USP, ChP

Available at: www.camag-laboratory.com (homepage)

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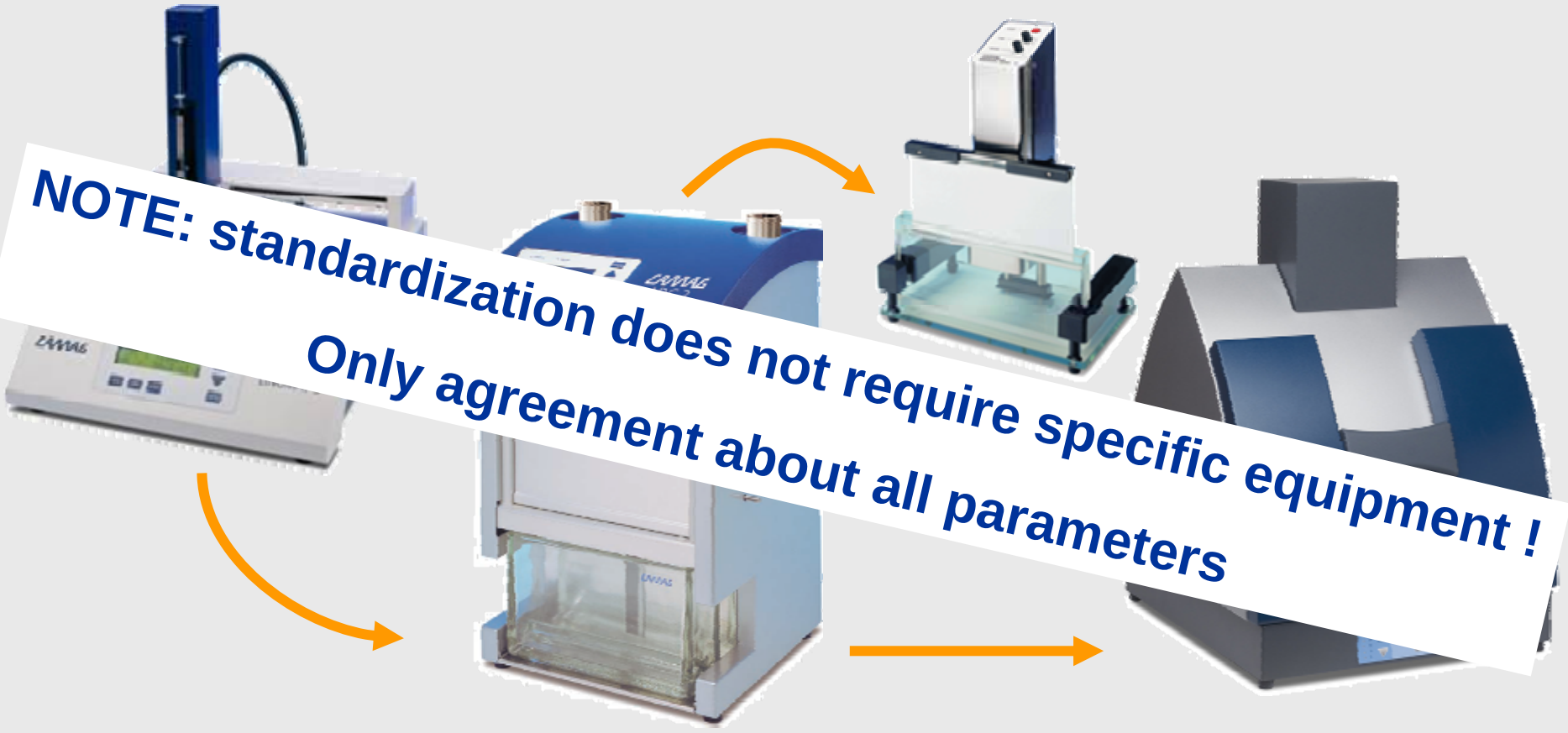
# The basic HPTLC setup

Application Development Derivatization Documentation



**NOTE: standardization does not require specific equipment !**

**Only agreement about all parameters**

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# Standardized basic equipment

- Clear specification of HPTLC plates (type, format, manufacturer)
- Software control for “absolute” reproducibility of all parameters
- Independence from environmental factors (humidity !)
- Eliminating human factors

- Emulation by “manual” operation still possible to a certain degree

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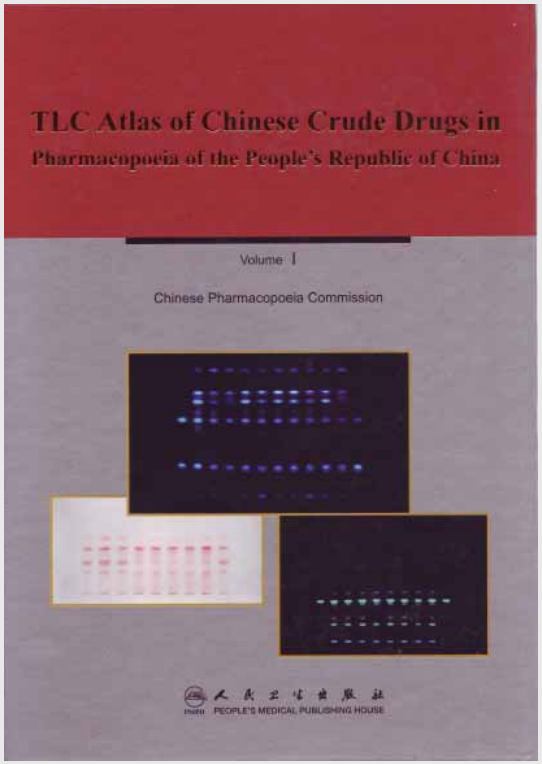
# Sources of methods

- European Pharmacopoeia (EP)
  - New monographs feature TLC and HPTLC in parallel
- British, French, German, Swiss Pharmacopoeias
  - Offer specific monographs not found in EP
- The USP Dietary Supplement Compendium
  - TLC and state of the art HPTLC
- Chinese Pharmacopoeia
  - HPTLC atlas as a supplement of 2005 ed.
- Japanese Pharmacopoeia
  - Only simple TLC



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# More sources of methods

- American Herbal Pharmacopoeia (19 monographs)
- Quality Standards of Indian Medicinal Plants

  (8 volumes)
- Indian Herbal Pharmacopoeia (54 monographs)
- Quality Standards of Traditional Chinese Medicines

  (Chinese only)
- Wagner, H. and Bladt, S. „Plant Drug Analysis“

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# Converting existing TLC methods to HPTLC

Assumptions:

- Results on HPTLC and TLC plates are similar if
  - No changes are made to chromatographic system
  - Same equipment is used
  - Original TLC method was optimized
- Due to the higher separation power HPTLC plates
  - Usually give improved result
  - Require shorter developing distance → less time
- HPTLC with no instruments looks bad, but so does TLC

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# Converting existing TLC methods to HPTLC

Practical aspects (I)

- Do not change chromatographic system (chamber configuration, mobile phase, stationary phase)
- Reduce application volume (generally) to 1/5 (typically 2 µL)
- Employ standardized methodology (based on SOP):
  - Fixed (x, y) application positions, (e.g.) 8 mm bands
  - Use 60 mm developing distance
  - Fixed drying time and temperature
  - Use dipping instead of spraying if possible
  - Fixed waiting times between derivatization and evaluation
  - Obtain multiple images (if possible) of plate (e.g. UV 254, UV 366 prior to derivatization, and white light, UV 366 after derivatization

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# Converting existing TLC methods to HPTLC

Practical aspects (II)

- Use same samples on TLC and HPTLC
- Evaluate whether changes in the result can
  - Fall under “additional weak zones may be seen”
  - Are due to natural variability of plant
- Color description is always subjective: e.g.

  blue, bluish

  blue white

  blue green, etc.
- Rf is predictable only for validated methods!

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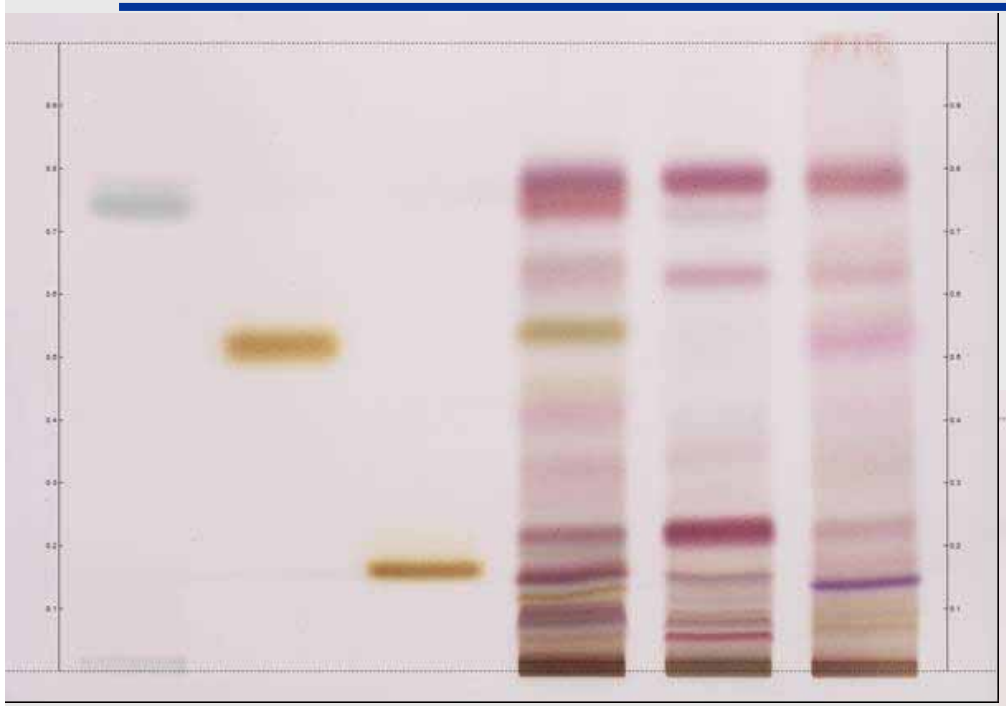
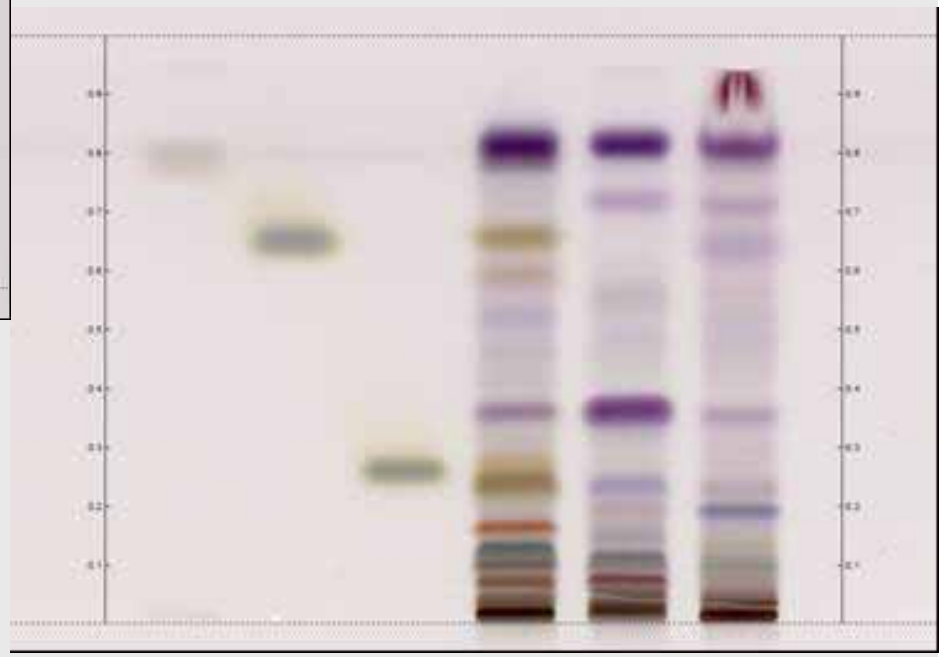
# Example: USP method for chamomile



TLC



HPTLC

application volume 2 µL

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# Developing an ID method from scratch

- Review literature for related plants
- Obtain multiple samples form different accessions
- Obtain samples of related plants and known adulterants
- Optimize sample preparation and detection
- Avoid toxic solvents
- Start with silica gel, select mobile phase

Reich, E., Schibli, A.:HPTLC for the analysis of medicinal plants, chapter 5, Thieme 2007

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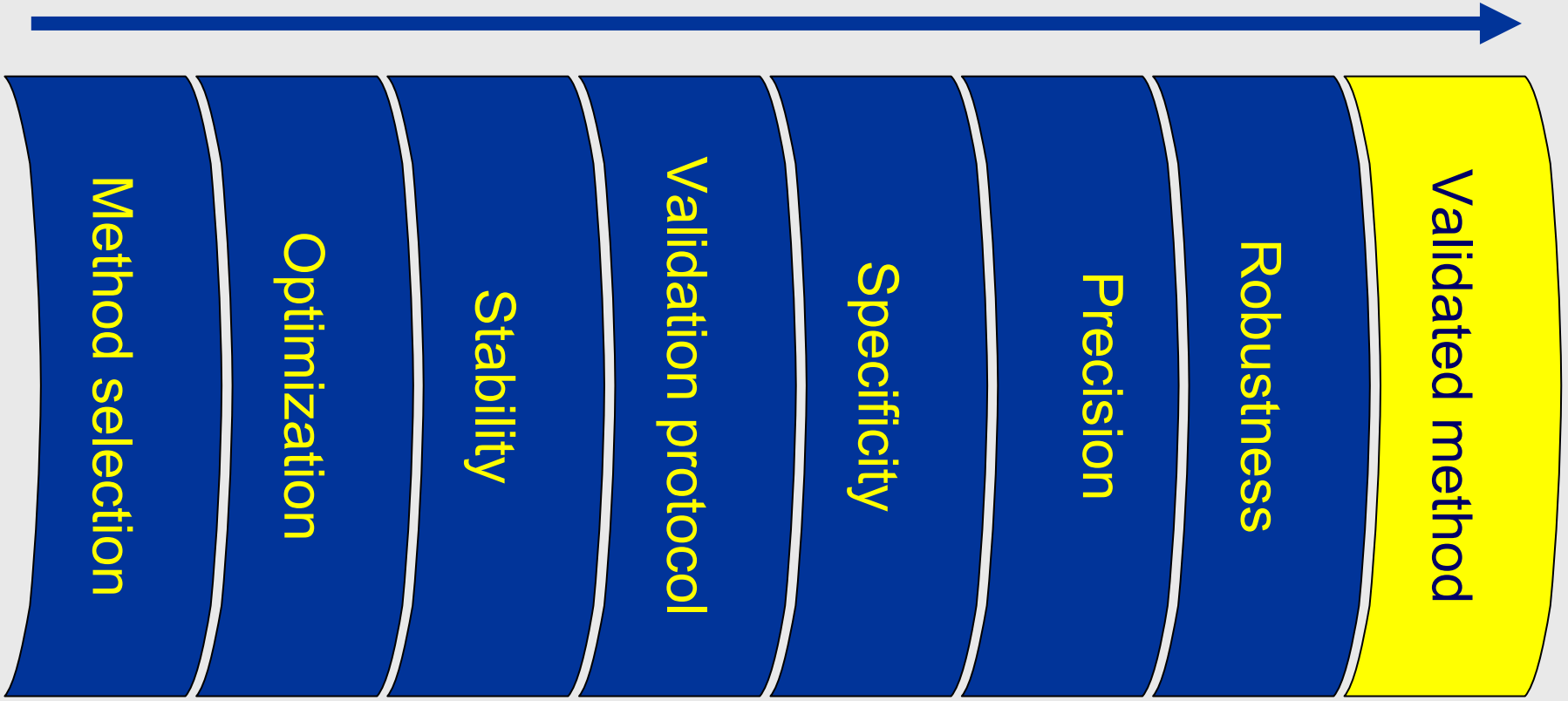
# Validation of qualitative methods




Validation of high-performance thin-layer chromatographic methods for the identification of botanicals in a cGMP environment. Reich, E., Schibli, A., DeBatt, A. (2008) J. AOAC Int. 91, 13-20.

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# Envisioning the Future...

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# Methods for identification of plants

- SOP is the basis for an HPTLC method template (instruments)

  → software template

- A (PhEur, USP, etc.) monograph or validated methods is basis for HPTLC method document →Method for identification of Thyme
- Result table/description is replaced by a reference image
- Corresponding instrument methods are derived from software template

  → instrument method

- Results on plate are qualified by a System Suitability Test (SST)
- Comparison of results with reference images

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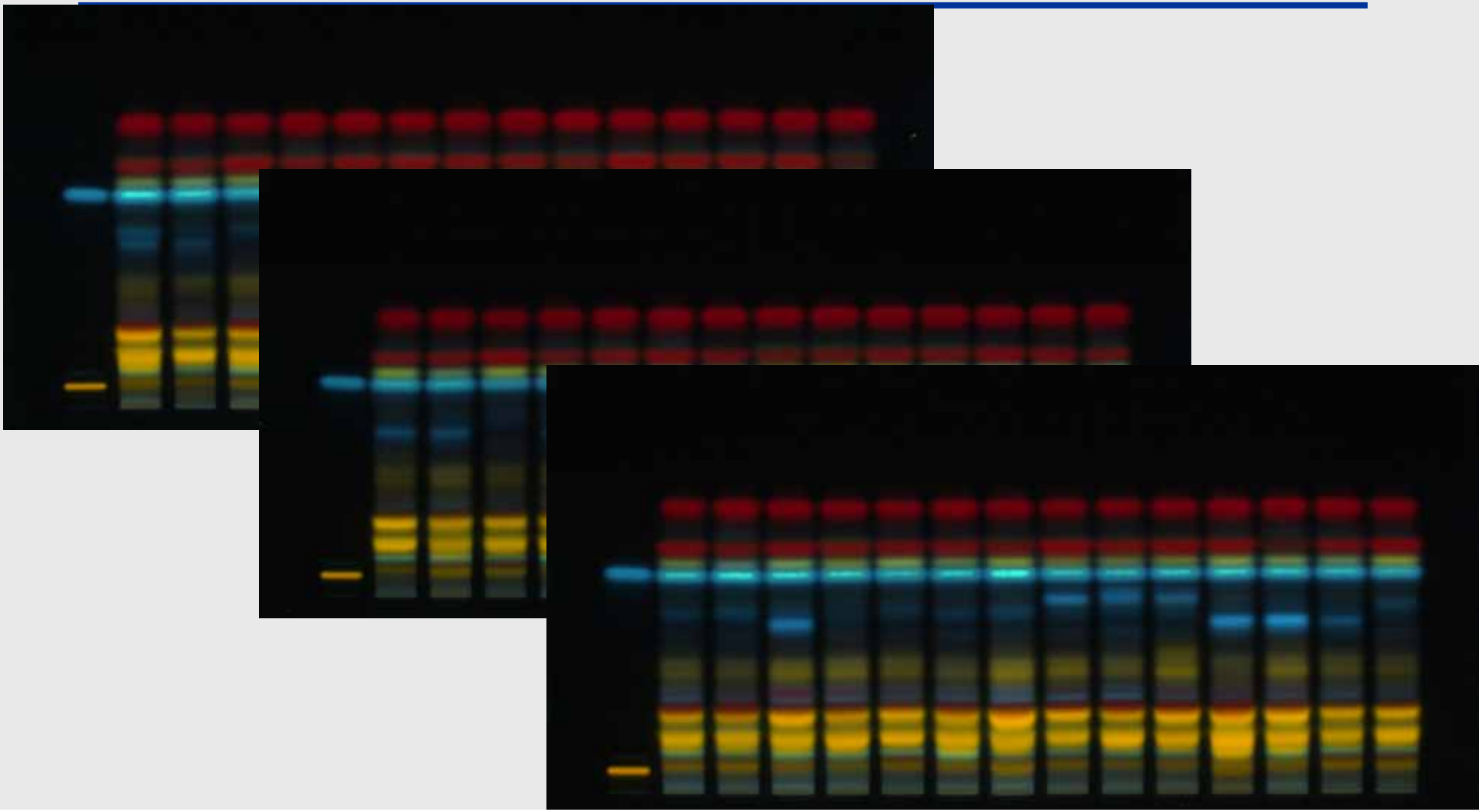
# Thyme leaf plates 1 - 3



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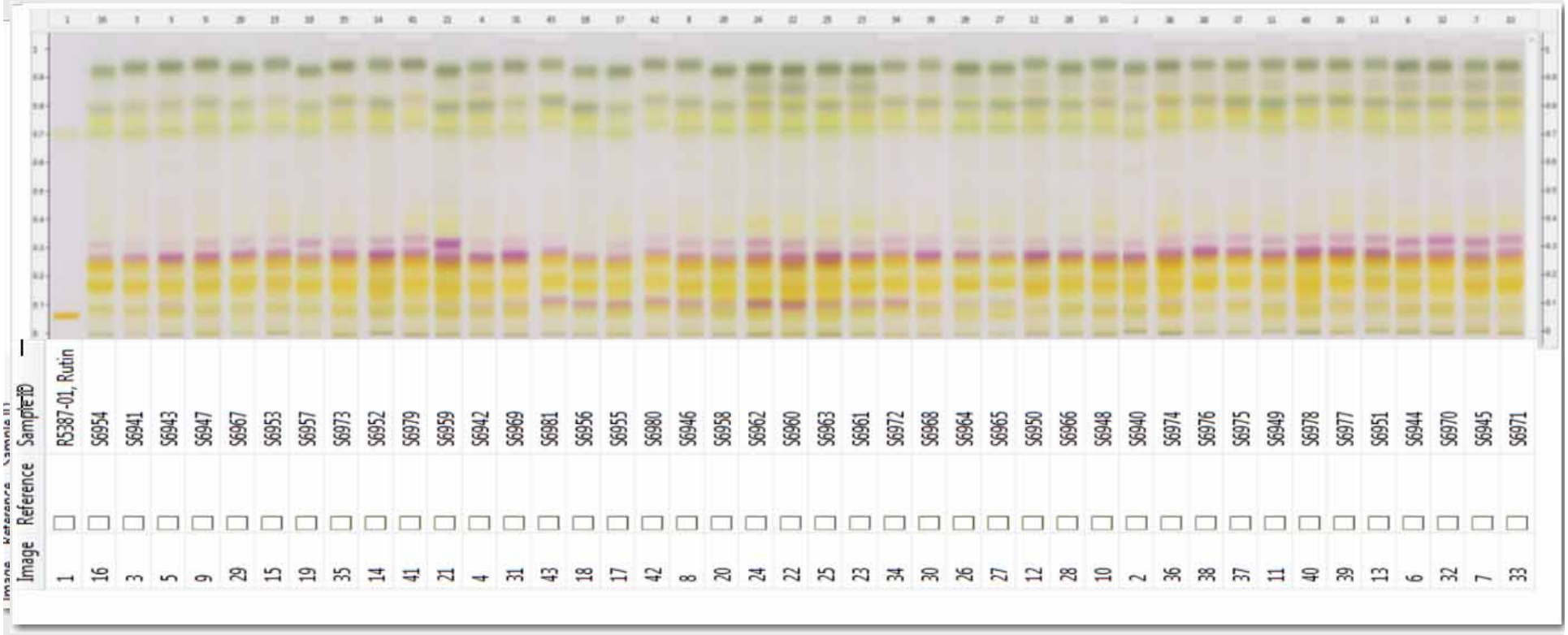
# Thyme leaf



# Goal:
# The CAMAG HPTLC method collection

- Identification of 150 (+) plants from East and West
- Predictable results everywhere
- Convenient comparison against reference images
- Compatible with (current and future) harmonized description of HPTLC
- Based on ATS4, ADC2, Visualizer and Software

# Goal:
# CAMAG Laboratory Network

- Collaboration on development and validation of HPTLC methods for identification of plants
- Setting global quality standards for HPTLC
- Providing training and research opportunities
- Offering analytical services for customers

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# Who is part of the network?

- CAMAG Laboratory Muttenz
- CAMAG Scientific Inc. Wilmington (NC)
- Shanghai University of TCM (Prof. Wang)
- University of Applied Sciences Wädenswil (Prof. Meier)
- University of Regensburg (Prof. Heilmann)
- University of Barcelona (Prof. Canigueral)
- University Sapienza Rome (Prof. Nicoletti)
- University of Graz (Prof. Bauer)

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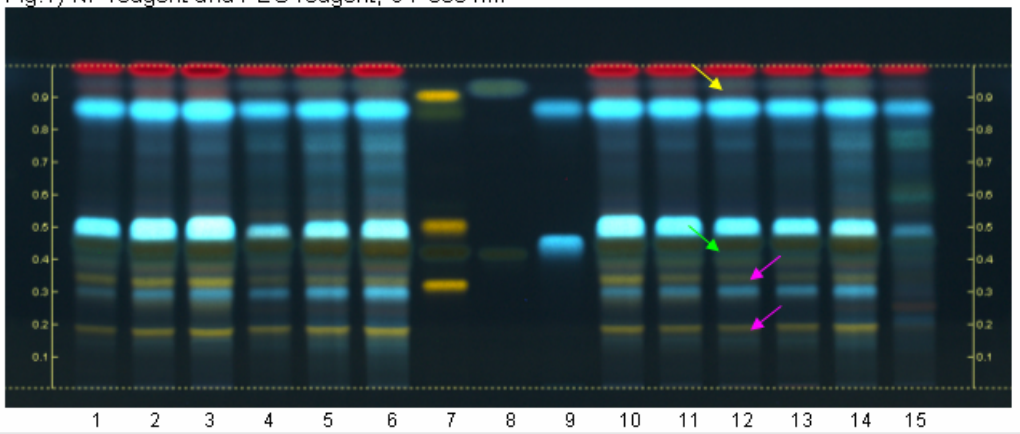
# Examples from other labs (HS Wädenswil)

## Oswego tea

**4. Results**

Fig.1) NP reagent and PEG reagent, UV 366 nm




| Track | Volume | Sample | Track | Volume | Sample |
|---|---|---|---|---|---|
| 1 | 2 µL | Oswego tea; herb 1 | 9 | 2 / 5 µL | Chlorogenic acid, rosmarinic acid (with increasing Rf) |
| 2 | 4 µL | Oswego tea; herb 1 | 10 | 4 µL | Oswego tea; herb 1 |
| 3 | 6 µL | Oswego tea; herb 1 | 11 | 4 µL | Oswego tea; herb 2 |
| 4 | 2 µL | Oswego tea; herb with flower | **12** | **4 µL** | **Oswego tea; herb 3** |
| 5 | 4 µL | Oswego tea; herb with flower | 13 | 4 µL | Oswego tea; herb 4 |
| 6 | 6 µL | Oswego tea; herb with flower | 14 | 4 µL | Oswego tea; herb with flower |
| 7 | 2 µL | Rutin, narirutin, hyperoside, quercetin (with increasing Rf) | 15 | 4 µL | Oswego tea; flower |
| 8 | 2 µL | Naringin, naringenin (with increasing Rf) | | | |

**System suitability test**

Rutin: orange fluorescent zone at Rf ~ 0.32 (UV 366 nm).

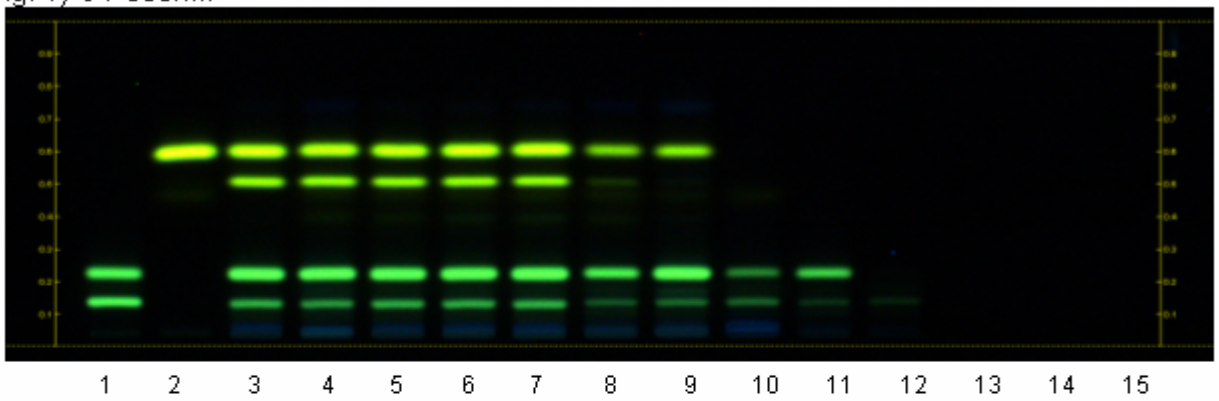
Hyperoside: orange fluorescent zone at Rf ~ 0.50 (UV 366 nm).

# Examples from other labs (Uni  Regensburg)

## Coptis rhiz.

**4. Results**

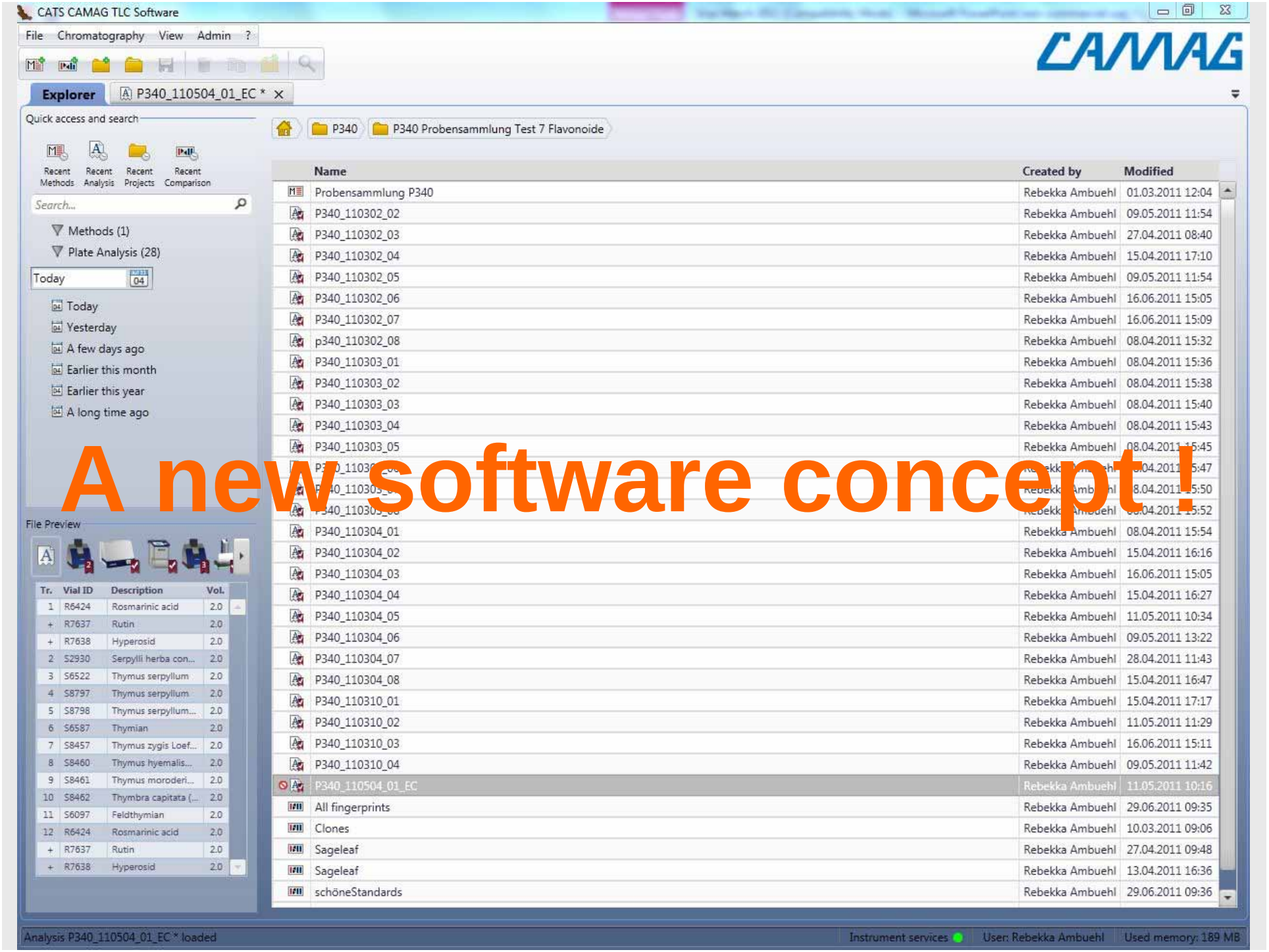
Fig. 1) UV 366nm




| Track | Volume | Sample | Track | Volume | Sample |
|---|---|---|---|---|---|
| 1 | 10 µL | Palmatine and berberine | **9** | **10 µL** | **Coptis teeta rhizome** |
| 2 | 10 µL | Coptisine | 10 | 10 µL | Chinese corktree bark |
| **3** | **10 µL** | **Coptis rhizome # 1** | 11 | 10 µL | Chinese mahonia bark |
| **4** | **10 µL** | **Coptis rhizome # 2** | 12 | 10 µL | Tinospora root |
| **5** | **10 µL** | **Coptis rhizome # 3** | 13 | | blank |
| **6** | **10 µL** | **Coptis rhizome # 4** | 14 | | blank |
| **7** | **10 µL** | **Coptis rhizome # 5** | 15 | | blank |
| **8** | **10 µL** | **Coptis deltoidea rhizome** | | | |

**System suitability test**

Palmatine: fluorescent zone at Rf ~ 0.14.
Berberine: fluorescent zone at Rf ~ 0.23.
Coptisine: fluorescent zone at Rf ~ 0.60.


CATS CAMAG TLC Software
File Chromatography View Admin ?
CAMAG
Explorer
P340_110504_01_EC *
Quick access and search
Recent Methods
Recent Analysis
Recent Projects
Recent Comparison
Search...
Methods (1)
Plate Analysis (28)
Today
Today
Yesterday
A few days ago
Earlier this month
Earlier this year
A long time ago
P340
P340 Probensammlung Test 7 Flavonoide
Name
Created by
Modified
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P340_110302_02 Rebekka Ambuehl 09.05.2011 11:54
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Clones Rebekka Ambuehl 10.03.2011 09:06
Sageleaf Rebekka Ambuehl 27.04.2011 09:48
Sageleaf Rebekka Ambuehl 13.04.2011 16:36
schöneStandards Rebekka Ambuehl 29.06.2011 09:36
File Preview
Tr. Vial ID Description Vol.
1 R6424 Rosmarinic acid 2.0
+ R7637 Rutin 2.0
+ R7638 Hyperosid 2.0
2 S2930 Serpylli herba con... 2.0
3 S6522 Thymus serpyllum 2.0
4 S8797 Thymus serpyllum 2.0
5 S8798 Thymus serpyllum... 2.0
6 S6587 Thymian 2.0
7 S8457 Thymus zygis Loef... 2.0
8 S8460 Thymus hyemalis... 2.0
9 S8461 Thymus moroderi... 2.0
10 S8462 Thymbra capitata (... 2.0
11 S6097 Feldthymian 2.0
12 R6424 Rosmarinic acid 2.0
+ R7637 Rutin 2.0
+ R7638 Hyperosid 2.0
A new software concept!
Analysis P340_110504_01_EC * loaded
Instrument services
User: Rebekka Ambuehl
Used memory: 189 MB

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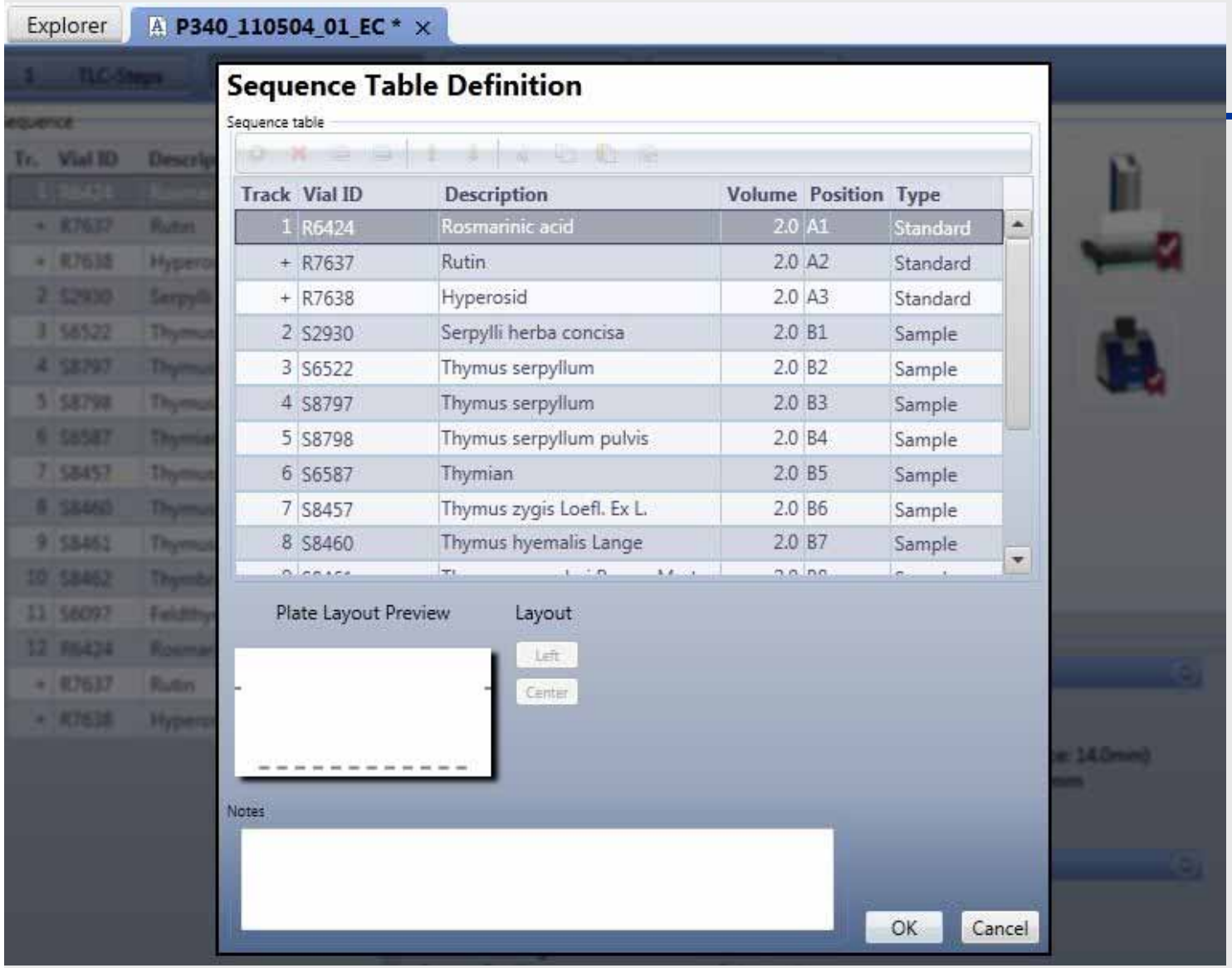

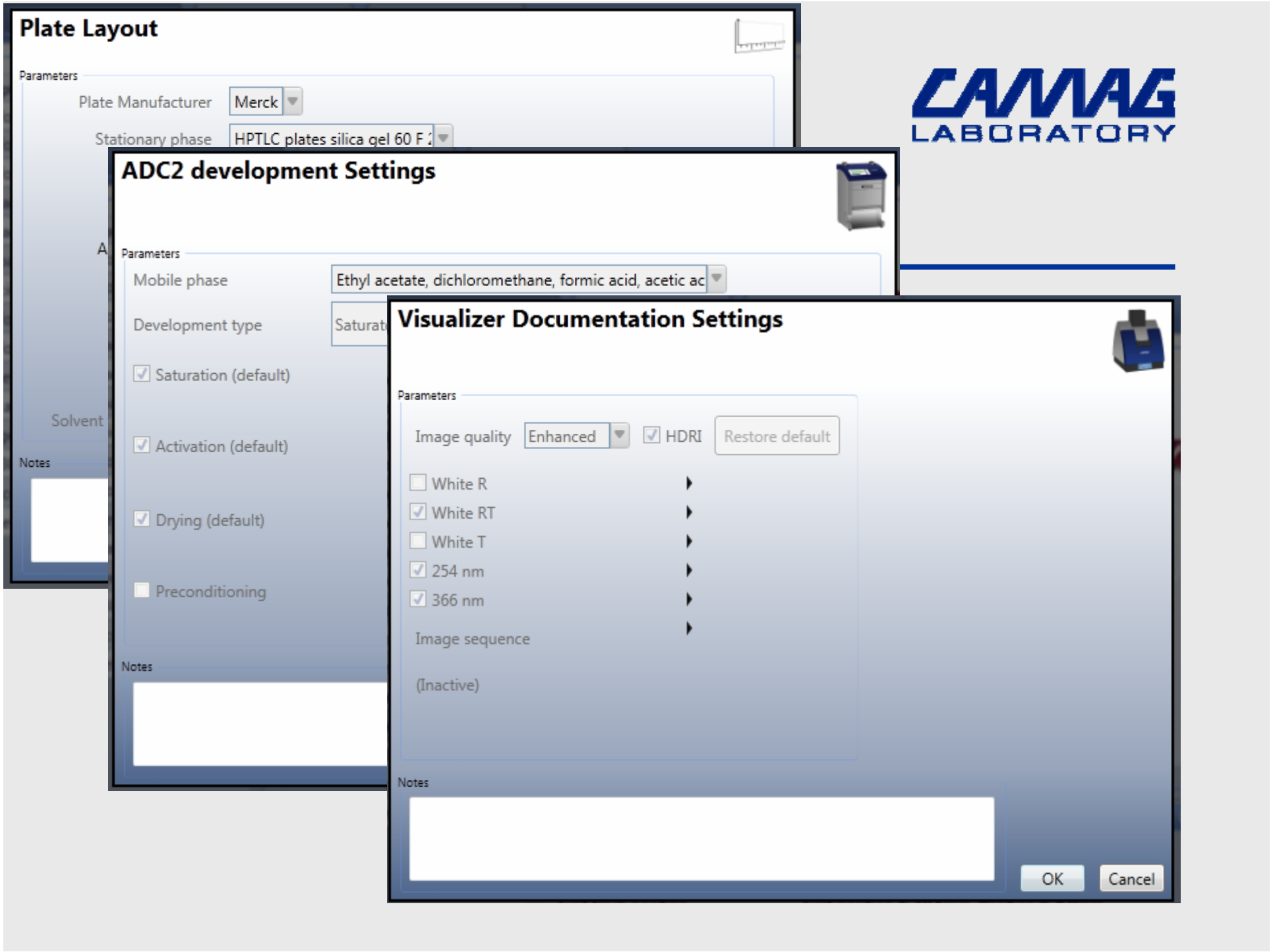
Plate Layout
Parameters
Plate Manufacturer
Merck
Stationary phase
HPTLC plates silica gel 60 F
Solvent
Notes
ADC2 development Settings
Parameters
Mobile phase
Ethyl acetate, dichloromethane, formic acid, acetic ac
Development type
Saturat
Saturation (default)
Activation (default)
Drying (default)
Preconditioning
Notes
Visualizer Documentation Settings
Parameters
Image quality
Enhanced
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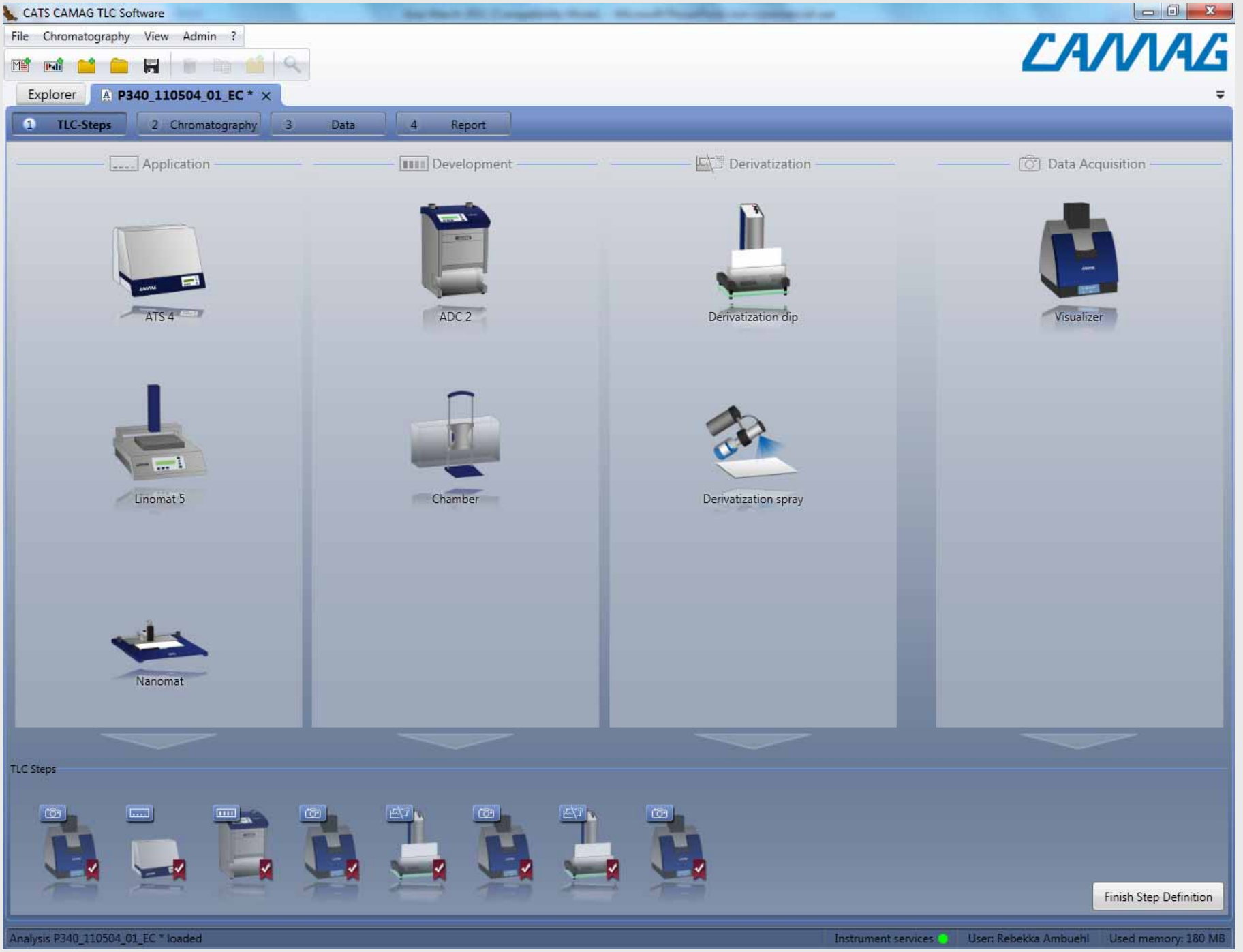
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CAMAG
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P340_110504_01_EC *
1 TLC-Steps
2 Chromatography
3 Data
4 Report
Application
Development
Derivatization
Data Acquisition
ATS 4
ADC 2
Derivatization dip
Visualizer
Linomat 5
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Nanomat
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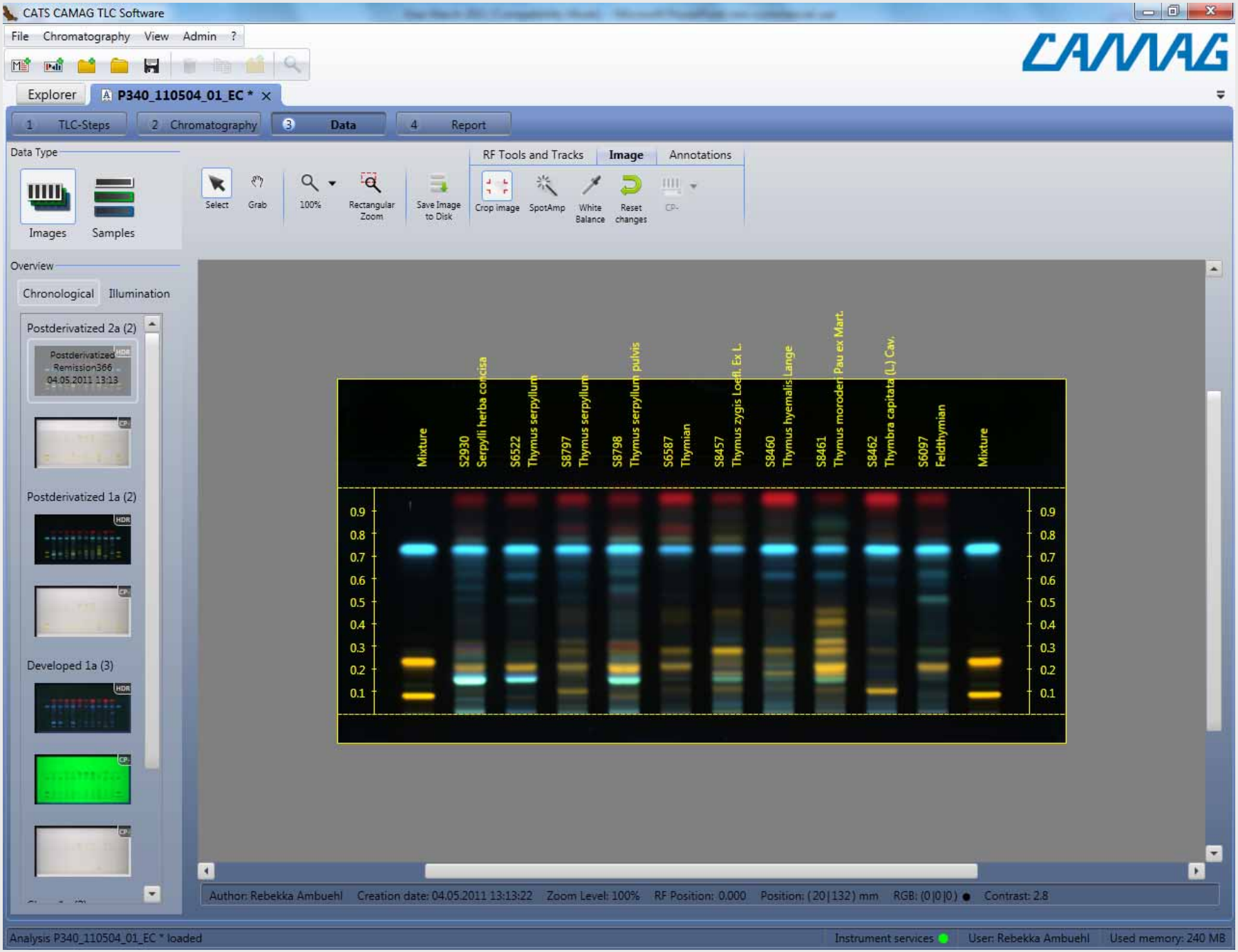
CATS CAMAG TLC Software
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CAMAG
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P340_110504_01_EC *
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4 Report
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Postderivatized 2a (2)
Postderivatized Remission366 04.05.2011 13:13
Postderivatized 1a (2)
Developed 1a (3)
Mixture
S2930 Serpylli herba concisa
S6522 Thymus serpyllum
S8797 Thymus serpyllum
S8798 Thymus serpyllum pulvis
S6587 Thymian
S8457 Thymus zygis Loefl. Ex L.
S8460 Thymus hyemalis Lange
S8461 Thymus moroderi Pau ex Mart.
S8462 Thymbra capitata (L.) Cav.
S6097 Feldthymian
Mixture
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Author: Rebekka Ambuehl Creation date: 04.05.2011 13:13:22 Zoom Level: 100% RF Position: 0.000 Position: (20|132) mm RGB: (0|0|0) Contrast: 2.8
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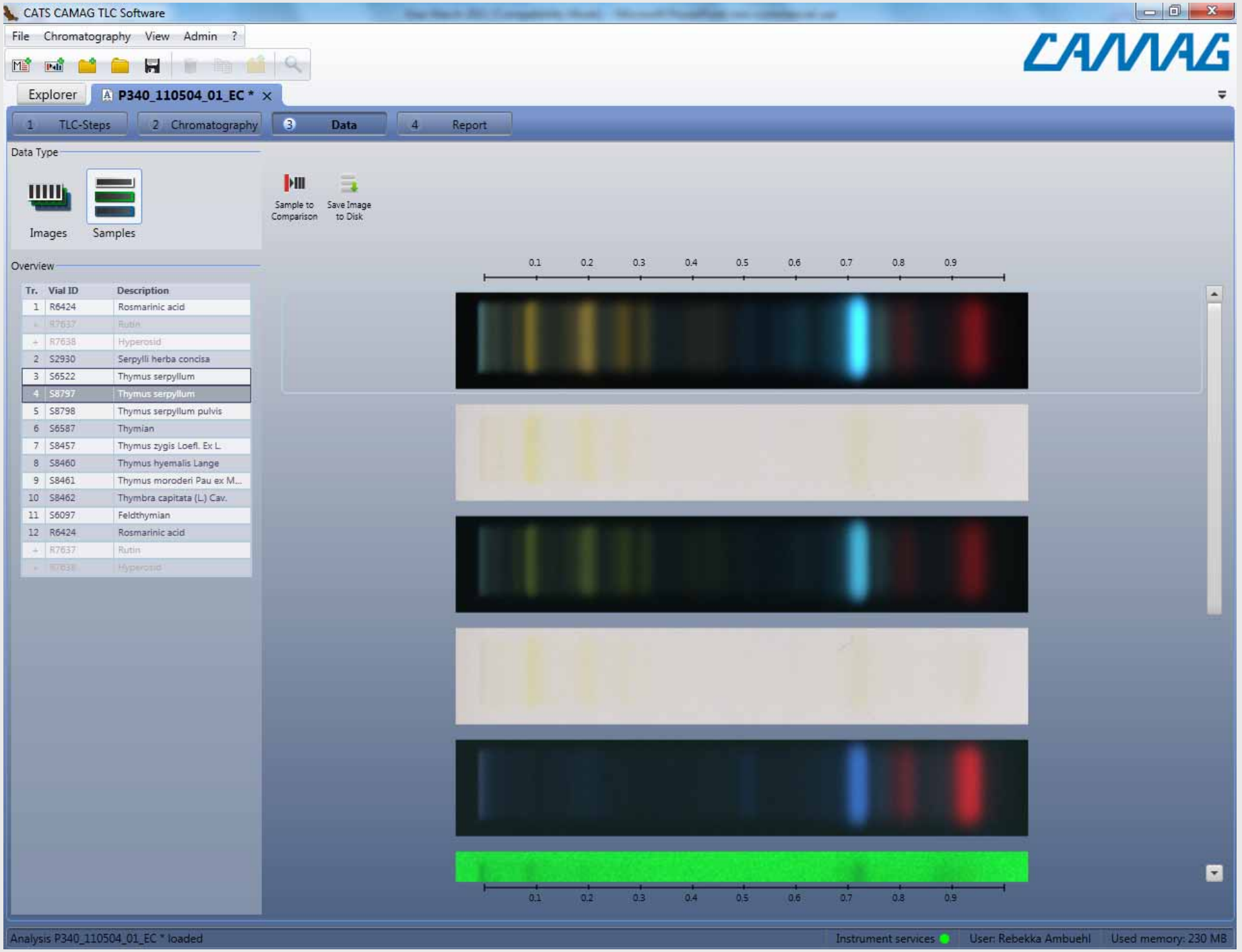
CATS CAMAG TLC Software
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Explorer
P340_110504_01_EC *
1 TLC-Steps
2 Chromatography
3 Data
4 Report
Data Type
Images
Samples
Sample to Comparison
Save Image to Disk
Overview
Tr. Vial ID Description
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+ R7637 Rutin
+ R7638 Hyperosid
2 S2930 Serpylli herba concisa
3 S6522 Thymus serpyllum
4 S8797 Thymus serpyllum
5 S8798 Thymus serpyllum pulvis
6 S6587 Thymian
7 S8457 Thymus zygis Loefl. Ex L.
8 S8460 Thymus hyemalis Lange
9 S8461 Thymus moroderi Pau ex M...
10 S8462 Thymbra capitata (L.) Cav.
11 S6097 Feldthymian
12 R6424 Rosmarinic acid
+ R7637 Rutin
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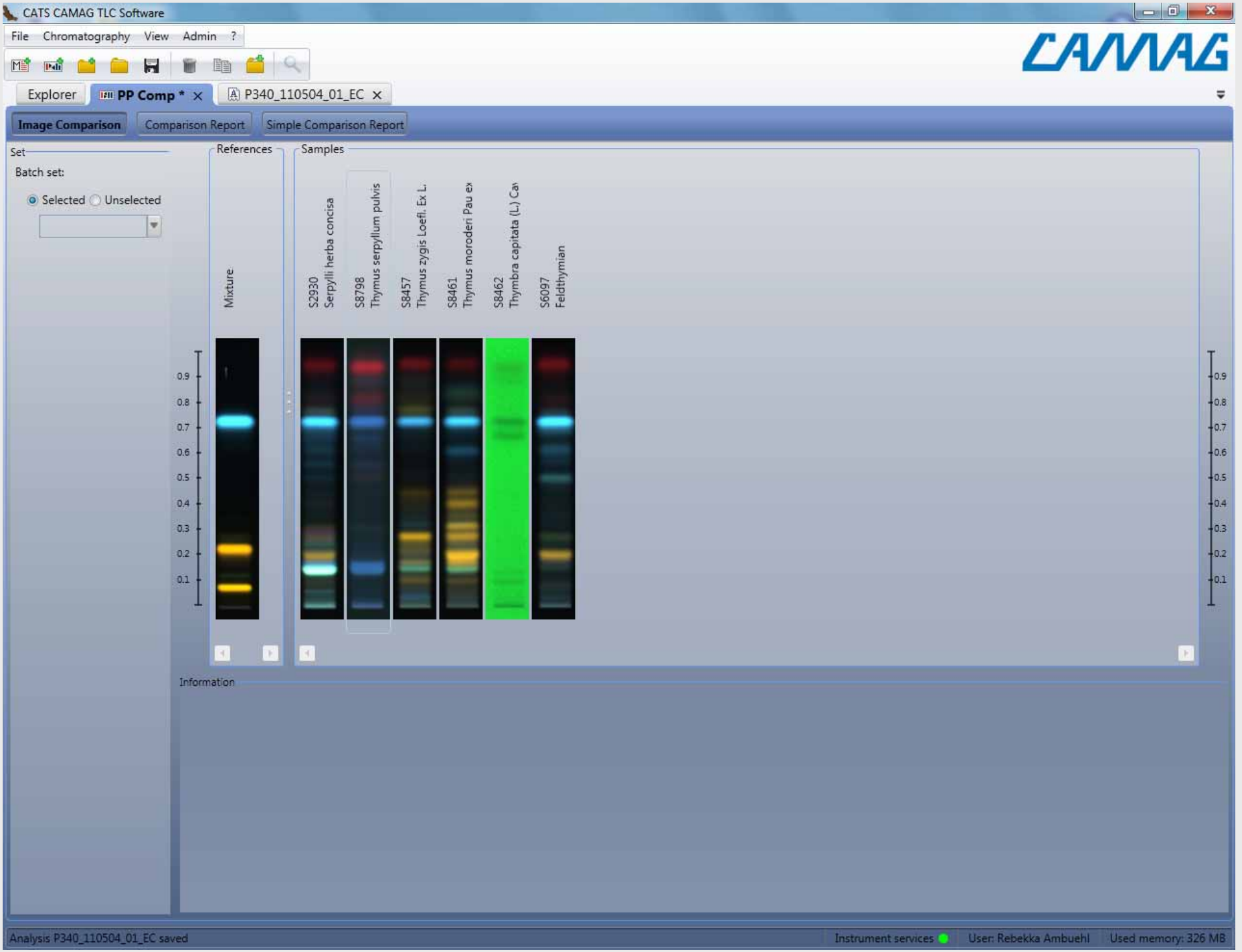
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S8457 Thymus zygis Loefl. Ex L.
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S8462 Thymbra capitata (L.) Ca
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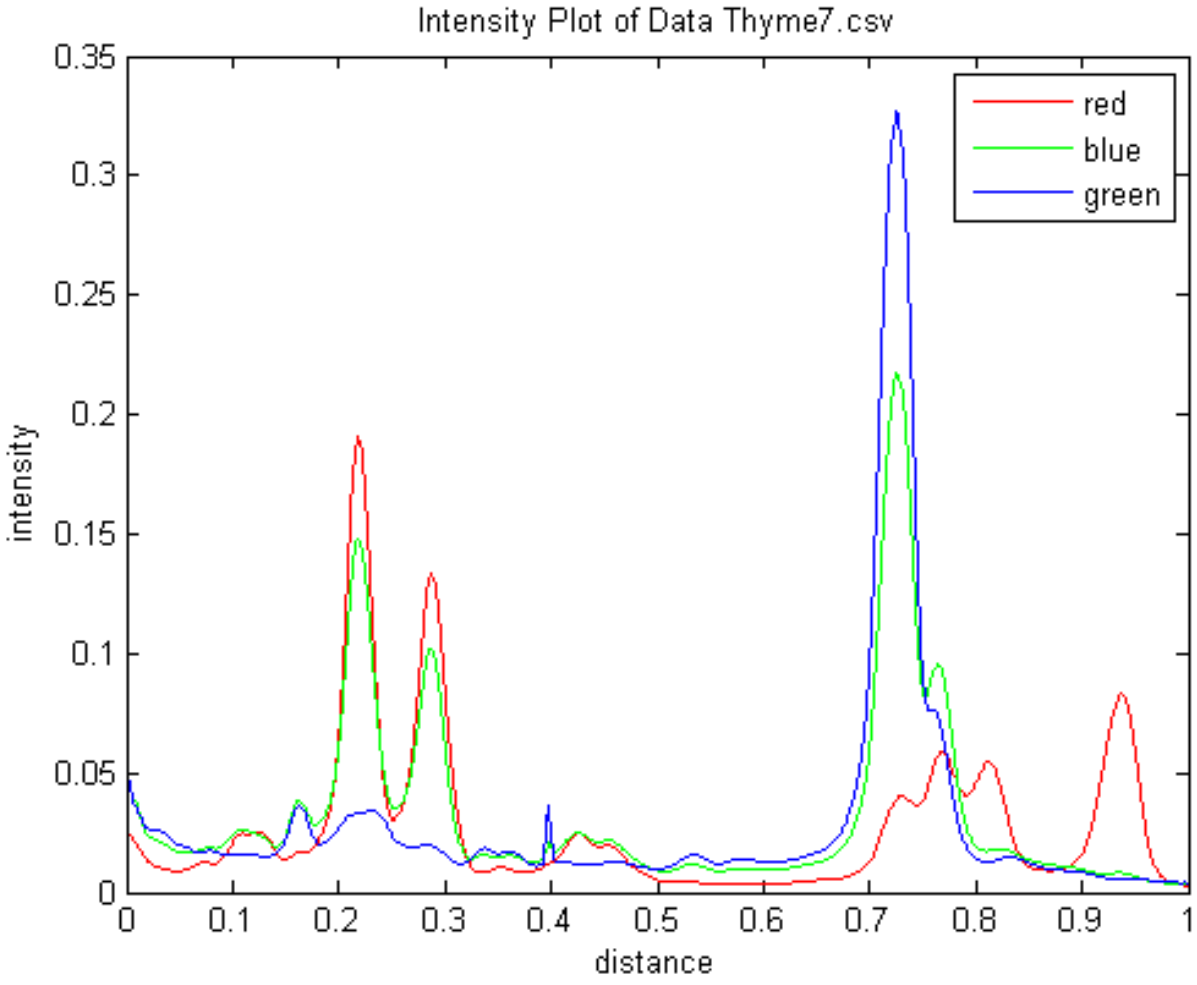
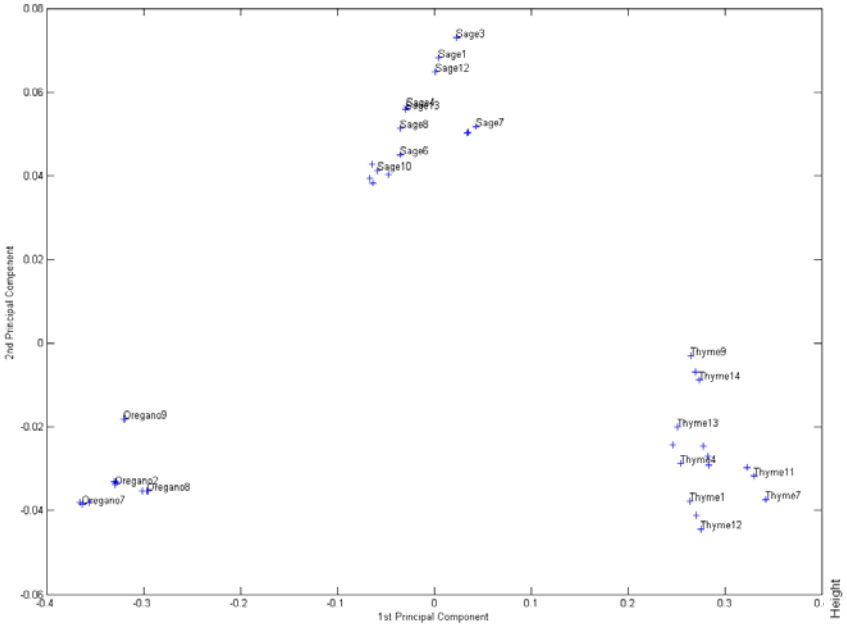
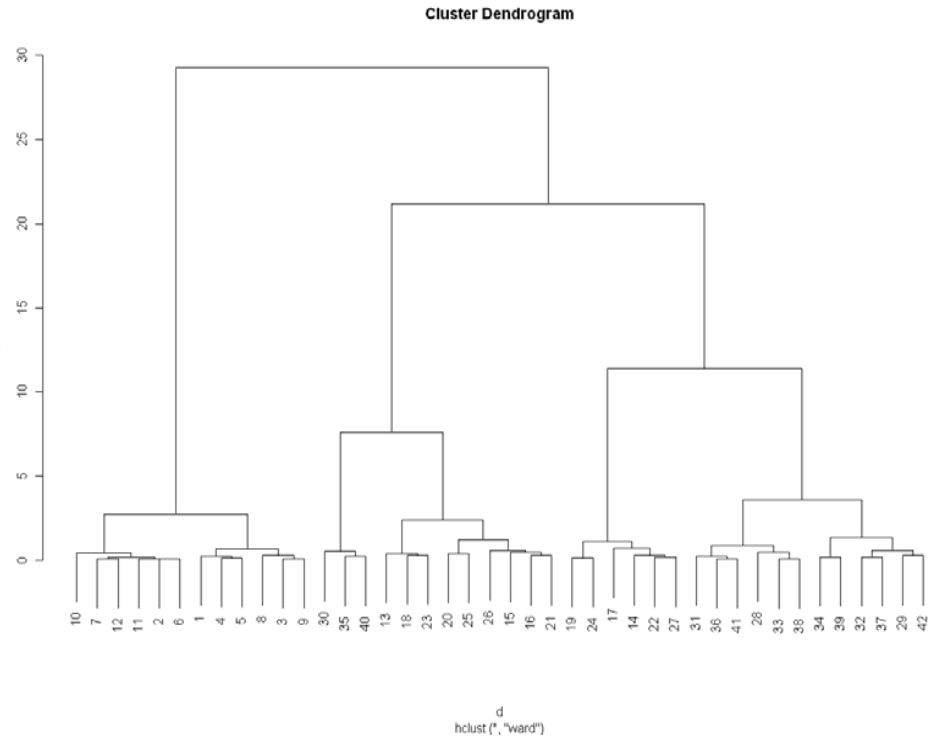
# Three channels per track



# PCA (Master thesis R. Ambühl, Uni Basel)



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# HPTLC as research tool

- Evaluation of column fractions
- Identification by MS
- Screening for bio-activity

# Application of HPTLC-MS for the identification of flavonoids in herbal extracts

Bauer R[1], Meier M[1], Pferschy-Wenzig E[1], Woelkart K[1], Reich E[2]
[1] Institute of Pharmaceutical Sciences, Karl-Franzens-University Graz, Universitätsplatz 4, 8010 Graz, Austria
[2] CAMAG-Laboratory, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland

## Introduction

Recently, a HPTLC-MS interface became available, which semi-automatically can extract zones of interest from TLC/HPTLC plates and direct them into a LC-MS system for substance identification or structure elucidation [1]. So far, the method has hardly been applied for the analysis of plant extracts [2]. Flavonoids are very abundant in plants and play an important role as anti-oxidants. Therefore, we now have evaluated the application of the HPTLC-MS interface for the investigation of flavonoid containing herbal drugs.

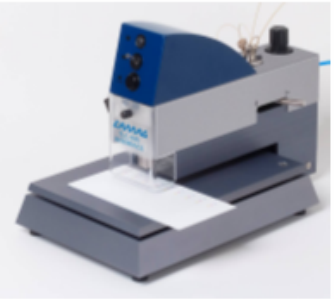
## Summary

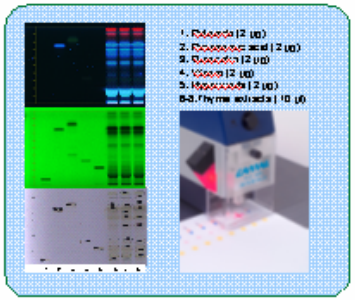
Rutoside, hyperoside, vitexin, quercetin and rosmarinic acid as pure substances were used to optimize extraction, detection and identification by HPTLC-MS. It was possible to identify hyperoside, vitexin, quercetin and rosmarinic acid also in an extract of Thymus vulgaris. The CAMAG HPTLC-MS interface proved to be a quick and powerful tool for the on-line identification of flavonoids in TLC/HPTLC separations. It can complement the classical TLC detection tools and can help to identify individual zones of a TLC/HPTLC separation.

## Principle

The instrument is used to isolate compounds from a TLC/HPTLC plate and transfer them into a mass spectrometer for identification or structure elucidation. CAMAG TLC-MS Interface can be connected to any brand of LC-coupled mass spectrometer.

- Semi-automatic instrument involving automatic piston movement for pressure seal of the TLC/HPTLC zone on both glass plates and aluminium foils
- Extraction directly from the plate using a suitable solvent delivered by a HPLC pump
- Online transfer into the mass spectrometer
- Automatic cleaning of the piston between the extractions.



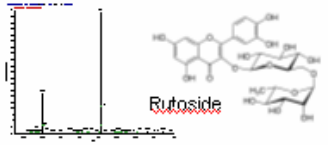


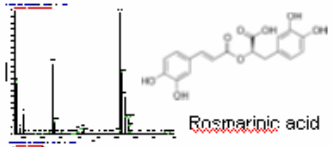

## Results and Discussion

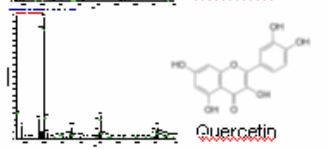
Rutoside, hyperoside, vitexin, quercetin and rosmarinic could be extracted from TLC/HPTLC silica plates by the CAMAG TLC-MS Interface using acetonitrile as solvent delivered by an HPLC pump at 100 µl/min. After application of 2 µg of each compound, significant mass spectra were obtained by electro spray ionization (ESI) in the negative ion mode. Mass spectrometric signals were obtained within a minute per substance zone. The spectra were suitable for identification of the compounds.

It was possible to identify hyperoside, vitexin, quercetin and rosmarinic acid also in an methanolic extract of *Thymus vulgaris*. The applied amount corresponded to 1 mg plant equivalent and was comparable to regular amounts in HPTLC separations. The CAMAG HPTLC-MS interface proved to be a quick and powerful tool for the on-line identification of flavonoids in TLC/HPTLC separations. It can complement the classical TLC detection tools.

The TLC-MS Interface can also be used for extraction into vials for NMR, (ATR-)FTIR, static nanospray, direct inlet EI-MS, or MALDI. Scraping off the plate is no more necessary.



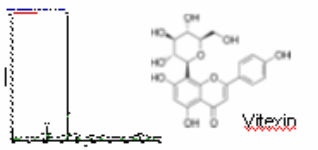



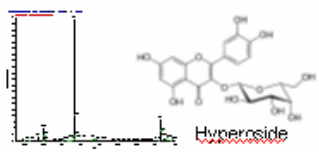




## Experimental

Extracts of *Thymus vulgaris* (500 mg extracted with 5 ml methanol in a subcritic bath for 10 min) and pure flavonoids have been applied as bands onto HPTLC plates (Merck) using an automatic TLC sampler ATS 4 (CAMAG). Chromatography was performed in an automatic developing chamber ADC 2 with humidity control (CAMAG). Humidity control: saturated MgCl2 solution (Saturation: 20 min); developing distance from application position/ lower edge: 62/70; developing solvent: ethyl acetate, formic acid, water (15/1/1); developing time: 18 min; plate drying: 5 min; derivatization reagent: NP-Reagent/PEG.

Separated zones were eluted from the plate with the CAMAG TLC-MS Interface using acetonitrile as solvent delivered by an HPLC pump (at 100µl/min). The interface was hyphenated to a Finnigan LCQ Deca XP Plus ion trap mass spectrometer equipped with an electro spray ionization (ESI) source in negative ion mode.




## References

1. Luftmann, H. et al. (2007) Rapid Commun. Mass Spectrom. 21:3772-3776.
2. Reich E, Widmer V. (2009) Planta Med. 75(7): 711-718.

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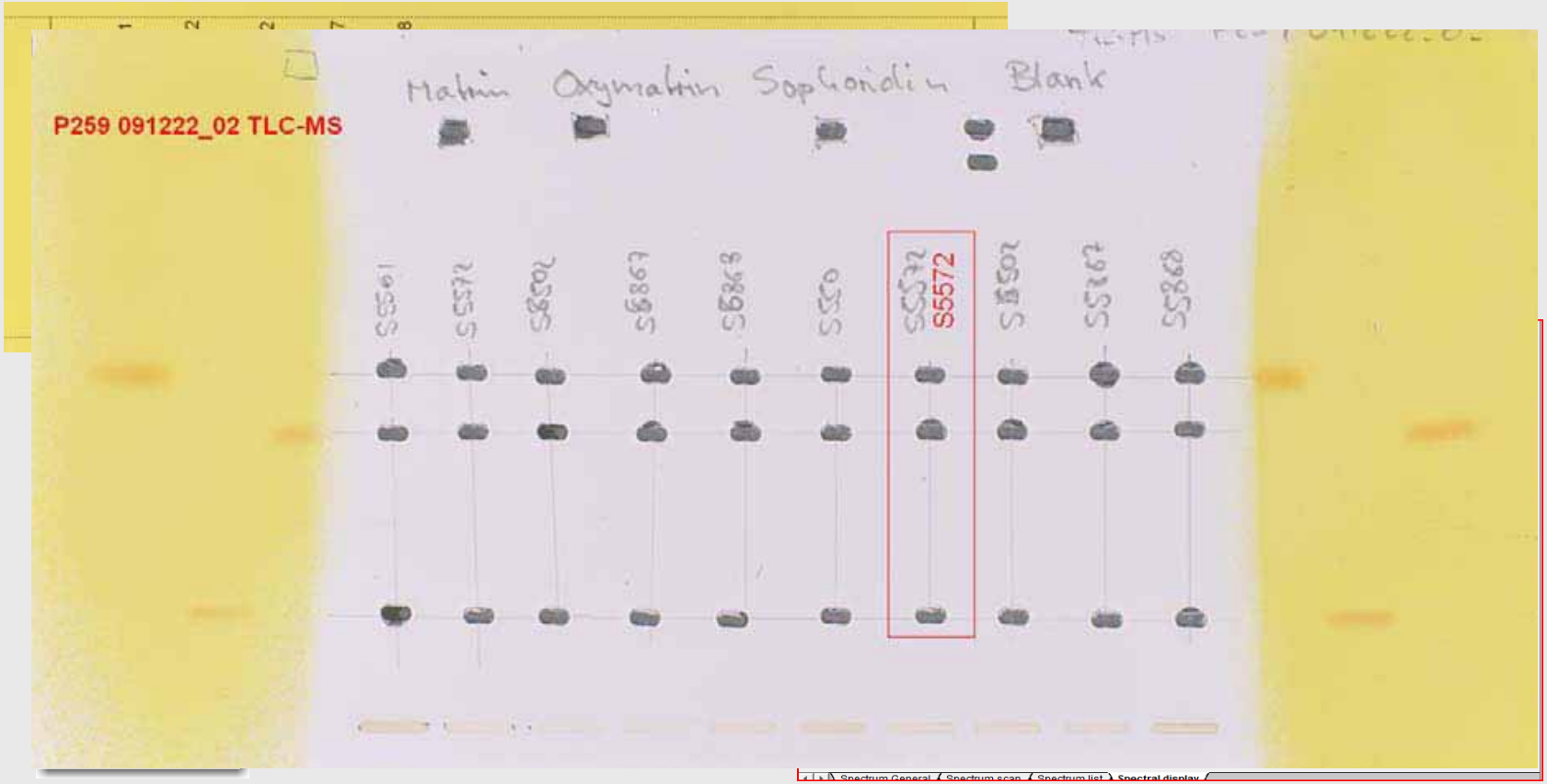
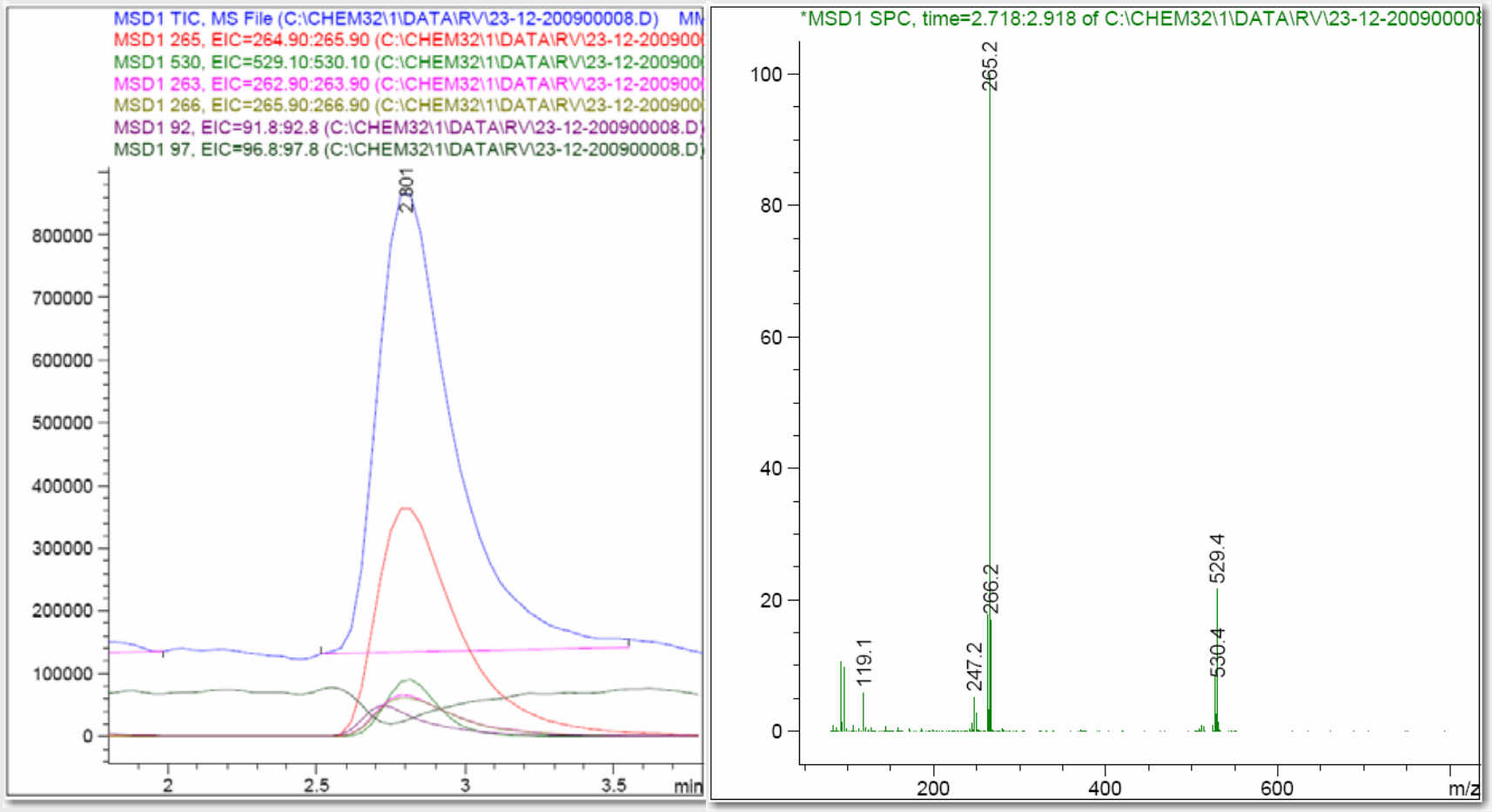
# Uni Graz

# Diploma Thesis R. Vizzini (CAMAG)
# Identification of alkaloids in *Sophora flavescens*



# Identification of alkaloids in *Sophora flavescens*



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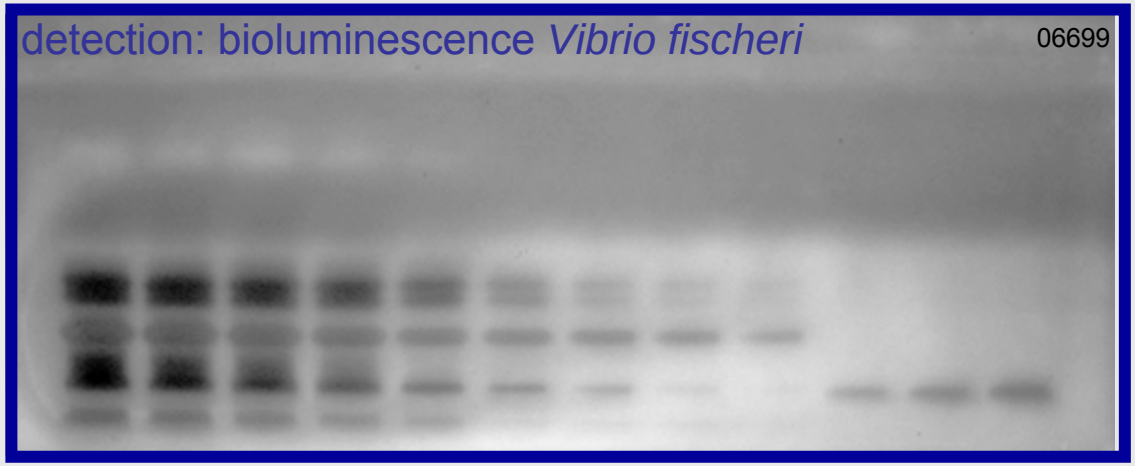
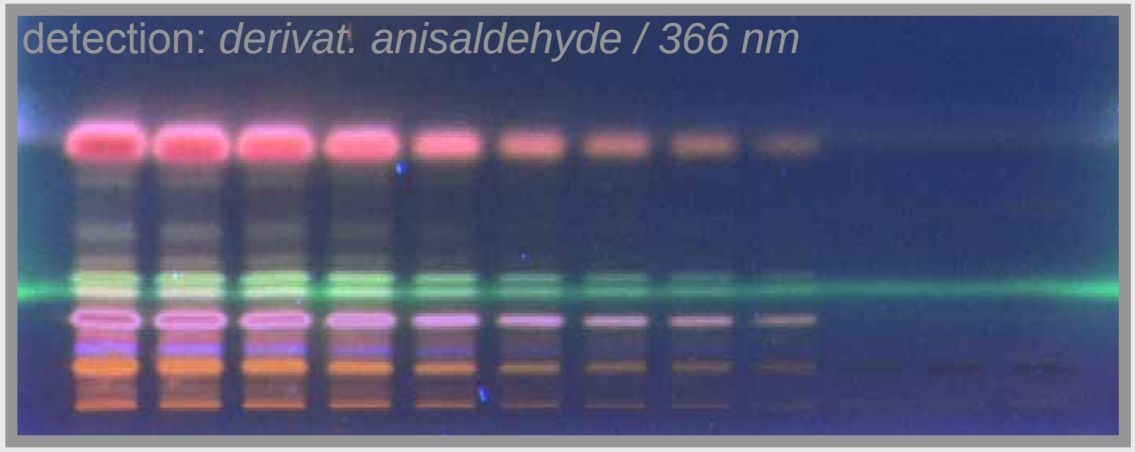
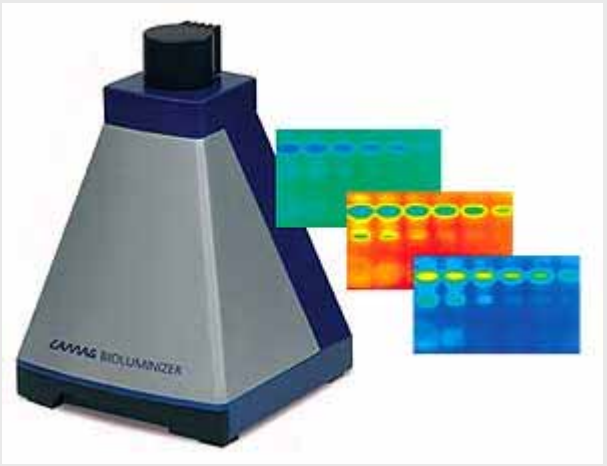
# BioLumineX - BioLuminizer




A B C D E F G H I np np np



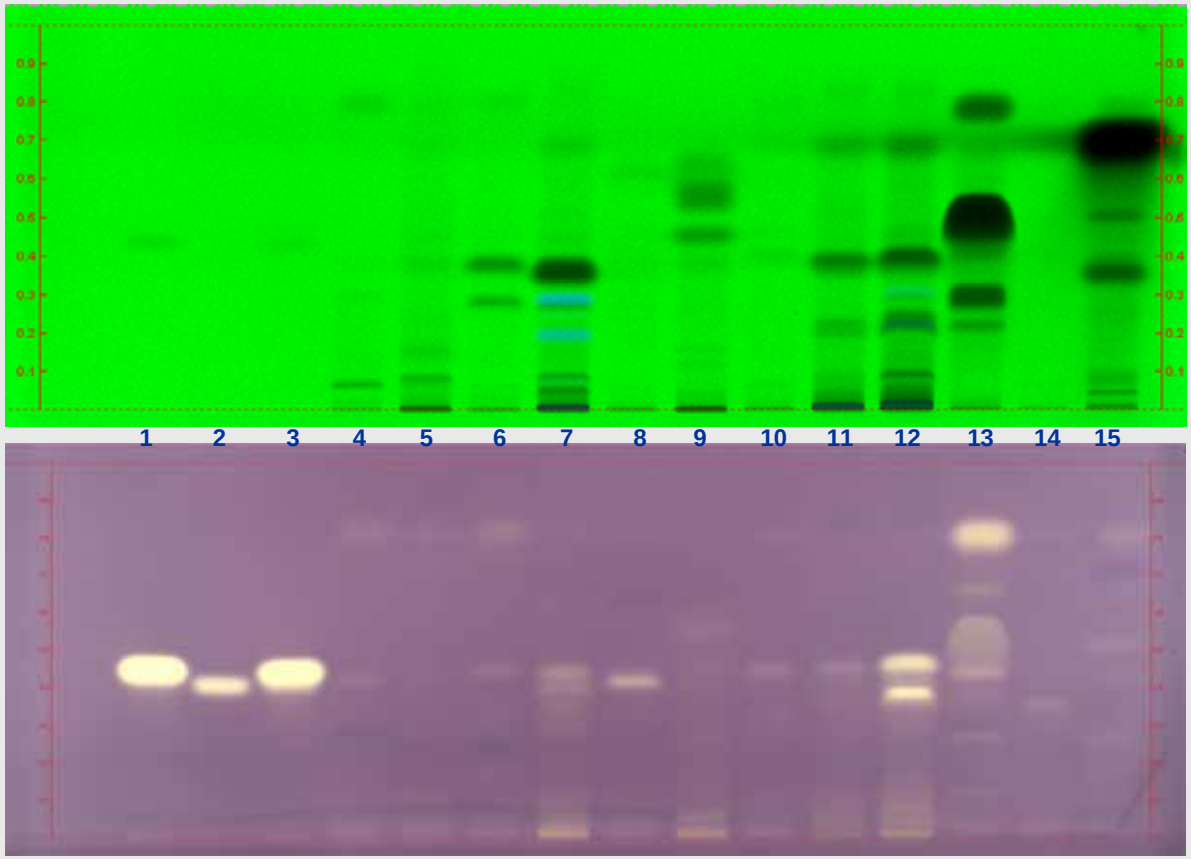
Bayer

ChromaDex™

A - I : chamomile oil np : 4-nitrophenol

# Bio-assays: DPPH

## Anti-oxidative properties of esential oils



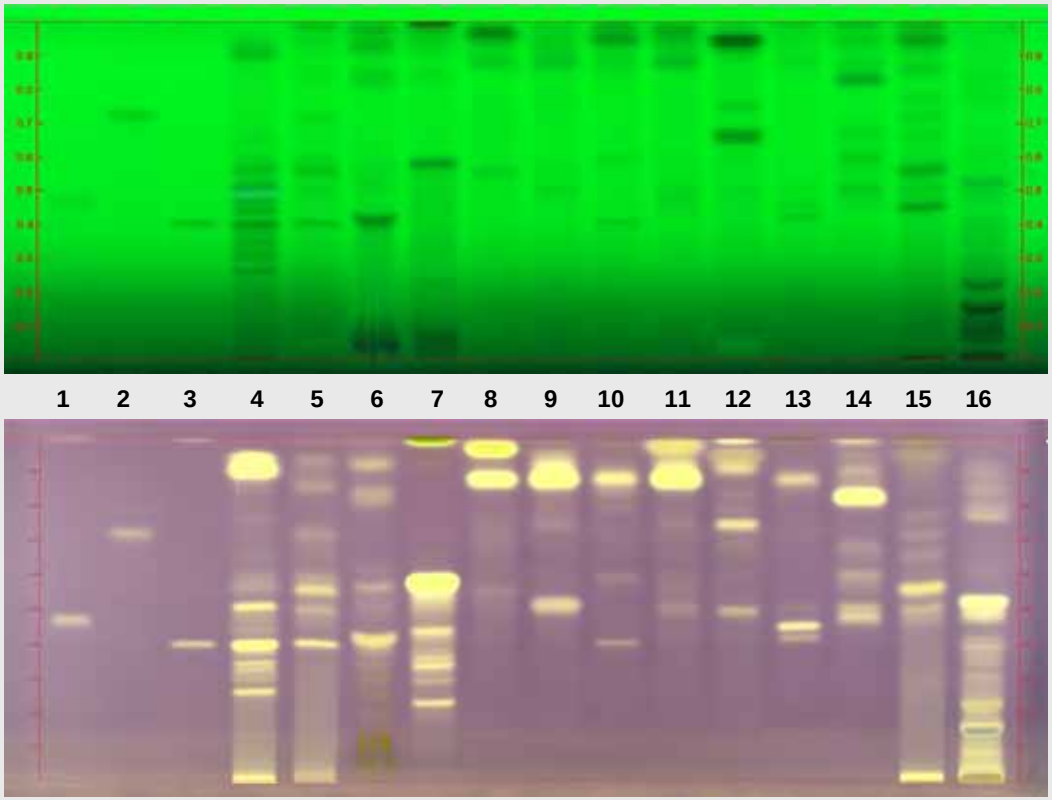

**Silica gel 60 $F_{254}$**

**Toluene : ethyl acetate 95:5**

**1: carvacrol methylester; 2: thymol; 3: carvacrol; 4: thyme oil; 5:sage oil; 6:neroli oil; 7: lemon, oil; 8: peppermint oil; 9: rosemary oil; 10: chamomile oil; 11:sweet orange oil; 12: manuka oil; 13: tea tree oil; 14: pine oil; 15: niaouli oil;**

# Bio-assays: DPPH

## Anti-oxidative properties of flavonoids




**Silica gel 60 $F_{254}$**

**Ethyl acetate, acetic acid, formic acid, water 100:11:11: 27**

**1: chlorogenic acid; 2: quercitrin; 3: rutin; 4: ginkgo leaf extract; 5: St. John's wort; 6: great mullein; 7: ribwort plantain; 8: rosemary; 9: majoram; 10: basil; 11: thyme, 12: chamomile; 13; peppermint; 14: arnica; 15: birch; 16: hibiscus**

# Thank you!

eike.reich@camag.com

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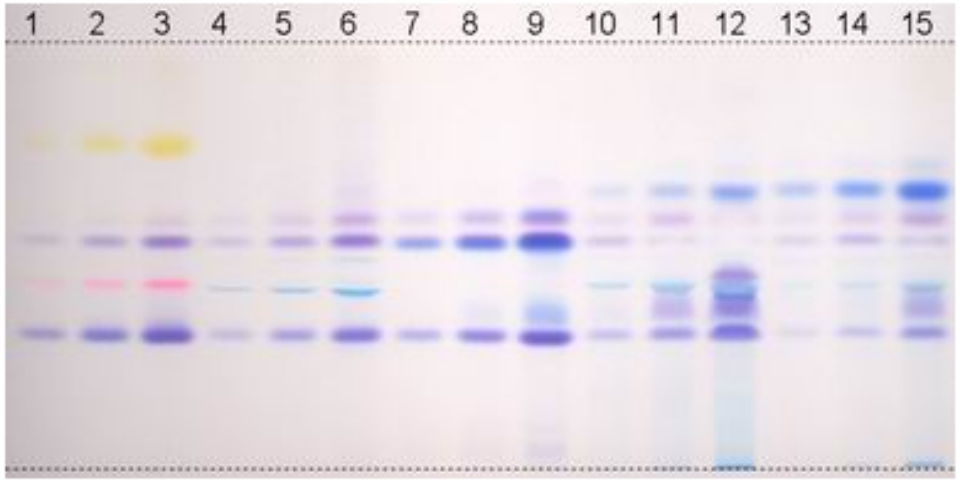
Determination of apolar lipids from human skin by HPTLC >>
Determination of ceramides from human skin by HPTLC >>

**New Publications**
Quality control of multicomponent herbal drugs: example from TCM >>

**HPTLC of dyes**




**New CAMAG TLC-MS Interface:**
For direct extraction of compounds from TLC/HPTLC layers into MS.

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a center for applied HPTLC

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- Dedicated to research
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- Separation of phospholipids by HPTLC
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