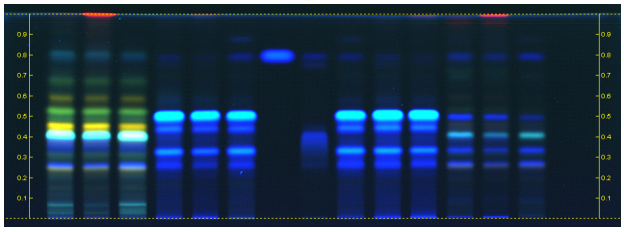
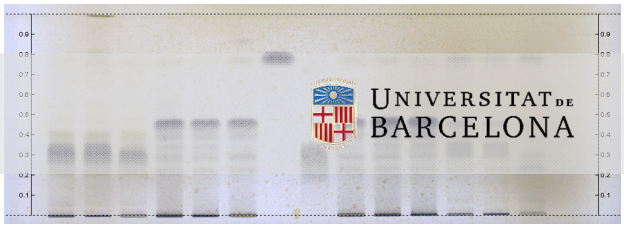
Séminaire Cub CCM
Université d'Avignon et des Pays du Vaucluse
Avignon, 12-13 Avril 2017





L'HPTLC dans le control de qua-lité des produits de plantes dans la Pharmacopée Européenne

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Unitat de Farmacologia, Farmacognòsia i Terapèutica
Facultat de Farmàcia i Ciències de l'Alimentació

L'HPTLC dans le control de qualité des produits de plantes dans la Pharmacopée Européenne


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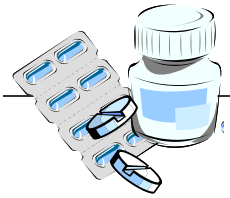
HPTLC in the quality control of herbal drugs and herbal preparations

Content

1. Herbal medicinal products (HMP)
2. Quality of herbal medicinal products
3. European Pharmacopoeia monographs
4. TLC/HPTLC in quality control of herbal products
5. The issues and the improvements: Chapter *2.8.25* of the *Ph. Eur.*
6. Development of an HPTLC method
7. Future prospects



Herbal medicinal products

Active substance + **Dosage form**

Herbal preparations

- ✓Herbal drugs
- ✓Extracts
- ✓Essential oils
- ✓Etc...

Complexity !

- ✓Tablets, pills
- ✓Capsules
- ✓Drops, syrups
- ✓Creams, ointments
- ✓Suppositories
- ✓Tee preparations
- ✓Soluble tee preparations

Etc.

Herbal drug

European Pharmacopoeia

Herbal drugs are mainly whole, fragmented or cut, plants, parts of plants, algae, fungi, lichen in an unprocessed state, usually in dried form but sometimes fresh.

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)

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

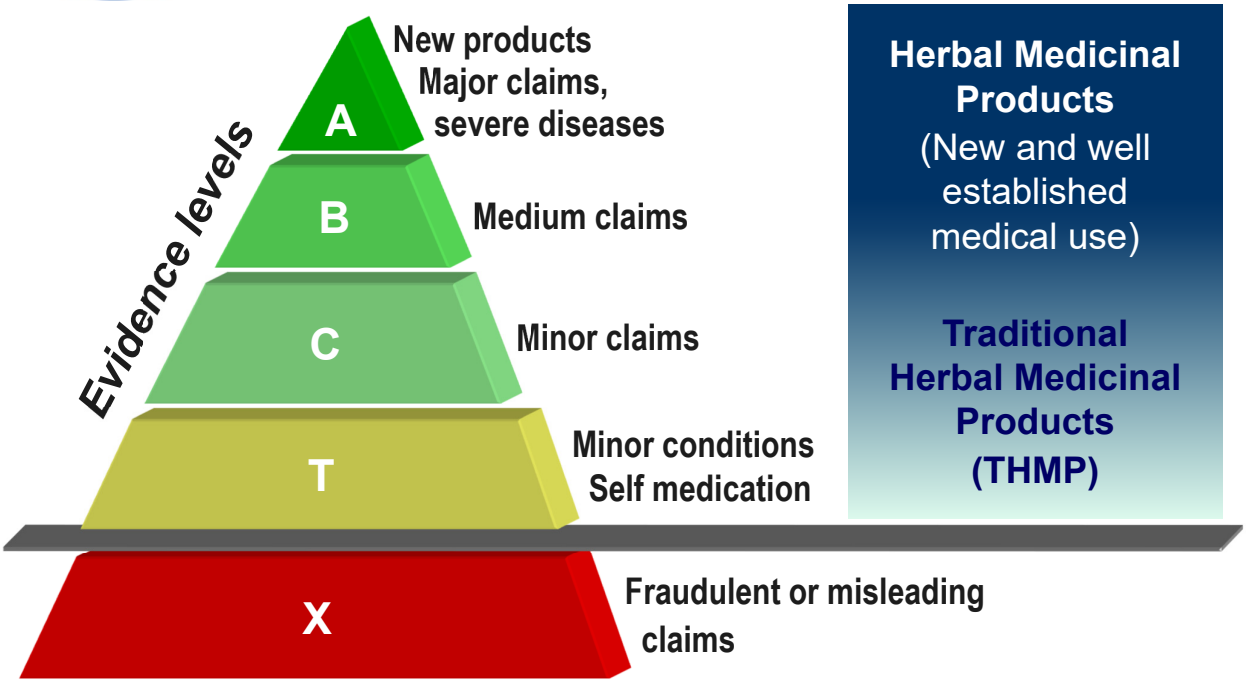
= **Herbal preparations** (Directive 2004/24/CE)

Herbal medicinal products

Types

- **Herbal medicinal products**
  - ✓ **New**
  - ✓ Based on **well established medical use** (WEU)
- **Traditional herbal medicinal products (THMP)**

Evidence levels

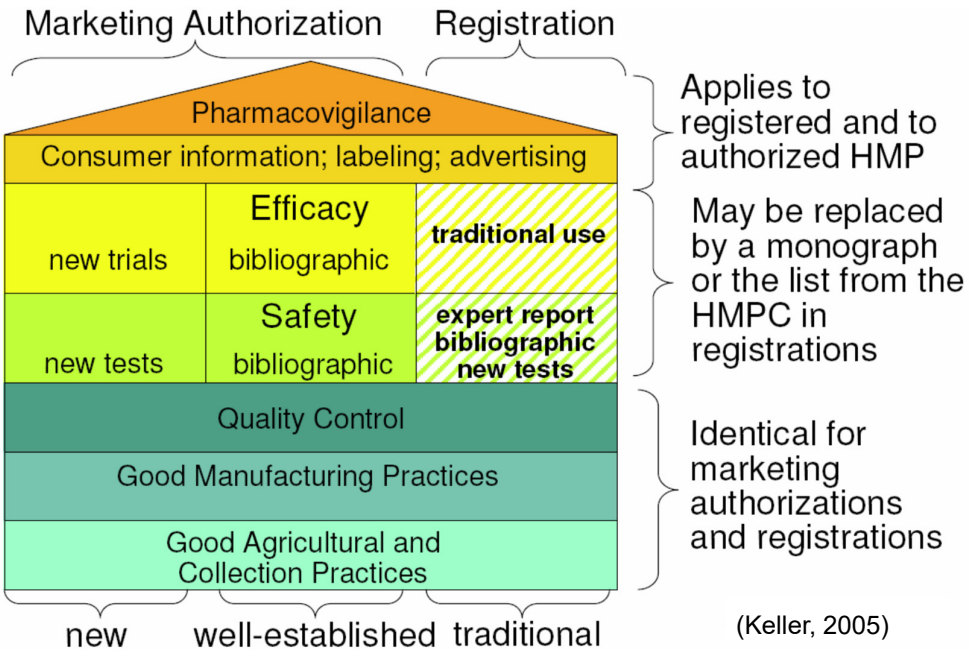


Herbal Medicinal Products
(New and well established medical use)

Traditional Herbal Medicinal Products (THMP)

Requirements for HMP

EU regulations



(Keller, 2005)

Herbal medicinal products

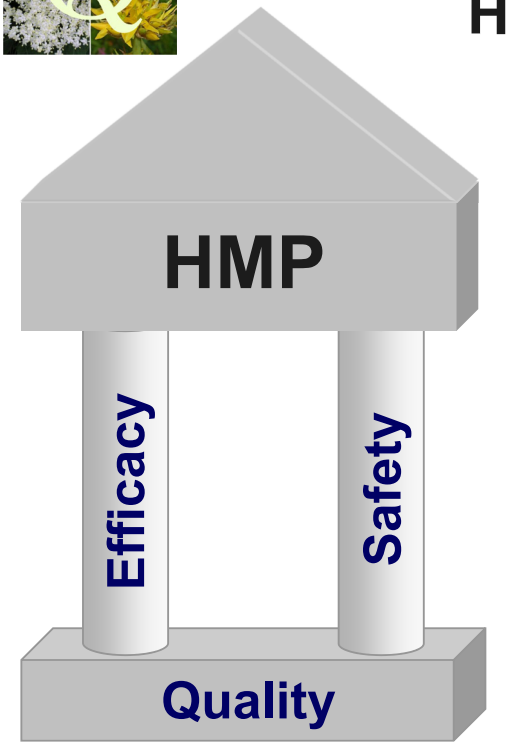
Quality requirements

Directive 2004/24/EC

*"The **quality** aspect of the medicinal product is **independent of** its **traditional use** so that **no derogation** should be made **with regard to the necessary physicochemical, biological and microbiological tests**."*

Quality

Herbal Medicinal Products (HMP)



(Based on Bauer, 1998)

Quality is the basis for the reproducibility of **safety** and **efficacy**.

Herbal medicinal products

Production

Medicinal plant

↓ Harvest and post-harvest processing

Herbal drug

↓ Milling / extraction

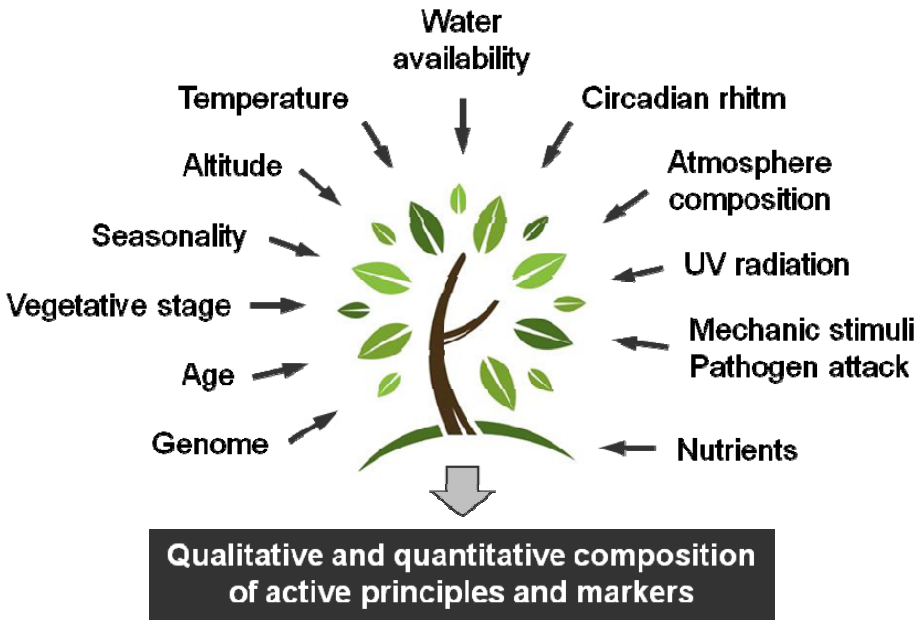
Herbal preparation

↓ Formulation

Herbal medicinal product

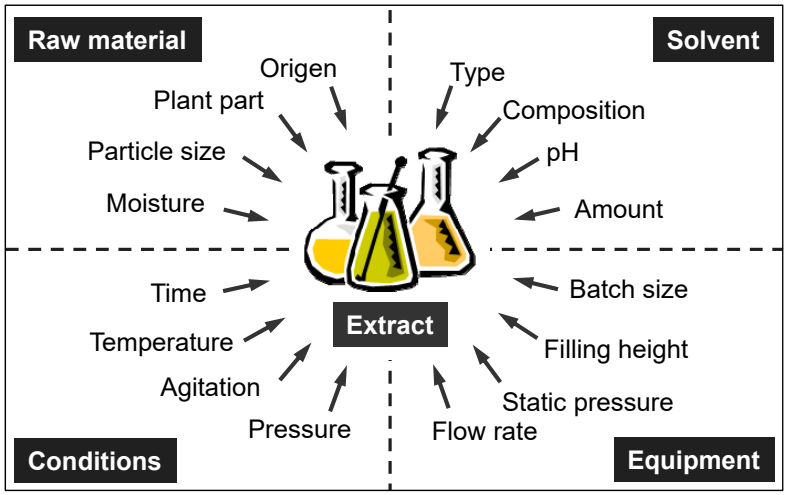
Variability of plant material

Factors



Extraction with solvents

Factors to consider



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.)

Herbal medicinal products

Overcoming the difficulties

Seeking the global quality

- ✓ Medicinal plant
- ✓ Herbal drug
- ✓ Herbal preparation (extract, ...)
- ✓ Production processes
- ✓ Traceability
- ✓ Medicinal product

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 quantity of active principles or markers is within the accepted limits.

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

Declaration of herbal substances and preparations in HMP and THMP

"... the herbal drug or herbal drug preparation in its **entirety** is regarded as the active substance.."

Data given depend on if they are standardised, quantified or other herbal substance or herbal preparation

Medicinal products

Quality specifications and analytical methods

Directive 2004/24/EC

*"**Products should comply** with quality standards in relevant **European Pharmacopoeia monographs or** those in the **pharmacopoeia of a Member State**."*

Pharmacopoeia

What is it?

A Pharmacopeia is a **regulatory document**, mandatory in a specific geographical area, on the **quality requirements of medicinal products**.

It mainly contains the **specifications**, which include **analytical methods and acceptation criteria**, for active substances, excipients and dosage forms.

Not in a Pharmacopoeia?

If **no monograph is given in a Pharmacopoeia** … "a comprehensive specification for the herbal substance must be supplied and should be set out in the same way where practicable, as the monographs on herbal substance in the European Pharmacopoeia."

Analytical procedures not given in a Pharmacopoeia should be validated in accordance with the corresponding ICH guideline.

Herbal medicinal products

Production

Medicinal plant

↓ Harvest and post-harvest processing

→ Herbal drug

↓ Milling / extraction

→ Herbal preparation

↓ Formulation

⇨ **Herbal medinal product**

European Pharmacopoeia

http://www.edqm.eu

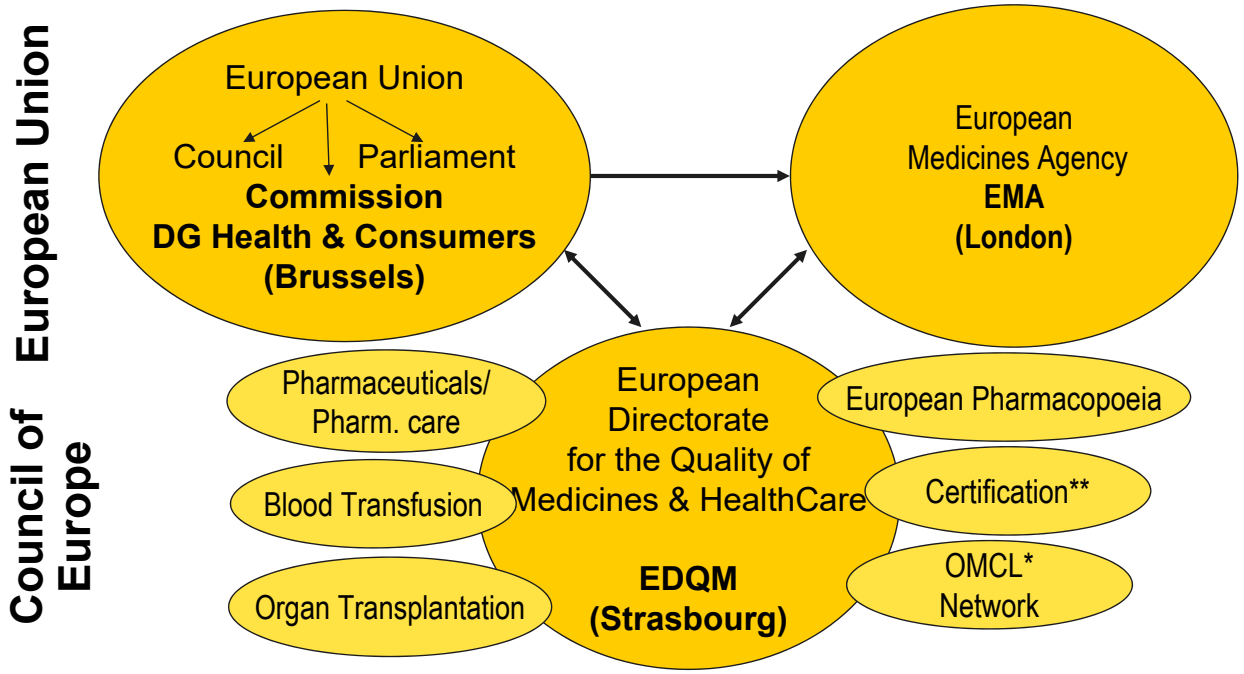
European Pharmacopoeia is part of the

European Directorate for the Quality of Medicines & Health Care (EDQM)

The EDQM belongs to the **Council of Europe** (DG III)

Headquaters of the EDQM (Strasnbourg, France)

European regulatory network



***OMCL:** Official Medicines Control Laboratories
****Certification:** Certification of Suitability of Monographs of the European Pharmacopoeia

European Pharmacopoeia

edqm Since 1964

http://www.edqm.eu

European Directorate for the Quality of Medicines & Health Care (EDQM)

2017

Members: 38

- ✓37 European states
- ✓European Union

Observers: 30

- ✓8 European countries
- ✓20 non-european countries
- ✓WHO, Taiwan FDA

European Pharmacopoeia

For whom?

- ✓ Producers of herbal drugs
- ✓ Manufacturers of herbal drug preparations
- ✓ Pharmaceutical industry
- ✓ Regulatory authorities
- ✓ Official Medicines Control Laboratories (OMCLs)
- ✓ Community pharmacies
- ✓ Others...

European Pharmacopoeia

Editions

Edition	Year of publication
1st	1969
2nd	1981
3rd	1997
4th	2001
5th	2004
6th	2007
7th	2010
8th	2013
9th	2016

European Pharmacopoeia

9th Edition

Volume 9.0

- ✓ More than **2300 monographs** of ingredients and pharmaceutical forms.
- ✓ More than **350 general texts**.

- ✓ More than **60 groups** of experts and working parties.
- ✓ More than **800 experts** of all over Europe.

European Pharmacopoeia

General monographs related to herbals (1)

Requirements apply to the entire class!

Herbal drugs	*Plantae medicinales*
Herbal drug preparations	*Plantae medicinales praeparatore*
Herbal teas	*Plantae ad ptisanam*
Extracts	*Extracta*
Fatty oils	*Olea herbaria*
Essential oils	*Aetherolea*

European Pharmacopoeia

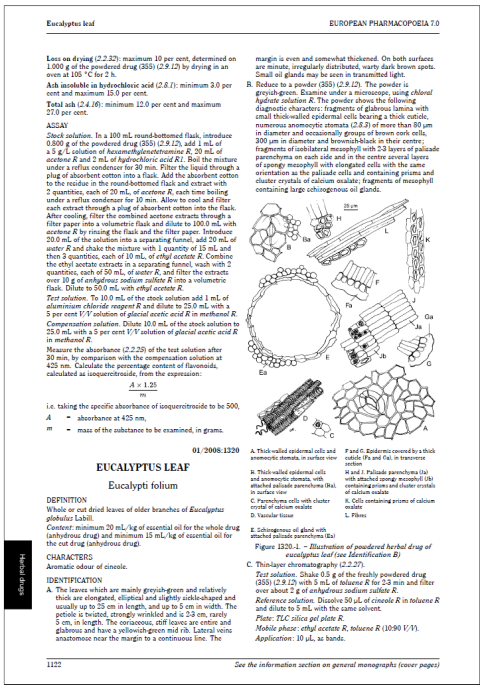
General monographs related to herbals (2)

Requirements apply to the entire class!

Herbal drugs for homoeopathic preparations	*Plantae medicinales ad praeparationes homoeopathicas*
Mother tinctures for homoeopathic preparations	Tincturae maternae ad praeparationes homoeopathicas

European Pharmacopoeia

Individual monographs



Eucalyptus leaf EUROPEAN PHARMACOPOEIA 7.0

Loss on drying (2.2.32): maximum 10 per cent, determined on 1.000 g of the powdered drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

Ash insoluble in hydrochloric acid (*2.8.1*): minimum 3.0 per cent and maximum 15.0 per cent.

Total ash (*2.4.16*): minimum 12.0 per cent and maximum 27.0 per cent.

ASSAY

Stock solution. In a 100 mL round-bottomed flask, introduce 0.800 g of the powdered drug (355) (*2.9.12*), add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool and filter each extract through a plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a filter paper into a volumetric flask and dilute to 100.0 mL with *acetone R* by rinsing the flask and the filter paper. Introduce 20.0 mL of the solution into a separating funnel, add 50 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 mL, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask. Dilute to 50.0 mL with *ethyl acetate R*.

Test solution. To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min, by comparison with the compensation solution at 425 nm. Calculate the percentage content of flavonoids, calculated as isoquercitroside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of isoquercitroside to be 500,

A = absorbance at 425 nm,

m = mass of the substance to be examined, in grams.

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EUCALYPTUS LEAF

Eucalypti folium

DEFINITION

Whole or cut dried leaves of older branches of *Eucalyptus globulus* Labill.

Content: minimum 20 mL/kg of essential oil for the whole drug (anhydrous drug) and minimum 15 mL/kg of essential oil for the cut drug (anhydrous drug).

CHARACTERS

Aromatic odour of cineole.

IDENTIFICATION

A. The leaves which are mainly greyish-green and relatively thick are elongated, elliptical and slightly sickle-shaped and usually up to 25 cm in length, and up to 5 cm in width. The petiole is twisted, strongly wrinkled and ≤ 2.0 cm, rarely 5 cm, in length. The coriaceous, stiff leaves are entire and glabrous and have a yellowish-green mid rib. Lateral veins anastomose near the margin to a continuous line. The margin is even and somewhat thickened. On both surfaces are minute, irregularly distributed, warty dark brown spots. Small oil glands may be seen in transmitted light.

B. Reduce to a powder (355) (*2.9.12*). The powder is greyish-green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of glabrous lamina with small thick-walled epidermal cells bearing a thick cuticle, numerous anomocytic stomata (*2.8.3*) of more than 80 µm in diameter and occasionally groups of brown cork cells, 300 µm in diameter and brownish-black in their centre; fragments of isobilateral mesophyll with 2-3 layers of palisade parenchyma on each side and in the centre several layers of spongy mesophyll with elongated cells with the same orientation as the palisade cells and containing prisms and cluster crystals of calcium oxalate; fragments of mesophyll containing large schizogenous oil glands.

Figure 1320.-1 – *Illustration of powdered herbal drug of eucalyptus leaf (see Identification B)*

A. Thick-walled epidermal cells and anomocytic stomata, in surface view
B. Thick-walled epidermal cells and anomocytic stomata, with attached palisade parenchyma (Ba), in surface view
C. Parenchyma cells with cluster crystal of calcium oxalate
D. Vascular tissue
E. Schizogenous oil gland with attached palisade parenchyma (Ea)
F and G. Epidermis covered by a thick cuticle (Fa and Ga), in transverse section
H and J. Palisade parenchyma (Ha) with attached spongy mesophyll (Hb) containing prisms and cluster crystals of calcium oxalate
K. Cells containing prisms of calcium oxalate
L. Fibres

C. Thin-layer chromatography (*2.2.27*).

Test solution. Shake 0.5 g of the finely powdered drug (355) (*2.9.12*) with 5 mL of *toluene R* for 2-3 min and filter over about 2 g of *anhydrous sodium sulfate R*.

Reference solution. Dissolve 50 µL of *cineole R* in *toluene R* and dilute to 5 mL with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: ethyl acetate R, toluene R (10:90 V/V).

Application: 10 µL, as bands.

1122 *See the information section on general monographs (cover pages)*

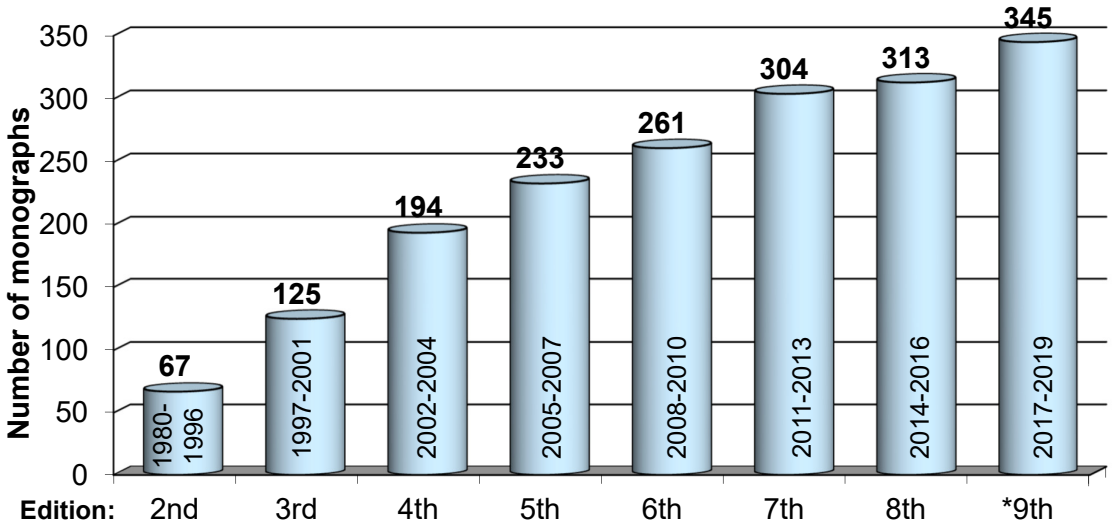
Herbal drugs

Index of an individual monograph

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

European Pharmacopoeia

Monographs on herbal drugs and herbal preparations

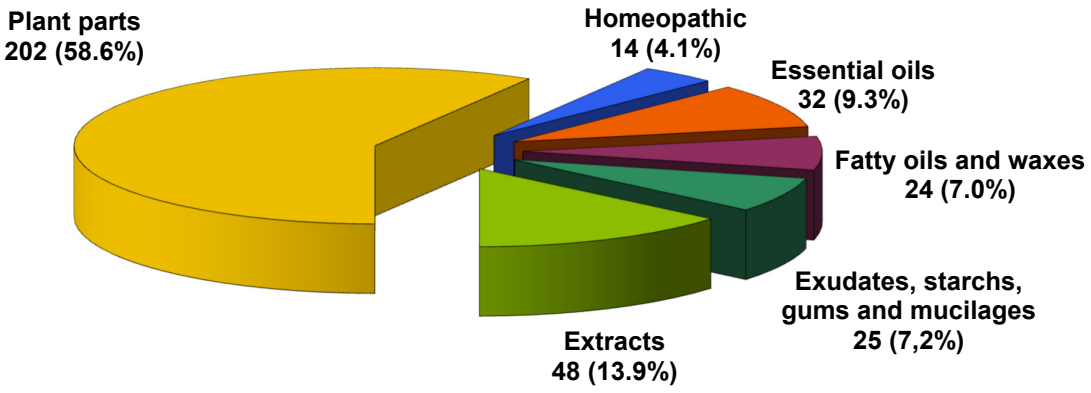


*) Including supplements until 9.2

European Pharmacopoeia

Monographs on herbal drugs and herbal drug preparations

9th Edition* (2017-2019)
345 monographs



*) Including supplement 9.2

European Pharmacopoeia

Working groups

On herbal drugs and herbal drug preparations (2017)

Groups of experts (permanent)	
13A	Phytochemistry A
13B	Phytochemistry B
13H	Fatty oils and derivatives

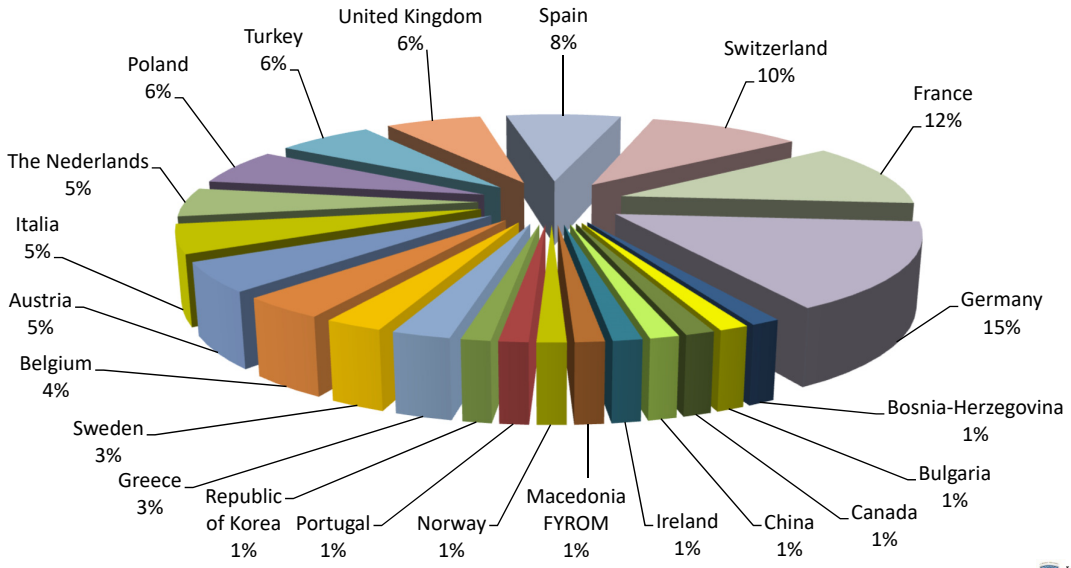
Ad hoc working parties (temporary)	
EXT	Extracts
MQH	Microbiological quality of herbal drugs
PST	Pesticides in herbal drugs
TCM	Traditional Chinese medicines
WXT	Water for the preparation of extracts

European Pharmacopoeia

Experts by country

Groups 13A, 13B, 13H, TCM, EXT, MQH, PST and WXT

78 experts of **22** countries (2017)

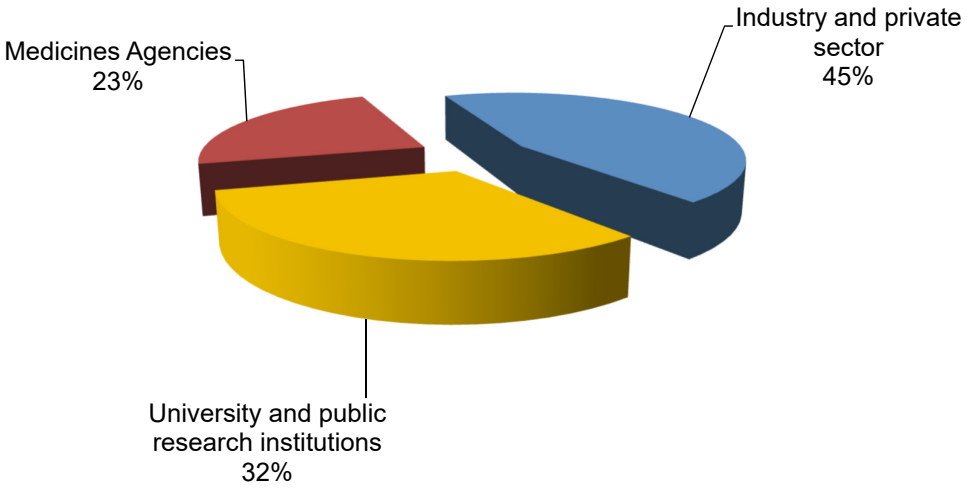


European Pharmacopoeia

Experts by profile

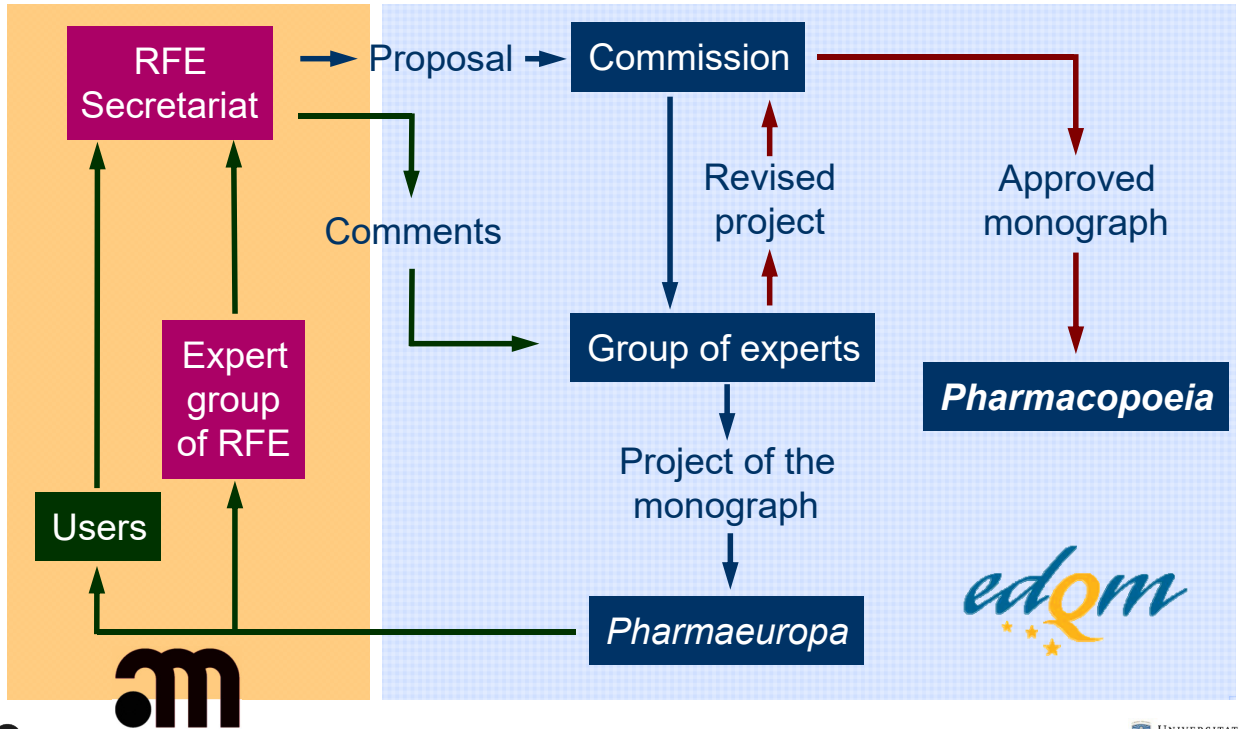
Groups 13A, 13B, 13H, TCM, EXT, MQH, PST and WXT

78 experts of **22** countries (2017)



European Pharmacopoeia

Elaboration of a monographs



European Pharmacopoeia

Reference substances

CRS: Chemical Reference Substances

HRS: **Herbal Reference Substances**

European Pharmacopoeia

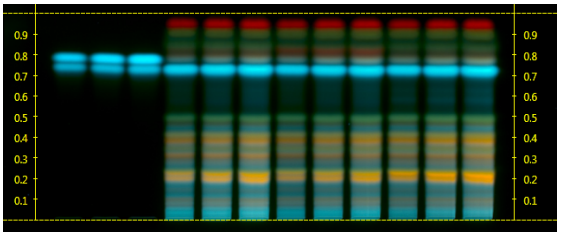
Reference substances

Uses	Ph. Eur. tasks
✓ Qualitative: screening for adulterants. ✓ Qualitative: peak identification and system suitability test in chromatography ✓ Quantitative: assay standard with assigned content	✓ Preparing CRS and HRS substances ✓ Establishment and adoption ✓ Monitoring their content and purity ✓ Supply

Quality of herbal drugs and herbal preparations

Objectives

HPTLC



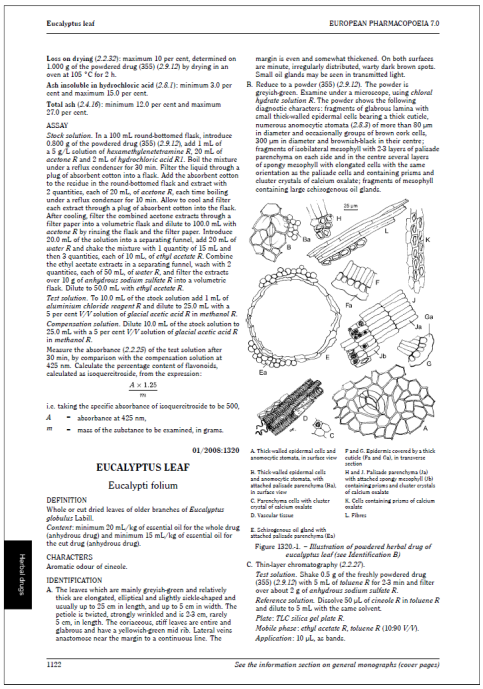
Stability

Quality control

- Identity
- Purity Contaminants
- Quantitative determination of active principles and markers

European Pharmacopoeia

Monographs



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EUCALYPTUS LEAF

Eucalypti folium

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CHARACTERS

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IDENTIFICATION

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K. Cells containing prisms of calcium oxalate
L. Fibres

Figure 1320.-1 – *Illustration of powdered herbal drug of eucalyptus leaf (see Identification B)*

C. Thin-layer chromatography (2.2.27).

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Reference solution. Dissolve 50 µL of *cineole R* in *toluene R* and dilute to 5 mL with the same solvent.

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Mobile phase: ethyl acetate R, toluene R (10:90 V/V).

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Herbal drugs

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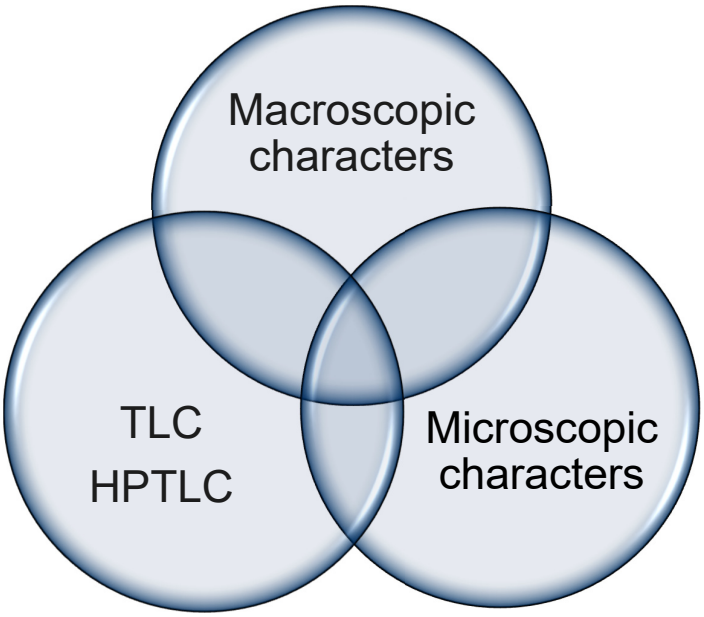
European Pharmacopoeia

Monograph

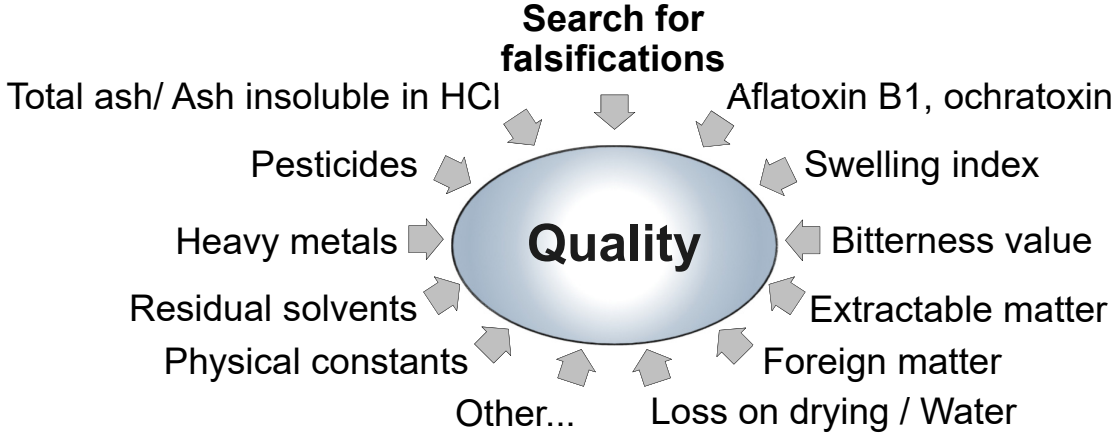
Identification



European Pharmacopoeia

Monograph

Tests*

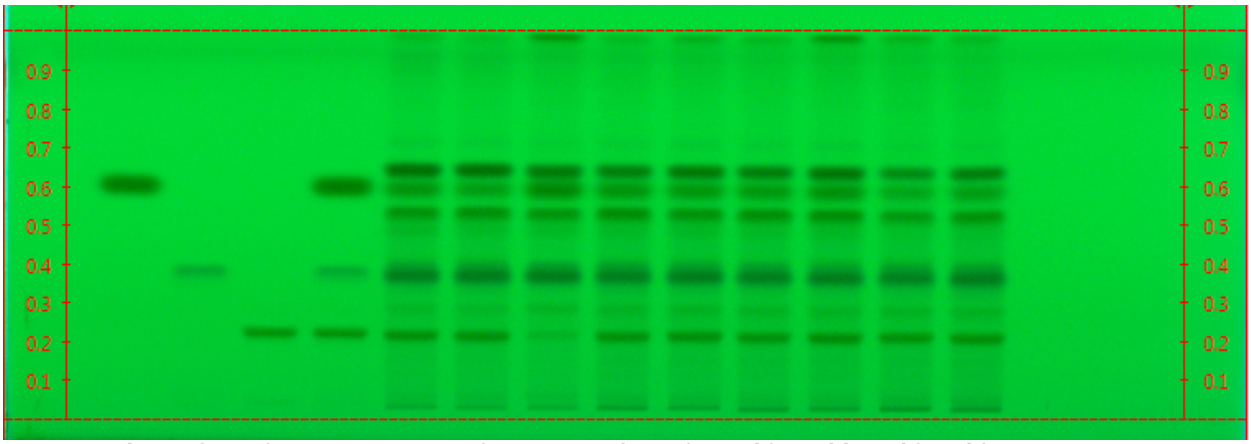


*) The tests applied will depend on the type of product (herbal drug, tincture, dry extract, essential oil, etc…)

Identification

Mate leaf (*Ilex paraguariensis*)

Detection 1: UV 254 nm **HPTLC**

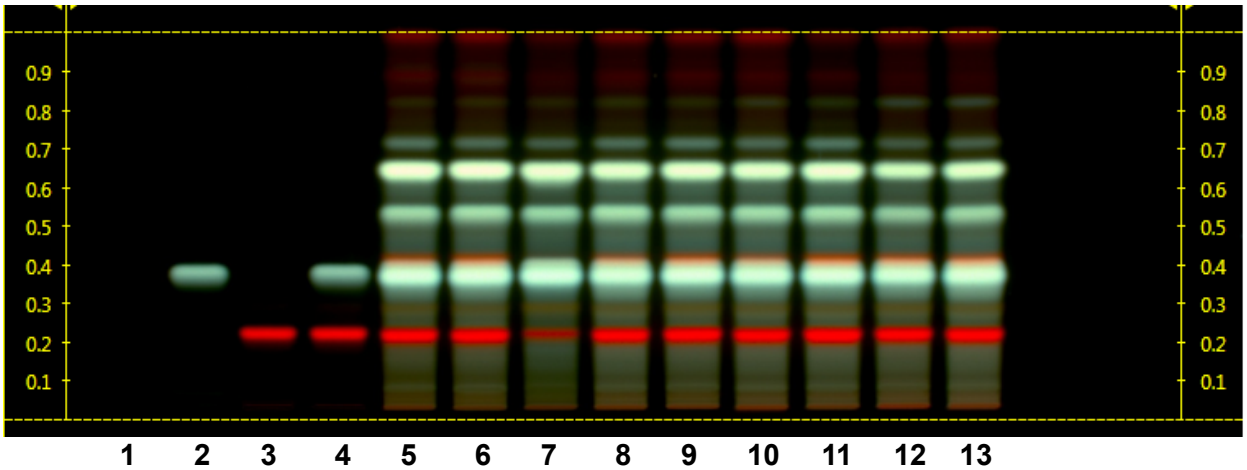


1. Caffeine
2. Chlorogenic acid
3. Rutin
5-13. Mate leaf samples

Identification

Mate leaf (*Ilex paraguariensis*)

Detección 2: NPR + PEG / UV 365 nm **HPTLC**

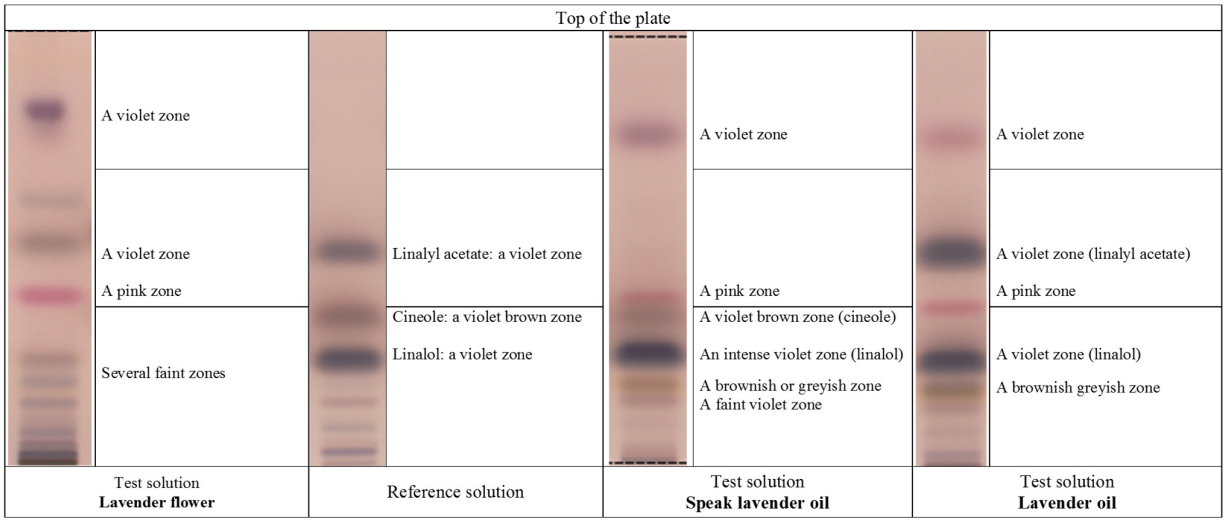


1. Caffeine
2. Chlorogenic acid
3. Rutin
5-13. Mate leaf samples

Identification

Methods harmonisation

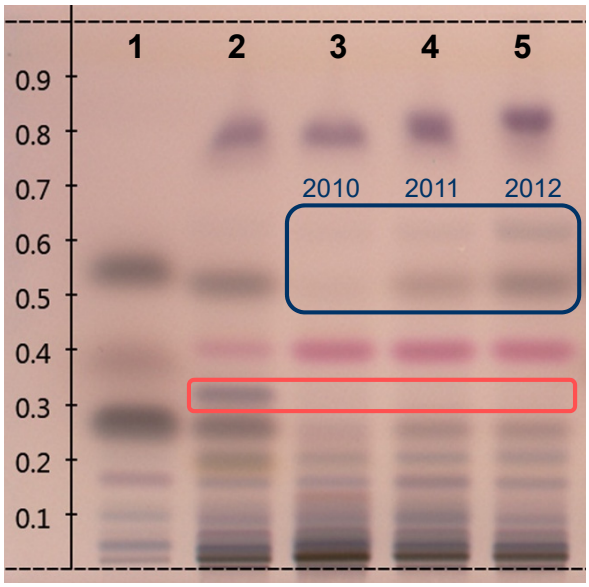
Lavender flower (*Lavandula angustifolia*), **Lavender oil and Speak lavender oil** (*Lavandula latifolia*)



Detection: anisaldehyde reagent, 100-105 °C (5-10 min) / daylight

Identification and stability

Lavender flower (*Lavandula angustifolia*)



Detection: anisaldehyde reagent, 100-105 °C (5-10 min) / daylight

HPTLC

1. Reference solution
2. Lavandin flower
3. Lavender flower (2010)
4. Lavender flower (2011)
5. Lavender flower (2012)

Purity: possible falsifications

Hamamelis leaf and hamamelis bark

Hamamelis leaf (*Hamamelidis folium*)

Whole or cut, dried leaf of *Hamamelis virginiana* L.

Hamamelis bark (*Hamamelidis cortex*)

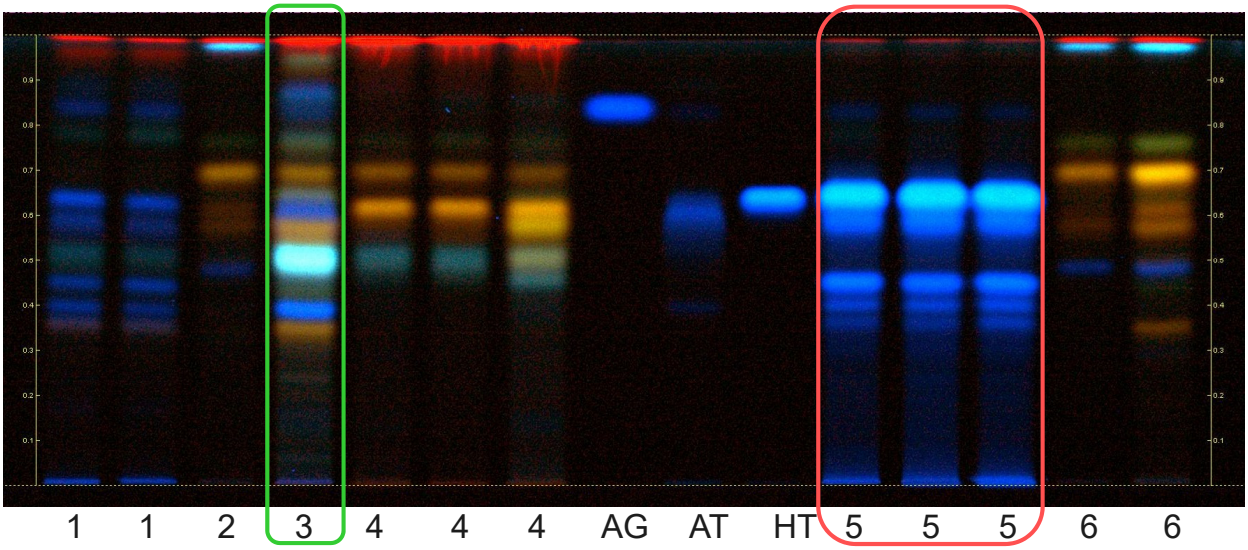
Cut, dried bark from the trunk and branches of *Hamamelis virginiana* L.



Purity: possible falsifications

Hamamelis leaf and hamamelis bark

Detection: NPR + PEG / UV 365 nm



1: Hamamelis stem (*H. virginiana)*
2: Hazelnut stem (*C. avellana*)
3: Hamamelis leaf
4: Hazelnut leaf
AG: Gallic acid
AT: Tannic acid
HT: Hamamelitanin
5: Hamamelis bark
6: Hazelnut bark

Purity: possible falsifications

Arnica flower

Arnica flower
(*Arnica montana*)

Arnica chamissonis flower

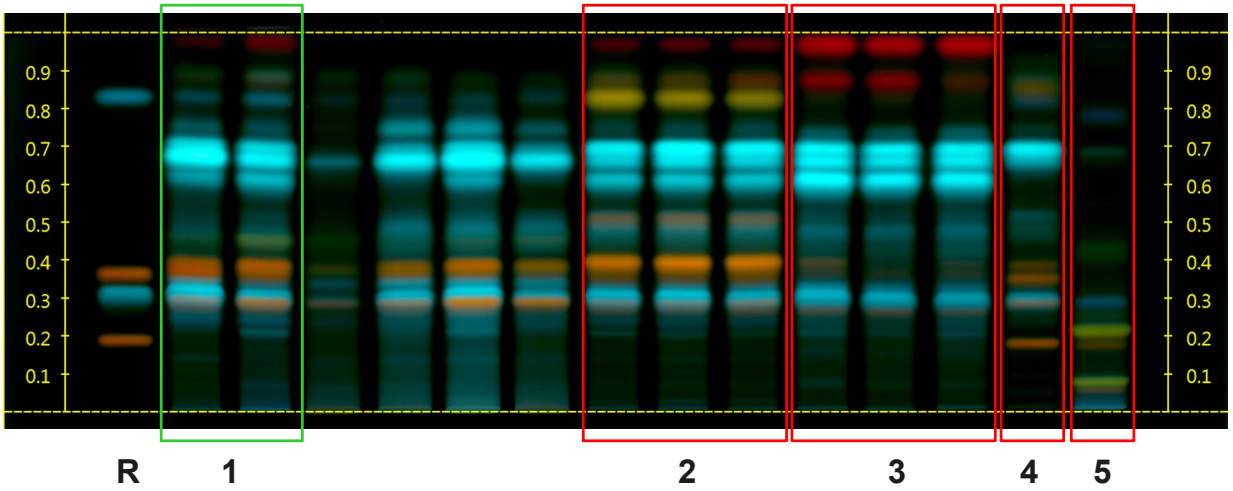
Mexican arnica flower
(*Heterotheca inuloides*)

Calendula flower
(*Calendula officinalis*)

Purity: possible falsifications

Arnica flower

Detection: NPR + PEG / UV 365 nm



R: Caffeic acid
Hyperoside
Chlorogenic acid
Rutin

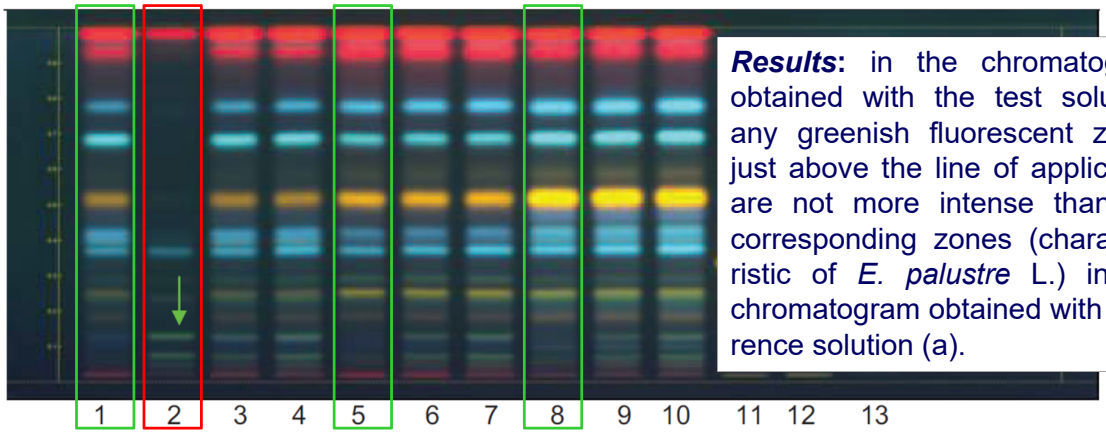
1: Flower of *Arnica montana*
2: Flower of *Arnica chamisonis*
3: Aerial part of *A. montana*

4: Flower of *Heterotheca inuloides* (Mexican arnica)
5: Flower of *Calendula officinalis*

Purity: possible falsifications

Equisetum stem (*Equisetum arvense* L.)

Test for *Equisetum palustre*



Results: in the chromatogram obtained with the test solution, any greenish fluorescent zones just above the line of application are not more intense than the corresponding zones (characteristic of *E. palustre* L.) in the chromatogram obtained with reference solution (a).

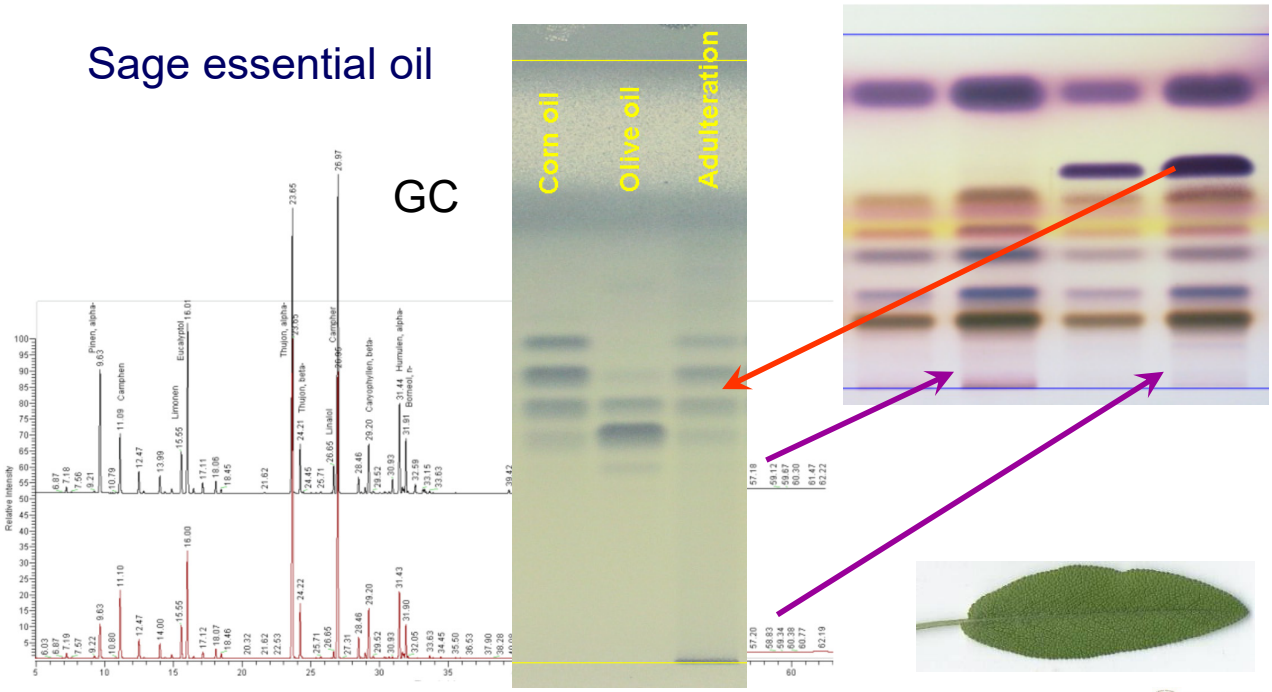
1: **Sample 1**
2: **Reference solution (a): *E. palustre* HRS**
3: Sample 1 + 5% *E. palustre*
4: Sample 1 + 10% *E. palustre*
5: **Sample 2**
6: Sample 2 + 5% *E. palustre*
7: Sample 2 + 10% *E. palustre*
8: **Sample 3**
9: Sample 3 + 5% *E. palustre*
10:Sample 3 + 10% *E. palustre*
11-13: Reference solution (b)

Purity: possible falsification

Essential oil adulterated with fatty oil

Sage essential oil

GC



Stability of HMP

Accepted variation in content during the proposed shelf-life

With constituents of known therapeutic activity	± **5%** of the **declared** assay value, unless justified
Constituents with known therapeutic activity are unknown	±**10%** of the **initial** assay value, if justified

Stability of HMP

Fingerprint chromatograms

HS or HP in its entirety is regarded as the active substance

→

- ✓ The stability of other substances should also be demonstrated.
- ✓ Their proportional content remains comparable to the initial

↑

Appropriate fingerprint chromatograms

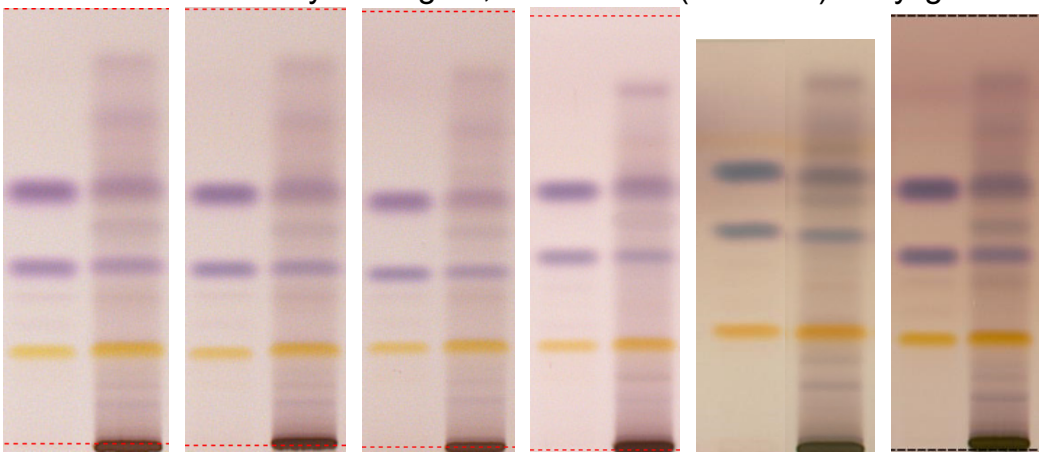
Stabylity: HPTLC profile

Combination of 3 herbal drugs in capsules

System 1

25ºC, 60% RH

Detection: Anisaldehyde reagent, 100-105 °C (5-10 min) / dayligth



0 months | 3 months | 6 months | 9 months | 12 months | 18 months

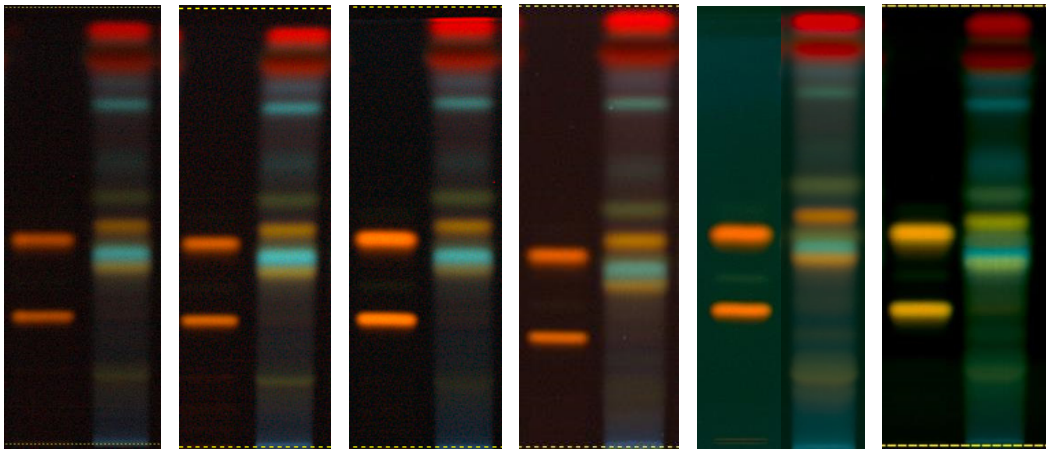
Stabylity: HPTLC profile

Combination of 3 herbal drugs in capsules

System 2

25ºC, 60% RH

Detection: NPR + PEG 400 / UV 365 nm

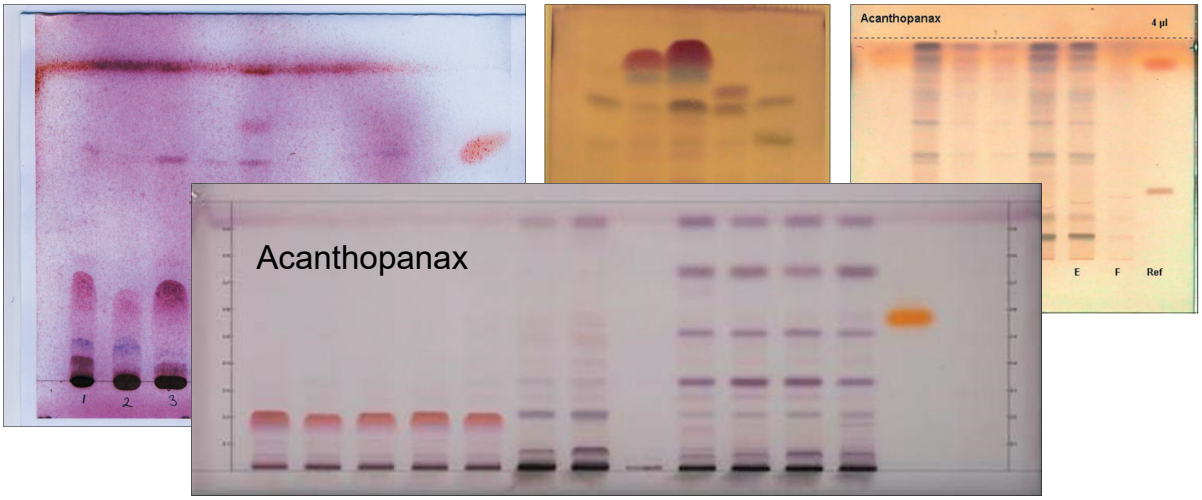


0 months | 3 months | 6 months | 9 months | 12 months | 18 months

Identification by TLC

Problems

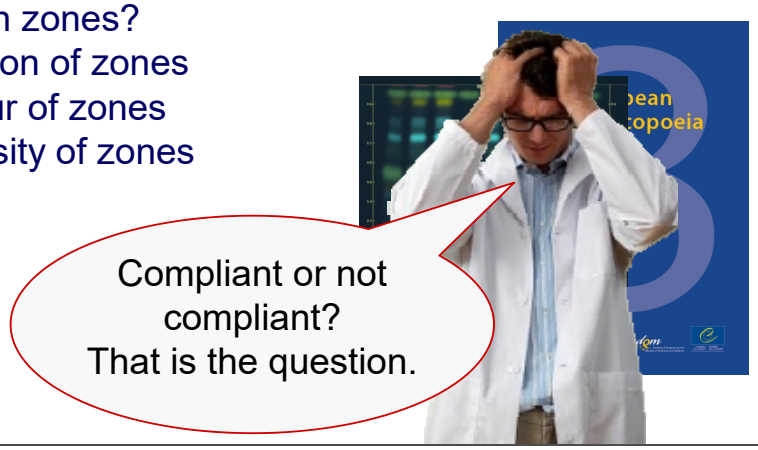
- ✓ **Variability of the chromatograms**
  - Variability of herbal drugs (differences between batches)
  - Lack of reproducibility intra- and inter-laboratory



Identification by TLC

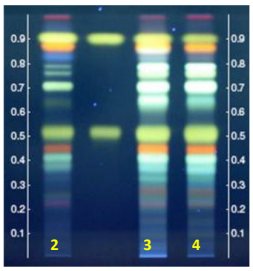
Problems

- ✓ **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

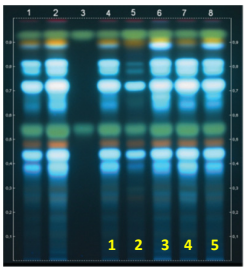


HPTLC *versus* TLC

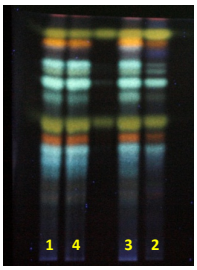
TLC reproducibility



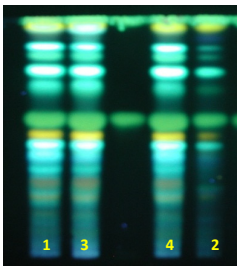
Laboratory 1
(glass plate F_{254})



Laboratory 2



Laboratory 3

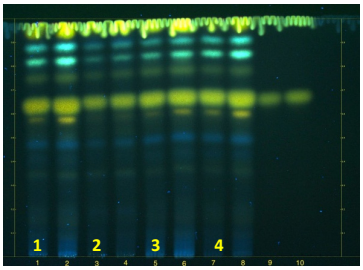


Laboratory 4

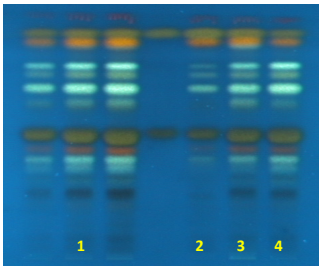
Roman chamomile flower

TLC

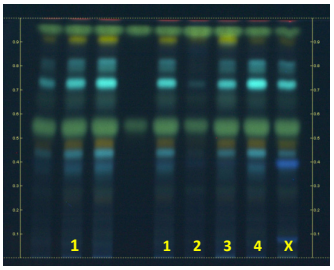
Ph. Eur. Interlaboratory trial



Laboratory 5
(the *1.5-fold* application vol is additionally applied)



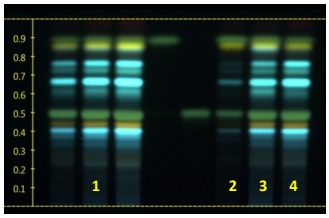
Laboratory 6



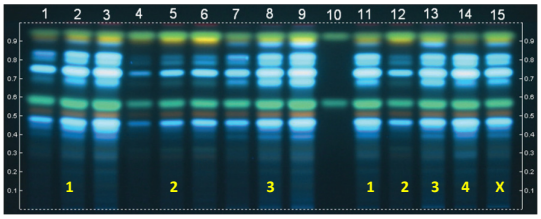
Laboratory 7

HPTLC *versus* TLC

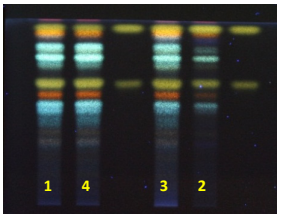
HPTLC reproducibility



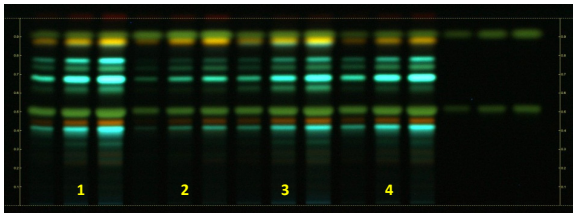
Laboratory 8



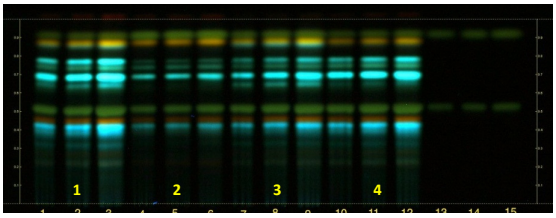
Laboratory 2



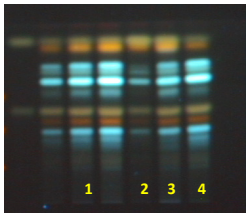
Laboratory 3



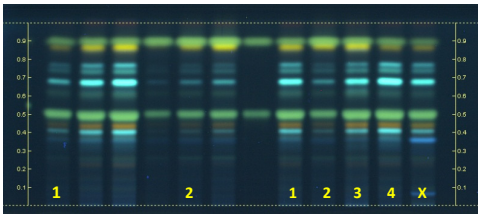
Laboratory 5 (instrumental)



Laboratory 5 (manual)



Labortory 6



Laboratory 7

Roman chamomile flower

HPTLC

Ph. Eur. Interlaboratory trial

HPTLC for identification of herbals

Ph. Eur. Improvements (*chapter 2.8.25*)

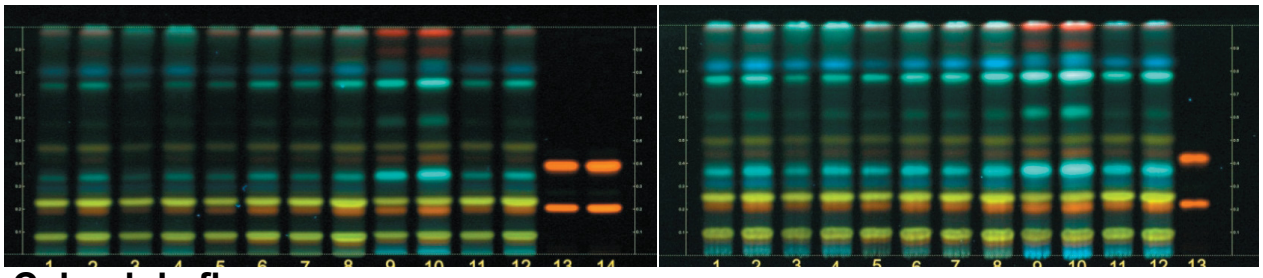
1. Improvement of reproducibility

- ✓ **Introduction of HPTLC**
  Instrumentation may help

Ph. Eur. collaborative trial

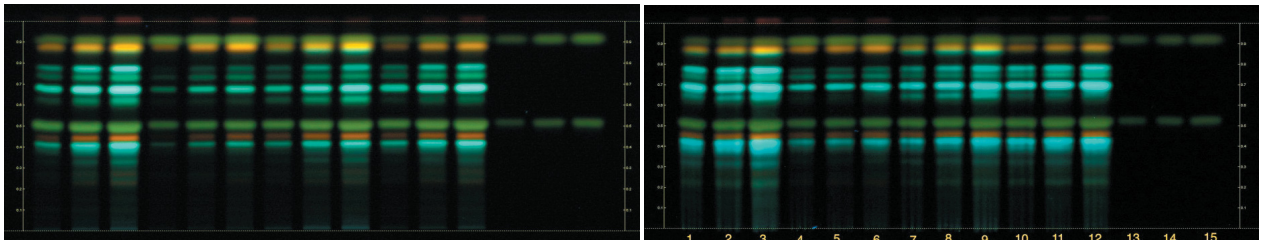
Instrumental *versus* manual HPTLC

Instrumental HPTLC | **Manual HPTLC**



Calendula flower

Roman chamomile flower



… Hawthorn leaf and flower; St. John's wort, Birch leaf, Passion flower …

HPTLC for identification of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

1. Improvement of reproducibility

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

Standard operating protocols (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

HPTLC for identification 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)

HPTLC chromatograms

Qualification of a plate

Ways to approach the qualification

USP

Based on two or more reference substances that have just separable R_F or **a reference extract (in most cases)**. Results should match description of colors and position (not exact).

European Pharmacopeia

System-specific suitability test (**SST**).

HPTLC Association

Based on reproducible and standardized HPTLC results, exact R_F values are defined.

System-specific suitability test (SST)

Ph. Eur. 2.8.25

SST

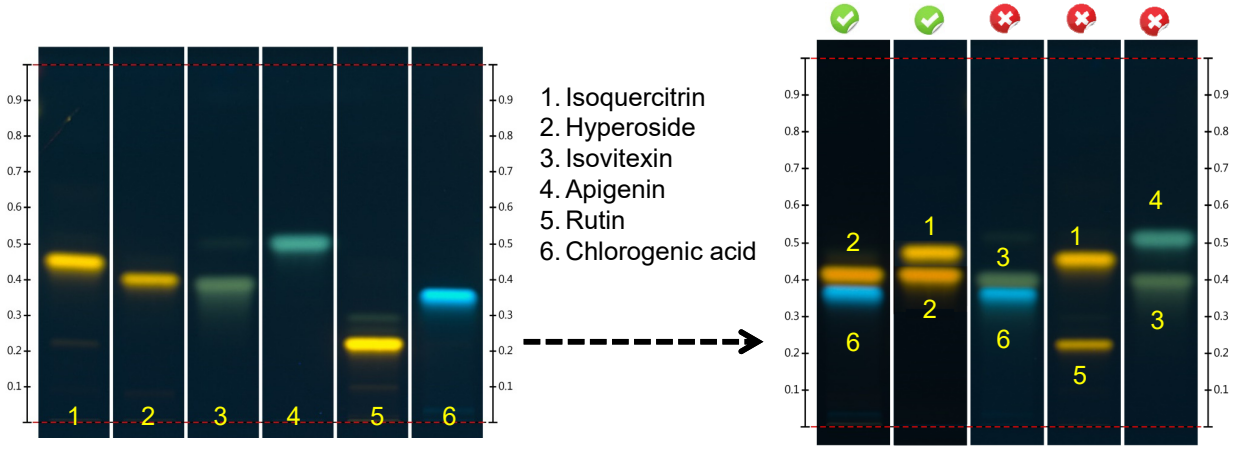
Test is based on the **separation of 2 substances** that have **similar retardation factors** (R_F values) **but** that are barely **separable** under the specified chromatographic conditions.

System-specific suitability test (SST)

Ph. Eur. 2.8.25

Flavonoids

Developing solvent: Ethyl acetate / formic acid / water (80:10:10)



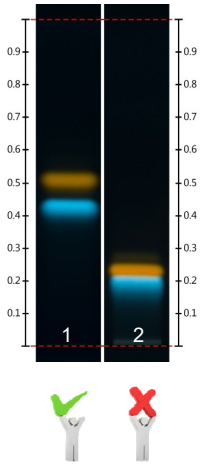
System-specific suitability test (SST)

Ph. Eur. 2.8.25

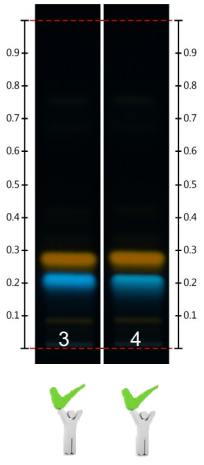
Flavonoids: Different developing solvents

Hyperoside
Chlorogenic acid

Ethyl acetate, formic acid, water (15:1:1)



1. Ethyl acetate, acetic acid, formic acid, water (100:11:11:27)
2. Ethyl acetate, formic acid, water (15:1:1)



3. Isoquercitrin and caftaric acid
4. Isoquercitrin and chlorogenic acid

HPTLC for identification of herbals

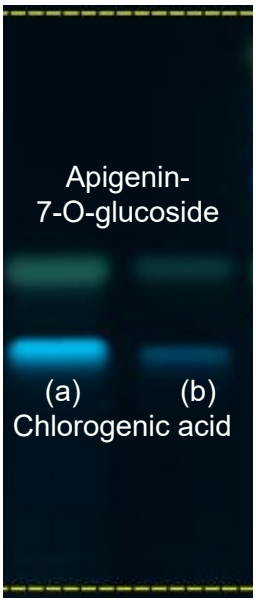
Ph. Eur. improvements (*chapter 2.8.25*)

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**

Introduction of an intensity marker

Ph. Eur. 2.8.25



R R¼

4-fold dilution

Example of intensity marker: chlorogenic acid (CA)

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 identification of herbals

Ph. Eur. improvements (*chapter 2.8.25*)

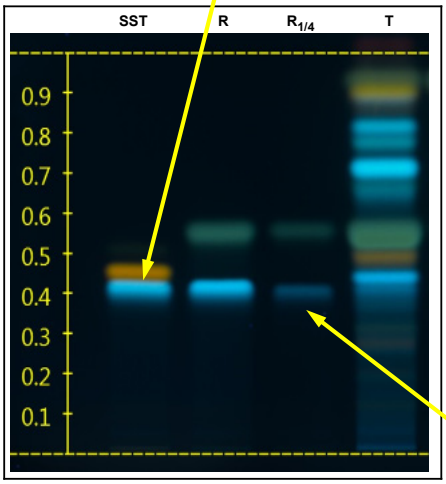
Roman chamomile flower

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

System-specific suitability test (SST)



Typical chromatogram

Intensity marker

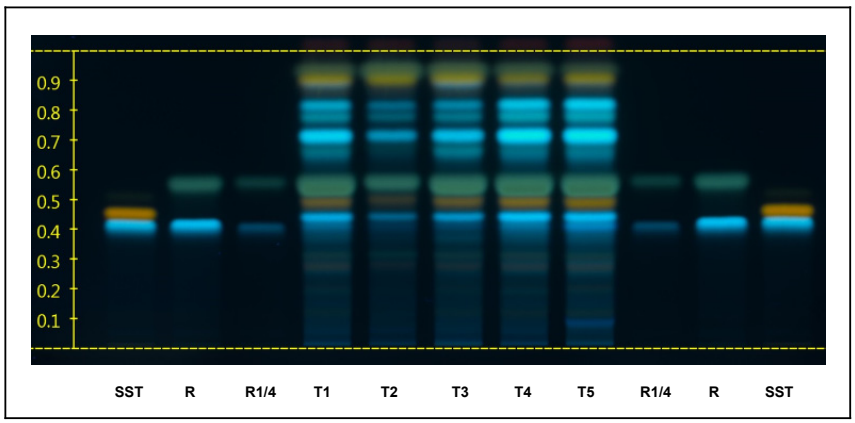
SST: Reference solution (c)
R: Reference solution (a)
$R_{¼}$: Reference solution (b). R diluted with factor 4
T: Test solution (T1)

HPTLC for identification 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),
$R_{1/4}$: Reference solution (b): R diluted with factor 4

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

HPTLC for identification of herbals

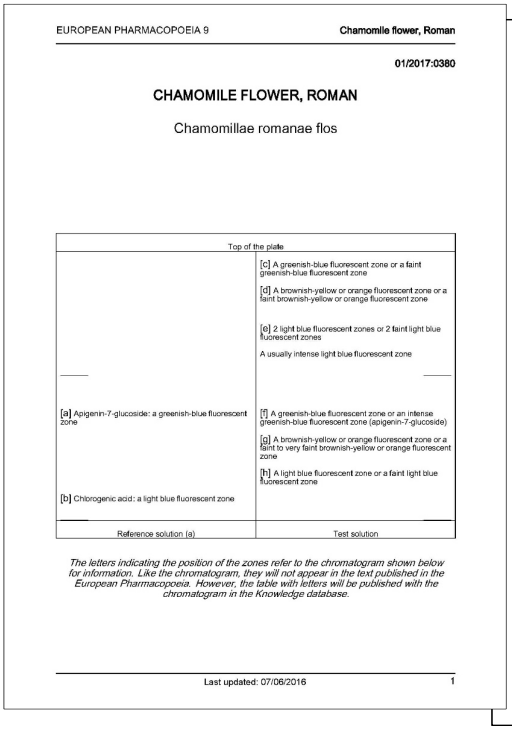
Ph. Eur. improvements (*chapter 2.8.25*)

2. Improvement of the description and interpretation of the chromatograms

- ✓ Sequence of 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.

HPTLC for identification of herbals

Ph. Eur. improvements (*chapter 2.8.25*)



EUROPEAN PHARMACOPOEIA 9 — Chamomile flower, Roman

01/2017:0380

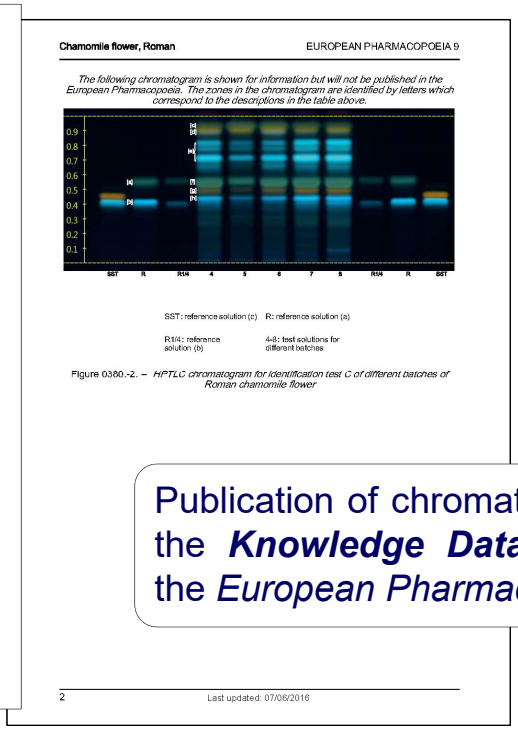
CHAMOMILE FLOWER, ROMAN

Chamomillae romanae flos

Top of the plate	
	[c] A greenish-blue fluorescent zone or a faint greenish-blue fluorescent zone
	[d] A brownish-yellow or orange fluorescent zone or a faint brownish-yellow or orange fluorescent zone
	[e] 2 light blue fluorescent zones or 2 faint light blue fluorescent zones A usually intense light blue fluorescent zone
[a] Apigenin-7-glucoside: a greenish-blue fluorescent zone	[f] A greenish-blue fluorescent zone or an intense greenish-blue fluorescent zone (apigenin-7-glucoside)
	[g] A brownish-yellow or orange fluorescent zone or a faint to very faint brownish-yellow or orange fluorescent zone
	[h] A light blue fluorescent zone or a faint light blue fluorescent zone
[b] Chlorogenic acid: a light blue fluorescent zone	
Reference solution (a)	Test solution

The letters indicating the position of the zones refer to the chromatogram shown below for information. Like the chromatogram, they will not appear in the text published in the European Pharmacopoeia. However, the table with letters will be published with the chromatogram in the Knowledge database.

Last updated: 07/06/2016 — 1



Chamomile flower, Roman — EUROPEAN PHARMACOPOEIA 9

The following chromatogram is shown for information but will not be published in the European Pharmacopoeia. The zones in the chromatogram are identified by letters which correspond to the descriptions in the table above.

SST: reference solution (c) R: reference solution (a)

R1/4: reference solution (b) 4-8: test solutions for different batches

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

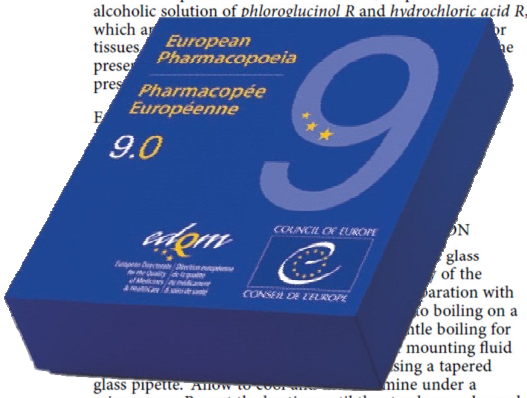
2 — Last updated: 07/06/2016

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

Standard operating procedures

Ph. Eur. 2.8.25

2.8.25 Qualitative 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… tissues… prese… pres…

…glass …of the …paration with …o boiling on a …ntle boiling for …mounting fluid …sing a tapered …mine 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 — 295

Standard operating procedures

Ph. Eur. 2.8.25

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

High-performance thin-layer chromatography for use in the qualitative evaluation of herbal drugs and herbal drug preparations is a thin-layer chromatographic technique (*2.2.27*) which, unless otherwise stated in an individual monograph, uses a glass plate coated with a uniform, typically 200 µm, layer of porous (average pore size 60 Å), irregular particles of silica gel with a size between 2 µm and 10 µm and an **average particle size of 5 µm**, a polymeric binder and a fluorescence indicator (F_{254}). The results are qualified using a system-specific suitability test.

EQUIPMENT

The equipment used for qualitative high-performance thin-layer chromatography typically consists of:

Glass plates, as described above, usually with a size of **20 x 10 cm**.

Devices suitable for the application of specified volumes of samples as bands and allowing control of dimension and position of application.

A device suitable for conditioning the relative humidity of the stationary phase.

A suitable chromatographic chamber (for example, a twin trough chamber).

A device suitable for reproducible drying of the developed plate.

Devices suitable for reagent application to and heating of the plate as part of the derivatization procedure.

A system suitable for electronic documentation of chromatograms under UV 254 nm, UV 366 nm, and white light.

Note: conventional thin-layer chromatographic methods using glass plates or sheets coated with particles of 5-20 µm or high-performance thin-layer chromatographic aluminum backed sheets may be used, provided that the results obtained fulfil the general system suitability criteria of the bands having developed perpendicular to the lower edge of the plate and the solvent front being parallel to the upper edge of the plate and satisfy the system-specific suitability test stated in the individual monograph (where a monograph exists).

Standard operating procedures

Ph. Eur. 2.8.25

Methods

PREPARATION OF TEST SOLUTIONS

Unless otherwise stated in the individual monograph (where one exists), the test solution is usually prepared as follows. For dry herbal drugs or dry extracts, weigh 0.5 g of the powdered drug or 0.1 g of the dry extract and sonicate for 15 min with 5 ml of methanol R. After filtration or centrifugation the filtrate or supernatant is used as the test solution. For essential oils, dissolve 50 uL in 1 mL of toluene R and use this solution as the test solution.

PREPARATION OF REFERENCE SOLUTIONS

Unless otherwise stated in the individual monograph (where one exists), reference solutions are usually prepared as follows: prepare a 1mg/mL solution of the reference substance in *methanol R* or, for essential oil, in *toluene R*; prepare a 1 to 4 dilution of this reference solution using the same solvent for use as intensity reference (diluted reference solution).

INTENSITY MARKER

One of the substances of the reference solution and the diluted reference solution is used as intensity marker.

PREPARATION OF SYSTEM-SPECIFIC SUITABILITY SOLUTION

Prepare the solution as stated in the individual monograph (where one exists).

SAMPLE APPLICATION AND PLATE LAYOUT

Unless otherwise stated in the individual monograph (where one exists), samples are applied as narrow bands of 8 mm length at a distance of 8 mm from the lower edge of the plate. The center of the first track, which is used for the system-specific suitability solution, is positioned 20 mm from the left edge of the plate. The minimum distance between tracks (center to center) is 11 mm. A maximum of 15 tracks are applied onto a standard plate. If no electronic solvent front detection device is used the developing distance is marked with a pencil close to the right or left edge of the plate.

Standard operating procedures

Ph. Eur. 2.8.25

Methods (cont.)

CONDITIONING OF THE PLATE

Following sample application and unless otherwise stated in the individual monograph (where one exists), the plate is conditioned at a **relative humidity of 33%** for a sufficient time to reach equilibrium (for example, by standing in a closed chamber containing a saturated magnesium chloride solution or by the use of preconditioned air).

PREPARATION OF THE DEVELOPING CHAMBER AND DEVELOPMENT OF THE PLATE

Unless otherwise stated in the individual monograph (where one exists), the chromatographic separation is performed in a saturated chamber. Where a **twin trough chamber** is used, the **rear trough is fitted with a filter paper**. The chamber is charged with a sufficient amount of developing solvent to wet the filter paper completely and achieve a **level of 5 mm in both troughs**. With the lid closed the chamber is left **20 min for saturation**. The plate is introduced in a vertical position into the front trough of the chamber so that the coating layer faces the filter paper. When the mobile phase has reached 70 mm (corresponding to a development path of 6 cm) the plate is removed from the chamber and dried in a vertical position in a stream of cold air. Other chamber configurations and developing distances may be specified in an individual monograph.

Note: other development chambers may be employed if the results obtained fulfil all of the system suitability criteria.

VISUALISATION

Chromatograms on the plate are visualised as stated in the individual monograph (where one exists). Where derivatization reagents are used, typically 3.5 mL of reagent in solution per plate of 20 x 10 cm are homogenously sprayed onto the plate or the plate is immersed into the reagent solution typically at a speed of 5 mm per sec for a dwell time of usually 1 sec. Observation may be performed under UV 254 nm, UV 366 nm or white light prior to and/or after derivatization. When electronic images are taken, exposure is adjusted on the track with the system specific suitability solution.

Standard operating procedures

Ph. Eur. 2.8.25

Methods (cont.)

SYSTEM-SPECIFIC SUITABILITY TEST

This test is based on the **separation of two substances that have similar but just separable R_F values** under the specified chromatographic conditions (for example, chlorogenic acid and hyperoside in chromatographic systems used for flavonoids). The results for the test and reference solutions are only valid when the system-specific suitability solution satisfies the separation requirements stated in the individual monograph.

VISUAL EVALUATION

The chromatograms obtained with the test and reference solutions are compared against the description in the result table in the monograph with respect to zone **position** and **color**, as well as to **intensity** for the test solution. Zones of the test solution described in the result table **without a descriptor** have intensities similar to the zone of the intensity marker in the reference solution. Those described as '**intense**' zones are visually more intense than the zone of the intensity marker in the reference solution; '**faint**' zones are visually less intense than the zone of the intensity marker in the reference solution, but equal to or more intense than the zone of the intensity marker in the diluted reference solution; '**very faint**' zones are visually less intense than the zone of the intensity marker in the diluted reference solution.

Development of an HPTLC method

Polysaccharide containing herbals

Development steps

1. **Preparation of the test solution**
   a) Heating conditions for hydrolysis
   b) Concentration of the trifluoroacetic acid used for hydrolysis
   c) Sample treatment after hydrolysis
2. **Chromatographic separation**
   a) Number of developments
   b) Selection of mobile phase
   c) Chamber saturation
3. **Detection**
4. **System suitability test (SST)**
5. **Intensity marker**

Development of an HPTLC method

Polysaccharide containing herbals

Example: Acacia and Acacia spry/roller dried

IDENTIFICATION

C. High performance thin-layer chromatography (*2.8.25*).

Test solution: To **100 mg** of the powdered drug in a thick-walled centrifuge tube add 2 mL of a 100 g/L solution of *trifluoroacetic acid R*, and shake vigorously. Stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer 1 mL of the clear supernatant liquid into a 10 mL flask, and add 5 mL of *methanol R*.

Reference solution (a)*:** Dissolve 5 mg of ***galactose *R*, 5 mg of ***arabinose*** *R*, 5 mg of **rhamnose** *R*, 5 mg of ***glucose*** *R*, and 5 mg of ***xylose*** *R* in 1 mL of *water R* and dilute to 10 mL with *methanol R*.

***Reference solution (b)*:** Dilute 2.5 mL of *reference solution (a)* to 10 mL with *methanol R*.

Reference solution (c)*:** Dissolve 5 mg of ***galactose *R* and 5 mg of ***glucose*** *R* in 1 mL of *water R* and dilute to 10 mL with *methanol R*.

***Intensity marker*:** galactose.

Development of an HPTLC method

Polysaccharide containing herbals

Example: Acacia and Acacia spry/roller dried

IDENTIFICATION (Continuation)

Plate*: *TLC silica gel plate R (2-10 µm) F_{254}.

Mobile phase*: *acetonitrile R*, *water R (85:15 V/V).

***Application*:** 4 µL of test solution and of the reference solutions (a) and (b), and 2 µL of reference solution (c) as bands of 8 mm.

***Development* A:** over a path of 6 cm, without saturation of the chamber.

***Drying*:** in air.

***Development* B:** over a path of 6 cm, without saturation of the chamber, using newly prepared mobile phase.

Drying: in air.

***Detection*:** Treat with a solution prepared as follows: dissolve 4 g of *diphenylamine R* and 4 mL of *aniline R* in 160 mL of *acetone R*. Add *phosphoric acid R* until the precipitate formed dissolves again (about 30 mL). Examine in daylight after heating at 120 °C for 5-10 min.

Development of an HPTLC method

Polysaccharide containing herbals

Example: Acacia and Acacia spry/roller dried

IDENTIFICATION (Continuation)

System suitability: reference solution (c).

The chromatogram shows 2 distinct grey-blue zones in the middle third which may, however, be touching. The lower zone is galactose and the upper zone is glucose.

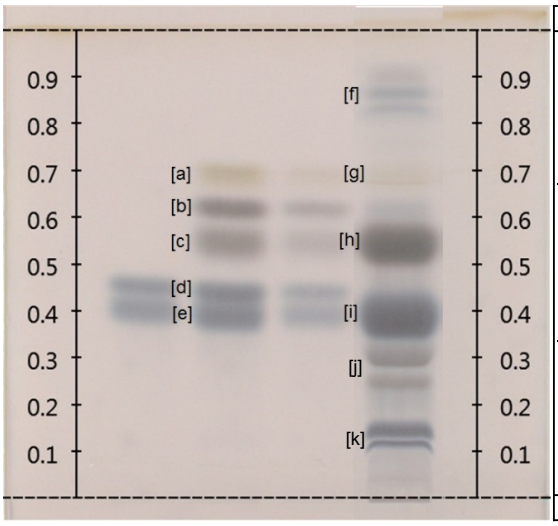
Results: See below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution.

Development of an HPTLC method

Polysaccharide containing herbals

Example: Acacia and Acacia spry/roller dried

IDENTIFICATION (Continuation) ***Results***



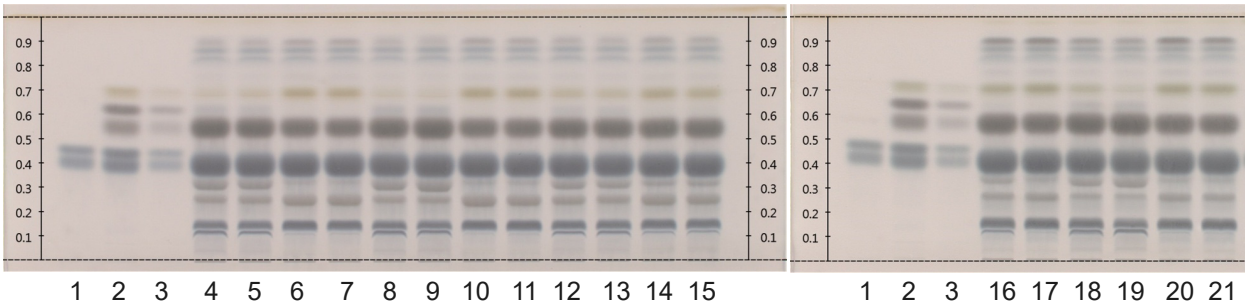
Top of the plate	
[a] Rhamnose: a greenish brown zone	[f] Three very faint blue zones
[b] Xylose: a brownish grey zone	[g] A very faint to equivalent greenish brown zone (rhamnose)
[c] Arabinose: a brownish grey zone	[h] An intense brownish grey zone (arabinose)
[d] Glucose: a grey-blue zone [e] Galactose: 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**

Development of an HPTLC method

Polysaccharide containing herbals

Example: Acacia and Acacia spry/roller dried

Batch chromatograms



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

Quality control of herbal products

Which compound(s) should be assayed?

Frequently, the active principles are totally or partially unknown

A	B	C
The compounds responsible of the therapeutic activity are known	The active compounds are partially known	The active compounds are unknown
Constituents with known therapeutic activity	**Active markers**	**Analytical markers**

Quality control of herbal products

Are analytical markers relevant?

Herbal medicinal products

"… the herbal drug or herbal drug preparation in its **entirety** is regarded as the active substance..."

- ✓ Quantification **can help** in the control of the **manufacturing process**
- ✓ In many cases, the content of analytical marker(s) is **not indicative of the suitability of the herbal drug for the intended use**
- ✓ **Does not guarantee the quality nor the stability** of the herbal drug.

Quality control of herbal products

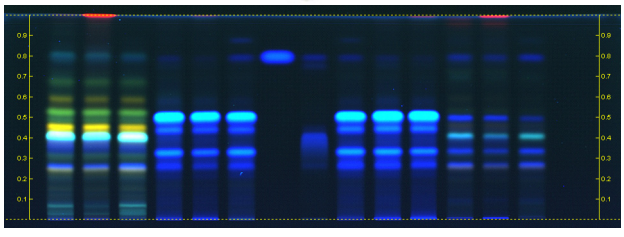
Is any better alternative to the assay of analytical markers?

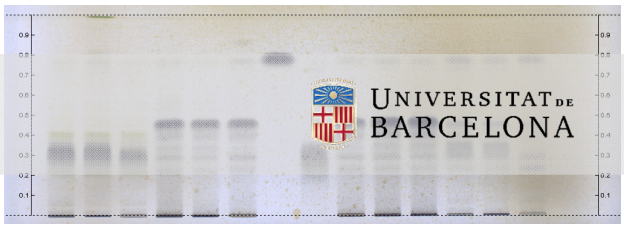
- ✓ A **more holistic approach** would be suitable.
- ✓ The approach would be able to evaluate the **strength** of the herbal drug / preparation / product.

To be considered: more in deep exploitation of HPTLC profiling.

Under discussion! Research and concept definitions are needed!

Séminaire Cub CCM
Université d'Avignon et des Pays du Vaucluse
Avignon, 12-13 Avril 2017





Merci beaucoup pour votre attention

L'HPTLC dans le control de qualité des produits de plantes dans la Pharmacopée Européenne

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