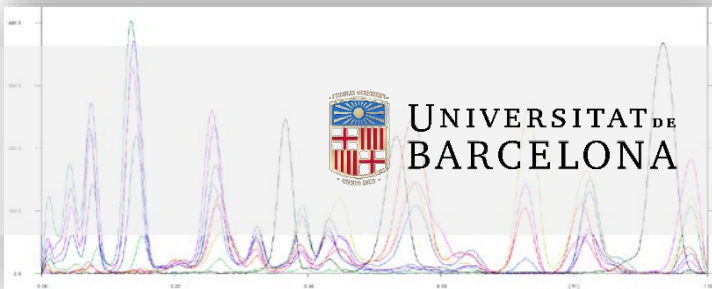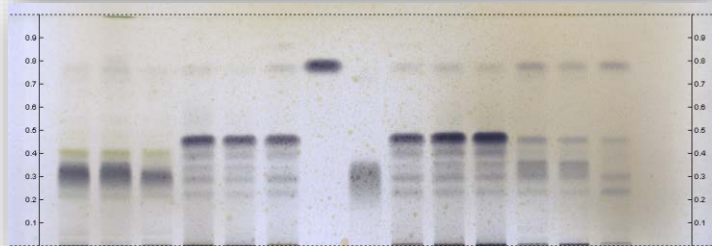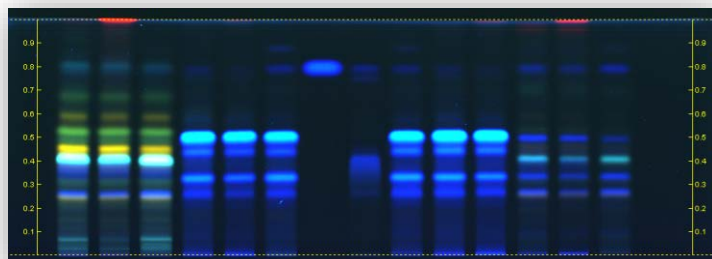# Le concept d'empreinte «fingerprint» basé sur l'HPTLC pour le control de qualité des drogues et préparations végétales dans la Pharmacopée Européenne

Salvador Cañigueral and **Roser Vila**
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Facultat de Farmàcia i Ciències de l'Alimentació

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Lavandula angustifolia

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HPTLC fingerprint in the quality control of herbal drugs and herbal preparations in the European Pharmacopoeia

Content

1. Herbal drugs and preparations

2. Quality control of herbals and *Ph. Eur.* monographs

3. TLC/HPTLC in quality control of herbal products

4. The issues and the improvements: Chapter *2.8.25* of the *Ph. Eur.*

5. Comprehensive HPTLC fingerprinting

6. Two examples

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Herbal drug

European Pharmacopoeia

Herbal drugs are mainly **whole, fragmented or broken plants or parts of plants** in an unprocessed state, usually in **dried** form but sometimes **fresh**. In this general monograph, the word "plant" is used in the broader sense to also include algae, fungi and lichens.

Certain **exudates** that have not been subjected to a specific treatment are also considered to be herbal drugs.

Herbal drug
(European Pharmacopoeia) **=** **Herbal substance**
(Directive 2004/24/CE)

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Herbal drug preparations

European Pharmacopoeia

Herbal drug preparations are homogeneous products obtained by subjecting herbal drugs to treatments such as **extraction, distillation, expression, fractionation, purification, concentration or fermentation**.

Include:
- Cut or powdered herbal drugs
- Extracts
- Essential oils
- Expressed juices
- Processed exudates

= **Herbal preparations** (Directive 2004/24/CE)

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Quality issues related to herbal (medicinal) products

- ✓ **Complexity** of the composition of herbal drugs and herbal preparations
- ✓ **Variability** of the biological materials (biodiversity, chemotypes, etc.).
- ✓ The **active principles** are sometimes **not identified** or only partially known
- ✓ Influence of the **collection and post-harvesting** processing (drying, storage, etc)
- ✓ Influence of the **extraction process**
- ✓ Possible **contaminations** (adulterations, heavy metals, pesticides, microbial, etc.)

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Quality

Herbal drugs and herbal preparations

Identity — Confirmation of the herbal drug

Purity — Detection of adulterations, falsifications, etc. Presence of contaminants

Strength — Confirmation that the **content** of active principles or markers is within the accepted limits.

And its preservation during the period of use: **Stability**

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European Pharmacopoeia
Monographs

Index of an individual monograph

1. Definition
2. Production
3. Characters
4. **Identification**
5. **Tests**
6. Assay
7. Conservation
8. Labelling

Monograph

Identification

Macroscopic characters

TLC
HPTLC

Microscopic characters

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Monograph

Tests*

Search for falsifications

Total ash/ Ash insoluble in HCl

Aflatoxin B1, ochratoxin

Pesticides

Swelling index

Heavy metals

Quality

Bitterness value

Residual solvents

Extractable matter

Physical constants

Foreign matter

Other...

Loss on drying / Water
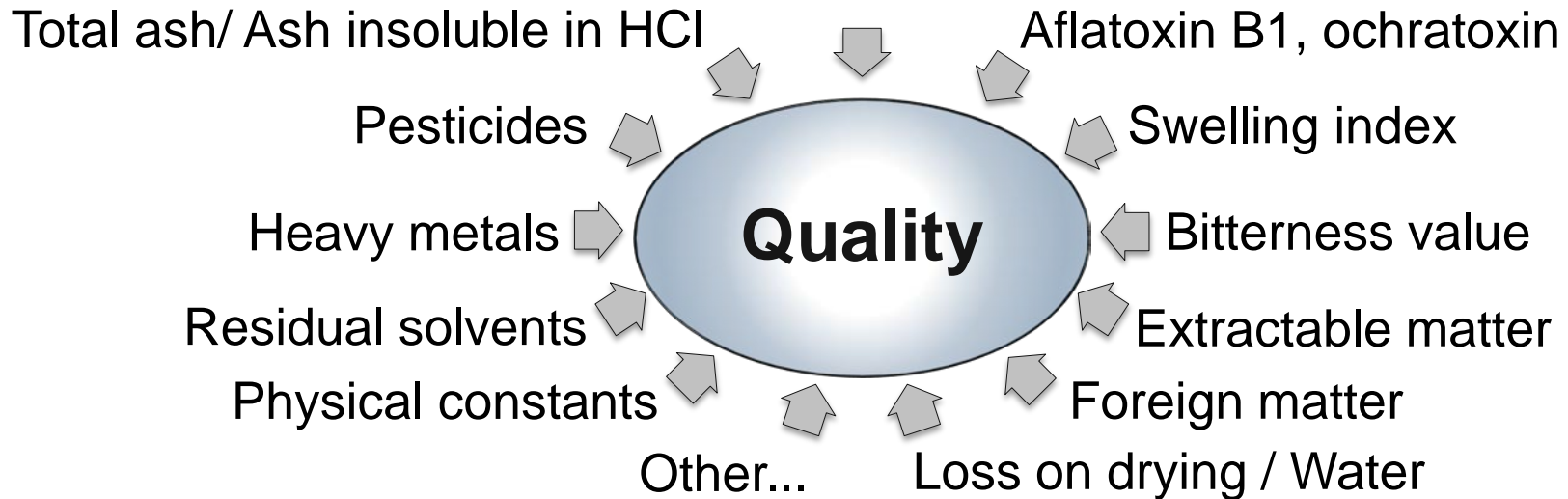
*) The tests applied will depend on the type of product (herbal drug, tincture, dry extract, essential oil, etc…)

Chromatographic herbal fingerprint

Definition

A chromatographic pattern of an herbal extract showing some common pharmacologically active and/or chemical characteristic com-pounds".
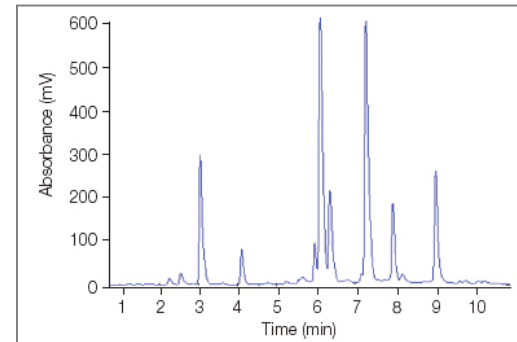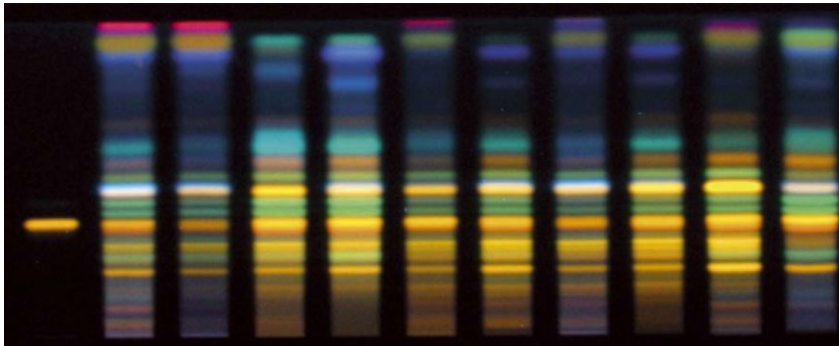
Van der Heyden *et al.* (2008) *LCGC Europe*, 21 (9): 438-443.

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TLC and quality control of herbals

A classical tool
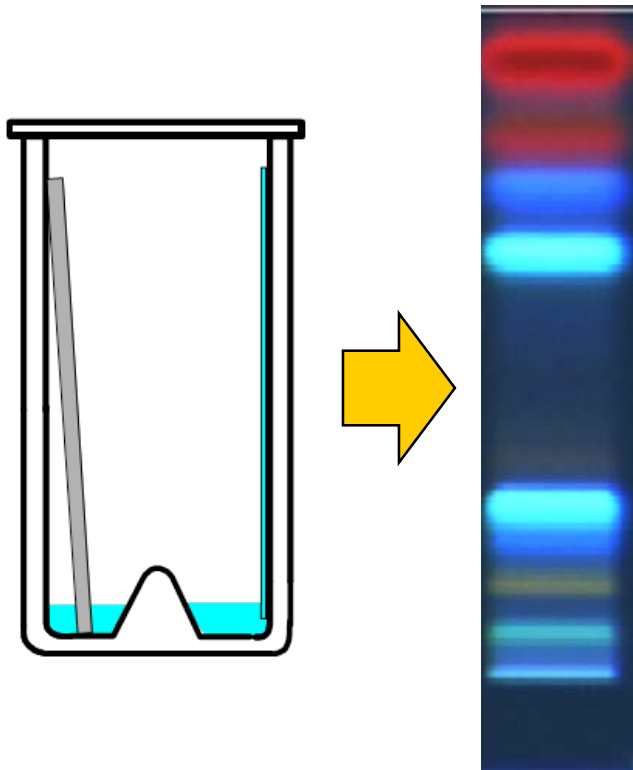
- ✓ Used since years.
- ✓ Included in most Pharmaco-poeias.
- ✓ Identification, detection of adulterations/falsifications
- ✓ Historically with a limited description of the analytical parameters.

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TLC/HPTLC fingerprint evaluation

Similarity

Sequence of zones	Visual comparison
✓ Number	✓ Description
✓ Position	✓ Images
✓ Color	✓ Analog chromato-grams
✓ Intensity	

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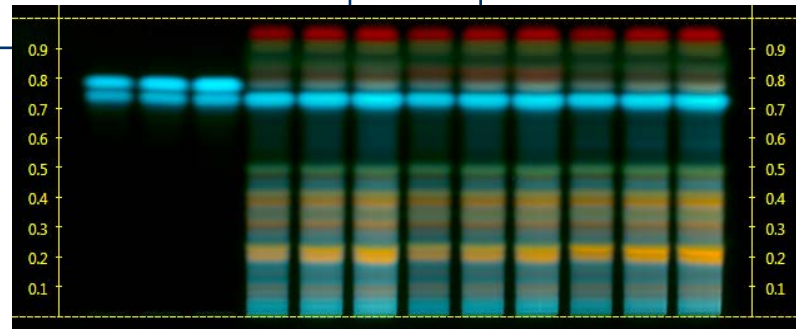
TLC and quality control of herbals

Problems

✓ **Variability of the chromatograms**
- Variability of herbal drugs (differences between batches)
- Lack of reproducibility intra- and inter-laboratory
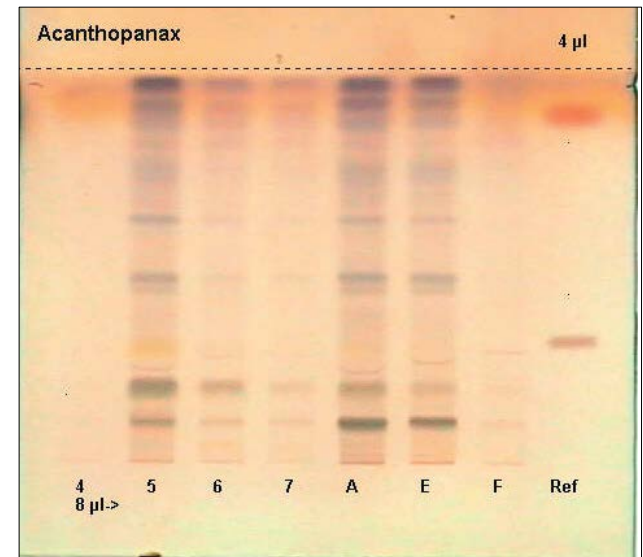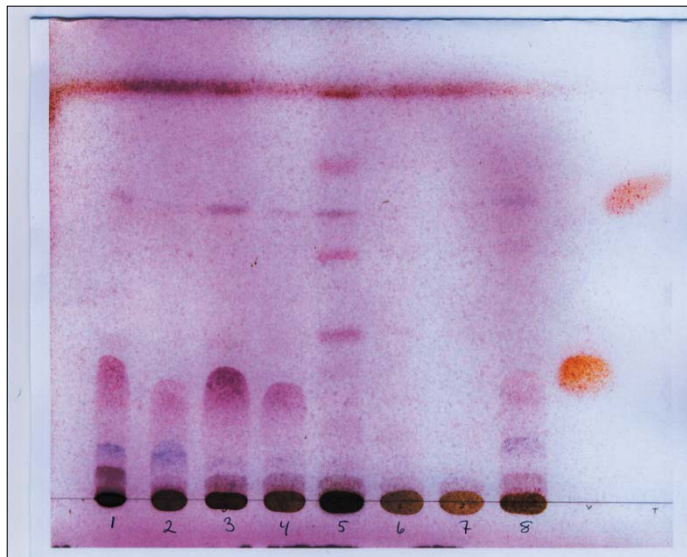
TLC and quality control of herbals

Problems

✓ **Interpretation of the chromatograms**
  – Difficulties for describing the natural variability in a single description
  – Is the chromatogram well done?
  – Difficulties for describing and interpreting:
    ‣ Which zones?
    ‣ Position of zones
    ‣ Colour of zones
    ‣ Intensity of zones

Compliant or not compliant?
That is the question.

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HPTLC *versus* TLC

TLC reproducibility
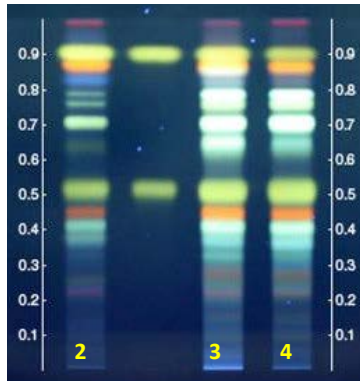
Laboratory 1
(glass plate F$_{254}$)
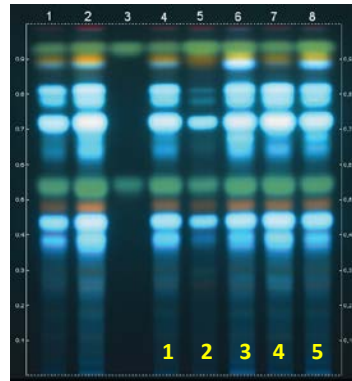
Laboratory 2
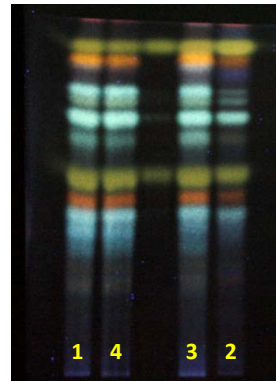
Laboratory 3
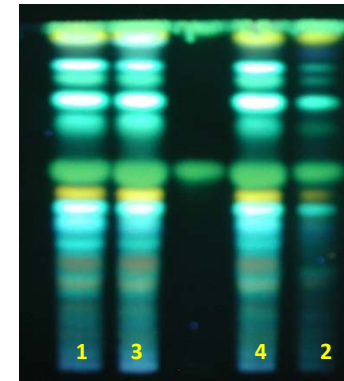
Laboratory 4

Roman chamo-mile flower

TLC

Ph. Eur. Inter-laboratory trial
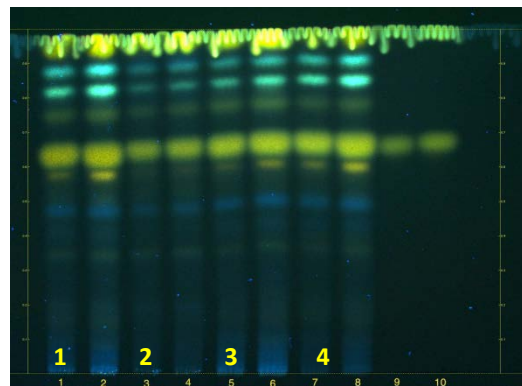
Laboratory 5
(the *1.5-fold* application vol is additionally applied)
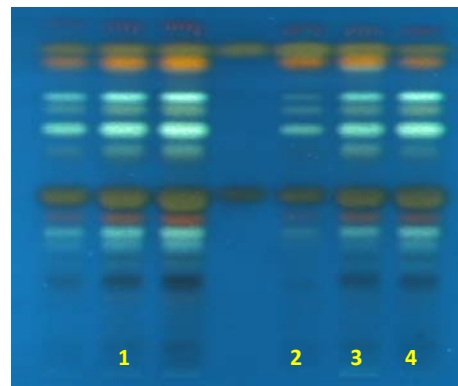
Laboratory 6

Laboratory 7

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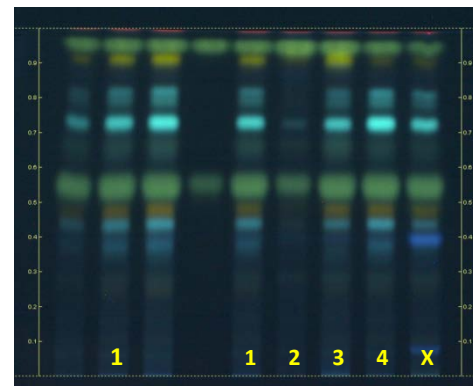
HPTLC *versus* TLC

HPTLC reproducibility
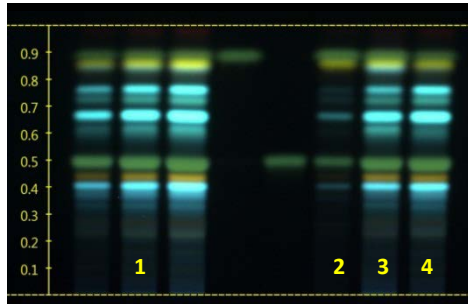
Laboratory 8
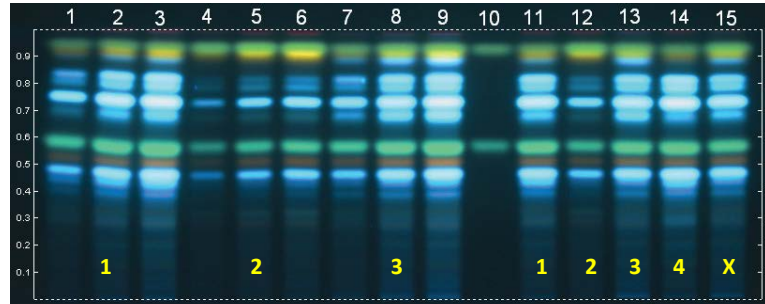
Laboratory 2
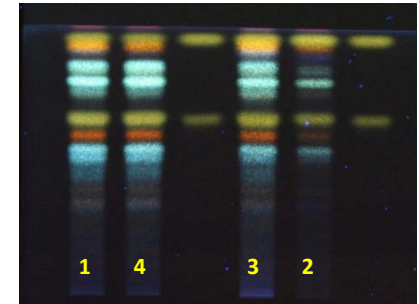
Laboratory 3
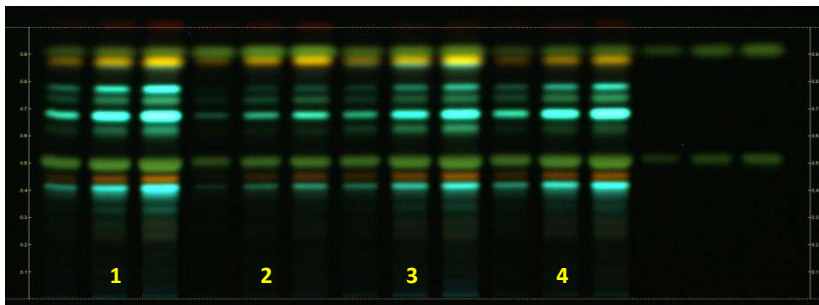
Laboratory 5 (instrumental)
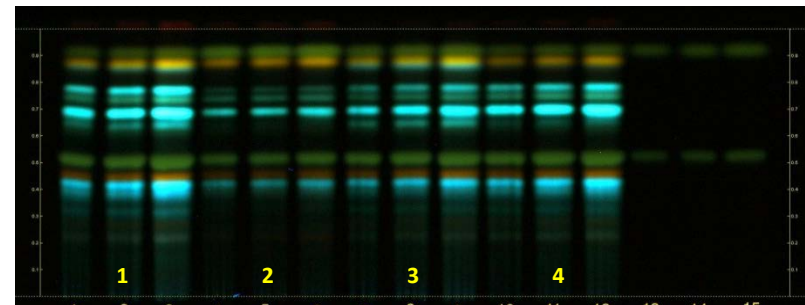
Laboratory 5 (manual)

Labortory 6
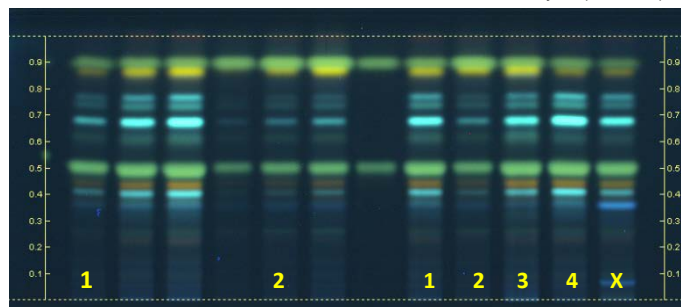
Laboratory 7

Roman chamomile flower

HPTLC

Ph. Eur. Interlaboratory trial

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HPTLC for quality control of herbals

Ph. Eur. Improvements (*chapter 2.8.25*)

1. Improvement of reproducibility
✓ **Introduction of HPTLC**

Instrumental HPTLC   **Manual HPTLC**
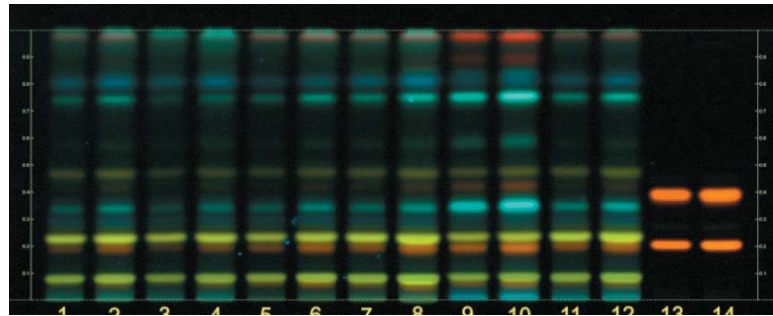
Calendula flower
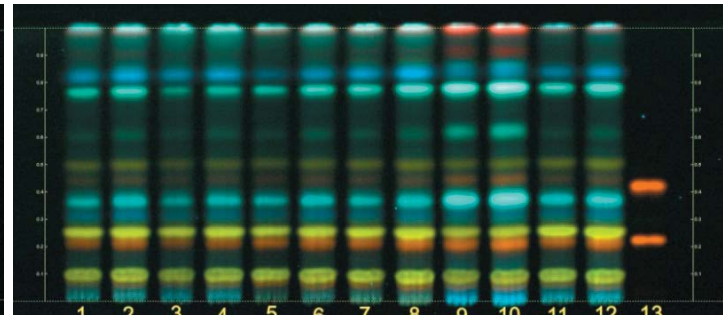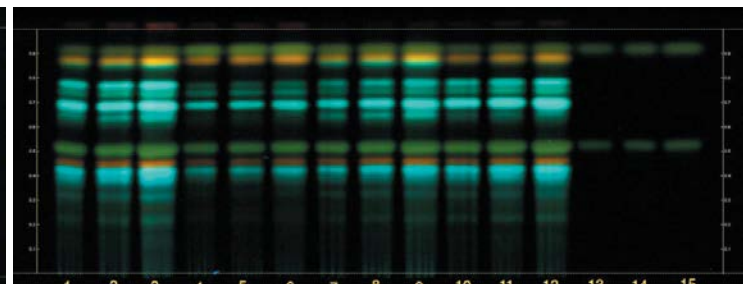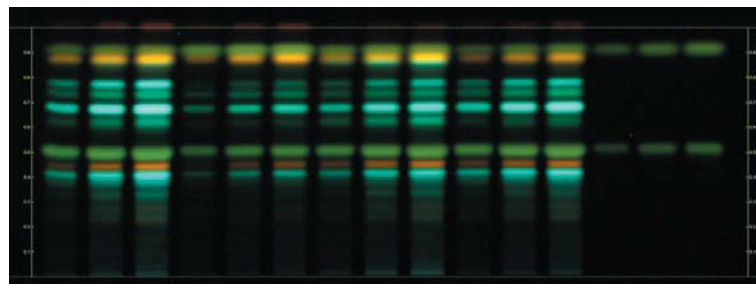
Roman chamomile flower

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HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

1. Improvement of reproducibility

  ✓ Introduction of HPTLC
  ✓ **Standardisation of methodology**

Set up of the HPTLC plate

Size: 20 x 10 cm
Labels
Position of samples

[a] = 20 mm   [b] = 10 mm

[c] = 8 mm   [d] = 8 mm

[e] = 62 mm   [f] = 70 mm

P17_050815_01

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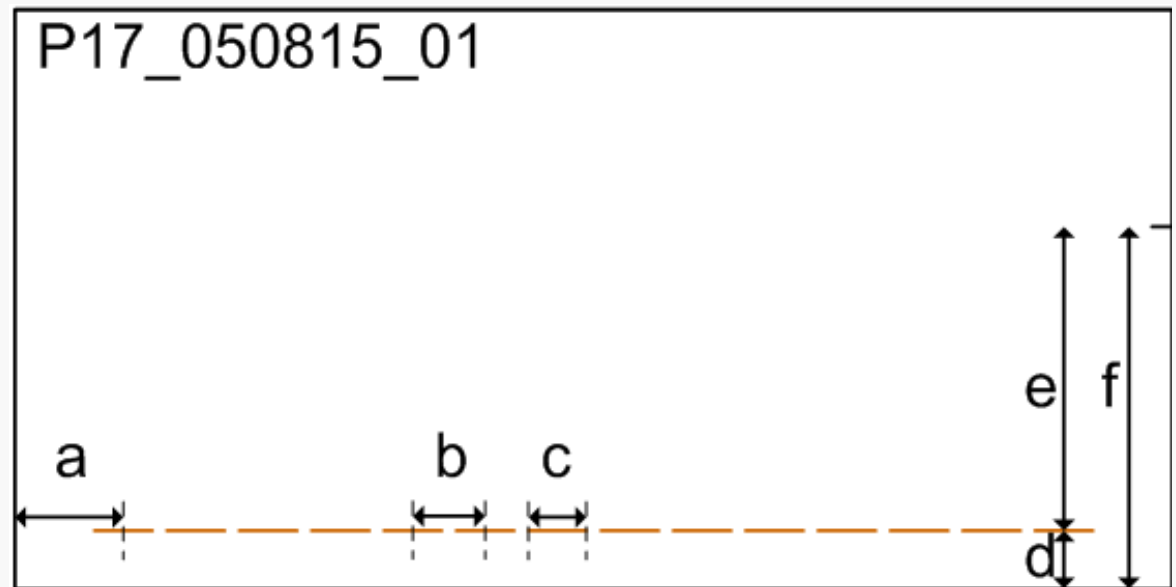
Standard operating procedures (SOP)

Reproducibility ⇐ Standardization of methodology ⇐ SOP

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

SOP

Reich, E., Schibli, A. (2004). J. Planar Chromatogr. 17, 438-443

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Standard operating procedures

Ph. Eur. 2.8.25

2.8.25 High-performance thin-layer chromatography of herbal drugs and herbal drug preparations

a monograph, for example: *lactic reagent R* which is used to show the presence of various features, 10 per cent *V/V* alcoholic solution of *phloroglucinol R* and *hydrochloric acid R*, which a[...] [...]or tissues[...] [...]e prese[...] pres[...]

[...] glass [...]y of the [...]aration with [...]to boiling on a [...]tle boiling for [...] mounting fluid [...]ing a tapered glass pipette. Allow to cool and then examine under a microscope. Repeat the heating until the starch granules and the water-soluble contents of the cells are no longer visible. Examine under a microscope.

01/2017:20825

2.8.25. HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF HERBAL DRUGS AND HERBAL DRUG PREPARATIONS

High-performance thin-layer chromatography (HPTLC) is used for qualitative analysis of herbal drugs and herbal drug preparations. It is a thin-layer chromatographic technique (2.2.27) that, unless otherwise stated in an individual monograph, uses a glass plate coated with a uniform, porous layer (average pore size 6 nm), typically 200 μm thick, of irregular particles of silica gel between 2 μm and 10 μm in size and with an average size of 5 μm, a polymeric binder and a fluorescence indicator (F_{254}). The results are qualified using a system-specific suitability test.

General Notices (1) apply to all monographs and other texts

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HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

1. Improvement of reproducibility

- ✓ Introduction of HPTLC
- ✓ Standardisation of methodology
- ✓ **Introduction of a system suitability test** (qualification of the plate)

Flavonoids
Developing solvent:
Ethyl acetate / formic acid / water (80:10:10)
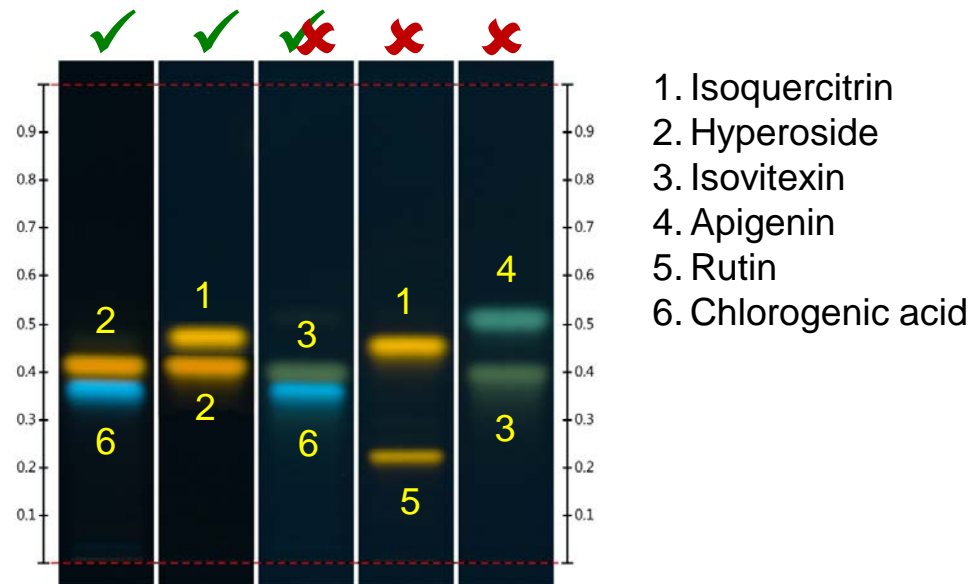
1. Isoquercitrin
2. Hyperoside
3. Isovitexin
4. Apigenin
5. Rutin
6. Chlorogenic acid

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HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

2. **Improvement of the description and interpretation of the chromatograms**

   ✓ **Sequence and characteristics of the zones**
   - ▸ Number
   - ▸ Position
   - ▸ Colour: encompassing description of zone colours
   - ▸ **Intensity: introduction of an intensity marker**

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Introduction of an intensity marker

Ph. Eur. 2.8.25

Example of intensity marker: chlorogenic acid (CA)

Apigenin-
7-O-glucoside

(a)   (b)
Chlorogenic acid

R   R¼

4-fold dilution

Visual intensity description
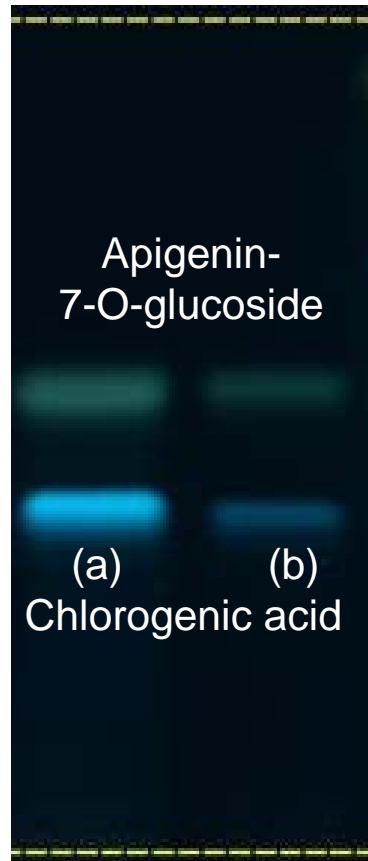
Intense zone:
More intense than CA zone intensity (a)

Zone with no descriptor for intensity:
Similar in intensity to CA zone intensity (a)

Faint zone:
Less intense than CA zone intensity (a) but equal to or more intense than CA zone intensity (b)

Very faint zone:
Less intense than CA zone intensity (b)

R and R¼: Reference solutions

HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

Roman chamomile flower

System-specific suitability test (SST)

Description table

Upper edge of plate	
	A greenish-blue fl zone (apigenin) A weak to equivalent brownish-yellow or orange fl zone
	Three light blue fl zones (upper two with a weak to equivalent intensity, the lowest usually intense)
_____	
Apigenin-7-glucoside : A greenish-blue fl zone	A equivalent to intense greenish-blue fl zone (apigenin-7-glucoside) A weak to equivalent brownish-yellow or orange fl zone
Chlorogenic acid: A light blue fl zone	A weak to equivalent light blue fl zone
_____	
Reference solution	**Test solution**

_____: Marks between upper, middle and lower third
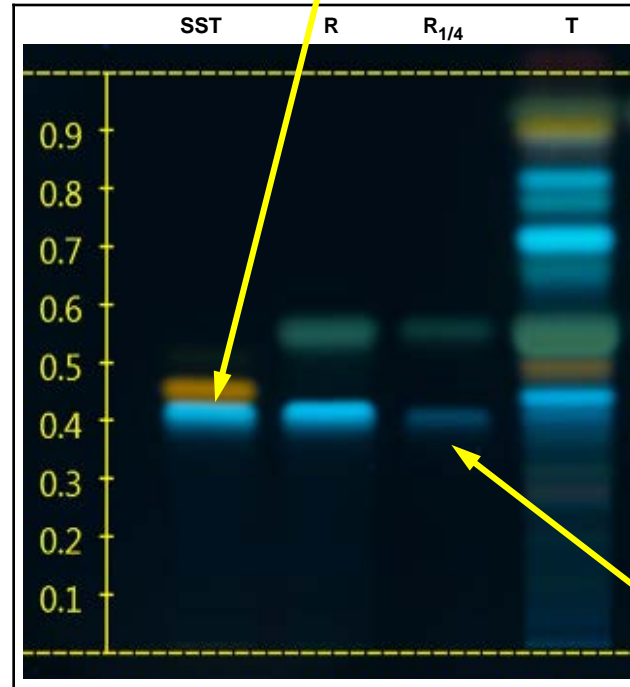
Typical chromatogram

Intensity marker

SST: Reference solution (c)
R: Reference solution (a)
R$_{1/4}$: Reference solution (b). R diluted with factor 4
T: Test solution (T1)

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HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

Example chromatograms of different batches

Roman chamomile flower

SST: Reference solution (c),
R: Reference solution (a),
R¼: Reference solution (b): R diluted with factor 4

T1-T5: Test solutions Chamomillae romanae flos
HPTLC-plate has been dipped for derivatization

HPTLC for quality control of herbals

Ph. Eur. improvements (chapter 2.8.25)

2. **Improvement of the description and interpretation of the chromatograms**

   ✓ Sequence and characteristics of the zones
   ✓ **Publication of colour pictures of chromatograms**
   ▸ Not in the Pharmacopeia itself but in the Knowledge database (available online for subscribers).
   ▸ Not mandatory, given only as information.
   ▸ Including several batches to show natural variability.

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HPTLC for quality control of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

Figure 0380.-2. – HPTLC chromatogram for identification test C of different batches of Roman chamomile flower

Publication of chromatograms in the ***Knowledge Data Base*** of the *European Pharmacopoeia*

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Polysaccharide containing herbals

HPTLC identification according to *Ph.Eur.* 2.8.25

Example: Acacia and Acacia spry/roller dried

Results

Top of the plate		
		[f] Three very faint blue zones
[a] Rhamnose: a greenish brown zone		[g] A very faint to equivalent greenish brown zone (rhamnose)
[b] Xylose: a brownish grey zone		
[c] Arabinose: a brownish grey zone		[h] An intense brownish grey zone (arabinose)
[d] Glucose: a grey-blue zone		
[e] Galactose: a grey-blue zone		[i] An intense grey-blue zone (galactose)
		[j] One or two equivalent to very faint brownish grey zones
		[k] One or two equivalent to faint blue zones
Reference solution (a)		Test solution

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Polysaccharide containing herbals

HPTLC identification according to *Ph.Eur. 2.8.25*

Example: Acacia and Acacia spry/roller dried

Batch chromatograms
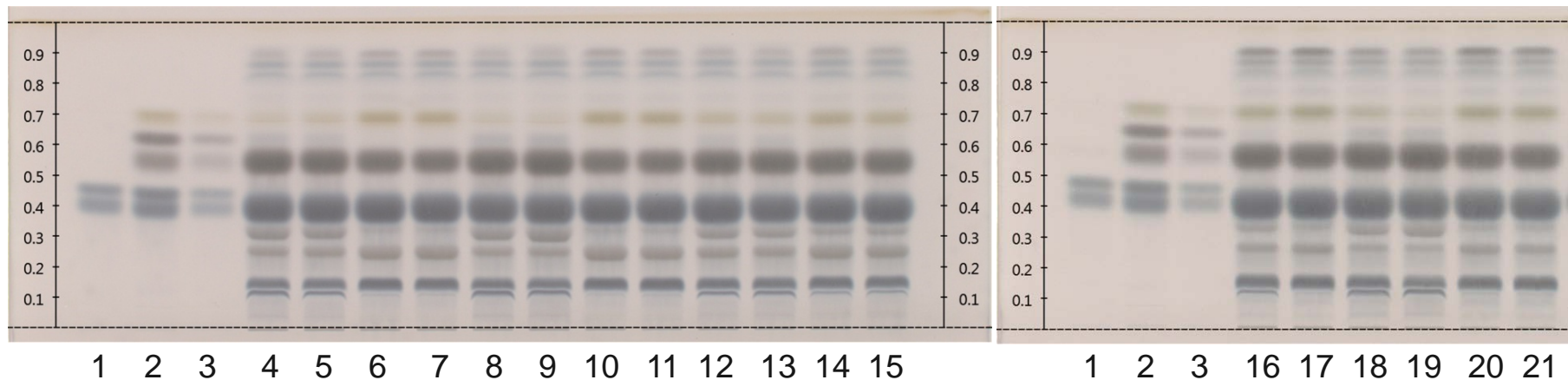
1: Reference solution (c) (SST)   **4-16:** Acacia, roller-dried
2: Reference solution (a) (R)   **17,18,21:** Acacia
3: Reference solution (b) (R/4)   **19,20:** Acacia, spry-dried

HPTLC fingerprint

The HPTLC **fingerprint** is the (electronic) image of the **visual** HPTLC - **chromatogram**

- ✓ Represents the **sample** (sample ID)

- ✓ Includes **application and front position**

- ✓ Consists of a **sequence** of (colored) zones

- ✓ May be a stack of multiple images in **different detection modes**

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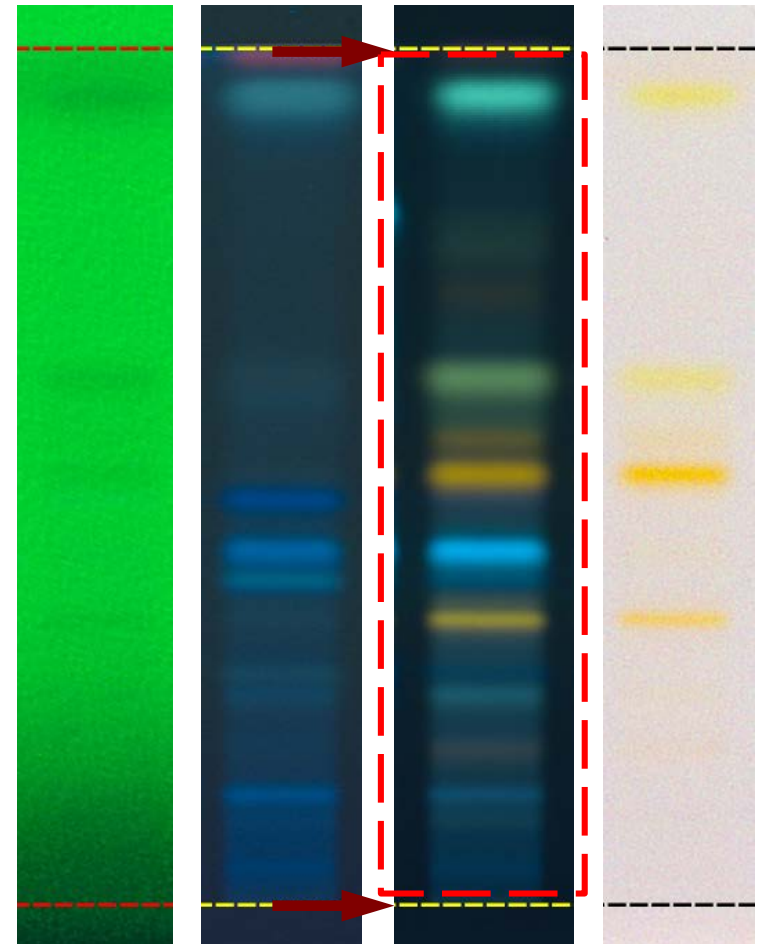
HPTLC fingerprint

2. The plate

The HPTLC fingerprint is part of the image taken of the entire HPTLC plate
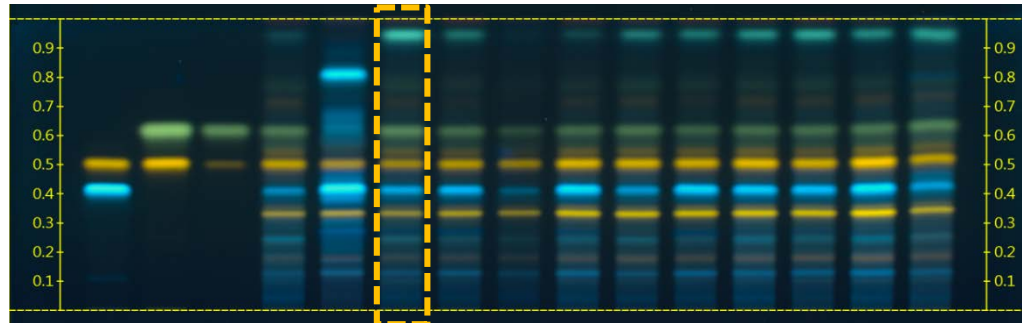
- ✓ More than one track: at least
  - – **System suitability test** (**SST**) (usually on first track)
  - – **Reference substances**
  - – **Sample**(s) Fingerprints of a number of samples may be on the same plate
- ✓ Clearly structured chromatograms, zones parallel and horizontal
- ✓ Through the image of the plate the fingerprint is linked to the plate identity (chromatographic conditions are recorded)

As long as the chromatographic conditions are the same and the SST is passed, fingerprints from different plates can be compared!

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HPTLC fingerprint

Image normalization based on SST
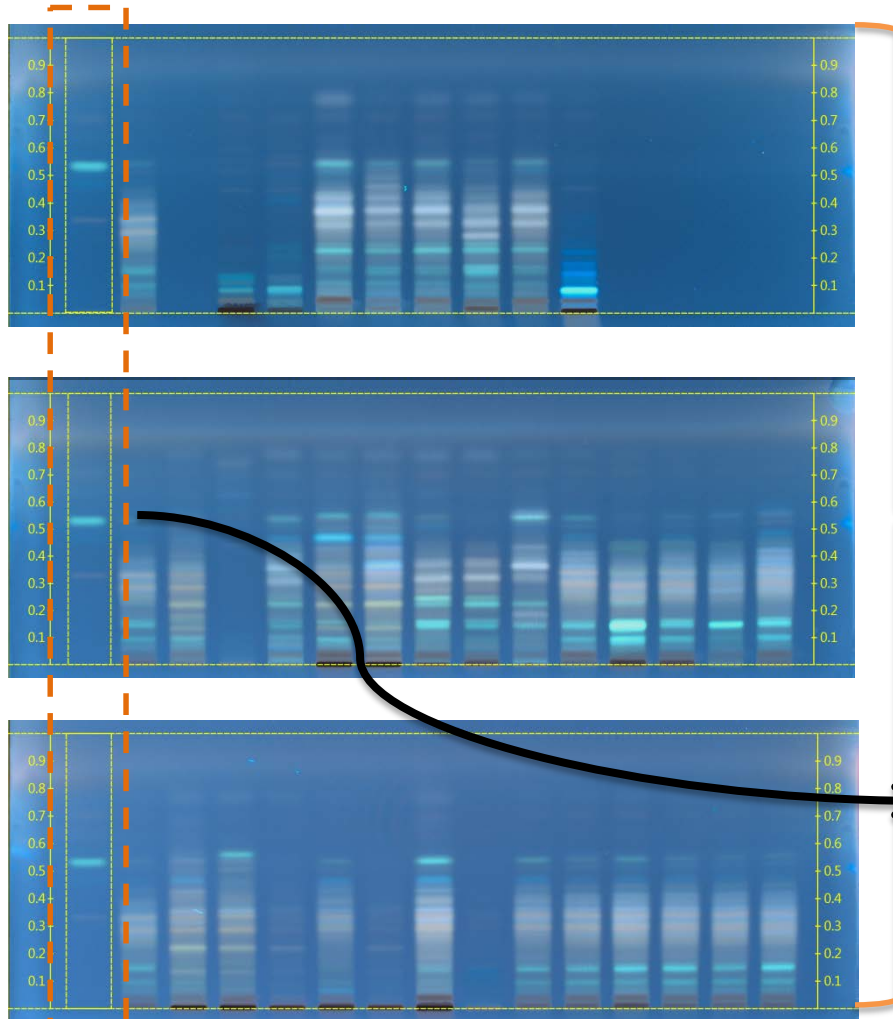
Comparing chromatograms from 3 different plates

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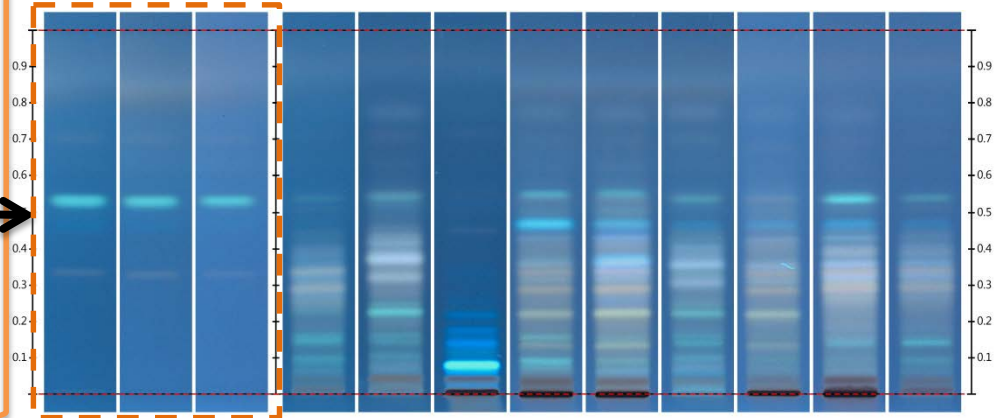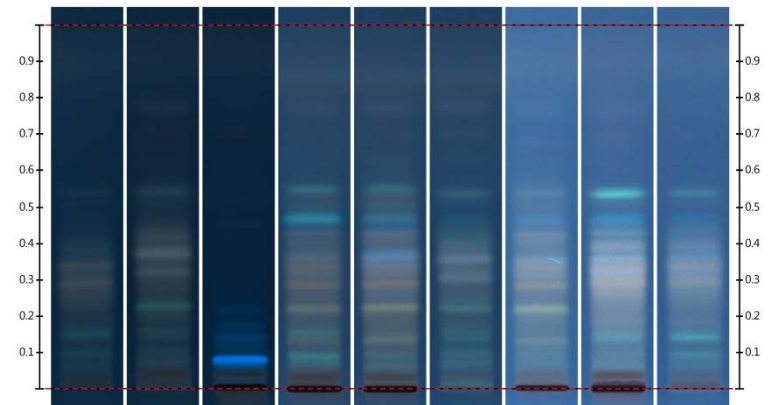
HPTLC fingerprint

HPTLC chromatograms can also be presented as profile

- Information from **scanning densitometry**
  - 200 nm to 800 nm
  - Absorption and/or fluorescence measurement

Black cohosh

200 nm
250 nm
300 nm
350 nm
366 nm
400 nm
450 nm
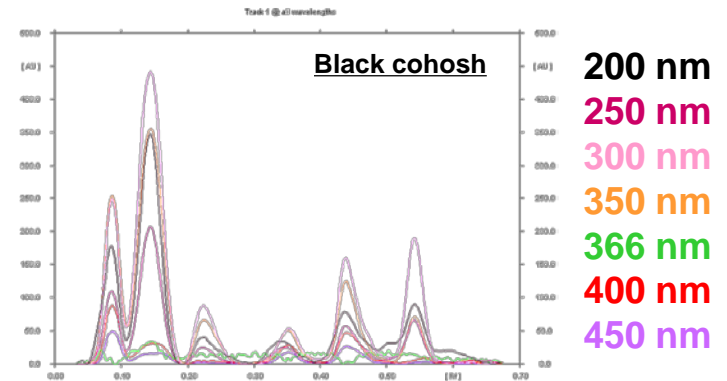
- Information from **image raw data**
  - UV 254 nm, UV 366 nm, (white light) prior to derivatization
  - (UV 254 nm), UV 366 nm, white light after derivatization

Black cohosh

Red = UV 254
Green = UV 366 nm
Brown = derivatized white light
Blue = derivatized UV 366nm

Fingerprints   Profiles obtained from images

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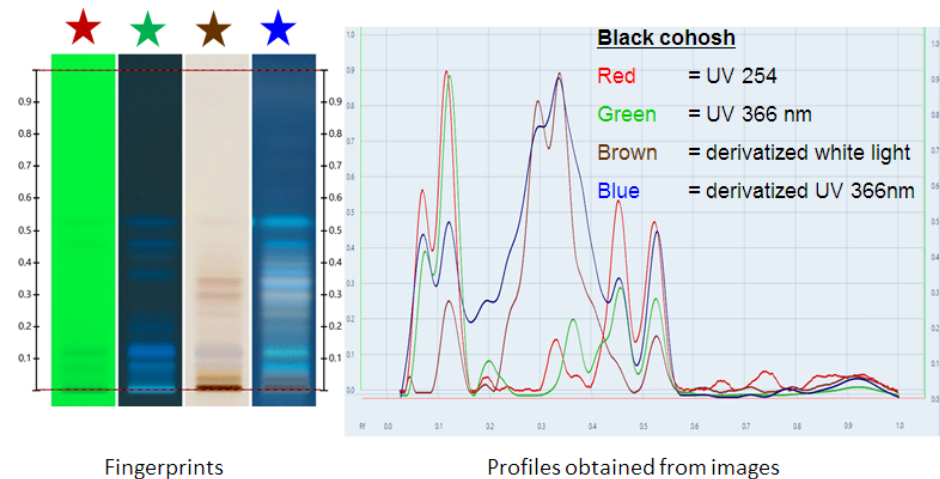
HPTLC fingerprint

Converting images into profiles

R: 223;
G: 160;
B: 003;

Pixels

Application zone

5 % below the application zone and
5% above the solvent front

Calculated on an aliquot
of the track (50 %)

For each R_F value the mean
luminance of the pixels (red, green,
blue) in the zone is calculated
$Y = 1/3\ R + 1/3\ G + 1/3\ B$

Plotting luminance as
function of R_F generates
a profile

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HPTLC fingerprint

4. Quantitative information
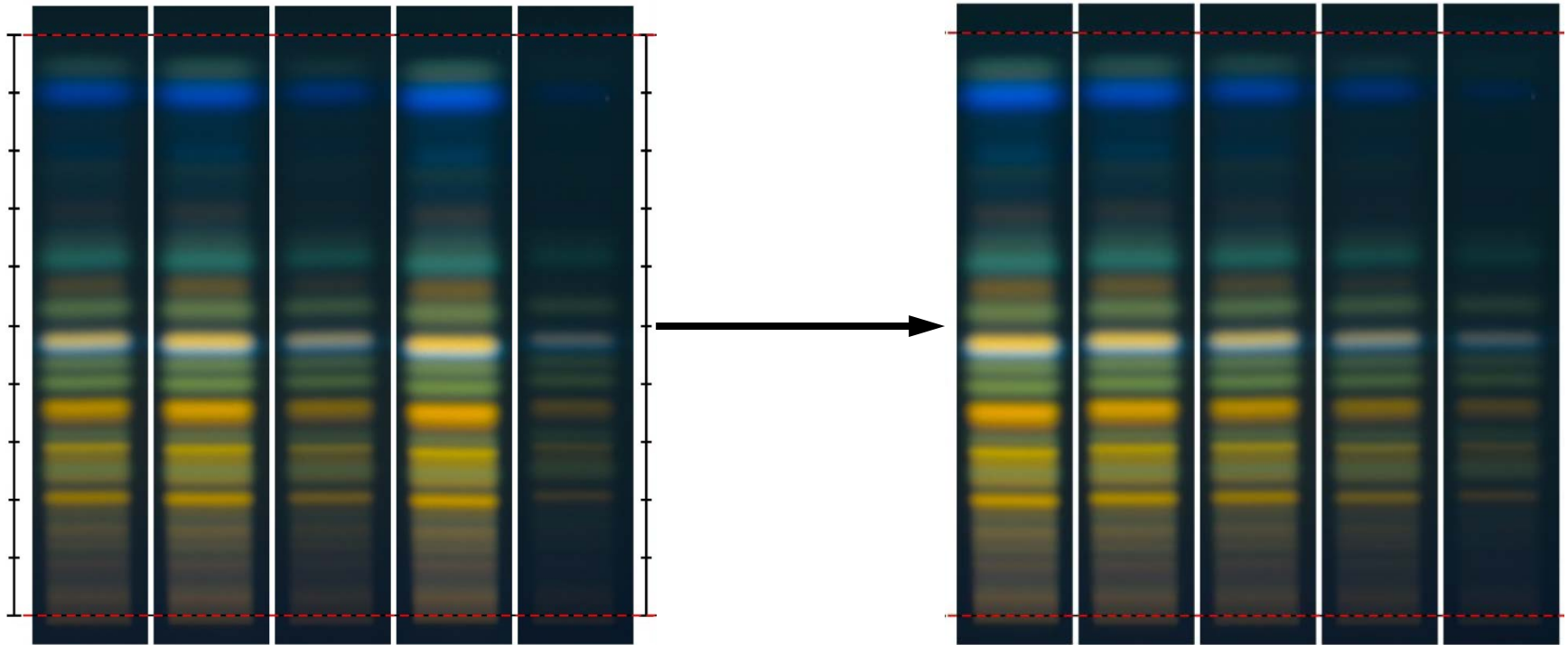
HPTLC fingerprint

Ginkgo biloba profile

Area

Regression mode: Polynomial

Calibration function:

$$y = -4.503 \times 10^{-14} x^2 + 4.080 \times 10^{-8} x - 6.787 \times 10^{-4}$$

Standard deviation of the calibration functic   Correlation coefficient:

$\sigma = 2.1512\%$ $R = 0.999395$
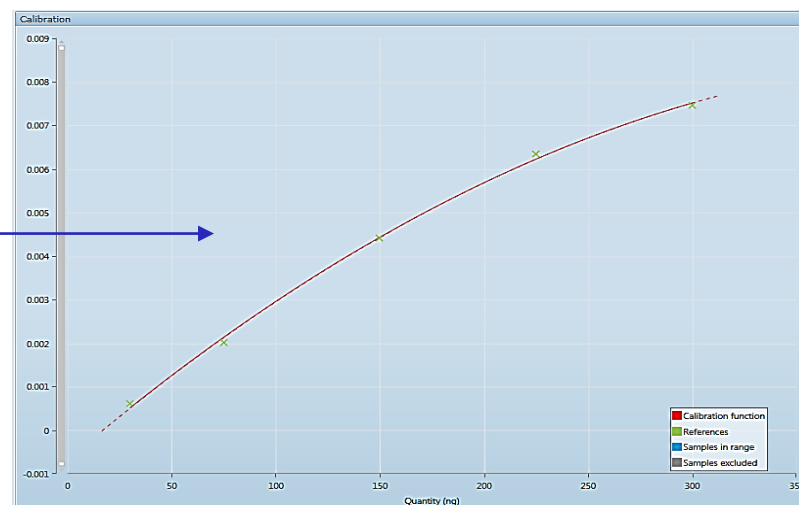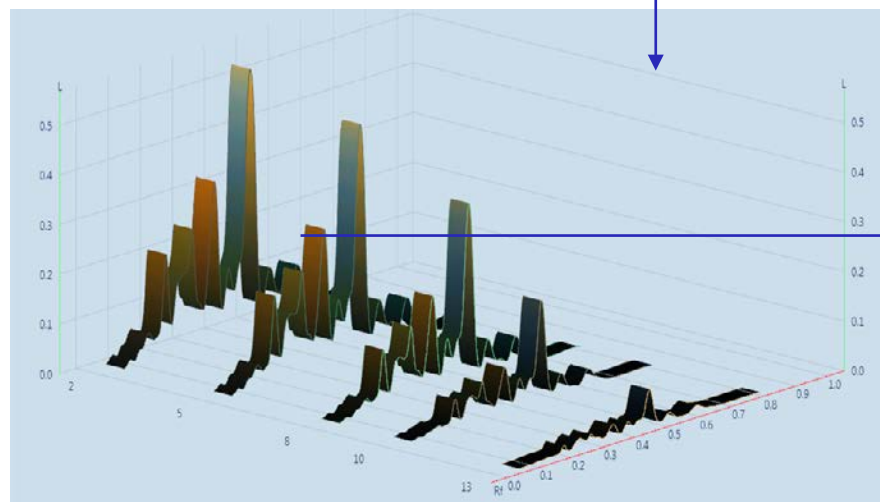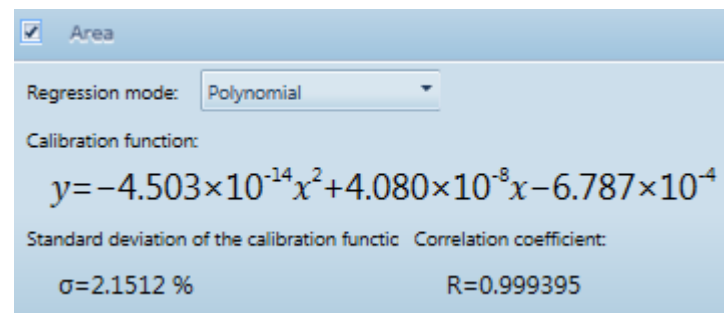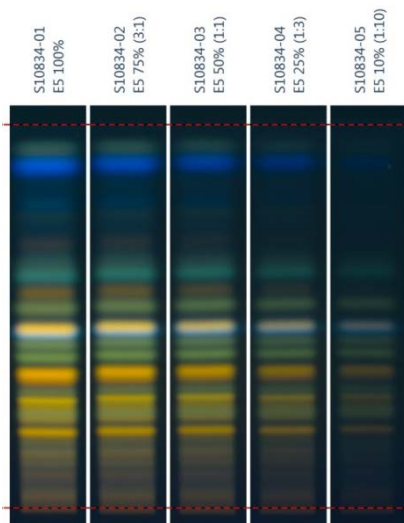
Comprehensive HPTLC profiling

What it means?

HPTLC fingerprints (images), which are used for identification, are converted into peak profiles and the intensities of selected zones are quantitatively compared to those of the corresponding zones of the reference material

A single analysis	➡	More information

HPTLC for ID

- ✓ Identification
- ✓ Purity
- ✓ Content

Arruda Frommenwiler D, Kim J, Yook CS, Trang Tran TT, Cañigueral S, Reich E (2018) Planta Medica (e-first / in press)

Two examples

Not related to the *European Pharmacopoeia*
- ✓ Ginkgo products
- ✓ Angelica gigas root

Ginkgo products

Dietary supplements from UK market

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)

Unitat de Farmacologia, Farmacognòsia i Terapèutica   -   Facultat de Farmàcia i Ciències de l'Alimentació - S. Cañigueral
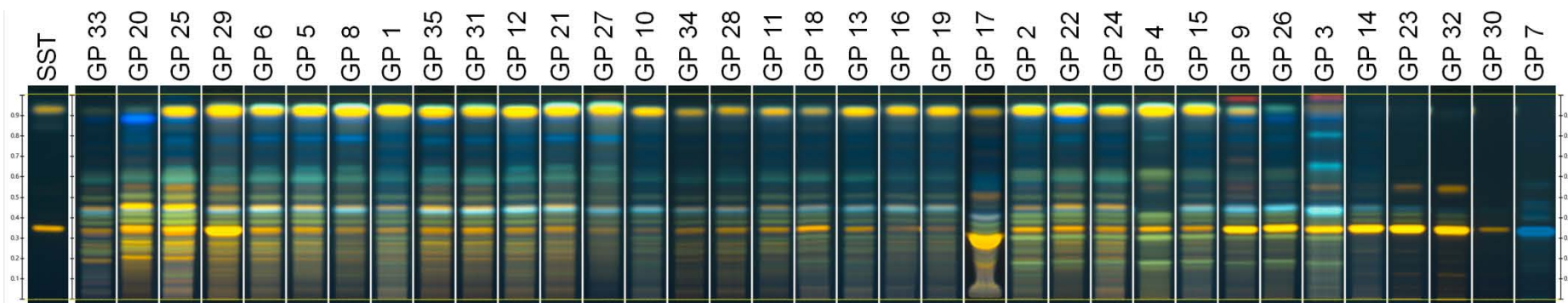
Ginkgo products

Dietary supplements from UK market
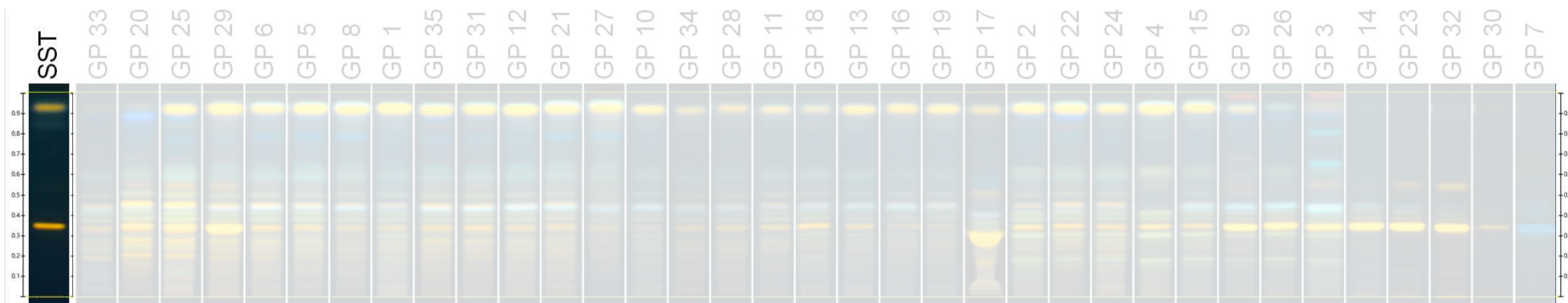
From the 35 products analysed:

- 2 Products comply with the required fingerprint (according to Pharmacopoeia)
- 2 Products show similar profile to the required, but a strong quercetin zone
- 17 Products have a weak fingerprint but strong zone due to quercetin
- 1 Product show strong zones due to quercetin and rutin
- 8 Products show an additional green zone / 6 of them have a high content of quercetin
- 4 Products show only a zone due to rutin
- 1 Product is not ginkgo

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)

Dietary supplements from UK market

Possible adulterants

- Rutin
- Quercetin
- Extracts from *Sophora japonica* flower
- Extracts from *Sophora japonica fruit*
- Extracts from buckwheat leaf

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)

Ginkgo dry extract purified

USP

Chemical identification

- HPTLC of flavonoids
- HPLC of flavonoids (from assay)

Assay

- Flavonoid glycosides by HPLC
- Terpene lactones by HPLC

Limit tests

- Quercetin ($\leq$ 0.5%) and rutin ($\leq$ 4%) by HPLC
- Ginkgolic acids by HPLC

Ginkgo products

HPTLC limit test for rutin

Flavonoid fingerprint under UV 254 nm is suitable

Rutin standard at concentration equivalent to 4%

27 Samples that are within the acceptance criterion (rutin ≤ 4%)

8 Samples that are above the acceptance criterion

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)
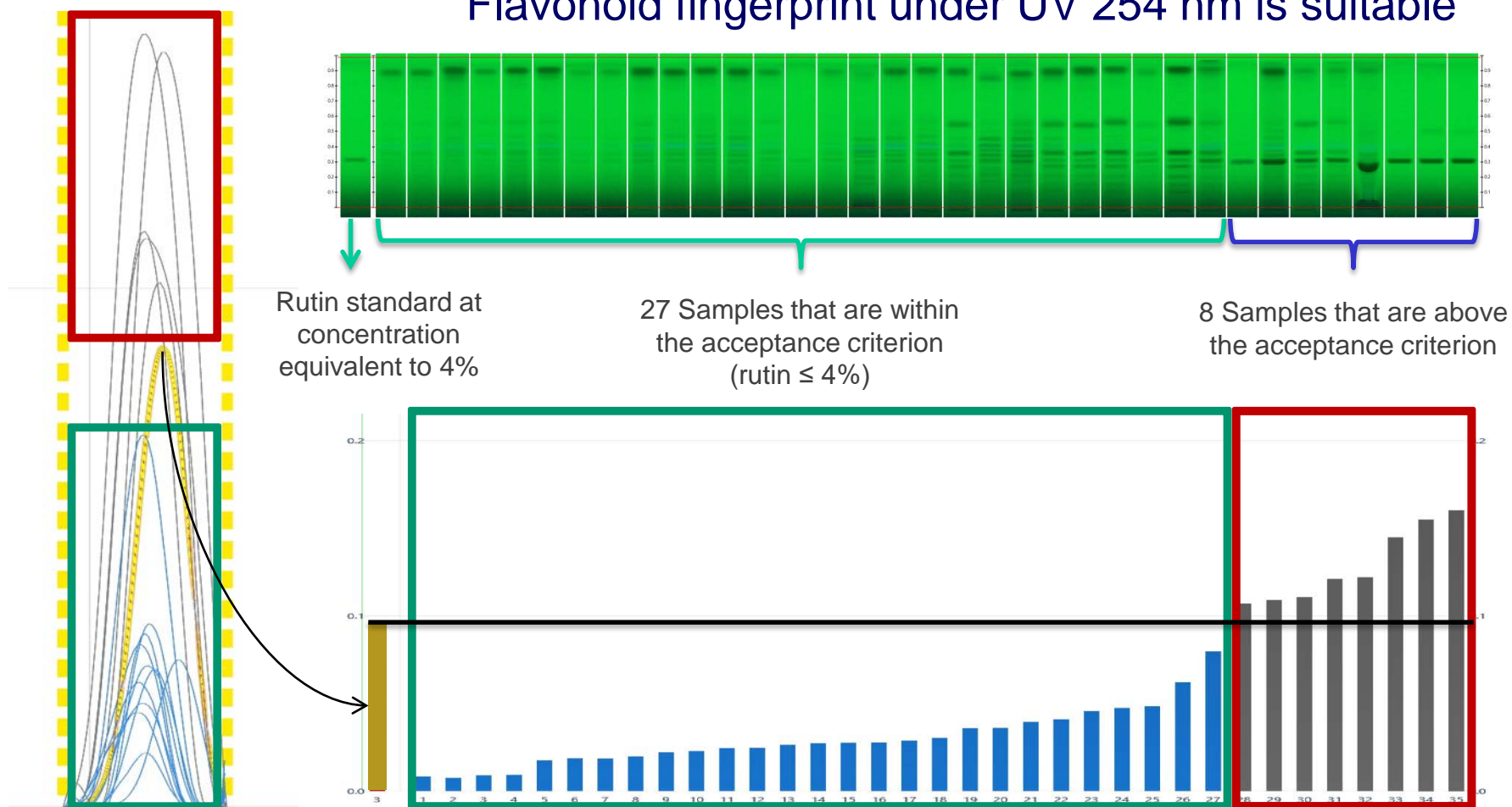
HPTLC limit test for quercetin

Flavonoid fingerprint (UV 365 after derivatisation) is suitable

9 samples are within
the acceptance criterion
(quercetin ≤ 0,5%)

26 samples are above the acceptance criterion

Sample containing
0.5% of quercetin
determined against
standard

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)

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Ginkgo products

HPTLC comprehensive profiling

A single identification analysis (HPTLC of flavonoids)

- ✓ Allows identification

- ✓ Allows the detection of adulterations and identify the adulterant.

- ✓ Avoids one HPLC analysis prescribed by the USP for limits of quercetin and rutin

Frommenwiler DA, Booker A, Heinrich M, Reich E, Cañigueral S (2017) GA Congress (Basel, Switzerland)

Comprehensive HPTLC fingerprinting

Angelica gigas root

✓ The roots of many *Angelica* species have a long use as traditional medicine.

✓ Some are traded in East Asian herbal markets under the same common name "Dang gui":
  – *Angelica sinensis* (Oliv.) Diels, used in China
  – *Angelica acutiloba* (Siebold & Zucc.) Kitag., used in Japan
  – *Angelica gigas* Nakai, used in Republic of Korea

Can ***comprehensive HPTLC fingerprinting*** simplify quality control *A. gigas* root, giving information on identity, purity and content?

FHH — Forum for the Harmonization of Herbal Medicines

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Identification test

28 *Angelica* species and related can be distinguished with this method
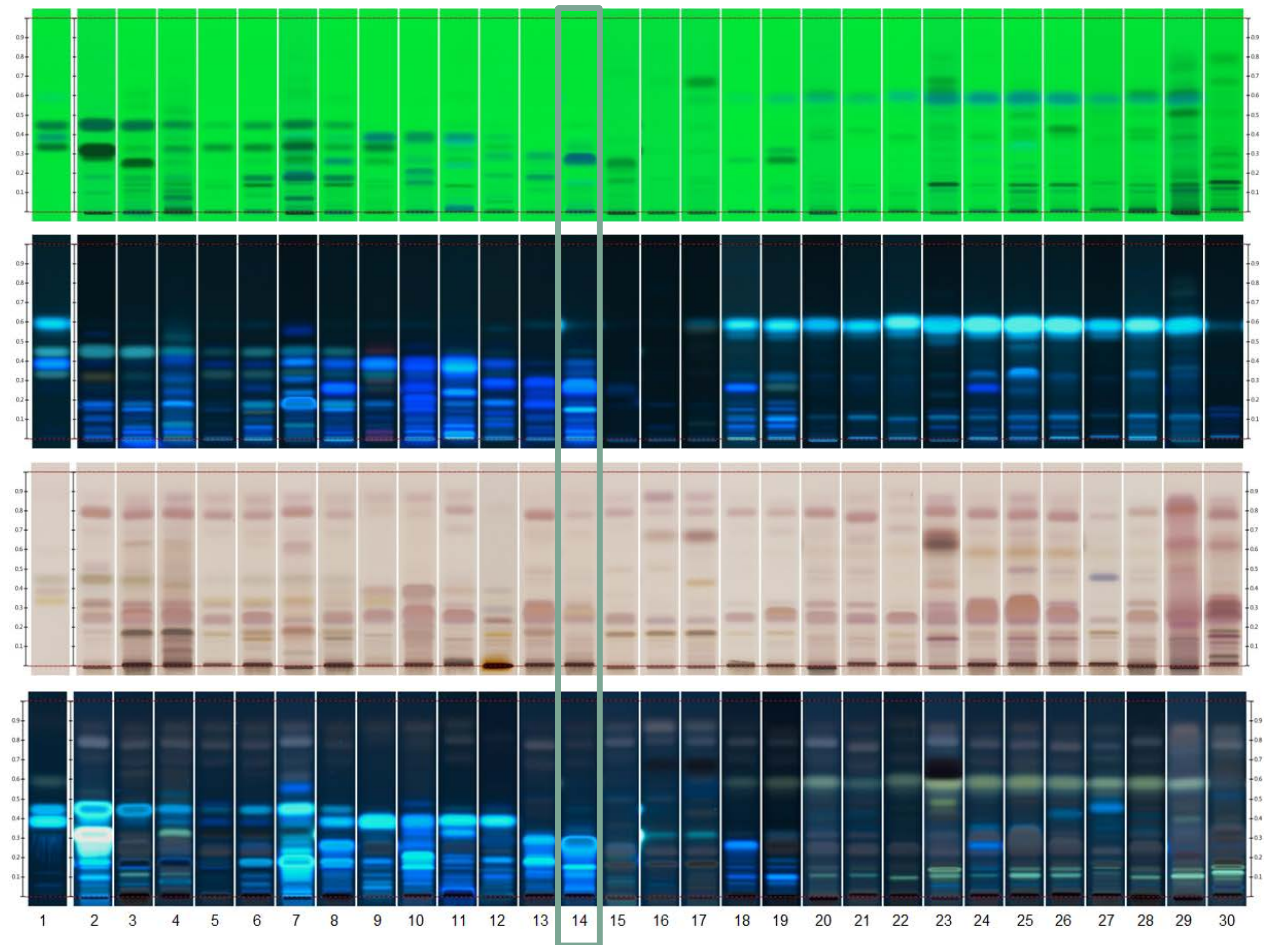
Angelica gigas

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Purity test

Can mixtures with other species be detect?
Test performed with the 3 main *Angelica* species

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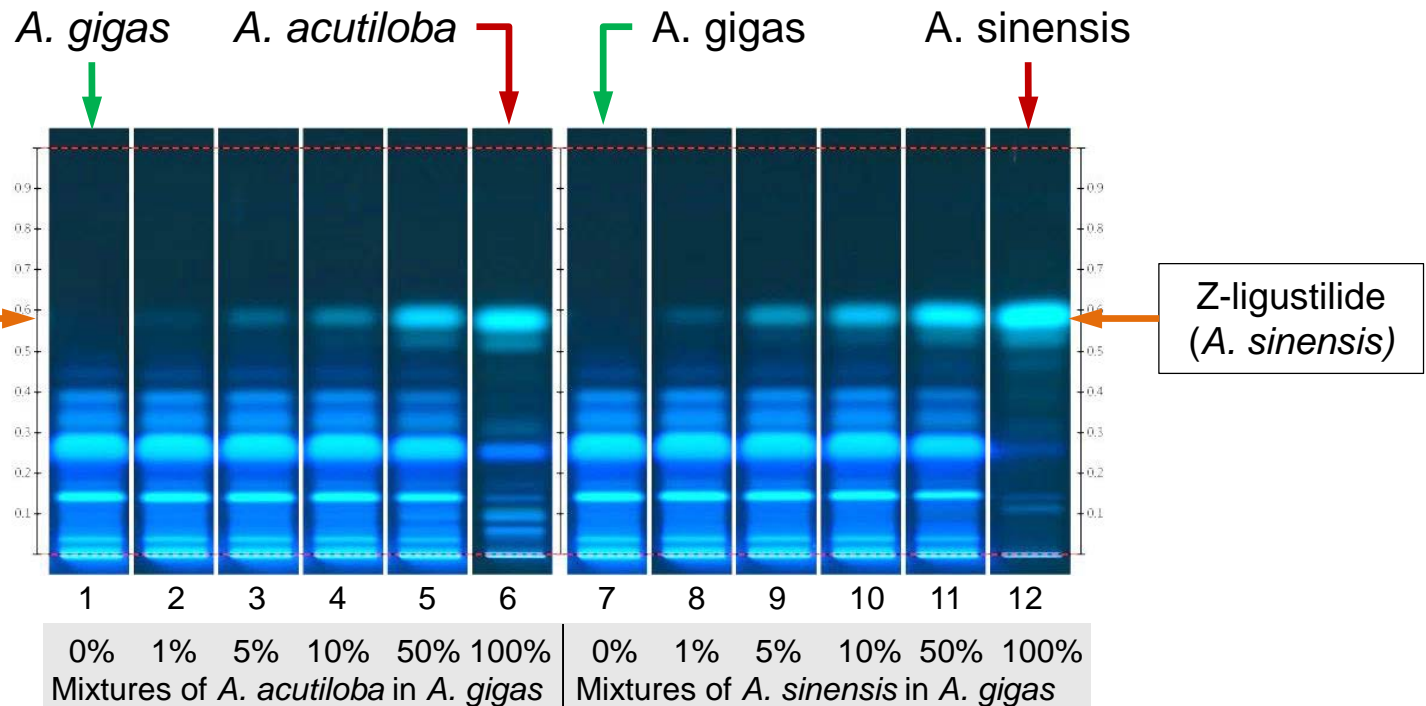
Comprehensive HPTLC fingerprinting

Angelica gigas root

Conversion of electronic images into "Peak profile from image" (PPI)

$$L = \left(\frac{1}{3}R\right) + \left(\frac{1}{3}G\right) + \left(\frac{1}{3}B\right)$$
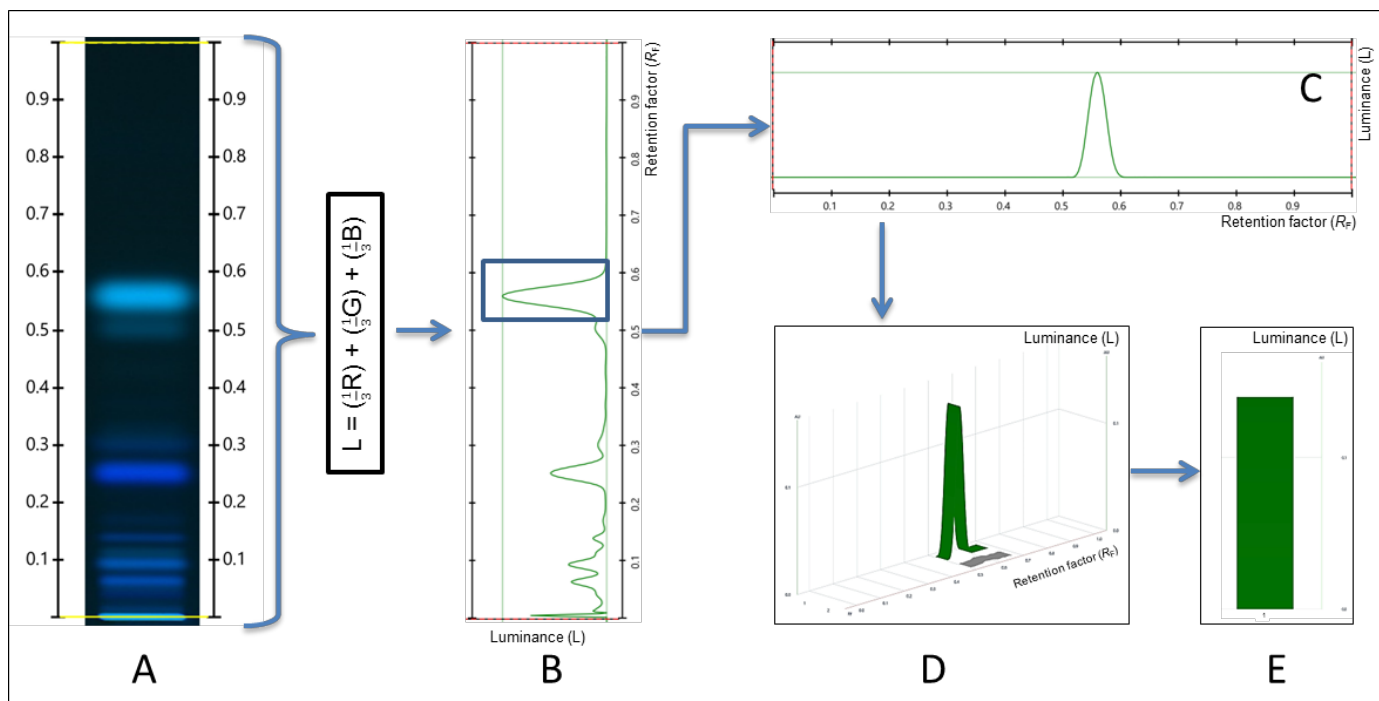
A. Fingerprint (image) under UV 366 nm prior to derivatization.
B. Fingerprint converted into PPI.
C. Peak due to Z-ligustilide after adjusting the integration range to exclude other peaks.

D. Isometric view of the peak due to Z-ligustilide in the PPI.
E. Bar graph representing the peak height due to Z-ligustilide.

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Purity test

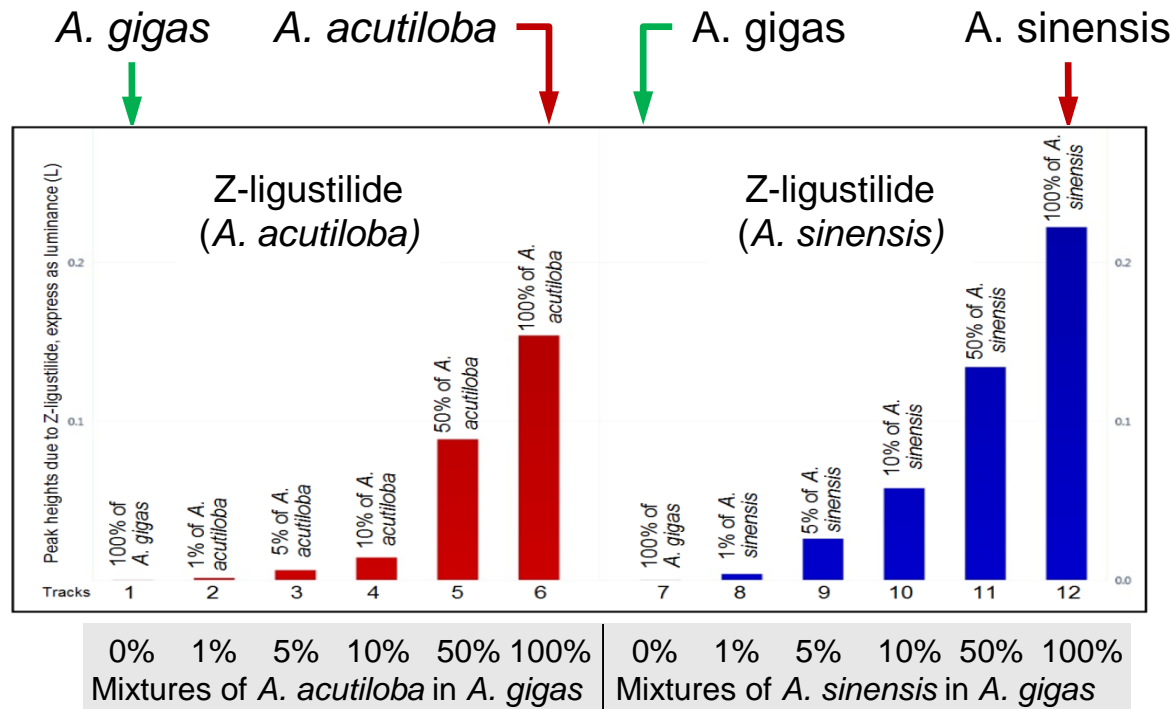**Can mixtures with other species be detect?
Test performed with the 3 main *Angelica* species**

A. gigas   A. acutiloba   A. gigas   A. sinensis

Z-ligustilide (*A. acutiloba*)   Z-ligustilide (*A. sinensis*)

Peak heights due to Z-ligustilide, express as luminance (L)

Tracks 1 2 3 4 5 6   7 8 9 10 11 12

100% of A. gigas | 1% of A. acutiloba | 5% of A. acutiloba | 10% of A. acutiloba | 50% of A. acutiloba | 100% of A. acutiloba | 100% of A. gigas | 1% of A. sinensis | 5% of A. sinensis | 10% of A. sinensis | 50% of A. sinensis | 100% of A. sinensis

0%   1%   5%   10%   50%   100%   0%   1%   5%   10%   50%   100%
Mixtures of *A. acutiloba* in *A. gigas* | Mixtures of *A. sinensis* in *A. gigas*

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Minimum content

Selection of analytical marker(s)
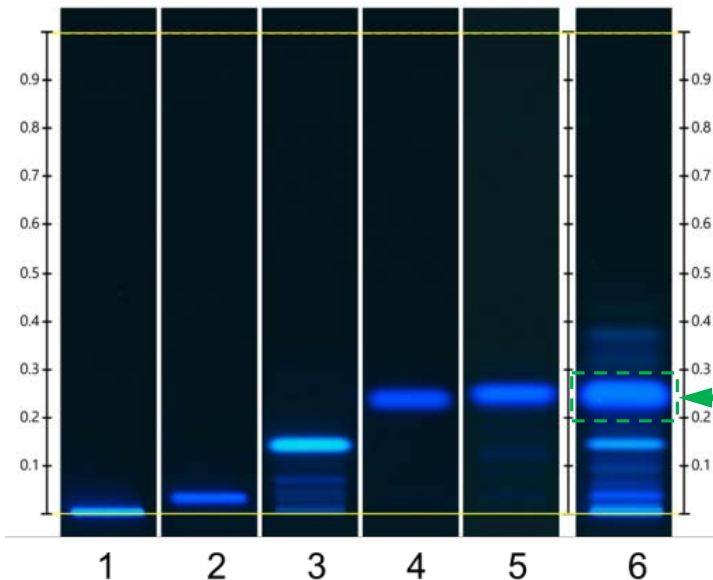
Identification of zones in the fingerprint of the root of *Angelica gigas*:

1: Nodakenin
2: Decursinol
3: 7-Demethylsubero-
   rosine (RF 0.15)

4: Decursin
5: Decursinol angelate
6: *Angelica gigas* root

← Decursin + Decursinol angelate

Detection: UV 366 nm prior derivatization
Concentration of reference substances:
1 mg/mL in methanol

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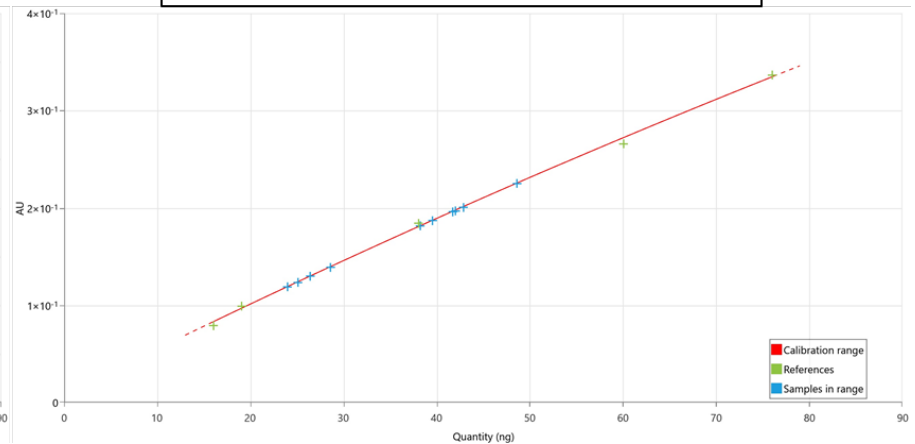# Comprehensive HPTLC fingerprinting

Angelica gigas root

Minimum content

Calibration curves for decursin

Calibration curve based on PPSD area (linear function)
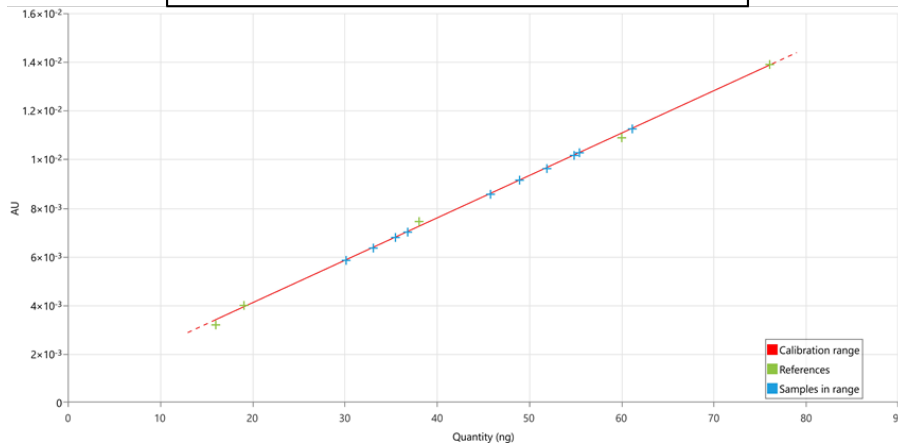

Calibration curve based on PPI height (polynomial function)

Peak profile from scan densitometry (PPSD)

Peak profile from image (PPI)

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Test for the minimum content of decursin (D) plus decursinol angelate (DA) in *A. gigas* samples for HRM.
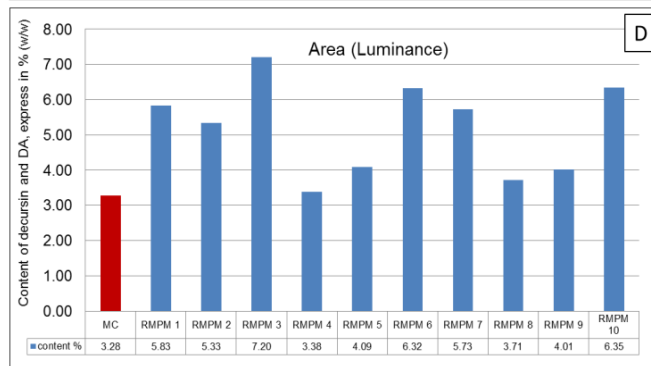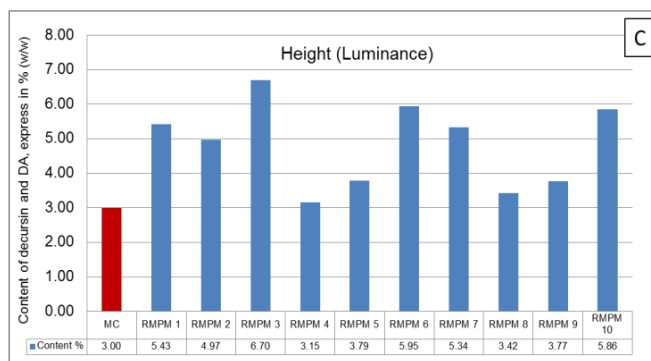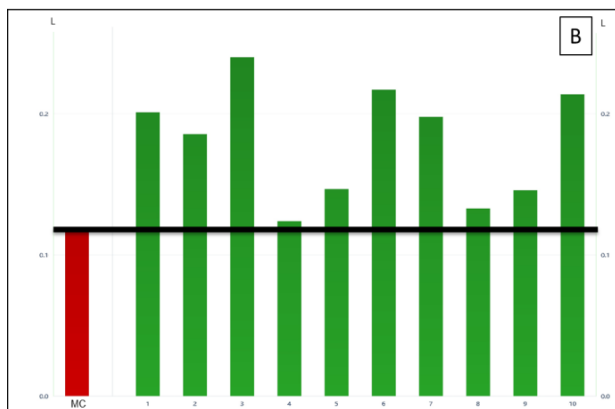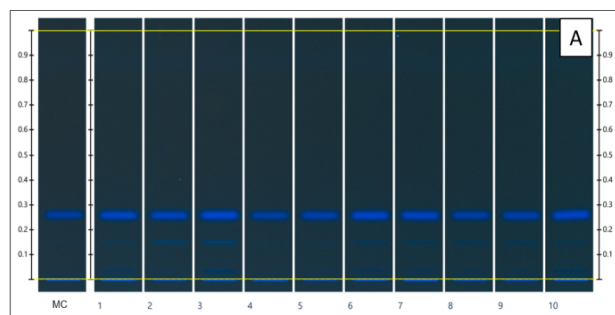
A: Visual comparison of the fingerprints.

B: Visual evaluation based on PPI.

C: Content (%) of D+DA calculated on PPI via height

D: Content (%) of D+DA calculated on PPI via area

MC: Minimum content (reference solution HRM-4)

Tracks 1–10: *A. gigas* root HRMs 1-10.

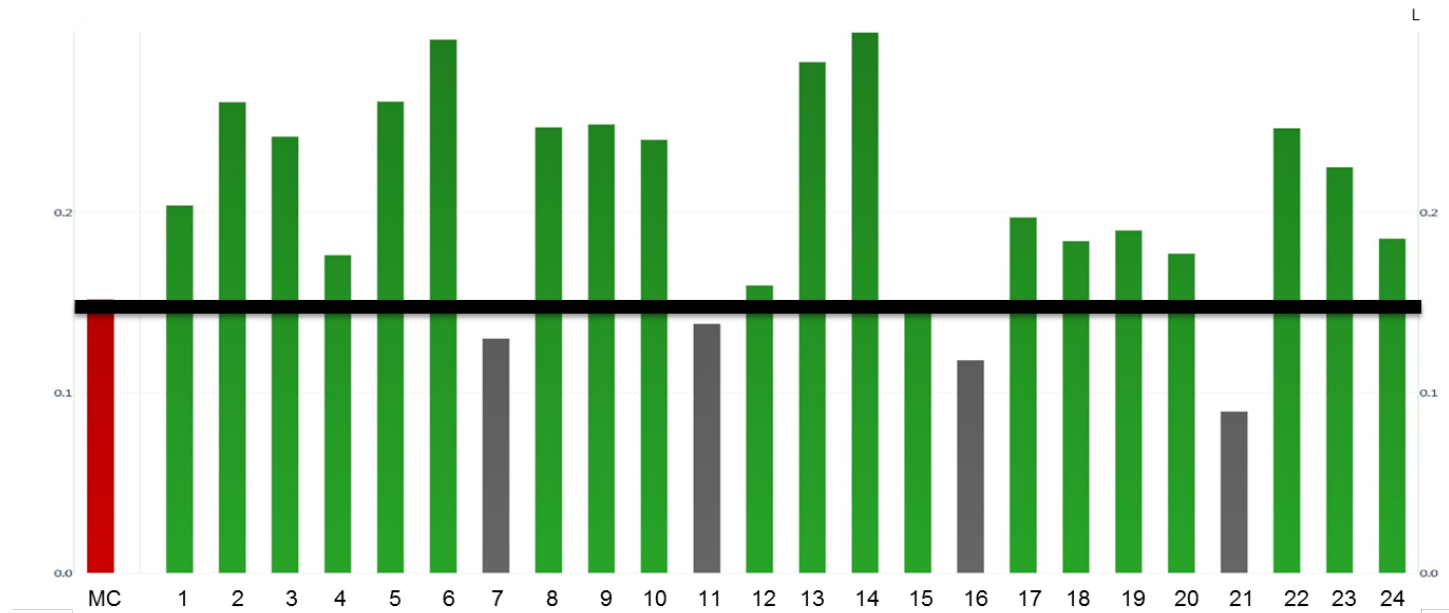Minimum content of D+DA expressed as D: **3.0%**

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Comprehensive HPTLC fingerprinting

Angelica gigas root

Minimum content
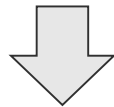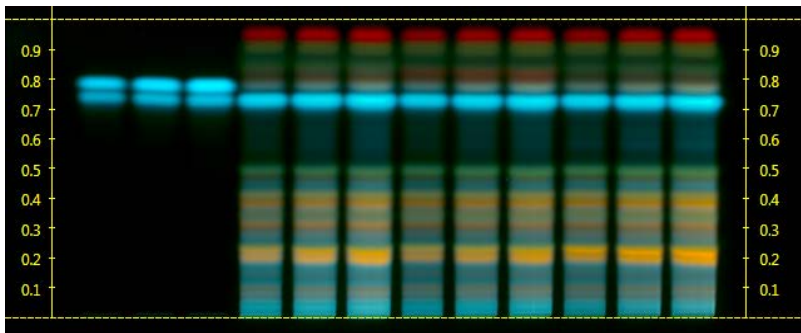
Visual test for minimum content of D+DA based on PPI

MC: Minimum content (reference solution HRM-4)
1-24: Angelica gigas root samples

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Quality of herbal drugs and herbal preparations

Objectives

Quality control

HPTLC

| Identity |

| Purity Contaminants |

| Quantitative determination of active principles and markers |

Stability

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Merci beaucoup pour votre attention

Le concept d'empreinte «fingerprint» basé sur l'HPTLC pour le control de qualité des drogues et préparations végétales dans la Pharmacopée Européenne

Salvador Cañigueral and **Roser Vila**
Unitat de Farmacologia, Farmacognòsia i Terapèutica
Facultat de Farmàcia i Ciències de l'Alimentació

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