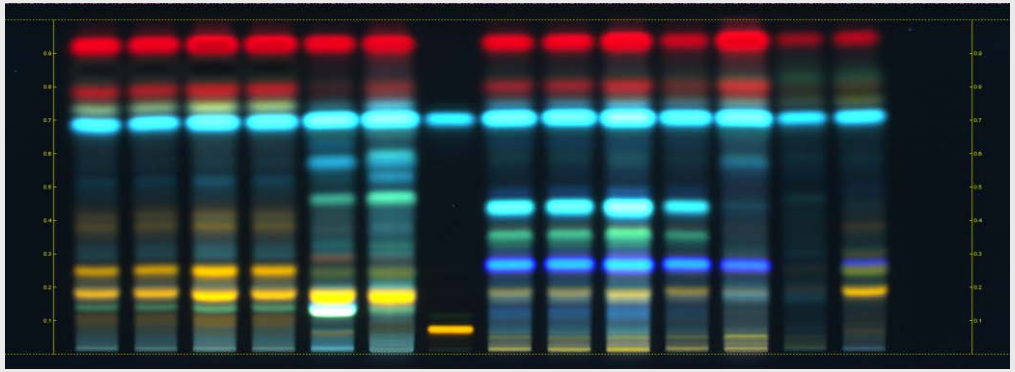
HPTLC of medicinal plants

Identification and quantification

Eike Reich

CAMAG Laboratory
Sonnenmattstrasse 11
4132 Muttenz / Switzerland



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The current situation for TLC

- Thin-layer chromatography (TLC) always was and still remains an important tool for the analysis of plants. The same is true to some degree for essential oils.
- 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 enormous flexibility.
- For decades TLC is integral part of monographs for medicinal plants in all pharmacopoeias and the primary method of identification.

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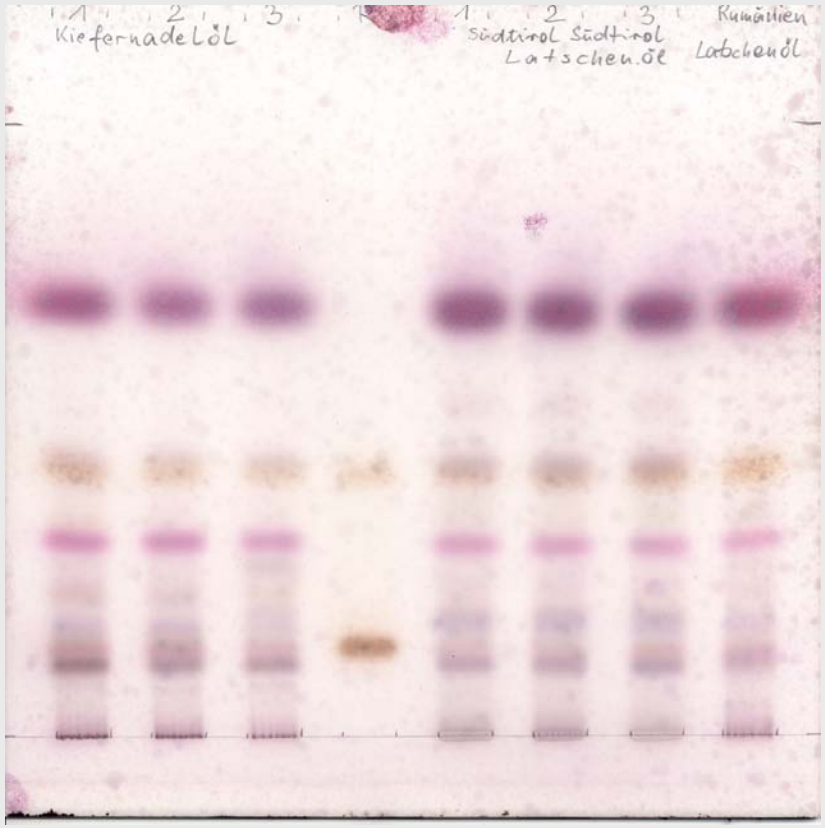
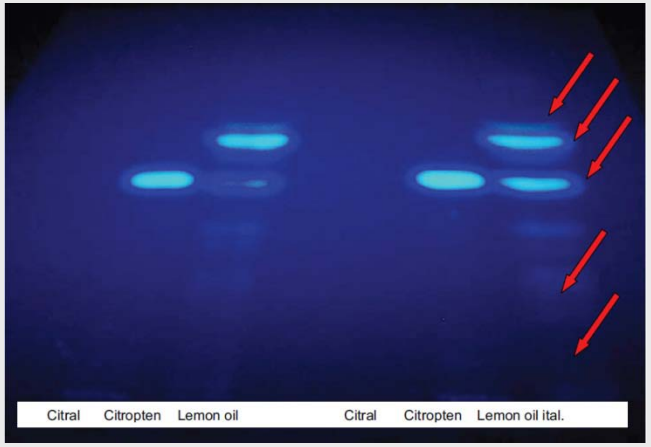
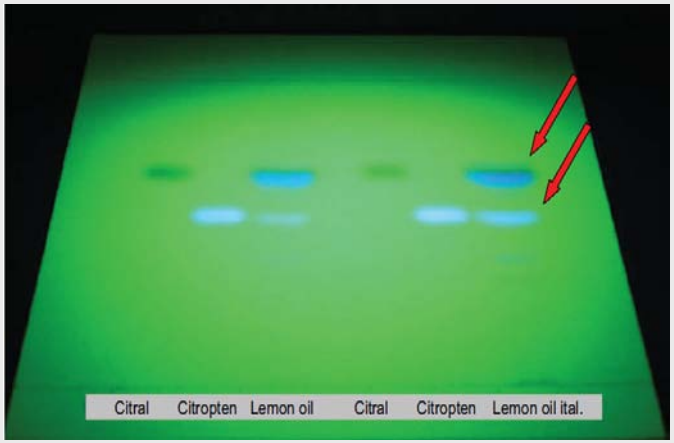
The current situation for TLC

- Growing expectation regarding performance characteristics have brought TLC methods to the limits.
- Since the turn of the century pharmacopoeias and the analytical community recognize the technical progress in instrumentation and improvements offered by high performance plates.
- Most recently HPTLC is being discussed as a modern technique and an alternative to classical TLC.
- Globalization of TLC?

TLC of essential oils as of today…







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Quality and reproducibility of results?

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.
- Many parameters that can be chosen freely affect the final result.
- There is little guidance available: the general method description in the Pharmacopoeias (EP 2.2.27, USP <201>, <621>, PhPRC ap. VI, JPXV 2.03) define „suitable” equipment and give ranges instead of values.
- 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 and uncertainty about what to expect.→ ATLAS?

Most text books are even worse …

- Example: how can a color be described correctly?

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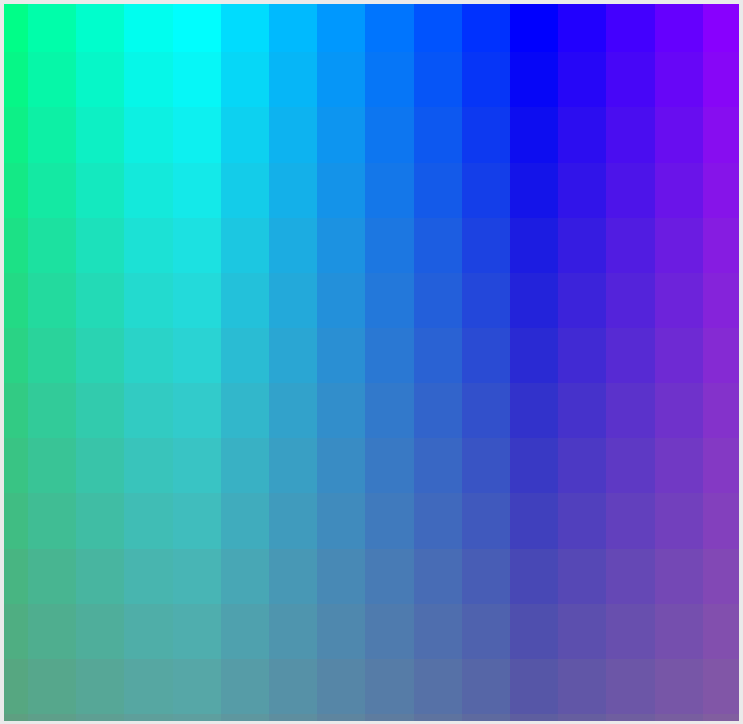
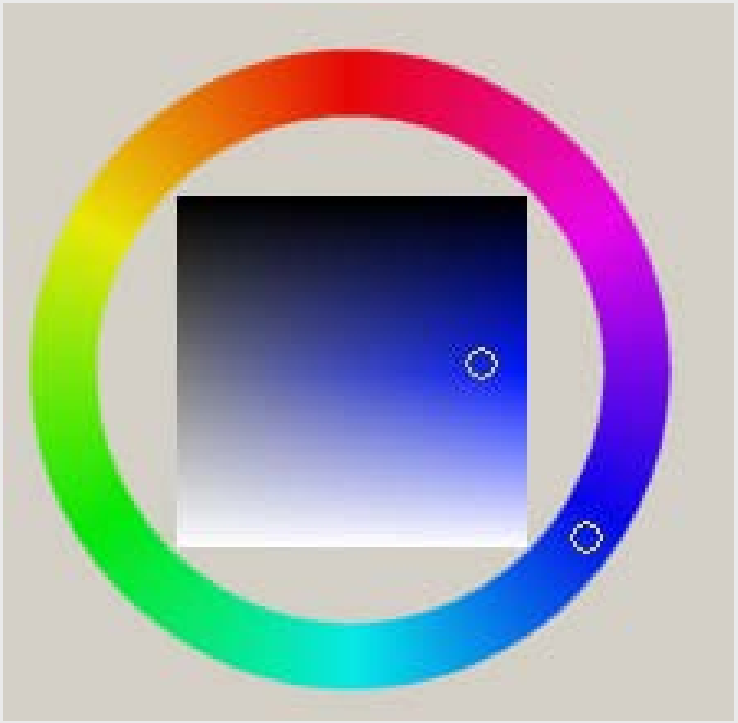
What is blue?





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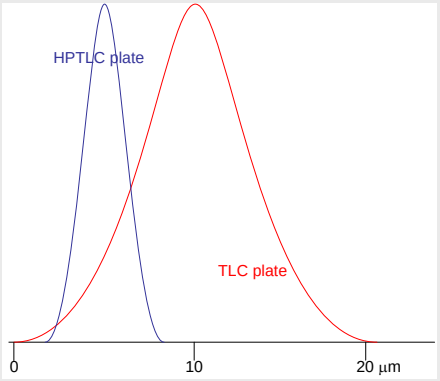
The „official“ versions of TLC

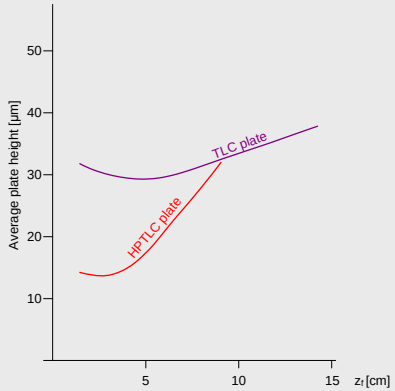
- Various textbook
  - Emphasis on simplicity and flexibility
- ChP
  - Revised Chapter on TLC
  - HPTLC Atlas in color
- USP
  - Revised Chapter <621 Chromatography>
  - TLC and HPTLC distinguished
- EP
  - Revised Chapter 2.2.27 TLC
  - TLC and HPTLC distinguished
  - New botanical monographs TLC and HPTLC parallel

HARMONIZATION

TLC in chapter 2.2.27 / <621>

- General description of (suitable) instrumentation for simple TLC
  - No details about specifications, performance criteria, and definition what “suitable” is
- Description of the individual steps
  - Non-specific instruction giving ranges instead of exact values
- PHEUR: In a monograph, where both normal and high-performance plates may be used, the working conditions for high-performance plates are given in the brackets [ ] after those for normal plates.
- TLC vs. HPTLC is primarily seen in the plate, yet similar results are expected





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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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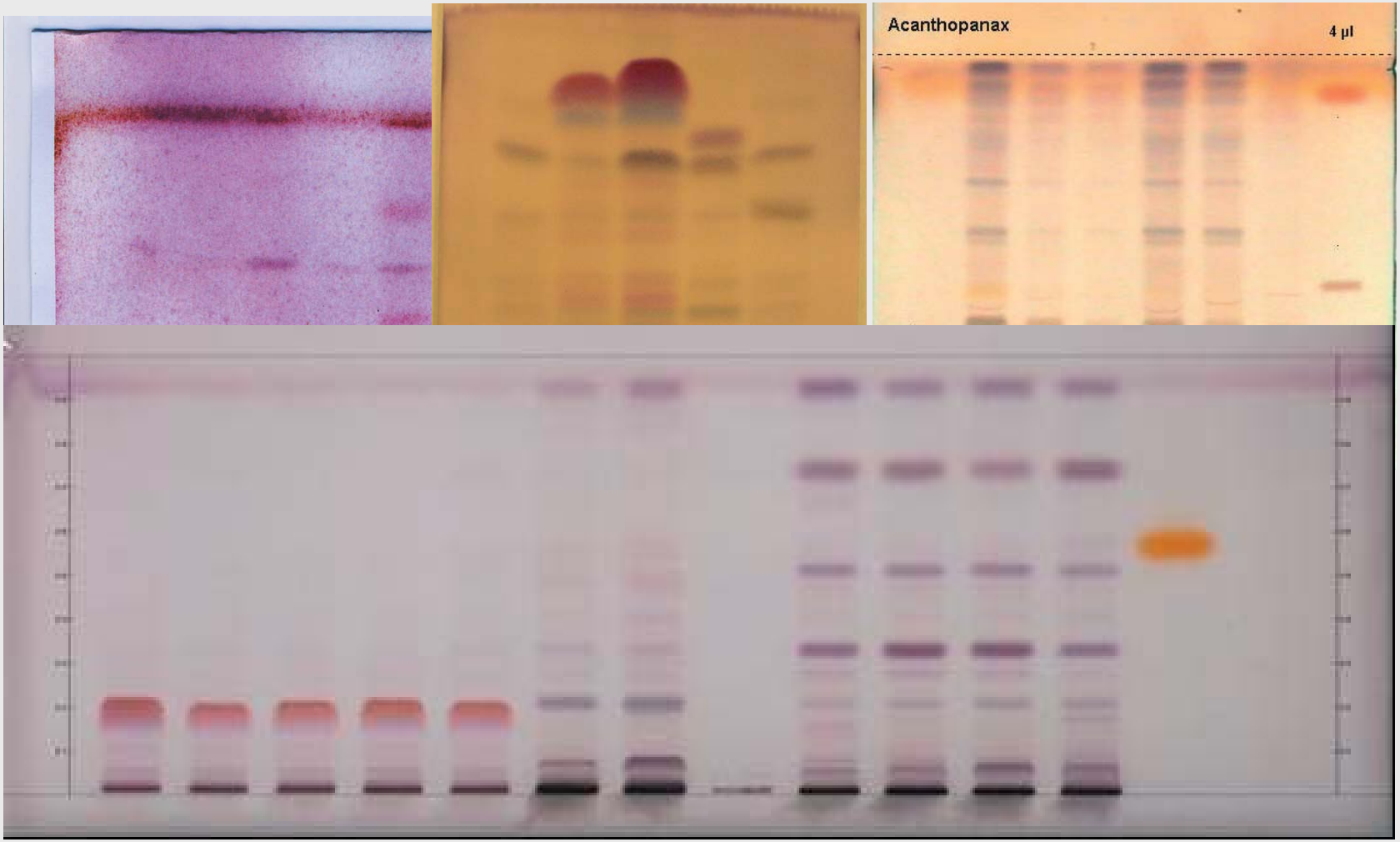
Identification of *Acanthopanax*



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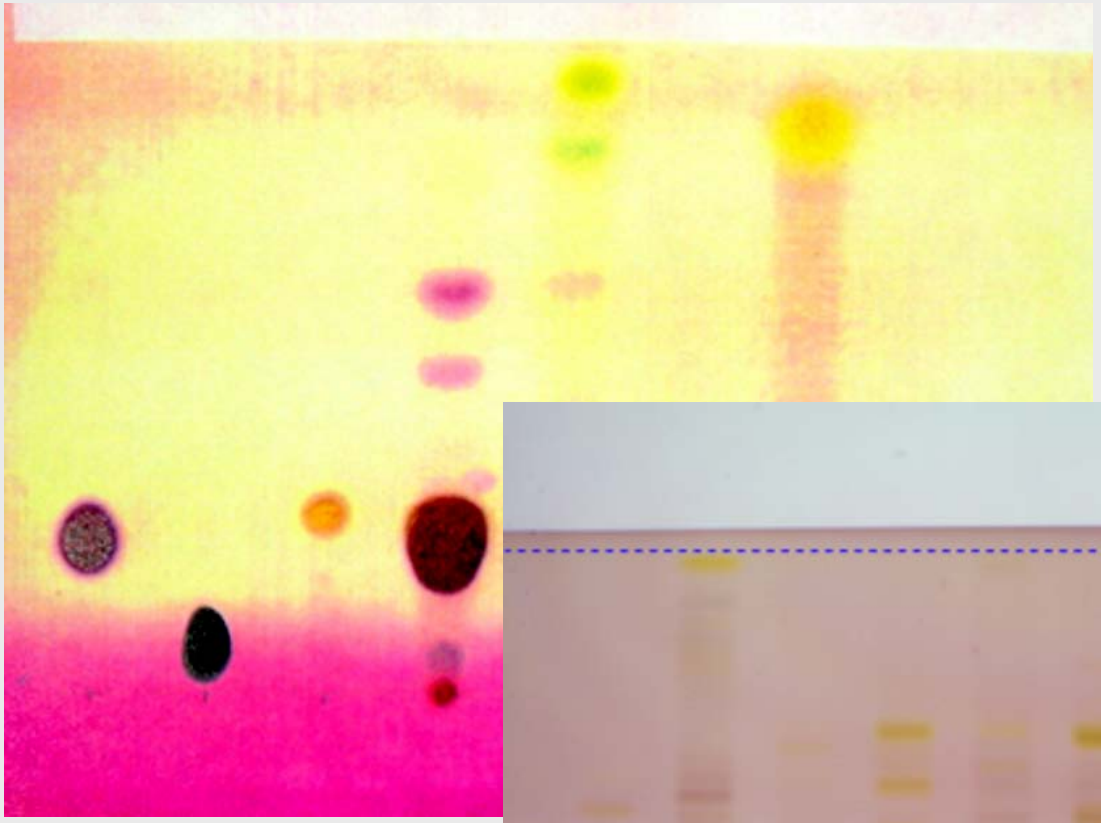
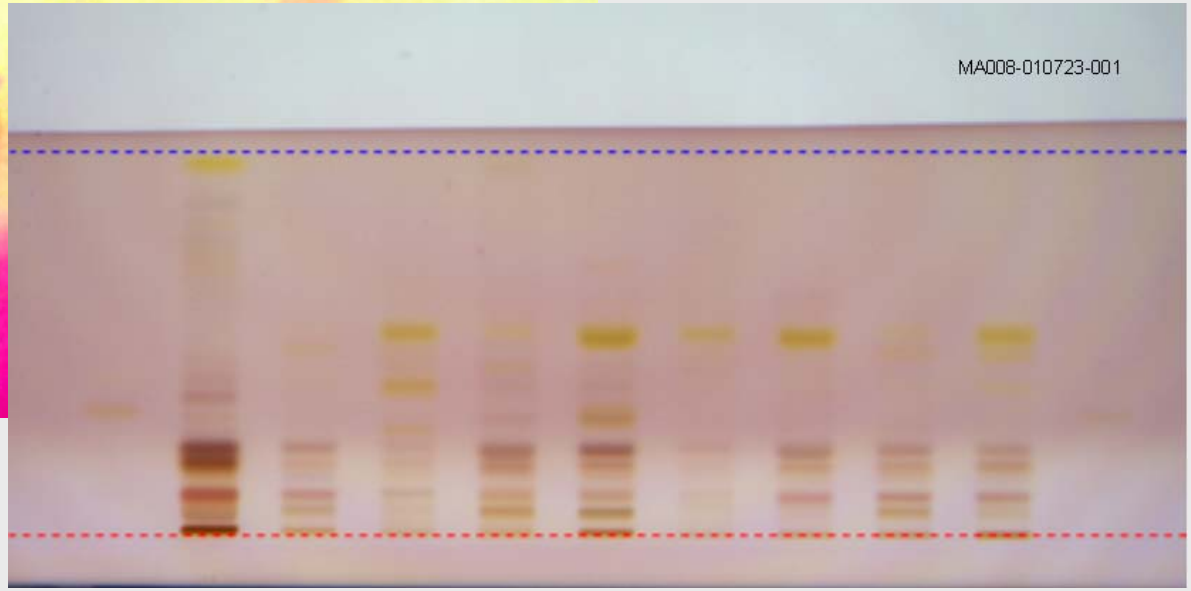
TLC or HPTLC?





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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

- Instruments
- Scientific basis
- Standardized methodology
- Validated methods

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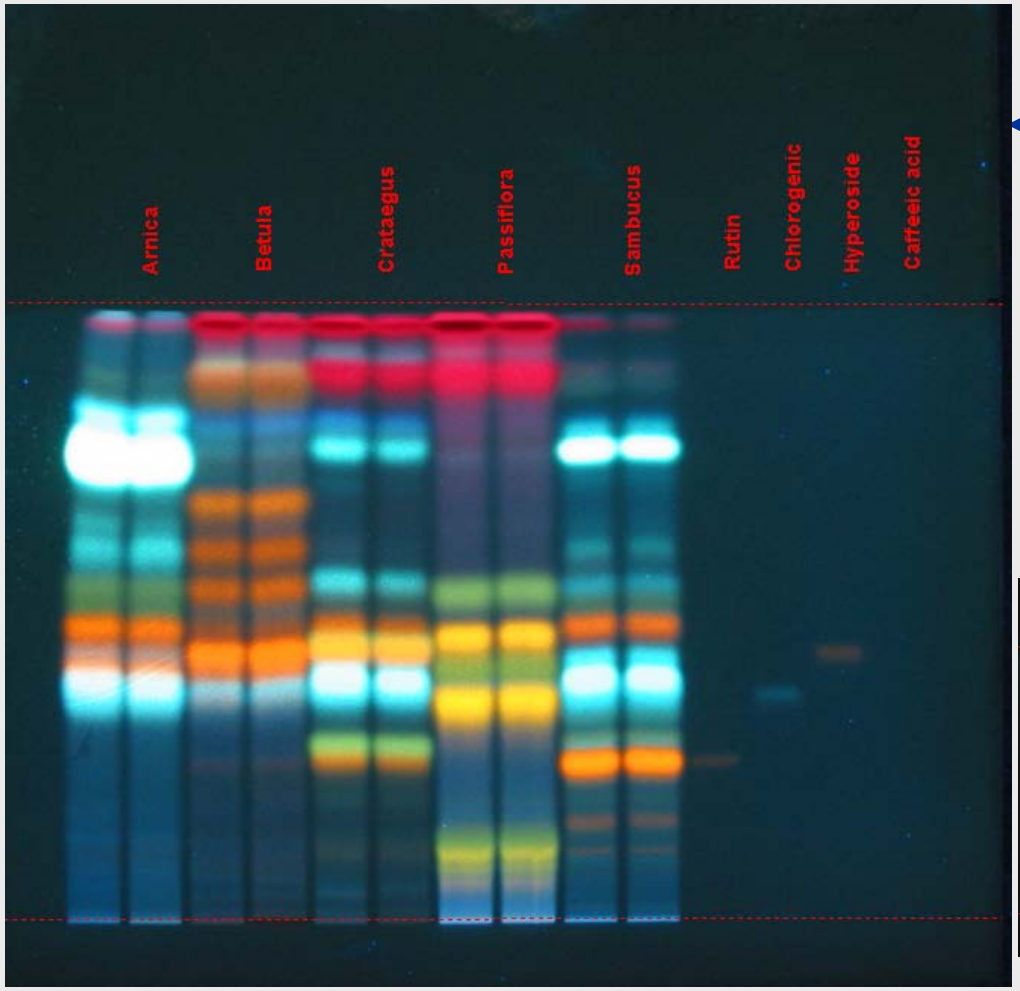
Comparison of «HPTLC»

on TLC and HPTLC plates under optimized conditions

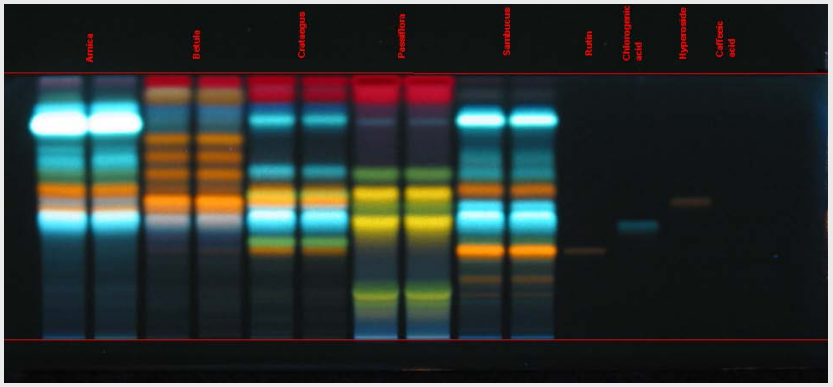


← TLC plate 20 x 20 cm

(135 mm)

HPTLC plate 20 x 10 cm

(60 mm) ↓



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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 process

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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
  - Top down
  - International collaboration
  - Publication

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Standardization and international harmonization of methodology?

- Agreement
  - Definitions
  - Parameters → SOP
- Guidance
  - General chapters
  - Style guide for monographs / template
  - Illustration (atlas)
- Enforcement

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Proposal (HPTLC-Association)

High Performance Thin-Layer Chromatography (HPTLC)

HPTLC is the High-Performance version of Thin Layer Chromatography. It features significantly shorter developing times, lower solvent consumptions and improved resolution. Highly reproducible results and traceable records are achieved through a standardized methodology and the use of suitable instruments (typically controlled by software) for all steps of the analysis. A system suitability test is used to qualify results.

Unless otherwise stated in the individual monograph the stationary phase is a *HPTLC plate* F_{254} (a glass plate coated with a uniform, typically 200 µm thin layer of porous irregular particles with a size between 2 and 10 µm and an average particle size of 5 µm. The layer typically consists of silica gel with a pore size of 60 Angstroms, a polymeric binder and a so called fluorescence indicator F_{254}). The standard format of the plate is 20×10 cm.

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Apparatus

- A device suitable for application of samples as bands providing control of dimension and position of the **application** as well as applied sample volume
- A suitable chromatographic chamber (typically a twin trough chamber) providing control of **saturation** and **developing distance**
- A device suitable for controlling the **activity** of the stationary phase via relative humidity
- A device suitable for reproducible **drying** of the developed plate
- Suitable devices for **reagent transfer** and **heating** as part of the derivatization procedure
- A device suitable for **electronic documentation** of chromatograms under UV 254 nm, UV 366 nm, and white light
- For **quantitative determinations** a densitometer or image evaluation software

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Procedure: →SOP

Sample application: Solutions are applied in small volumes as narrow bands of 8 mm width, **8 mm above** the lower edge of the plate. The **left** and **right** margins of the plate are at least **15 mm**, the minimum space **between** bands is **2 mm**.

Other application patterns may be specified in a monograph.

Chromatogram development

1. **Saturate** the chamber for **20 min** using a filter paper saturated with developing solvent and positioned against the rear wall of the chamber. The **solvent level** in the chamber must be **5 mm**.
2. Condition the plate at a **relative humidity between 30 and 40%**
3. Place the plate in the front trough of the chamber in a **vertical position** so that the stationary phase faces the filter paper.
4. Develop the plate to a distance of **70 mm** from the lower edge, then remove it from the chamber and dry it.

[Note: other chamber configurations or developments may be described by a monograph]

5. Visualize, document and evaluate the chromatograms as prescribed

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The acceptance of HPTLC: USP

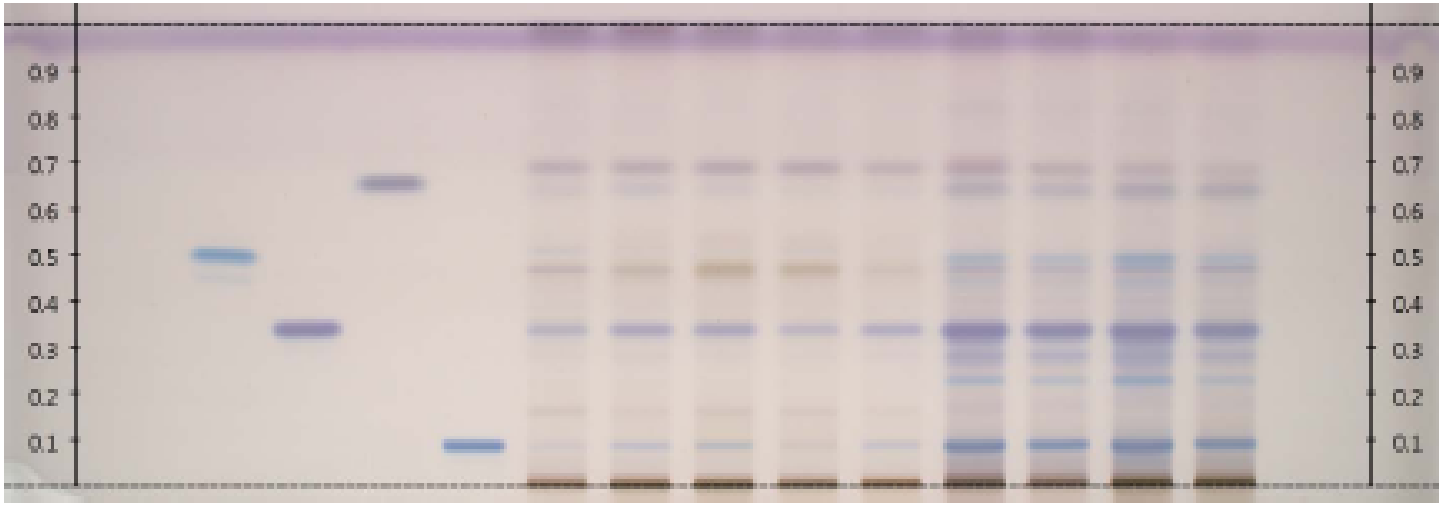
- Standard method for identification of herbal medicine ingredients listed in the Herbal Medicin Compendium (HMC)

  http://hmc.usp.org

- General chapter on HPTLC for ID of herbal medicine ingredients planned under “General Notices/Resources” for Fall 2013
  - Definition, equipment, methods
  - Theoretical basis
  - Explanation of parameters
  - SOP

Lagerstroemia speciosa Leaf — Identification

Thin-Layer Chromatography



Typical HPTLC Chromatograms

These chromatograms are supplied for information only

Track assignment: 1) virgatic acid (0.2 mg/mL); 2) USP Corosolic Acid RS (0.2 mg/mL); 3) oleanolic acid (0.2 mg/mL); 4) asiatic acid (0.2 mg/mL); 5-9) *Lagerstroemia speciosa* Leaf, commercial samples;10) USP *Lagerstroemia speciosa* Leaf Powdered Extract (20 mg/mL);11-13) *Lagerstroemia speciosa* Leaf powdered extract, commercial samples (20 mg/mL)

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The acceptance of HPTLC: PhEur

- All new monographs contain HPTLC conditions, either in addition to TLC, or alone
- Under discussion for inclusion as separate technique for ID of herbal materials 2.8. xx (→ Pharmeuropa Fall 2013)
  - Definition, equipment, methods
  - Rigorous standardization (SOP)
  - Introduction of system suitability test for qualification of data
  - Introduction of “intensity standards” for proper description of chromatograms
- No images / atlas yet

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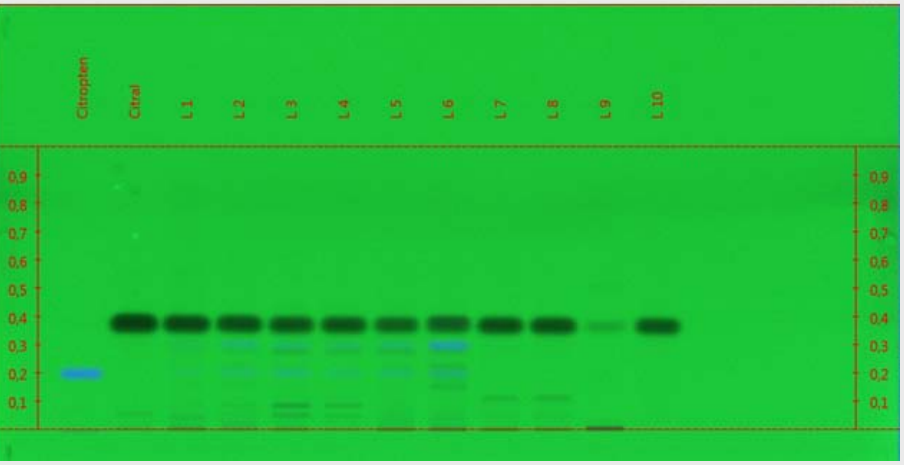
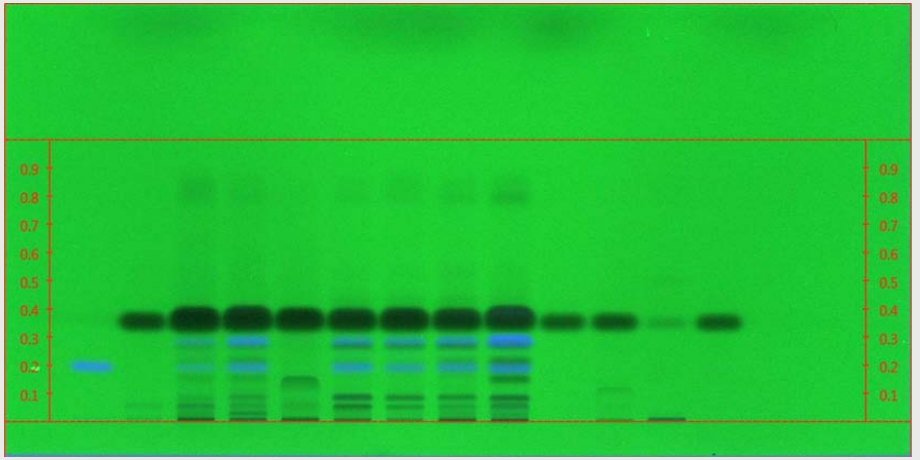
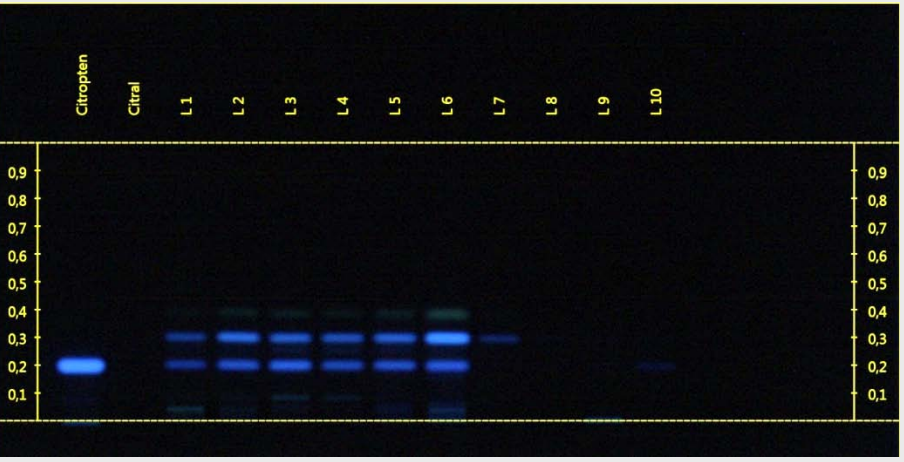
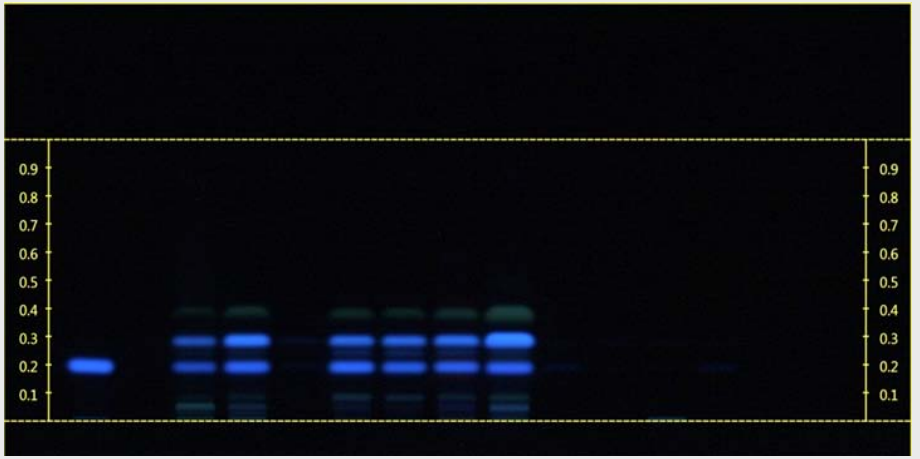
Collaborative trial Birch leaf (PhEur, 13B)

HPTLC of Lemon Oils: Different samples, two laboratories









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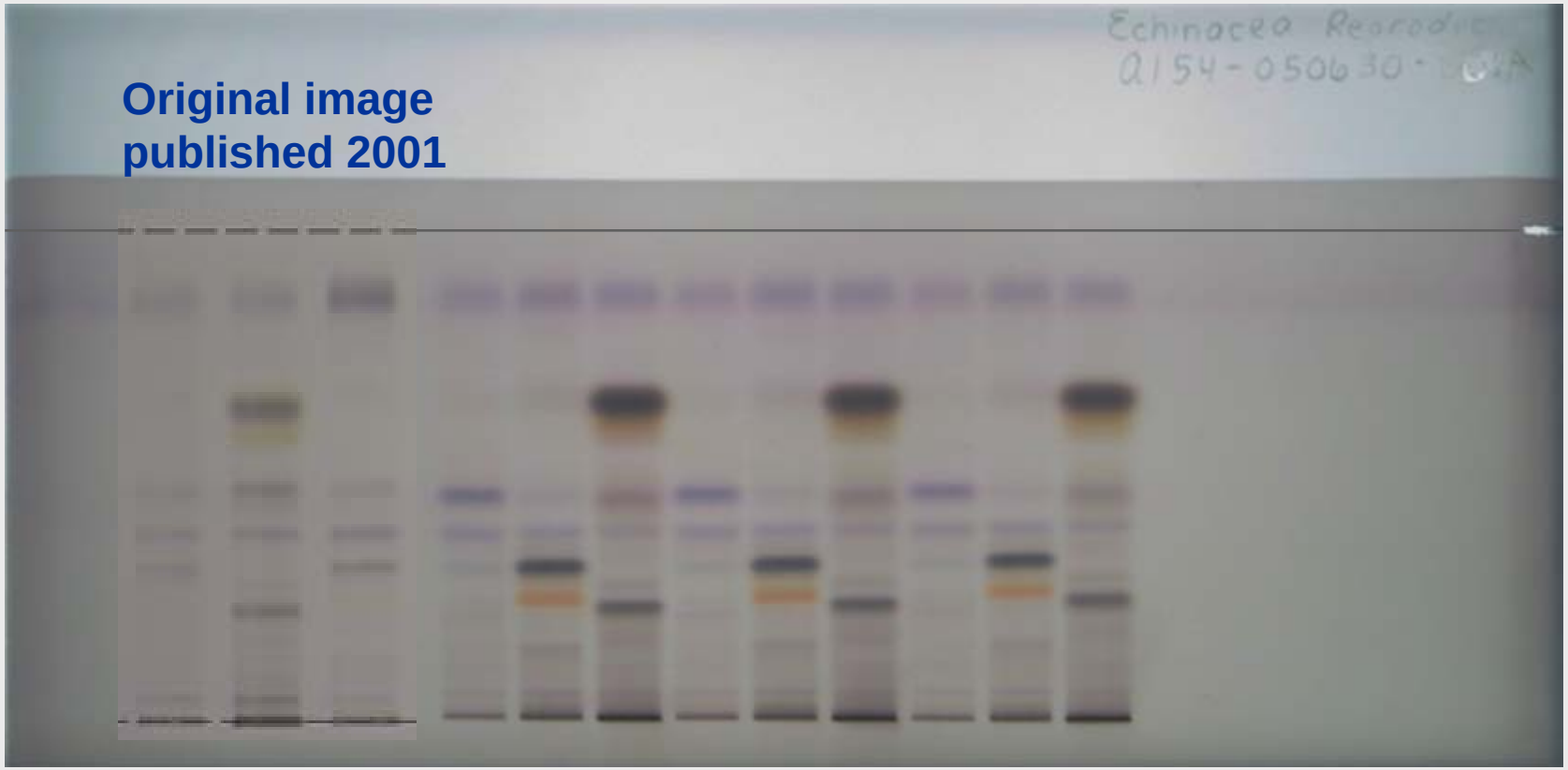
Successful standardization

Echinacea **June 30, 2005 – CSI Laboratory**



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Categories of herbal products

- Drugs / medicines (Pharmacopoeias)
  - Traditional Western, Traditional Chinese, Ayurvedic (AYUSH), Kampo, etc.
  - Dried single herbs or blends, encapsulated or for decoction
  - Tinctures, extracts, (polyherbal) formulations
- Natural Health Products (NHP) – Canada only
- Dietary supplements (section of the Food, Drug and Cosmetic Act)
- Ingredients of food or cosmetic products

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For dietary supplements:

- Compliance with label claim:
  - Identity
  - Amount
  - Declared content of marker
  - Quality
- Compliance with cGMP:
  - Identity of raw material
  - Control of production process
  - Batch to batch consistency
  - Stability (not yet an issue)

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Some regulatory aspects of quality

- Safety
- Identity
- Purity
- Strength / potency
- Efficacy

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Analytical aspects of quality

- Identity through fingerprints in addition to botanical ID
  - Natural variability
  - Adulteration with other species
  - „Bad“ quality
- Batch to batch consistency
  - Semi-quantitative (no absolute values) comparison of fingerprints
- Potency → assay of markers (absolute) quantitation of single or multiple substances

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HPTLC

- Is an ideal tool for identification of herbal materials, which are very complex mixures.
- Can be used for semi-quantitative comparison.
- Is able to provide quantitative results.

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Globalization for herbal materials

- Global sourcing of raw materials
- Different regulatory approaches
- Different specifications for «same» material
- Different quality management systems

- cGMP requirements

→ supply chain management
→ economic criteria

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HPTLC in a global supply chain

- Proper identification at growing site (DNA)
- Establishing the HPTLC fingerprint after harvest / drying / primary storage (part of CoA for a batch)
- Pooling batches from different growing / collection sites at whole saler / buyer; → specification, check for adulteration
- Identification as raw material at production site
- Monitoring of the fingerprint during production (extraction, blending, packaging)
- Identification as raw material for additional production steps

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HPTLC in a global supply chain

- All involved laboratories can use same
  - Validated method
  - Standard methodology
  - Specifications
  - Reference materials (images)

Examples

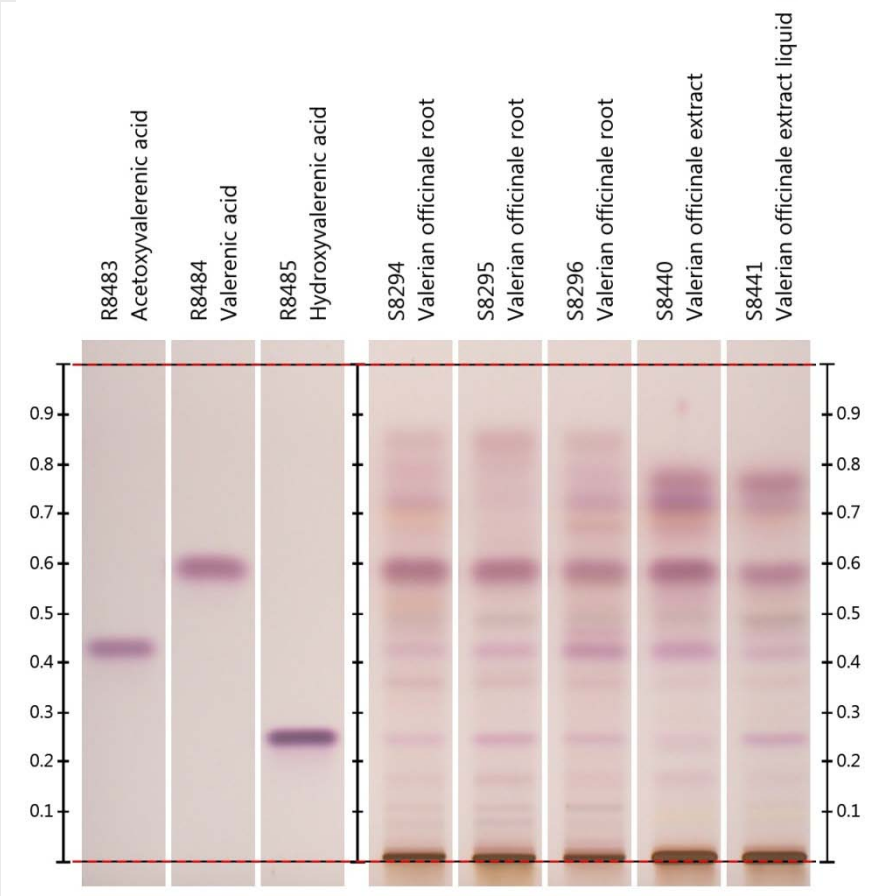
HPTLC fingerprints vs. content of markers

Identification of Valerian (PhEur)

Top of the plate	
Valerenic acid: a violet zone	A violet zone (valerenic acid)
Acetoxyvalerenic acid: a violet zone	A violet zone (acetoxyvalerenic acid)
	2 faint or very faint violet zones
Reference solution	**Test solution**

Valerian dry aqueous extract (PhEur)

Top of the plate	
Valerenic acid: a violet zone	
Acetoxyvalerenic acid: a violet zone	A violet zone (acetoxyvalerenic acid)
	A violet zone (hydroxyvalerenic acid)
Reference solution	**Test solution**



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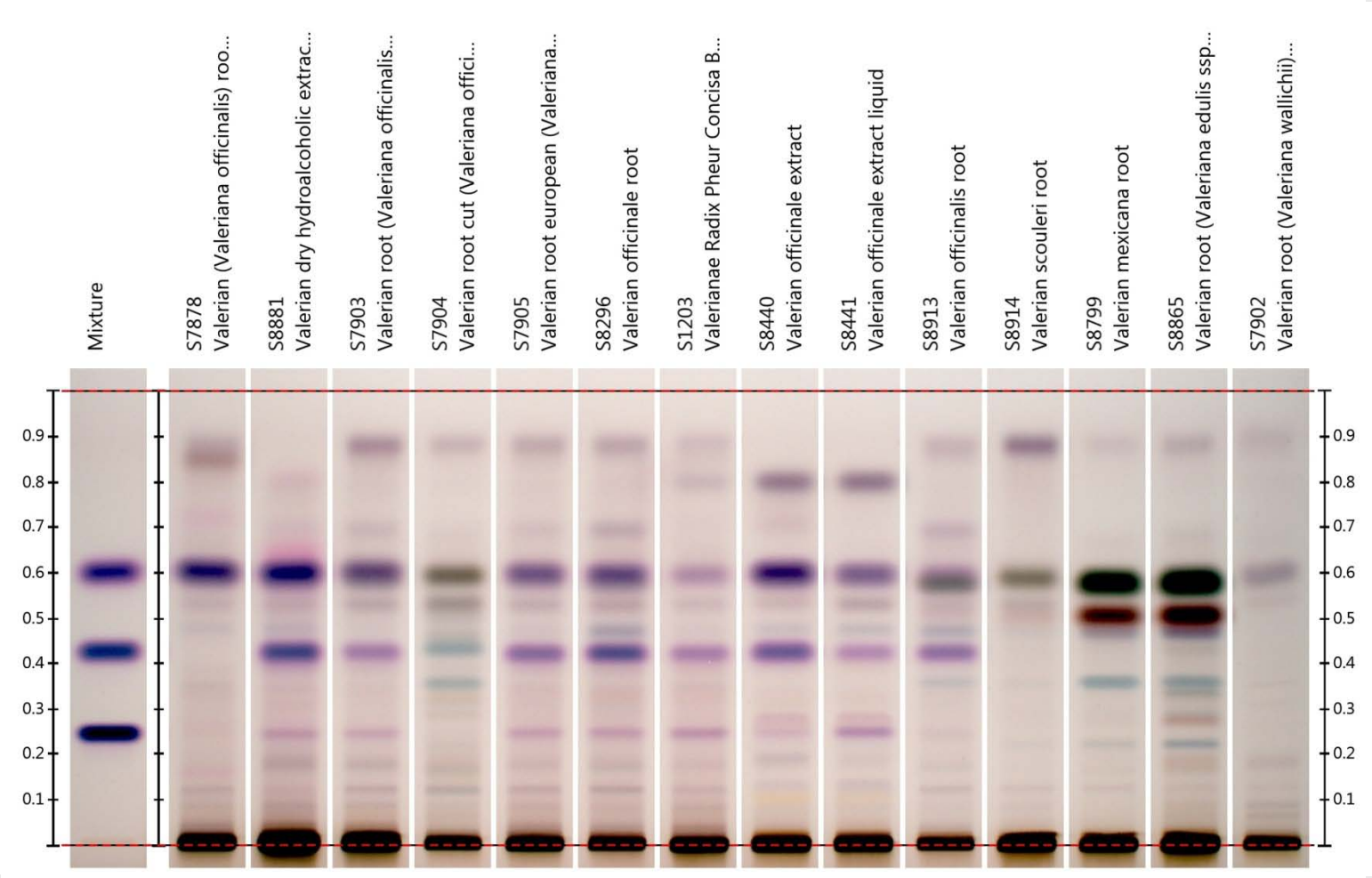
Valerian ↓ naturally variable



Guggul
Guggulsterone E and Z
alpha-Boswellic acid
beta-Boswellic acid
3-Acetyl-11-keto-beta-Boswellic acid
Acetyl-11-keto-beta-Boswellic acid
Boswellia
Carvone
Anethole
Linalool
Bornyl acetate
Myrrh
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1

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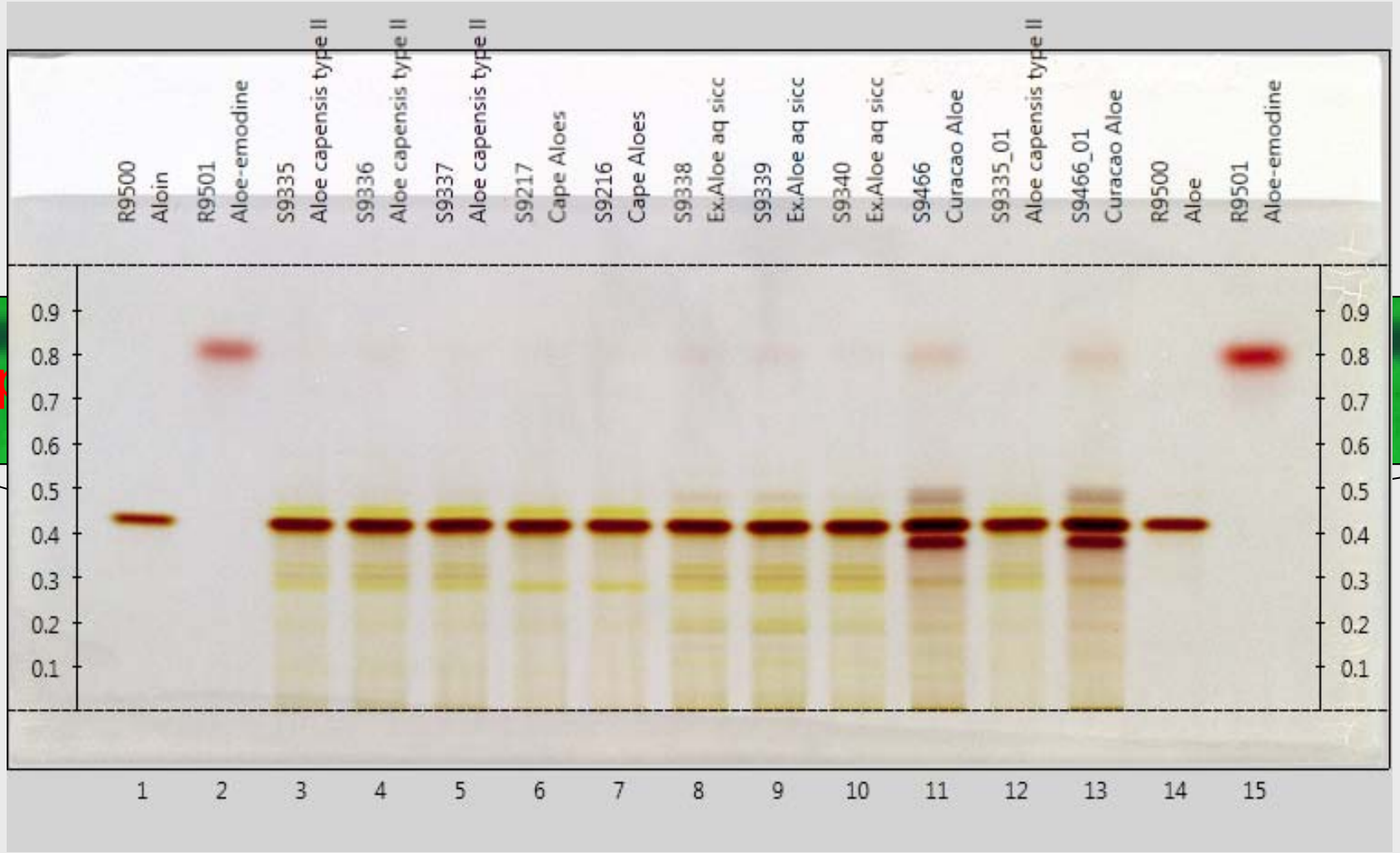
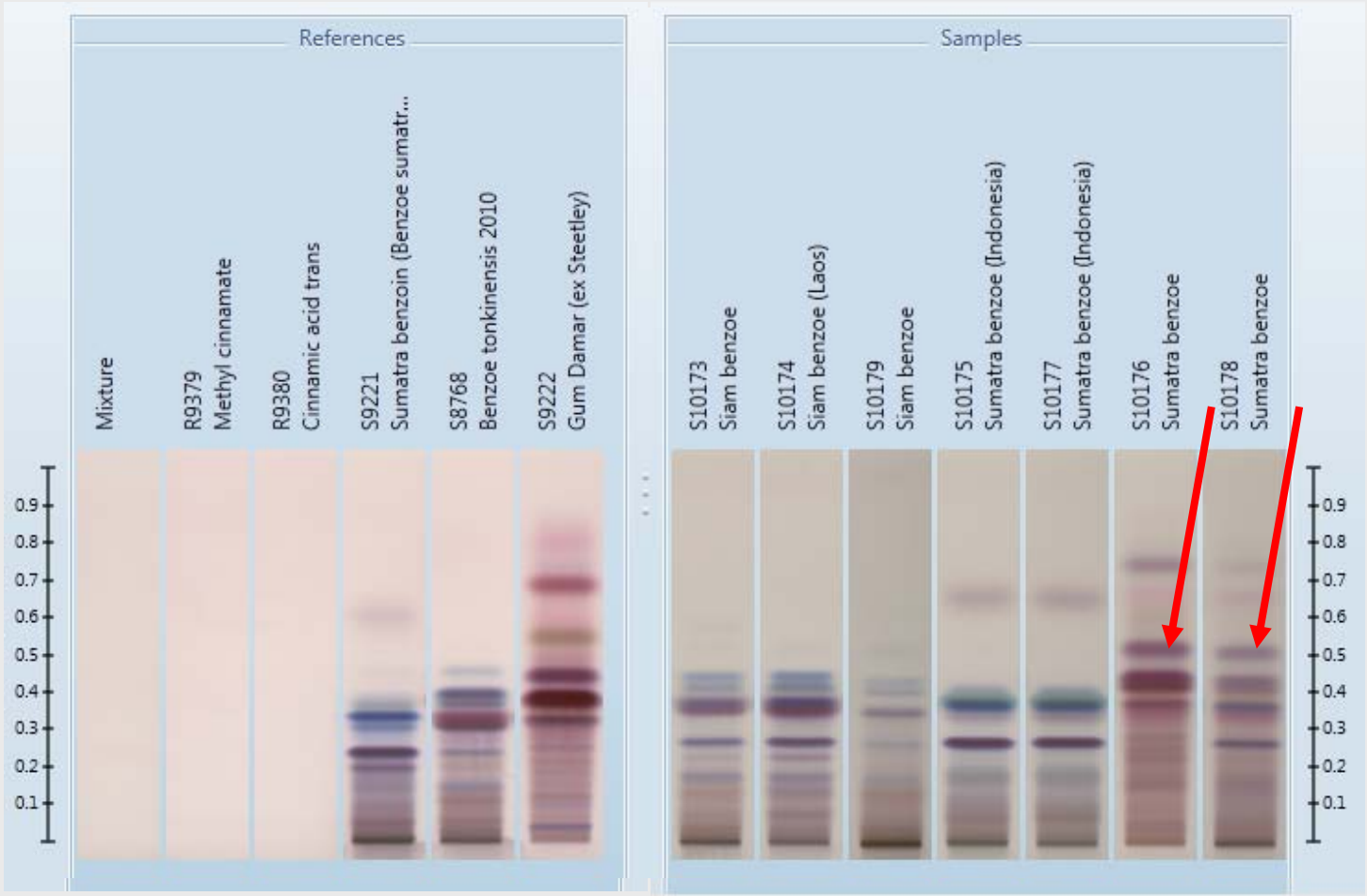
Aloe - PhEur



Identification of Benzoe (PhEur)



HPTLC Association

The International Association for the Advancement of High Performance Thin Layer Chromatography (HPTLC Association) promotes the use of HPTLC in plant analysis and other analytical fields. The Association brings together representatives from academia, industry, research, regulatory and standard setting bodies.

more about us

Methods

Latin plant name	Plant part	Date of publication
Achillea millefolium	flower	2012-04-19
Aesculus hippocastanum (Horse Chestnut)	seed	2012-12-20
Agrimonia eupatoria	flowering tops	2012-04-19
Alchemilla vulgare	herb	2012-04-19
Aloysia citriodora	leaf	2012-04-19
Alpinia officinarum	rhizome	2012-04-19
Alpinia oxyphylla	fruit	2012-04-19
Althaea officinalis (Marshmallow)	leaf	2013-02-23
Althaea officinalis (Marshmallow)	root	2013-02-23
Amomum krervanh, Amomum compactum	fruit	2012-04-19
Amomum villosum, Amomum longiligulare	fruit	2012-04-19
Angelica archangelica	root	2012-04-19
Angelica dahurica	root	2012-04-19
Angelica pubescens	root	2012-04-19
Angelica sinensis	root	2012-04-19
Arctostaphylos uva ursi	leaf	2012-04-19
Arnebia euchroma or Arnebia guttata	root	2012-04-19
Arnica montana	flower	2012-04-19
Artemisia annua	leaf	2012-04-19
Aspalathus linearis (Rooibos)	tea	2013-01-30
Astragalus membranaceus	root	2012-04-19
Avena sativa	herb	2012-04-19
Bacopa monnieri	herb	2012-04-19
Betula pendula and/or Betula pubescens	leaf	2012-04-19
Bupleurum chinense	root	2012-04-19
Calendula officinalis	flower	2012-04-19
Camellia sinensis	leaf	2012-04-19

Sweet wormwood leaf, qing hao (*Artemisia annua*)

1. Scope
This method identifies dried (and fresh) Sweet wormwood leaf (*Artemisia annua* L.) by HPTLC fingerprint and detects the adulterant Chinese wormwood leaf (*Artemisia apiacea* Hance).

2. Source of method
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3. Procedure

Sample preparation:	Mix 500 mg of powdered sample with 5 mL of methanol and sonicate for 10 minutes, then centrifuge or filter the solutions and use the supernatants / filtrates as test solutions.
Reference substances:	Dissolve 1 mg of chlorogenic acid in 1 mL of methanol. Dissolve 1 mg of caffeic acid in 1 mL of methanol. Optional: Dissolve 1 mg of rutin in 1 mL of methanol.
Stationary phase:	HPTLC Si 60 F_{254}
Application:	5 µL of references, 5 µL of test solutions
Mobile phase:	Ethyl acetate, water, acetic acid, formic acid 100:26:11:11 (v/v/v/v)
Development:	- Saturated chamber - Developing distance 70 mm from lower edge - Relative humidity 33%
Derivatization reagent:	NP reagent Preparation: 1 g of natural products reagent in 200 mL of ethyl acetate Use: Heat plate for 3 min at 100°C, then dip (time 0, speed 5)
Documentation:	1.) NP reagent, UV 366 nm

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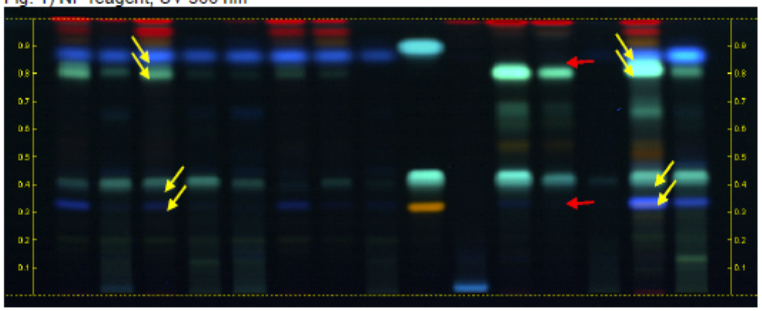
4. Results
Note: The images presented in this section are examples and are not intended to be used as basis for setting specifications for quality control purposes.

Fig. 1) NP reagent, UV 366 nm



Track	Volume	Sample	Track	Volume	Sample
1	5 µL	Sweet wormwood fresh leaf 1 (extracted with water)	9	5 µL	Rutin, chlorogenic acid, caffeic acid (with increasing Rf)
2	5 µL	Sweet wormwood fresh leaf 1 (extracted with ethanol)	10	5 µL	Chinese wormwood fresh leaf (extracted with water)
3	5 µL	Sweet wormwood fresh leaf 1 (extracted with methanol)	11	5 µL	Chinese wormwood fresh leaf (extracted with ethanol)
4	5 µL	Sweet wormwood fresh leaf 1 (extracted with hot water)	12	5 µL	Chinese wormwood fresh leaf (extracted with methanol)
5	5 µL	Sweet wormwood fresh leaf 2 (extracted with water)	13	5 µL	Chinese wormwood fresh leaf (extracted with hot water)
6	5 µL	Sweet wormwood fresh leaf 2 (extracted with ethanol)	**14**	**5 µL**	**Sweet wormwood dried leaf 3 (extracted with methanol)**
7	5 µL	Sweet wormwood fresh leaf 2 (extracted with methanol)	15	5 µL	Sweet wormwood dried leaf 3 (extracted with hot water)
8	5 µL	Sweet wormwood fresh leaf 2 (extracted with hot water)			

Sweet wormwood fresh leaf 1: source Switzerland; Sweet wormwood fresh leaf 2: source Korea

System suitability test
Chlorogenic acid: green fluorescent zone at Rf ~ 0.40.
Caffeic acid: green fluorescent zone at Rf ~ 0.90.

Identification
Compare result with reference images. The fingerprint of the test solution is similar to that of the corresponding botanical reference sample. Additional weak zones may be present. The chromatogram of the test solution shows a green fluorescent zone at Rf ~ 0.40 corresponding to reference chlorogenic acid and below it a blue zone at Rf ~ 0.32 (yellow arrows). In the upper part of the chromatogram there is an intense green zone at Rf ~ 0.81 and a blue one just above it at Rf ~ 0.86. Below the solvent front there are two red zones. Similar but fainter zones are seen in the fresh sample (track 3).

Test for adulteration
The blue zones at Rf ~ 0.32 and Rf ~ 0.86 are missing (red arrows, Chinese wormwood leaf).

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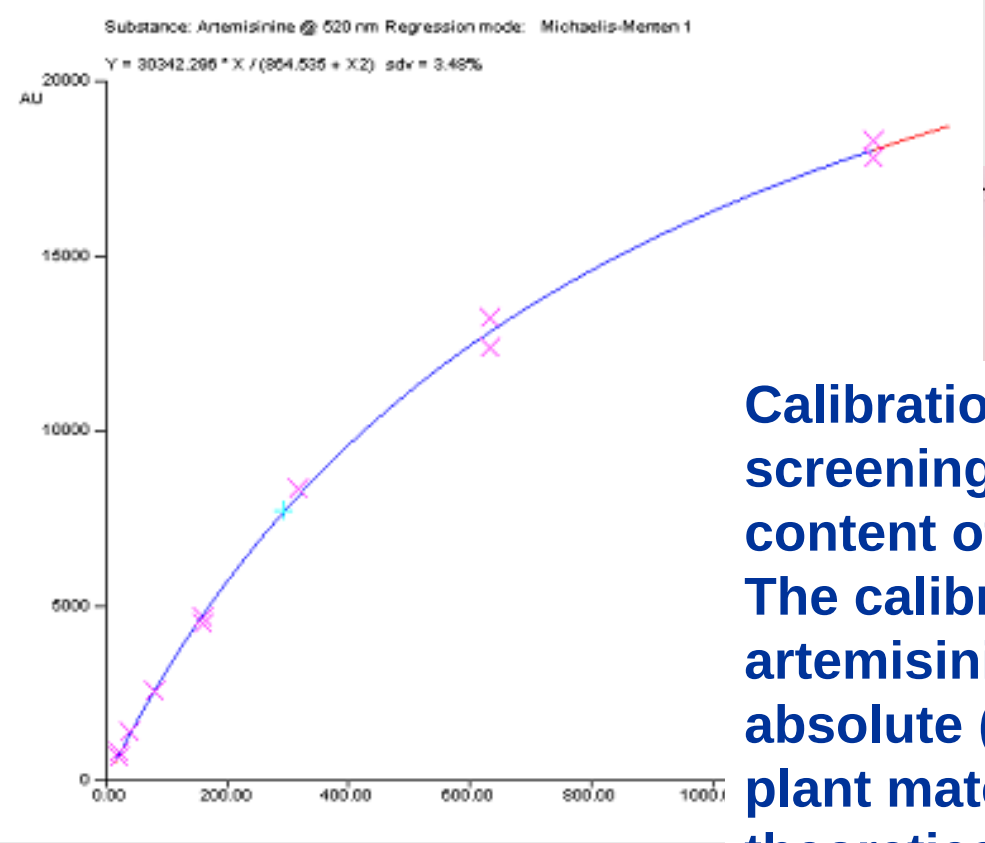
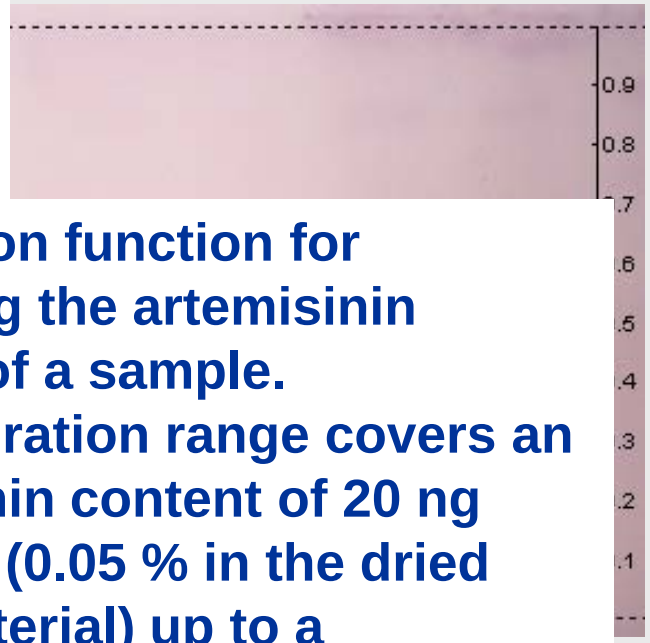
CAMAG LABORATORY

Artemisinin in *Artemisia annua*

- Quantitative method for screening and assay of marker contents in dried leaves of Artemisia
- Published 2007 in Journal of Liquid Chromatography and Related Technologies

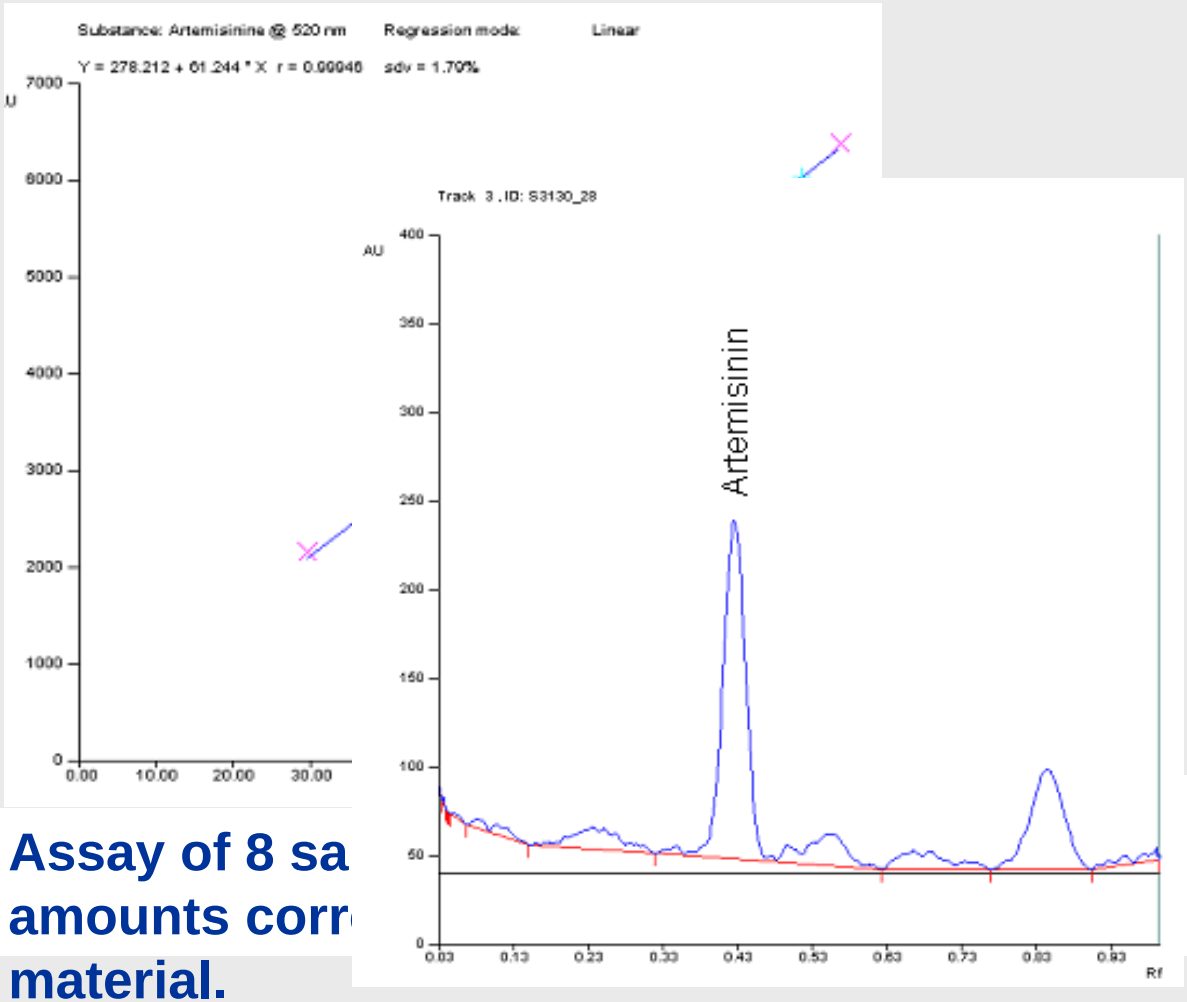
HPTLC Analysis of Artemisinin





Calibration function for screening the artemisinin content of a sample.
The calibration range covers an artemisinin content of 20 ng absolute (0.05 % in the dried plant material) up to a theoretical amount of 1300 ng (3.25 %).

HPTLC Analysis of Artemisinin

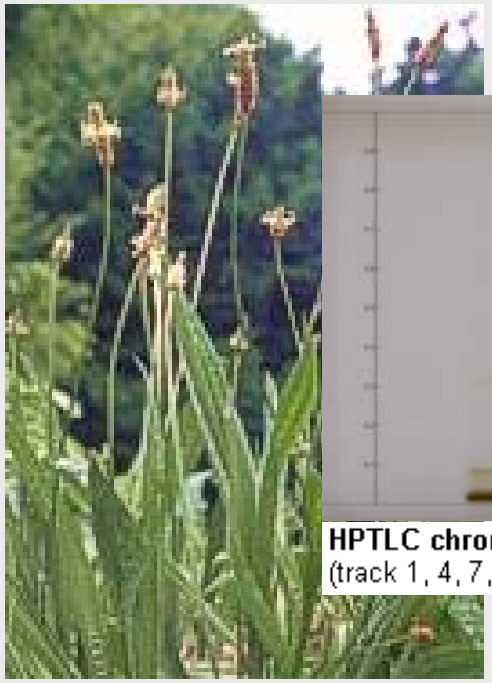


Assay of 8 sa
amounts corr
material.

Densitogram of an Artemisia sample
in the dried

A-87.1 Aucubin and catalpol in Plantain

- HPTLC quantification of aucubin and catalpol in leaves of Ribwort Plantain (Plantago lanceolata)



HPTLC chromatogram for determination of catalpol. Four samples of *Plantago lanceolata* (track 1, 4, 7, 10) and nine catalpol standards (track 2, 3, 5, 6, 8, 9, 11-13)

CAMAG LABORATORY

A-87.1 Aucubin and catalpol in Plantain

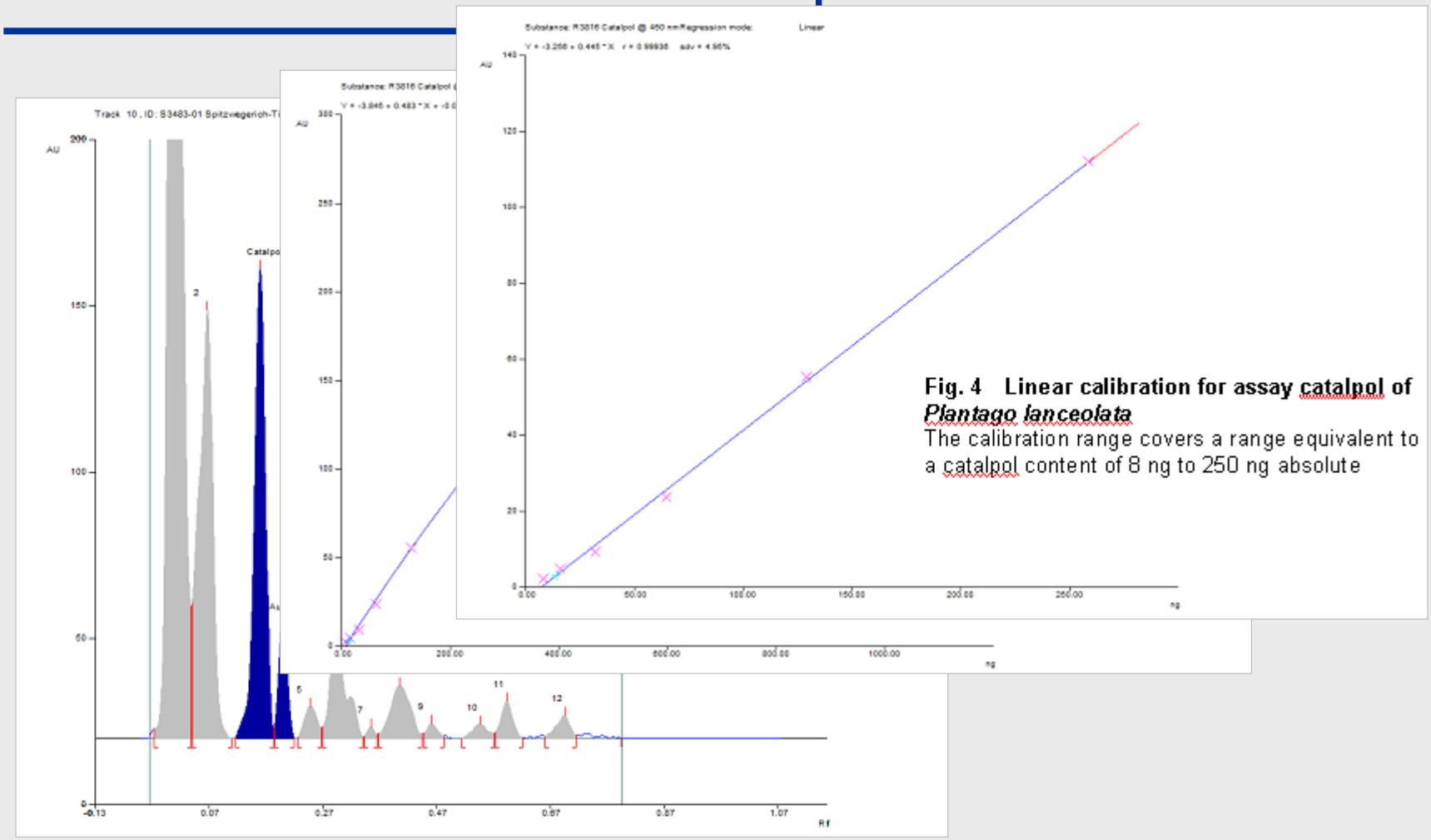
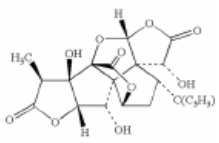


Fig. 4 Linear calibration for assay catalpol of *Plantago lanceolata*
The calibration range covers a range equivalent to a catalpol content of 8 ng to 250 ng absolute

Determination of ginkgolides A, B, and C and bilobalide in *Ginkgo biloba* dry extract by HPTLC

A-92.1



Key words:

HPTLC, densitometry, ginkgolide A, ginkgolide B, ginkgolide C, bilobalide, *Ginkgo biloba* extract

Scope:

This method is suitable for the quantification of ginkgolide A, ginkgolide B, ginkgolide C, and bilobalide in *Ginkgo biloba* dry extract.

Required or recommended CAMAG devices:

Automatic TLC Sampler 4 or Linomat 5, Automatic Developing Chamber ADC2 or Twin Trough Chamber 20 x 10 cm, TLC Scanner and winCATS software

Sample:

0.1 g of dry extract is sonicated with 10 mL of methanol for 10 min and filtered. The supernatant is used as test solution.

Standards:

A standard solution containing approx. 5 mg of bilobalide, 1 mg of ginkgolide A, 1 mg of ginkgolide B, and 1 mg of ginkgolide C in 20 µL of methanol.

Plate impregnation with sodium acetate solution:

8 g of sodium acetate are dissolved in 200 mL of ethanol, water 3:2. HPTLC plates are immersed into the solution for 2 seconds and allowed to dry at room temperature in the hood for 5 min. The plates are then heated at 90 °C for 30 min in an oven.

Derivatization reagent:

Acetic anhydride is directly used for spraying.

APPLICATION NOTES

CAMAG LABORATORY

Chromatography:

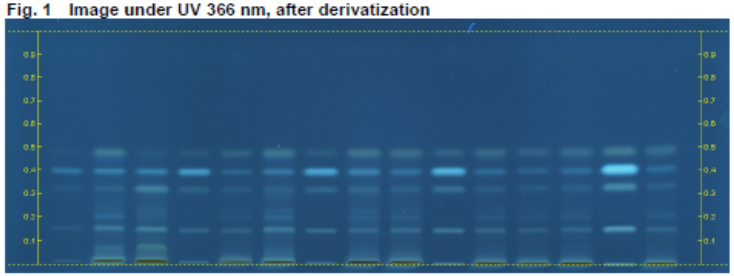
Stationary phase:	HPTLC Si 60 F_{254}, 20 x 10 cm (Merck), impregnated with sodium acetate (see above).
Sample application:	5-15 µL each of test solution and 2, 5, 7, 10, and 25 µL of standard are applied as 8 mm bands, min. 2 mm apart, 8 mm from lower edge of plate.
Developing solvent:	Toluene, ethyl acetate, acetone, methanol (20:10:10:1.2)
Development:	ADC2 or 20 x 10 cm Twin Trough Chamber, saturated for 20 min.
Developing distance:	70 mm from lower edge of plate.
Plate drying:	5 min in a stream of cold air.
Derivatization:	The plate is sprayed evenly with acetic anhydride and heated on the plate heater at 180 °C for 10 min.
Detection:	Examination under UV 366 nm.

Densitometry:

With CAMAG TLC Scanner and winCATS software in absorption mode at 300 nm (after derivatization) using a D2 lamp; evaluation via peak area, polynomial regression.

Results:

Fig. 1 Image under UV 366 nm, after derivatization



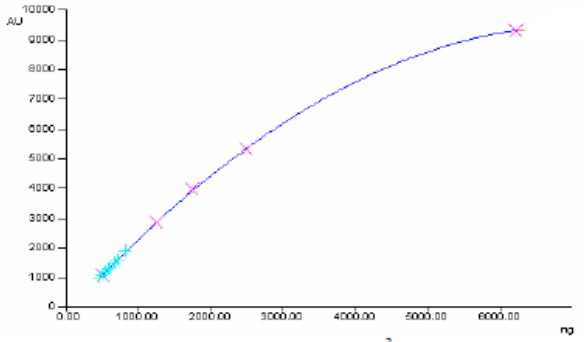
Track	Volume	Sample	Track	Volume	Sample
1	2 µL	Standard mix (bilobalide, ginkgolides A, B, and C (with decreasing Rf value)	9	15 µL	Ginkgo dry extract #6
2	5 µL	Ginkgo dry extract #1	10	10 µL	Standard mix
3	5 µL	Ginkgo dry extract #2	11	10 µL	Ginkgo dry extract #7
4	5 µL	Standard mix	12	15 µL	Ginkgo dry extract #3
5	15 µL	Ginkgo dry extract #3	13	10 µL	Ginkgo dry extract #7
6	5 µL	Ginkgo dry extract #4	14	25 µL	Standard mix
7	7 µL	Standard mix	15	10 µL	Ginkgo dry extract #7
8	5 µL	Ginkgo dry extract #5			

NOTE: The presented results are to be regarded as examples only!

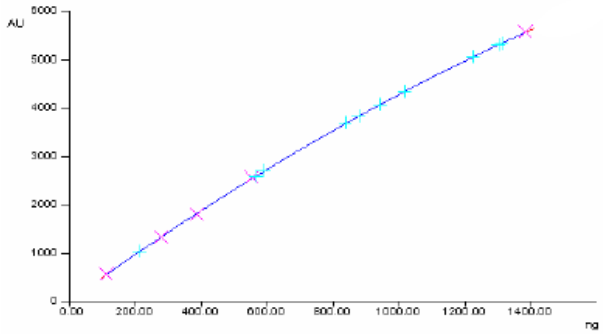
Please contact CAMAG for more application notes and products for analysis of herbals!

Fig. 2 Calibration functions measured at 300 nm after derivatization (polynomial regression via area)

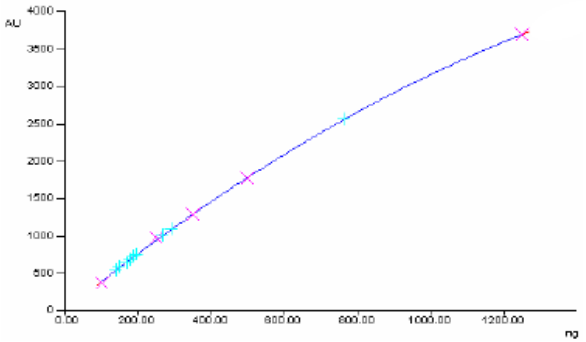
Ginkgolide A $Y = -231.6 + 2.702x - 0.0001878x^2$ r = 0.99996 sdv = 0.86%



Bilobalide $Y = 15.06 + 4.925x - 0.0006592x^2$ r = 0.99995 sdv = 1.11%



Ginkgolide B $Y = -1.006 + 3.969x - 0.0008115x^2$ r = 0.99988 sdv = 1.73%



APPLICATION NOTES

Ginkgolide C $Y = -30.2 + 2.889x - 0.0004281x^2$ r = 0.99994 sdv = 1.23%

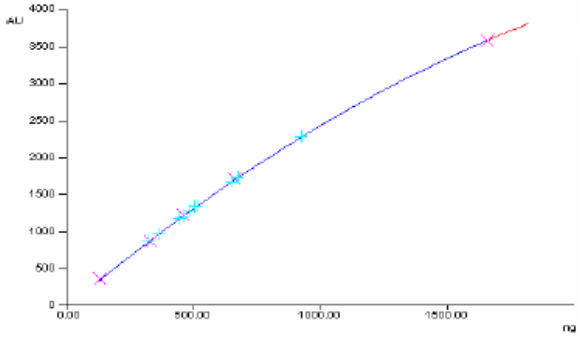
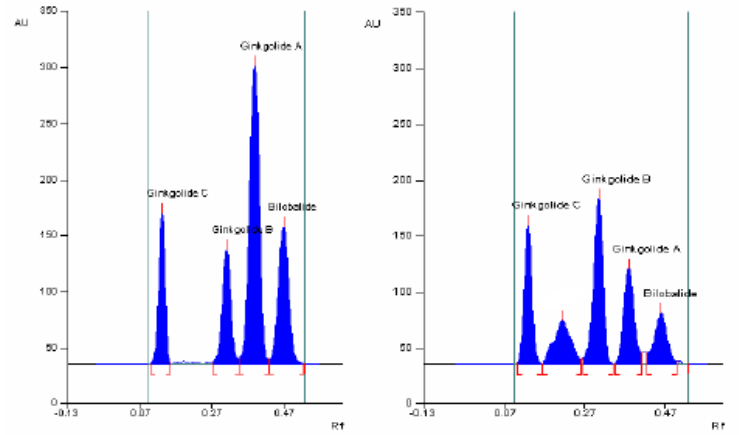


Fig. 3 Densitograms of standards (left) and a Ginkgo dry extract sample (right)



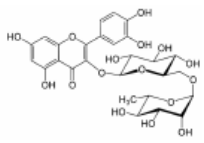
Literature

Based on the HPTLC method for identification of ginkgolides in Ginkgo, American Herbal Pharmacopoeia, 2003

NOTE: The presented results are to be regarded as examples only!
Please contact CAMAG for more application notes and products for analysis of herbals!

Determination of the flavonoid rutin in *Ginkgo biloba* dry extract by HPTLC

A-93.1



Key words:

HPTLC, densitometry, flavonoids, rutin *Ginkgo biloba* extract

Scope:

This method is suitable for the quantification of rutin in *Ginkgo biloba* dry extract. For additional visual evaluation of the HPTLC fingerprint the plate can be derivatized with natural products reagent.

Required or recommended CAMAG devices:

Automatic TLC Sampler 4 or Linomat 5, Automatic Developing Chamber ADC2 or Twin Trough Chamber 20 x 10 cm, TLC Scanner and winCATS software, Visualizer

Sample:

0.1 g of dry extract is sonicated with 10 mL of methanol for 10 min and filtered. The supernatant is used as test solution.

Standards:

A standard solution containing 0.1 mg/mL rutin in methanol.

Derivatization reagent (optional):

Natural Products reagent (NP reagent): 1 g of diphenylborinic acid aminoethylester is dissolved in 200 mL of ethyl acetate.

Macrogol reagent: 10 g of polyethylene glycol 400 (macrogol) are dissolved in 200 mL of dichloromethane.

NOTE: The presented results are to be regarded as examples only!

Please contact CAMAG for more application notes and products for analysis of herbals!

APPLICATION NOTES

Chromatography:

Stationary phase:	HPTLC Si 60 F_{254}, 20 x 10 cm (Merck).
Sample application:	2-5 µL each of test solution and 2, 4, 6, and 8 µL of standard are applied as 8 mm bands, min. 2 mm apart, 8 mm from lower edge of plate.
Developing solvent:	Ethyl acetate, glacial acetic acid, formic acid, water (100:11:11:27)
Development:	ADC2 or 20 x 10 cm Twin Trough Chamber, saturated for 20 min.
Developing distance:	70 mm from lower edge of plate.
Plate drying:	5 min in a stream of cold air.
Derivatization (optional):	the plate is heated at 100 °C for 3 min, dipped while still hot in NP reagent, dried in a stream of cold air and dipped in Macrogol reagent.
Detection:	a) UV 254 nm b) UV 366 nm c) (optional) UV 366 nm, derivatized with NP/Macrogol reagent

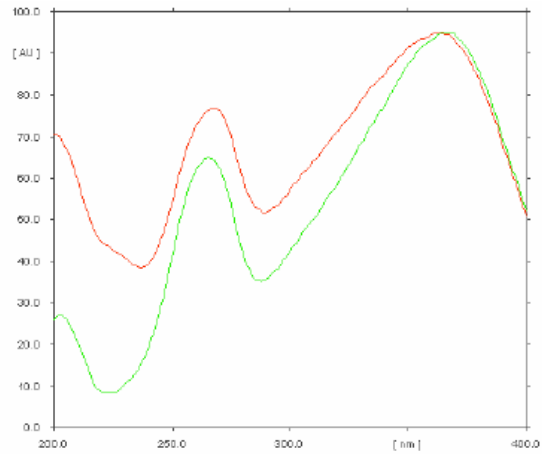
Densitometry:

With CAMAG TLC Scanner and winCATS software in absorption mode at 360 nm (prior to optional derivatization) using a D2 lamp; evaluation via peak height, polynomial regression.

Results:

Fig. 1 UV spectra of rutin standard (green) and corresponding zone in sample (red)

Based on the UV spectrum of rutin the UV_{max} of 360 nm was selected as measurement wavelength for quantification.



NOTE: The presented results are to be regarded as examples only!

Please contact CAMAG for more application notes and products for analysis of herbals!

Fig. 2 Linear calibration function of rutin in samples measured at 360 nm

$Y = -15.65 + 0.3664x - 8.598e-005x^2$ r = 0.99975 sdv = 1.96%

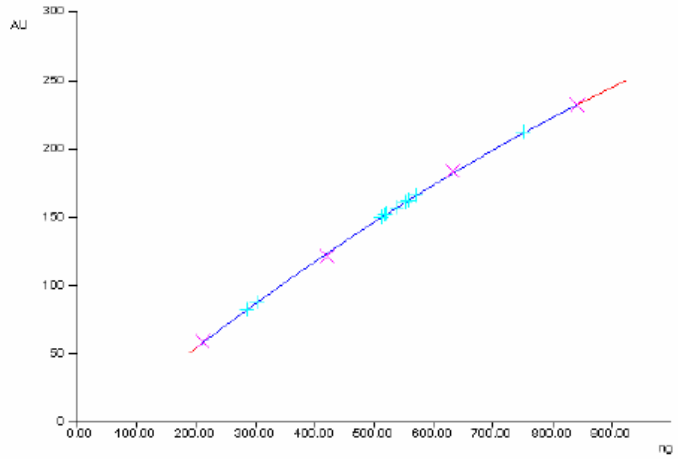
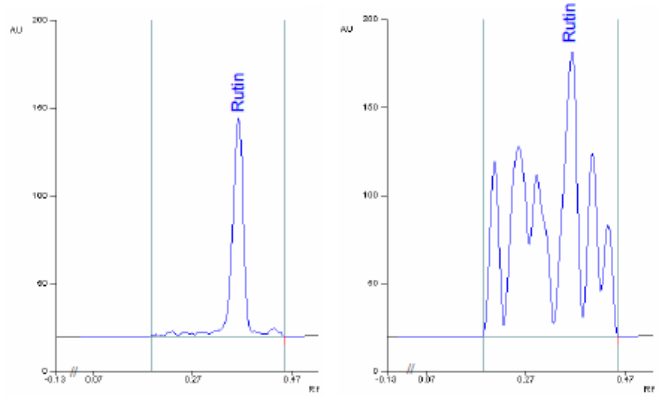
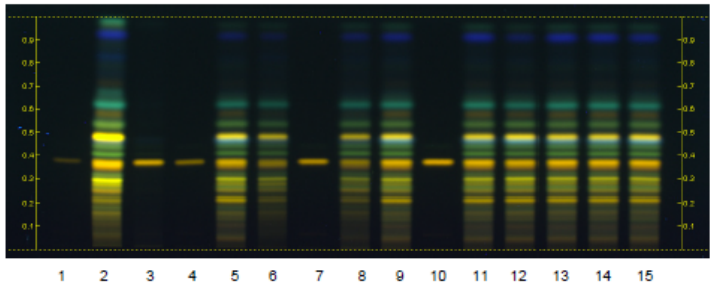


Fig. 3 Densitograms of standard (left) and a Ginkgo dry extract sample (right)



APPLICATION NOTES

Fig. 4 Image under UV 366 nm, after derivatization



Track	Volume	Sample	Track	Volume	Sample
1	2 µL	Rutin	9	4 µL	Ginkgo dry extract #6
2	2 µL	Ginkgo dry extract #1	10	8 µL	Rutin
3	2 µL	Ginkgo dry extract #2	11	4 µL	Ginkgo dry extract #7
4	4 µL	Rutin	12	7 µL	Ginkgo dry extract #3
5	7 µL	Ginkgo dry extract #3	13	4 µL	Ginkgo dry extract #7
6	5 µL	Ginkgo dry extract #4	14	4 µL	Ginkgo dry extract #7
7	6 µL	Rutin	15	4 µL	Ginkgo dry extract #6
8	6 µL	Ginkgo dry extract #5			

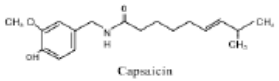
Literature

Based on the HPTLC method for identification of flavonoids in Ginkgo, American Herbal Pharmacopoeia, 2003.

NOTE: The presented results are to be regarded as examples only!
Please contact CAMAG for more application notes and products for analysis of herbals!

Determination of capsaicin in *Capsicum* tincture by HPTLC

A-85.1



Capsaicin

Key words:

HPTLC, densitometry, plant analysis, capsaicin, red pepper, cayenne pepper, *Capsicum annuum*, *Capsicum frutescens*

Introduction:

Capsaicin belongs to the active principles that cause the heat in *Capsicum* peppers. Capsaicinoids are powerful alkaloids, which retain their original potency despite time, cooking, or freezing. Although capsaicin has no odor or flavor, it is one of the most pungent compounds known, detectable to the palate in dilutions of one to seventeen million.

Scope:

This method is suitable for the quantitative evaluation of capsaicin in Capsicum tincture and the separation of the related capsaicinoid dihydrocapsaicin. The samples are chromatographed on RP-18 and evaluated densitometrically by absorption measurement at 200 nm.

Required or recommended CAMAG devices:

Automatic TLC Sampler 4 or Linomat 5, Automatic Developing Chamber ADC 2 or Twin Trough Chamber 20 x 10 cm, TLC Scanner 3 and winCATS software.

Sample:

10 mL of standardized Capsicum tincture PhEur (capsaicin content 0.020-0.060% m/m) or corresponding amount of sample is mixed with 10 mL of hexane, after separation the lower layer is used.

Standards:

Two standard solutions are prepared.
Solution I: 2.0 mg of capsaicin are dissolved in 100 mL of *tert*-butylmethylether (20 ng/µL).
Solution II: 5 mL of solution I are transferred in a 20 mL graduated flask and diluted with *tert*-butylmethylether to 20.0 mL (5 ng/µL).

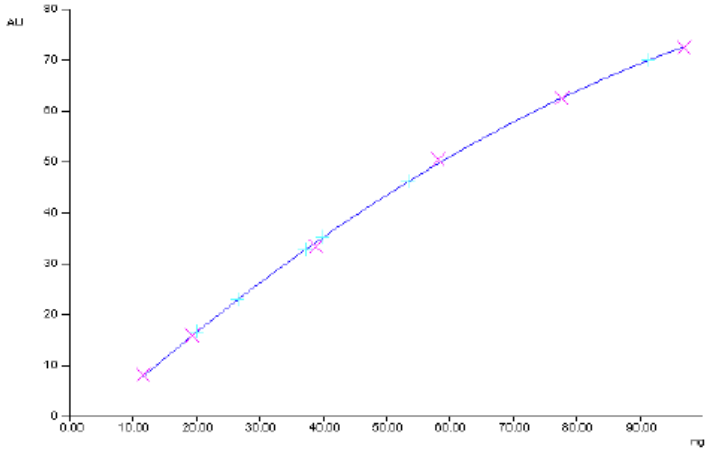
APPLICATION NOTES

CAMAG LABORATORY

Calibration curve for capsaicin (P86-041013-03)

Polynomial regression via peak height

$Y = -4.965 + 1.150x - 0.004x^2$ r = 0.99984 sdv = 1.48 %



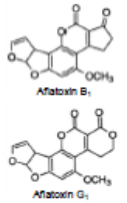
Literature:

Based on the HPTLC method for identification of standardized Capsicum tincture, European Pharmacopoeia 7.0

NOTE: The presented results are to be regarded as examples only!
Please contact CAMAG for more application notes and products!

Determination of aflatoxins B_1, G_1, B_2, and G_2 in tomato extract by HPTLC

A-97.1



Key words:

HPTLC, quantification, densitometry, immunoaffinity column, aflatoxin B_1, aflatoxin G_1, aflatoxin B_2, aflatoxin G_2, tomato extract

Introduction:

Aflatoxins are natural mycotoxins produced by *Aspergillus* fungi. High temperatures and humidity favor the occurrence of molds and thus the production of aflatoxins. The contamination of crops, nuts, dried fruits/vegetables, dried medicinal plants, and milk is quite common. Because of their strong carcinogenicity aflatoxins must be controlled in food and feeds.

Scope:

This method is suitable for the quantification of aflatoxins B_1, G_1, B_2, and G_2 in tomato extract according to the Test for Aflatoxins of chapter <561> Articles of Botanical Origin of USP 35 which limits aflatoxin B_1 to 5 ppb and the sum of B_1, G_1, B_2, and G_2 to 20 ppb. Chromatography is performed on HPTLC plates according to Method II.

Required or recommended CAMAG devices:

Automatic TLC Sampler 4 or Linomat 5, Automatic Developing Chamber ADC 2 or Twin Trough Chamber 20 x 10 cm, Visualizer, TLC Scanner and winCATS software.

Sample:

Transfer about 5 g of a representative powdered sample, accurately weighed, to a glass-stoppered flask. Add 20 mL of a mixture of methanol and water (17:3). Shake vigorously by mechanical means for not less than 30 minutes, and filter. Discard the first 5 mL of the filtrate, and collect the next 4 mL portion. Transfer the filtrate to a separatory funnel. Add 4 mL of sodium chloride solution (5 g of sodium chloride in 50 mL of water) and 2.5 mL of hexane, and shake for 1 minute. Allow the layers to separate, and transfer the lower aqueous layer to a second separatory funnel. Extract the aqueous layer in the separatory funnel twice, each time with 2.5 mL of methylene chloride, by shaking for 1 minute. Allow the layers to separate each time, separate the lower organic layer, and collect the

combined organic layers in a 50 mL conical flask. Evaporate the organic solvent on a water bath. Transfer the remaining extract to an appropriate sample tube and evaporate to dryness on a water bath. Cool the residue.
If interferences exist in the residue, proceed as directed for **Cleanup Procedure with immunoaffinity column (IAC)**; otherwise, dissolve the residue obtained above in 200 µL of acetonitrile, and shake by mechanical means if necessary.

Clean up with immunoaffinity column (IAC)
The residue of the above sample solution is dissolved in 5 mL of a mixture of methanol and water (60:40) and then diluted with 5 mL of water. This extract is applied onto a conditioned IAC. The IAC is rinsed twice with 10 mL of phosphate buffered saline (PBS) solution*, and the elution is performed slowly with 2 mL of methanol. Evaporate the eluate with nitrogen, and dissolve the residue in 200 µL of acetonitrile.
*Phosphate buffered saline (PBS) solution: Prepare 10 mM phosphate buffer solution containing 0.138 M sodium chloride and 0.0027 M potassium chloride in water, and adjust with 2 M sodium hydroxide to a pH of 7.4. A suitable powder mixture is available from Sigma as PBS P-3813

IAC preparation
Prior to conditioning, IAC should be adjusted to room temperature. For conditioning, apply 10 mL of PBS solution on each column and let pass through at a rate of 2 to 3 mL/min by gravity. Leave 0.5 mL of PBS buffer on top of column until Test Solution is applied.
For this application note the sample of tomato extract was extracted using an IAC from R-Biopharm.

Standards:

Accurately weighed standard solutions containing 0.05 µg/mL aflatoxin B_1 and aflatoxin G_1 and 0.01 µg/mL aflatoxin B_2 and aflatoxin G_2 in a mixture of chloroform and acetonitrile (9.8:0.2) are prepared.

Chromatography:

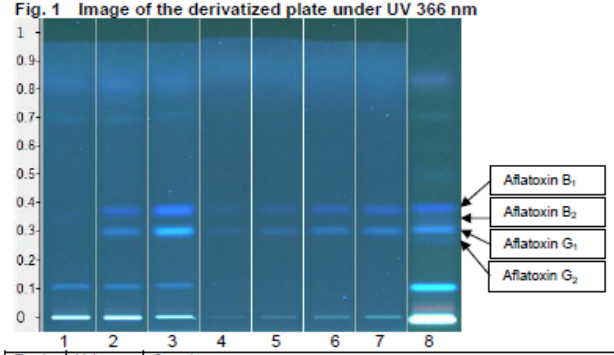
Stationary phase:	HPTLC Si 60 F_{254} 20 x 10 cm (Merck).
Sample application:	10 µL each of test solution and 20, 40 and 50 µL of standard are applied as 8 mm bands, min 2 mm apart, 8 mm from lower edge of plate.
Developing solvent:	Chloroform, acetone, water (140:20:0.3) (v/v/v)
Development:	20 x 10 cm Twin Trough Chamber or ADC 2, saturated for 20 min (filter paper), 10 mL developing solvent per trough, humidity control at 33 % relative humidity (using a saturated solution of $MgCl_2$).
Developing distance:	70 mm from lower edge of plate.
Plate drying:	5 min in a stream of cold air.
Derivatization:	optional: dip (time 0, speed 5) in paraffin, n-hexane (2:3), dry in air
Evaluation:	Examination under UV 366°nm.

Densitometry:

With CAMAG TLC Scanner and winCATS software in fluorescence mode at 366/>400 nm using a mercury lamp; evaluation via peak area, linear regression.

Results:

Fig. 1 Image of the derivatized plate under UV 366 nm



Track	Volume	Sample
1	10 µL	Tomato extract
2	10 µL	Tomato extract with 5 ppb aflatoxins B_1 and G_1 (spiked by overspotting with 50 µL of a 5 pg/µL standard solution)
3	10 µL	Tomato extract with 25 ppb aflatoxins B_1 and G_1 (spiked by mixing 125 ng each of aflatoxin B_1 and G_1 with 5 g of sample)
4	1 µL	Standards aflatoxin B_1 and G_1 (0.05 µg/mL → 50 pg absolute)
5	2 µL	Standards aflatoxin B_1 and G_1 (0.05 µg/mL → 100 pg absolute)
6	4 µL	Standards aflatoxin B_1 and G_1 (0.05 µg/mL → 200 pg absolute)
7	5 µL	Standards aflatoxin B_1 and G_1 (0.05 µg/mL → 250 pg absolute)
8	10 µL	Tomato paste, spiked with aflatoxins B_1, G_1, B_2, G_2 (B_2, G_2 show only faint zones)

Fig. 2 Densitogram of standards aflatoxin G_2, G_1, B_2, and B_1

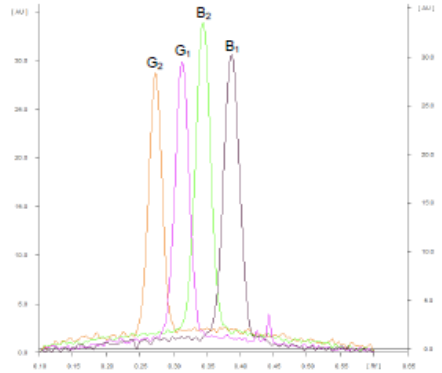


Fig. 3 Densitogram of a tomato extract sample (black) and the same sample spiked with 5 ppb of aflatoxins B_1 and G_1 (green)

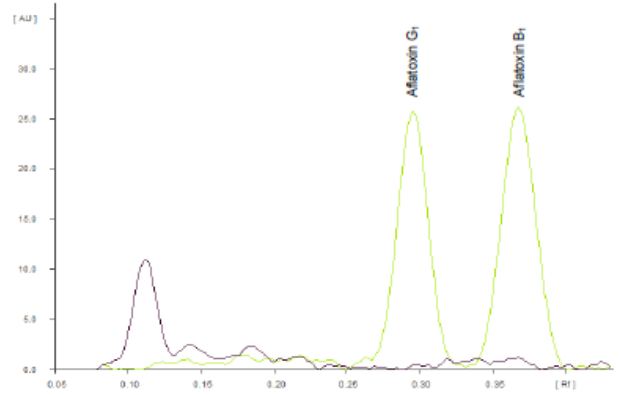
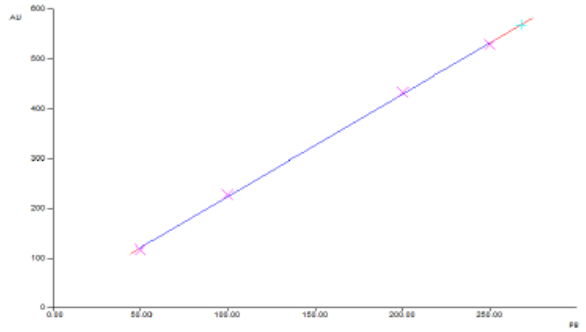


Fig. 4 Calibration function for aflatoxin B_1 measured at 366 nm
Regression via area y=17.653+2.057x r=0.99978 sdv=1.49 %



Literature

USP 35-2: Test for Aflatoxins" of chapter <561> Articles of Botanical Origin.

CAMAG LABORATORY

Pour conclure

- L’HPTLC est une méthode tout à fait utilisable dans le vaste domaine des plantes, en particulier médicinales sous certaines conditions
  - Méthodes mises au point (et validées)
  - Automatisation (pas de variation/opérateur)
  - Comparatif = standardisation

CAMAG LABORATORY

Pour conclure

- L’accompagnement dans la démarche HPTLC-Plantes est très étoffé
  - Notes d’application
  - Développement méthodes
  - HPTLC association
  - Réseaux de laboratoire
  - Documents (pannel discussion hptlc.com)
  - Motivation des acteurs

Merci de votre attention…