Broaden horizons in HPTLC

How advances in HPTLC standardization, combined with the use of data processing, are leading to new visions for cross-disciplinary applications

Tiên Do
CSO

CAMAG® 60 YEARS

Content

- Background
  - Comprehensive HPTLC fingerprinting, Test for minimum content by HPTLC
- Recent Developments
  - Case Studies by the Traditional Chinese Medicine Working Party (TCM WP)
  - Homoeopathic Working Party (HOM WP) Contributions: Standardization of HPTLC Methods
  - Universal HPTLC Mixture (UHM) for System Suitability Testing
- Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC
- Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications

Background

- Decision of 157Th session of Ph. Eur. Commission: TCM WP to conduct a pilot phase evaluating the suitability of “semi-quantitative HPTLC” as alternative to classical assays for TCM without marketing authorization.

“Comprehensive HPTLC Fingerprinting”

European Directorate for the Quality of Medicines & HealthCare

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Newsroom

Adoption of Fritillariae thunbergii bulbus describing alternative quality control test

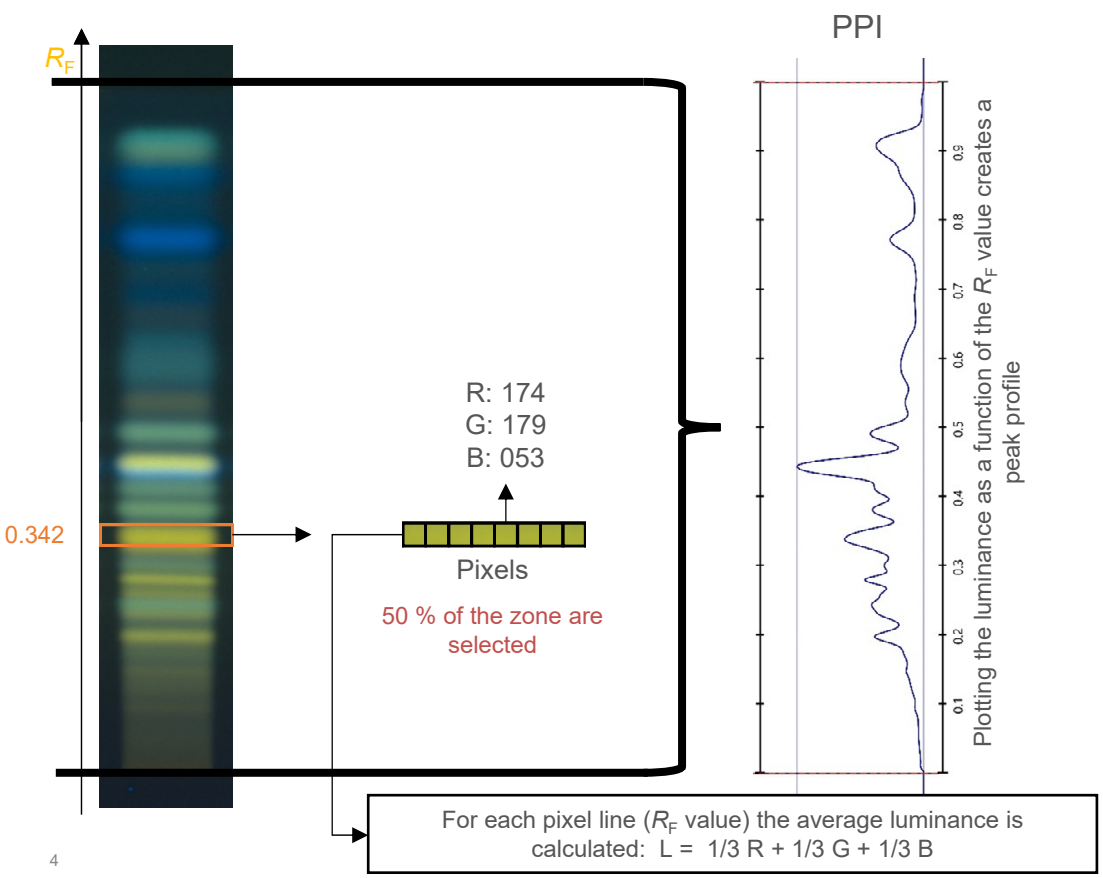
At its 168th session, the European Phamacopoeia (Ph. Eur.) Commission adopted Fritillariae thunbergii bulbus (2588), the first monograph to describe a test for minimum content of two markers (peimine and peiminine) by high-performance thin layer chromatography (HPTLC).

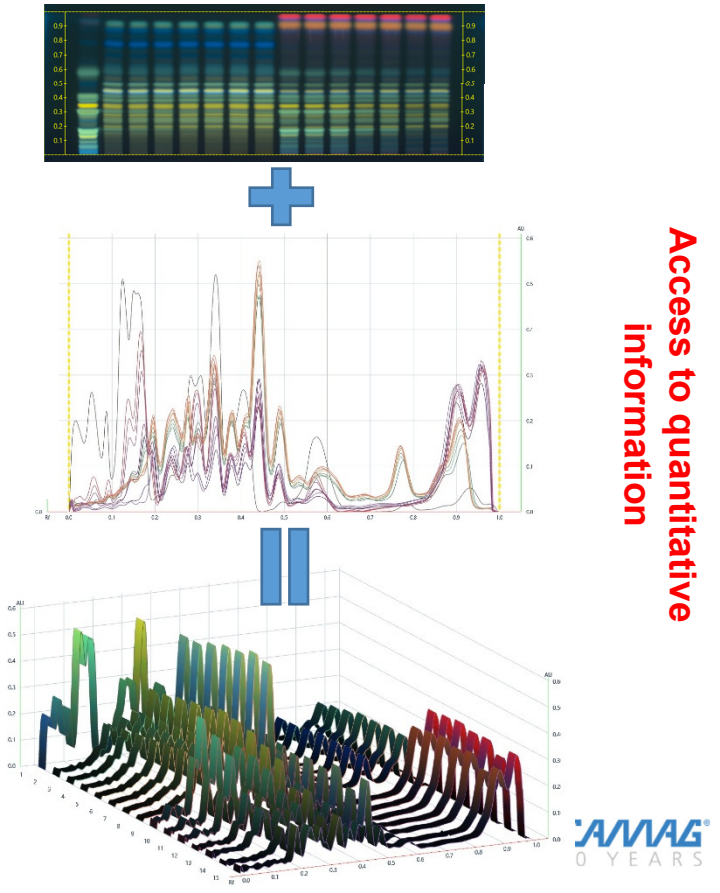
EDQM | STRASBOURG, FRANCE | 15/01/2021

At its 168th session, the European Phamacopoeia (Ph. Eur.) Commission adopted Fritillariae thunbergii bulbus (2588), the first monograph to describe a test for minimum content of two markers (peimine and peiminine) by high-performance thin layer chromatography (HPTLC). The decision to allow semi-quantitative HPTLC analysis as an alternative quality control test for non-authorised TCMs was taken by the Commission at its 163rd session (March 2019). At this session, the results of the pilot study carried out by the TCM Working Party (WP) were presented and showed that semi-quantitative HPTLC led to the same pass/fail results as an LC-assay, and therefore that it was a suitable method for quality control. The test can be carried out by the analyst in the context of the identification by HPTLC, thus it represents a user-friendly alternative that give the same information as the classical LC assay.

This approach will be used by the TCM WP for future elaboration of new monographs when deemed appropriate; it is limited to herbal drugs which do not have a marketing authorisation and to the determination of analytical markers.

Background

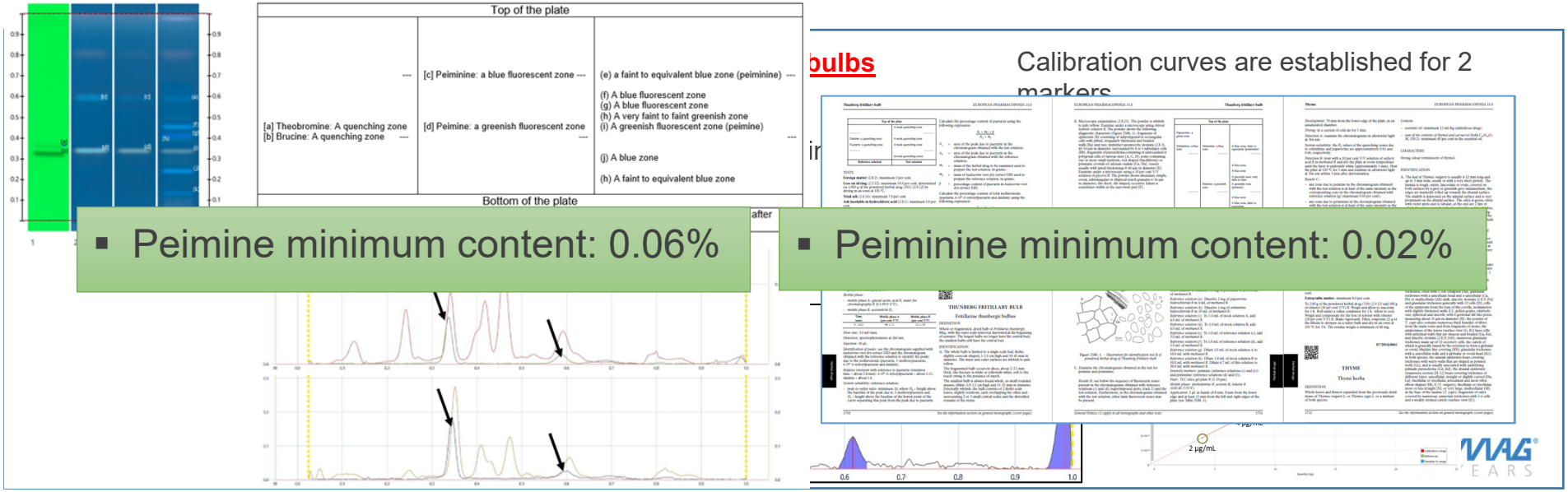




Access to quantitative information

Background

- At its 168th session, the European Phamacopoeia (Ph. Eur.) Commission adopted *Fritillariae thunbergii* bulbus (2588), the **first** monograph to describe a test for **minimum content of two markers** (peimine and peiminine) by high-performance thin layer chromatography (HPTLC).



- Peimine minimum content: 0.06%
- Peiminine minimum content: 0.02%

Recent development

- Case Studies by the TCM WP
- HOM Working Party Contributions
- Universal HPTLC Mixture (UHM) for System Suitability Testing

Recent development

CASE STUDIES BY THE TCM WP

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Recent development

CASE STUDIES BY THE TCM WP

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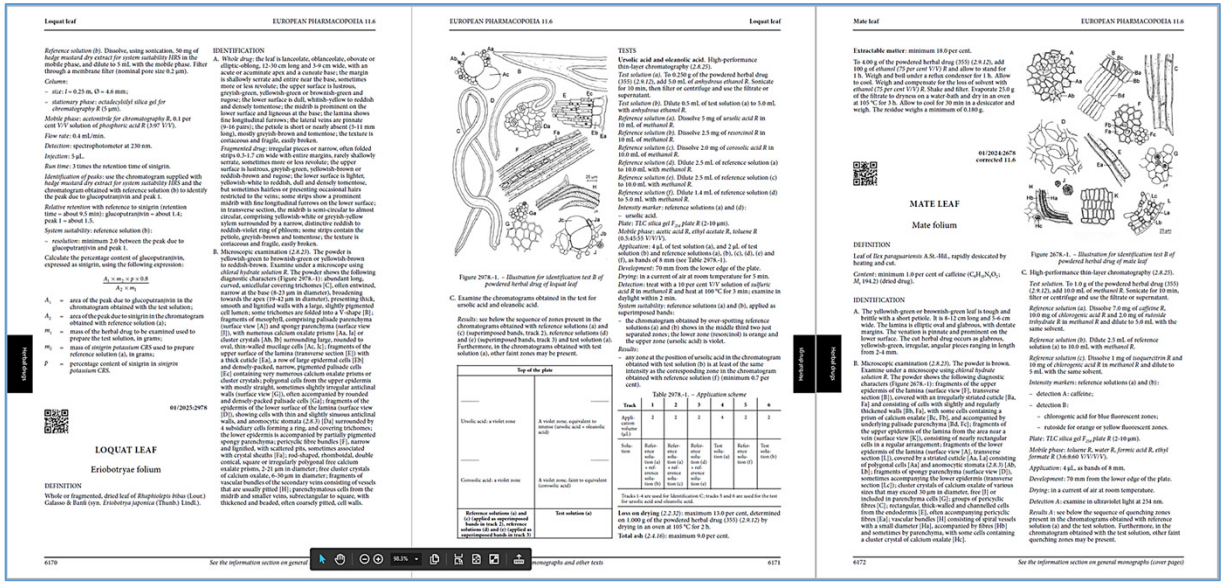
Eriobotryae folium (Loquat leaf) – published 01/2025:2978
Test for minimum content of ursolic acid



Loquat leaf EUROPEAN PHARMACOPOEIA 11.6

Reference solution (b). Dissolve, using sonication, 50 mg of *hedge mustard dry extract for system suitability HRS* in the mobile phase, and dilute to 5 mL with the mobile phase. Filter through a membrane filter (nominal pore size 0.2 µm).

Column:
- *size:* l = 0.25 m, Ø = 4.6 mm;
- *stationary phase: octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: acetonitrile for chromatography R, 0.1 per cent *V/V* solution of *phosphoric acid R* (3.97 *V/V*).

Flow rate: 0.4 mL/min.

Detection: spectrophotometer at 230 nm.

Injection: 5 µL.

Run time: 3 times the retention time of sinigrin.

Identification of peaks: use the chromatogram supplied with *hedge mustard dry extract for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to glucoputranjivin and peak 1.

Relative retention with reference to sinigrin (retention time = about 9.5 min): glucoputranjivin = about 1.4; peak 1 = about 1.5.

System suitability: reference solution (b):
- *resolution:* minimum 2.0 between the peak due to glucoputranjivin and peak 1.

Calculate the percentage content of glucoputranjivin, expressed as sinigrin, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.8}{A_2 \times m_1}$$

A_1 = area of the peak due to glucoputranjivin in the chromatogram obtained with the test solution;
A_2 = area of the peak due to sinigrin in the chromatogram obtained with reference solution (a);
m_1 = mass of the herbal drug to be examined used to prepare the test solution, in grams;
m_2 = mass of *sinigrin potassium CRS* used to prepare reference solution (a), in grams;
P = percentage content of sinigrin in *sinigrin potassium CRS*.

01/2025:2978

LOQUAT LEAF

Eriobotryae folium

DEFINITION

Whole or fragmented, dried leaf of *Rhaphiolepis bibas* (Lour.) Galasso & Banfi (syn. *Eriobotrya japonica* (Thunb.) Lindl.).

IDENTIFICATION

A. *Whole drug:* the leaf is lanceolate, oblanceolate, obovate or elliptic-oblong, 12-30 cm long and 3-9 cm wide, with an acute or acuminate apex and a cuneate base; the margin is shallowly serrate and entire near the base, sometimes more or less revolute; the upper surface is lustrous, greyish-green, yellowish-green or brownish-green and rugose; the lower surface is dull, whitish-yellow to reddish and densely tomentose; the midrib is prominent on the lower surface and ligneous at the base; the lamina shows fine longitudinal furrows; the lateral veins are pinnate (9-16 pairs); the petiole is short or nearly absent (5-11 mm long), mostly greyish-brown and tomentose; the texture is coriaceous and fragile, easily broken.

Fragmented drug: irregular pieces or narrow, often folded strips 0.3-1.7 cm wide with entire margins, rarely shallowly serrate, sometimes more or less revolute; the upper surface is lustrous, greyish-green, yellowish-brown or reddish-brown and rugose; the lower surface is lighter, yellowish-white to reddish, dull and densely tomentose, but sometimes hairless or presenting occasional hairs restricted to the veins; some strips show a prominent midrib with fine longitudinal furrows on the lower surface; in transverse section, the midrib is semi-circular to almost circular, comprising yellowish-white or greyish-yellow xylem surrounded by a narrow, distinctive reddish to reddish-violet ring of phloem; some strips contain the petiole, greyish-brown and tomentose; the texture is coriaceous and fragile, easily broken.

B. Microscopic examination (2.8.23). The powder is yellowish-green to brownish-green or yellowish-brown to reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2978.-1): abundant long, curved, unicellular covering trichomes [C], often entwined, narrow at the base (8-23 µm in diameter), broadening towards the apex (19-42 µm in diameter), presenting thick, smooth and lignified walls with a large, slightly pigmented cell lumen; some trichomes are folded into a V-shape [B]; fragments of mesophyll, comprising palisade parenchyma (surface view [A]) and spongy parenchyma (surface view [I]), with numerous calcium oxalate prisms [Aa, Ia] or cluster crystals (Ab, Ib) surrounding large, rounded to oval, thin-walled mucilage cells [Ac, Ic]; fragments of the upper surface of the lamina (transverse section [E]) with a thick cuticle [Ea], a row of large epidermal cells [Eb] and densely-packed, narrow, pigmented palisade cells [Ec] containing very numerous calcium oxalate prisms or cluster crystals; fragments of the lower surface of the lamina with mostly straight, sometimes slightly irregular anticlinal walls (surface view [G]), often accompanied by rounded and densely-packed palisade cells [Ga]; fragments of the epidermis of the lower surface of the lamina (surface view [D]), showing cells with thin and slightly sinuous anticlinal walls, and anomocytic stomata (2.8.3) [Da] surrounded by a subsidiary cells forming a ring, and covering trichomes; the lower epidermis is accompanied by partially pigmented spongy parenchyma; pericyclic fibre bundles [F], narrow and lignified, with scattered pits, sometimes associated with crystal sheaths [Fa]; rod-shaped, rhomboidal, double conical, square or irregularly polygonal free calcium oxalate prisms, 2-21 µm in diameter; free cluster crystals of calcium oxalate, 6-50 µm in diameter; fragments of vascular bundles of the secondary veins consisting of vessels that are usually pitted [H]; parenchymatous cells from the midrib and smaller veins, subrectangular to square, with thickened and beaded, often coarsely pitted, cell walls.

6170 *See the information section on general monographs and other texts*

EUROPEAN PHARMACOPOEIA 11.6 Loquat leaf

Figure 2978.-1. – *Illustration for identification test B of powdered herbal drug of loquat leaf*

C. Examine the chromatograms obtained in the test for ursolic acid and oleanolic acid.

Results: see below the sequence of zones present in the chromatograms obtained with reference solutions (a) and (c) (superimposed bands, track 2), reference solutions (d) and (e) (superimposed bands, track 3) and test solution (a). Furthermore, in the chromatograms obtained with test solution (a), other faint zones may be present.

TESTS

Ursolic acid and oleanolic acid. High-performance thin-layer chromatography (2.8.25).

Test solution (a). To 0.250 g of the powdered herbal drug (355) (2.9.12), add 5.0 mL of *anhydrous ethanol R*. Sonicate for 10 min, then filter or centrifuge and use the filtrate or supernatant.

Test solution (b). Dilute 0.5 mL of test solution (a) to 5.0 mL with *anhydrous ethanol R*.

Reference solution (a). Dissolve 5 mg of *ursolic acid R* in 10 mL of *methanol R*.

Reference solution (b). Dissolve 2.5 mg of *rosenmol R* in 10 mL of *methanol R*.

Reference solution (c). Dissolve 2.0 mg of *corosolic acid R* in 10.0 mL of *methanol R*.

Reference solution (d). Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

Reference solution (e). Dilute 2.5 mL of reference solution (c) to 10.0 mL with *methanol R*.

Reference solution (f). Dilute 1.4 mL of reference solution (d) to 5.0 mL with *methanol R*.

Intensity marker: reference solutions (a) and (d):
- ursolic acid.

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

Mobile phase: acetic acid R, ethyl acetate R, toluene R (0.5:45:55 *V/V/V*).

Application: 4 µL of test solution (a), and 2 µL of test solution (b) and reference solutions (a), (b), (c), (d), (e) and (f), as bands of 8 mm (see Table 2978.-1).

Development: 70 mm from the lower edge of the plate.

Drying: in a current of air at room temperature for 5 min.

Detection: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R* and heat at 100 °C for 3 min; examine in daylight within 2 min.

System suitability: reference solutions (a) and (b), applied as superimposed bands:
- the chromatogram obtained by over-spotting reference solutions (a) and (b) shows in the middle third two just separated zones; the lower zone (resorcinol) is orange and the upper zone (ursolic acid) is violet.

Results:
- any zone at the position of ursolic acid in the chromatogram obtained with test solution (b) is at least of the same intensity as the corresponding zone in the chromatogram obtained with reference solution (f) (minimum 0.7 per cent).

Table 2978.-1. – *Application scheme*

Track	1	2	3	4	5	6
Application volume (µL)	2	2	2	4	2	2
Solution	Reference solution (a) + reference solution (b)	Reference solution (a) + reference solution (b)	Reference solution (d) + reference solution (e)	Test solution (a)	Reference solution (f)	Test solution (b)

Tracks 1-4 are used for Identification C; tracks 5 and 6 are used for the test for ursolic acid and oleanolic acid.

Loss on drying (2.2.32): maximum 13.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (2.9.12) by drying in an oven at 103 °C for 2 h.

Total ash (2.4.16): maximum 9.0 per cent.

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Mate leaf EUROPEAN PHARMACOPOEIA 11.6

Extractable matter: minimum 18.0 per cent.

To 4.00 g of the powdered herbal drug (355) (2.9.12), add 100 g of *ethanol (75 per cent V/V) R* and allow to stand for 1 h. Weigh and boil under a reflux condenser for 1 h. Allow to cool. Weigh and compensate for the loss of solvent with *ethanol (75 per cent V/V) R*. Shake and filter. Evaporate 25.0 g of the filtrate to dryness on a water-bath and dry in an oven at 105 °C for 3 h. Allow to cool for 30 min in a desiccator and weigh. The residue weighs a minimum of 0.180 g.

01/2024:2678
corrected 11.6

MATE LEAF

Mate folium

DEFINITION

Leaf of *Ilex paraguariensis* A.St.-Hil., rapidly desiccated by heating and cut.

Content: minimum 1.0 per cent of caffeine ($C_8H_{10}N_4O_2$; M_r 194.2) (dried drug).

IDENTIFICATION

A. The yellowish-green or brownish-green leaf is tough and brittle with a short petiole. It is 6-12 cm long and 5-6 cm wide. The lamina is elliptic, oval and glabrous, with dentate margins. The venation is pinnate and prominent on the lower surface. The cut herbal drug occurs in pieces, yellowish-green, irregular, angular pieces ranging in length from 2-4 mm.

B. Microscopic examination (2.8.23). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2678.-1): fragments of the upper epidermis of the lamina (surface view [E], transverse section [B]), covered with an irregularly striated cuticle [Ba, Ea] and consisting of cells with slightly and regularly thickened walls [Bb, Ea], with some cells containing a prism of calcium oxalate [Bc, Eb], and accompanied by underlying palisade parenchyma [Bd, Ec]; fragments of the upper epidermis of the lamina from the area near a vein (surface view [K]), consisting of nearly rectangular cells in a regular arrangement; fragments of the lower epidermis of the lamina (surface view [A], transverse section [L]), covered by a striated cuticle [Aa, La] consisting of trigonal cells [Aa] and anomocytic stomata (2.8.3) [Ab, Lb]; fragments of spongy parenchyma (surface view [D]), sometimes accompanied by cluster crystals of calcium oxalate; transverse section [Lc]); cluster crystals of calcium oxalate of various sizes that may exceed 30 µm in diameter, free [I] or included in parenchyma cells [G]; groups of pericyclic fibres [C], rectangular, thick-walled and channelled cells from the endodermis [E], often accompanying pericyclic fibres [Ea]; vascular bundles [H] consisting of spiral vessels with a small diameter [Ha], accompanied by fibres [Hb] and sometimes by parenchyma, with some cells containing a cluster crystal of calcium oxalate [Hc].

Figure 2678.-1. – *Illustration for identification test B of powdered herbal drug of mate leaf*

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

Test solution. To 1.0 g of the powdered herbal drug (355) (2.9.12), add 10.0 mL of *methanol R*. Sonicate for 10 min, filter or centrifuge and use the filtrate or supernatant.

Reference solution (a). Dissolve 7.0 mg of *caffeine R*, 10.0 mg of *chlorogenic acid R* and 2.0 mg of *rutoside trihydrate R* in *methanol R* and dilute to 5.0 mL with the same solvent.

Reference solution (b). Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

Reference solution (c). Dissolve 1 mg of *isoquercitrin R* and 10 mg of *chlorogenic acid R* in *methanol R* and dilute to 5 mL with the same solvent.

Intensity markers: reference solutions (a) and (b):
- detection A: caffeine;
- detection B:
  - chlorogenic acid for blue fluorescent zones;
  - rutoside for orange or yellow fluorescent zones.

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

Mobile phase: toluene R, water R, formic acid R, ethyl formate R (3.6:6.6:60 *V/V/V/V*).

Application: 4 µL, as bands of 8 mm.

Development: 70 mm from the lower edge of the plate.

Drying: in a current of air at room temperature.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of quenching zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint quenching zones may be present.

6172 *See the information section on general monographs (cover pages)*

CAMAG 60 YEARS

Recent development

CASE STUDIES BY THE TCM WP

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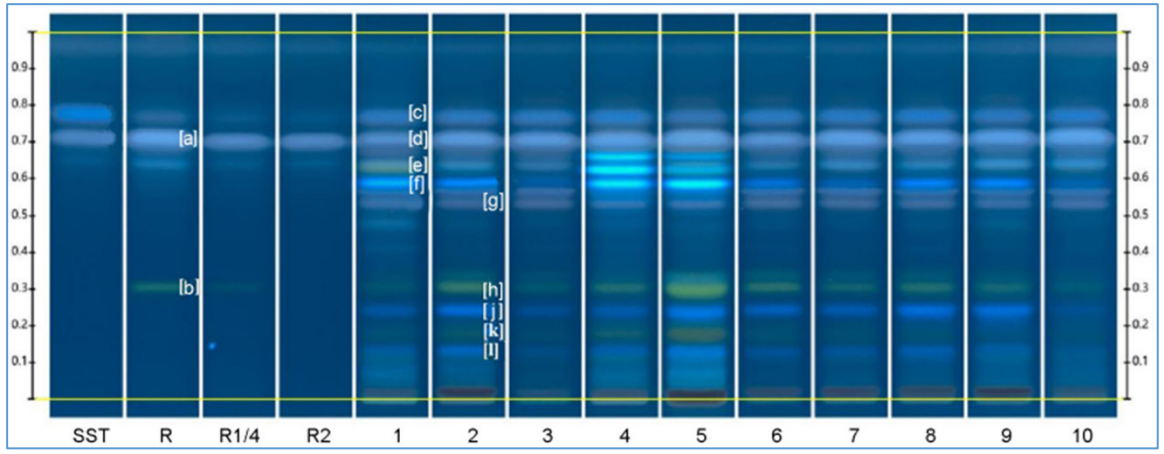
Pinelliae rhizomae praeparatum – draft monograph 2655
Consolidated comments from Pharmeuropa 35.4 and final discussion



CAMAG 60 YEARS

Recent development

CASE STUDIES BY THE TCM WP

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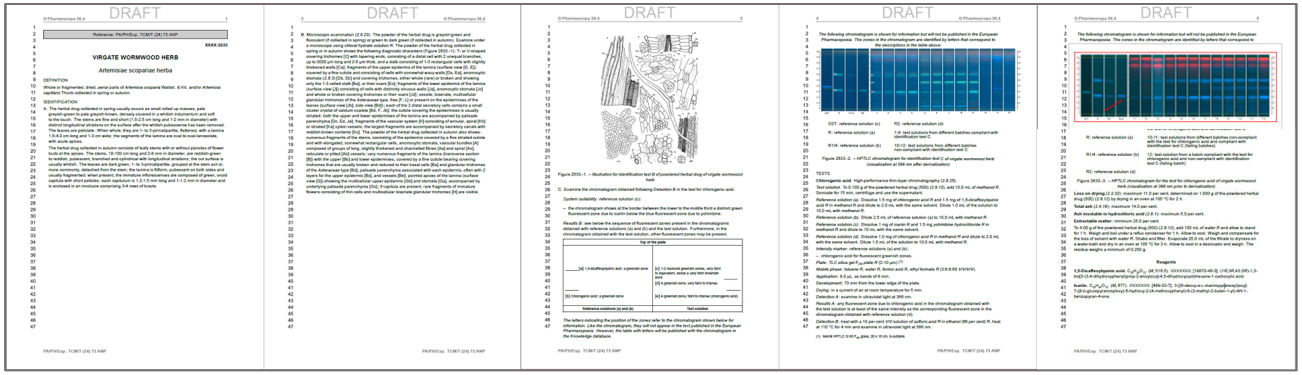
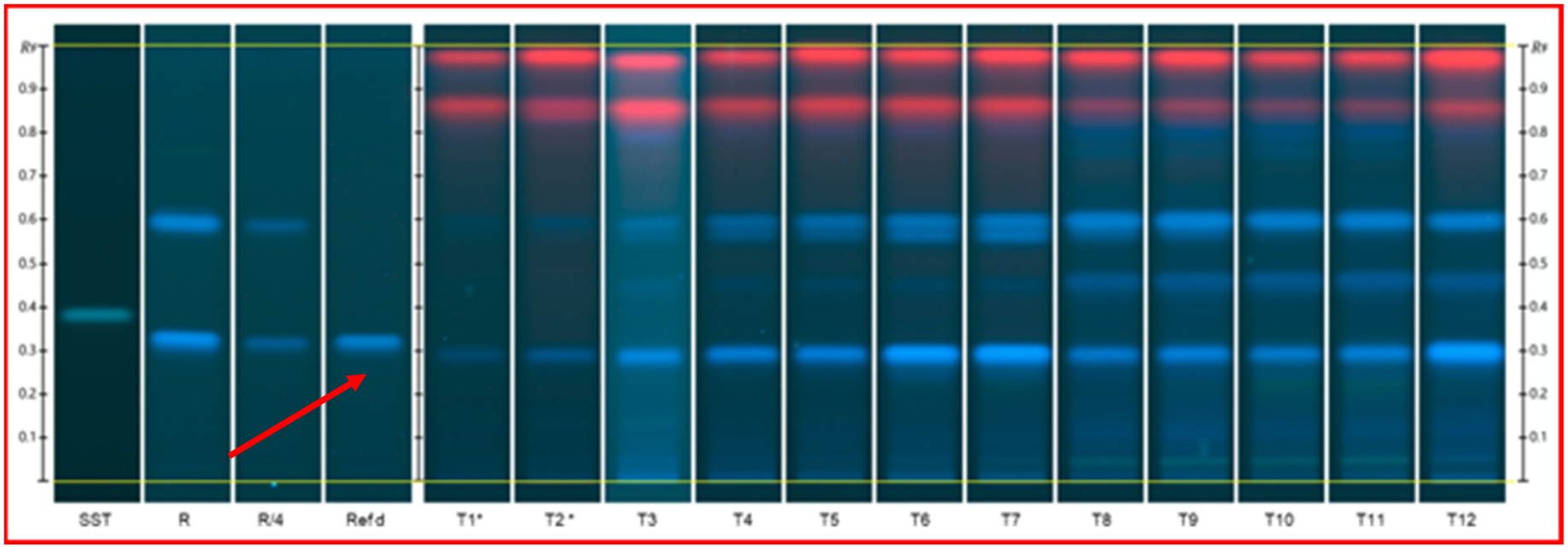


Artemisiae scopariae herba – in Pharmeuropa for comment (31.12.2024)

Test for minimum content of chlorogenic acid


Rf
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
SST
R
R/4
Ref d
T1*
T2*
T3
T4
T5
T6
T7
T8
T9
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T11
T12

Recent development

CASE STUDIES BY THE TCM WP

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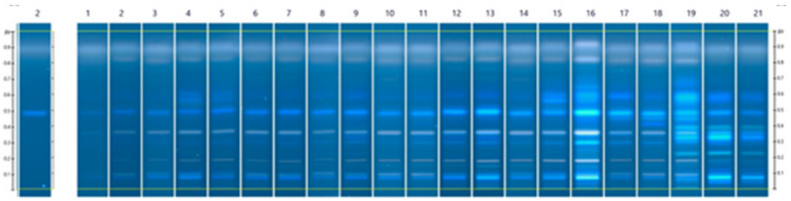
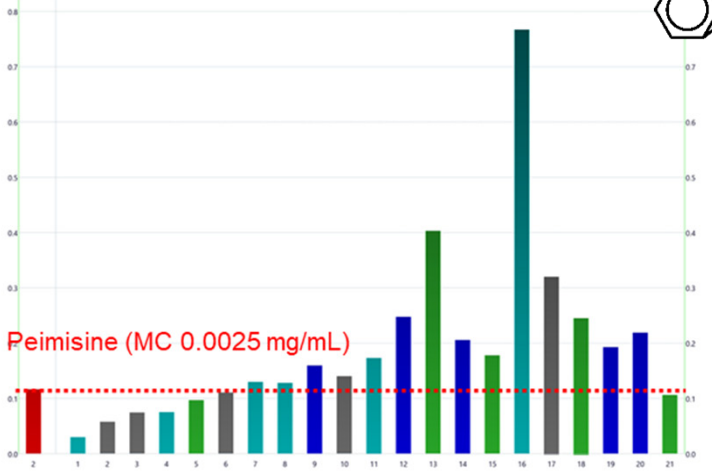
Identification



Peak profile from images (PPI) and fingerprints of all samples

Test for minimum content



Fritillaria cirrhosae bulbous – in progress
Test for minimum content of peimisine

Recent development

CASE STUDIES BY THE TCM WP

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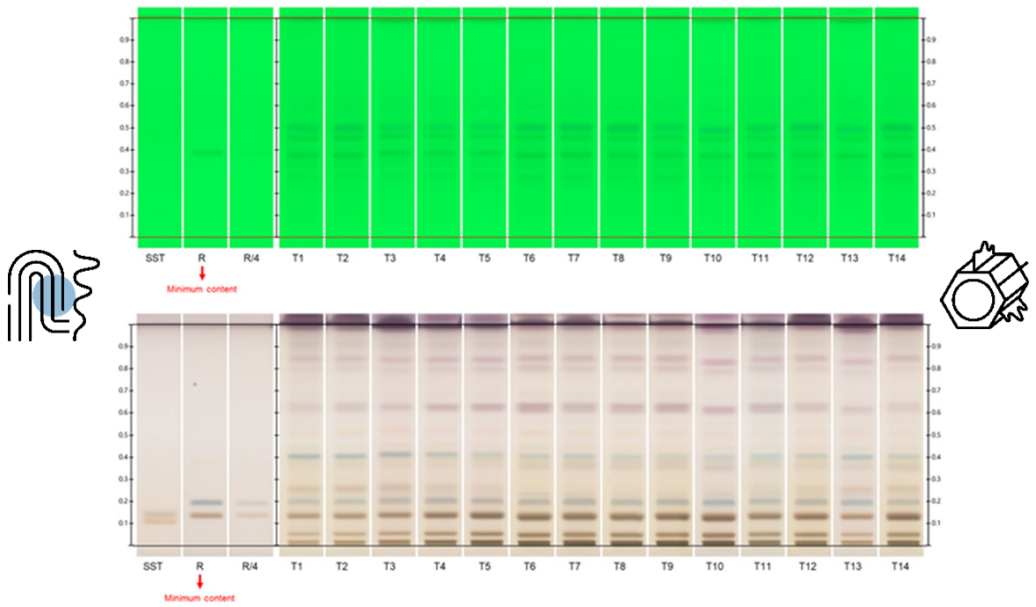


Ziziphi spinosae semen (Spine date seed) – in progress
Test for minimum content of jujuboside A and spinosin

Recent development

HOM WP CONTRIBUTIONS

Many discussions on the use of HPTLC in the HOM WP

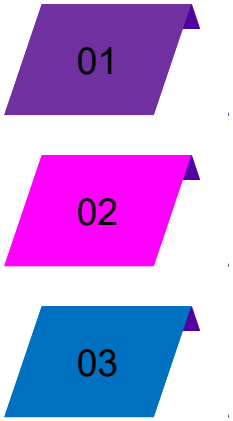
Published in the Pharmeuropa 36.2 April 2024

In an attempt to harmonise the current practices in Europe, the European Pharmacopoeia Commission has decided to launch a pilot study on the use of the High-Performance Thin-Layer Chromatography (HPTLC) method for a semi-quantitative evaluation of the levels of defined markers as quality control in monographs on homoeopathic preparations containing non-toxic compounds.

Recent development

HOM WP CONTRIBUTIONS

- Pilot study



Recent development

HOM WP CONTRIBUTIONS

- Pilot study

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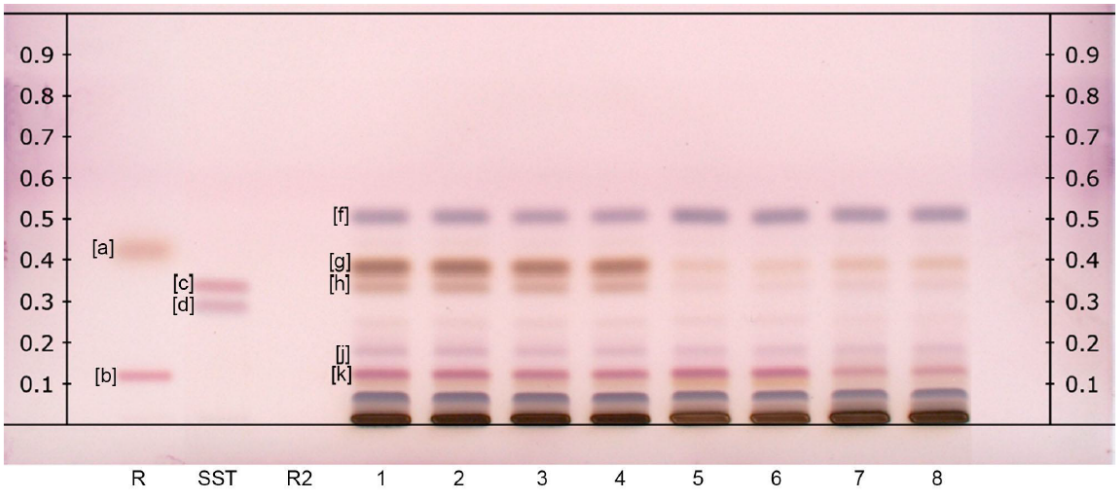
Chamomilla for homoeopatic preparations in Pharmeuropa xxxx:2493



R: reference solution (a)

SST: reference solution (b)

R2: reference solution (c), used for detection A

1-8: test solutions from different batches of mother tincture.

Recent development

HOM WP CONTRIBUTIONS

- Pilot study

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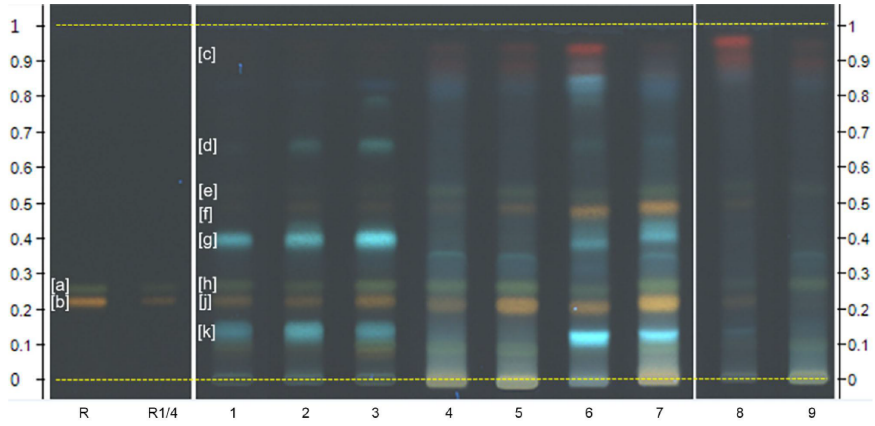
Calendula for homoeopatic preparations in Pharmeuropa xxxx:2493

R: reference solution (a)	1-3: test solutions from different batches of mother tinctures according to method 1.1.10
R1/4: reference solution (b)	4-5: test solutions from different batches of mother tinctures produced using method 1.1.3
	6: test solution from a batch of mother tincture produced using method 1.1.5
	7: test solution from a batch of mother tincture produced using method 1.1.3
	8: test solution from a batch of mother tincture produced using method 1.1.5 out of specification
	9: test solution from a batch of mother tincture produced using method 1.1.3 out of specification



Recent development

HOM WP CONTRIBUTIONS

- Pilot study

01

02

03 *Arnica planta tota for homoeopatic preparations*
In progress

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

A System Suitability Test (SST) shall verify that analytical method was suitable for its intended purpose when the analysis was performed.

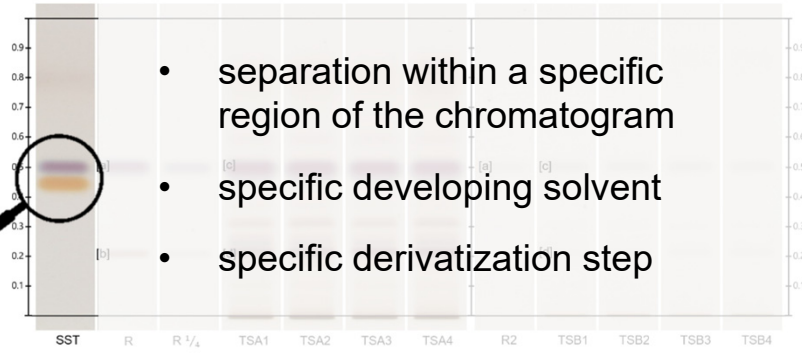
In 2.8.25

System-specific suitability test. This 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 (for example, chlorogenic acid and hyperoside in chromatographic systems used for onoids). The results for the test and reference solutions only [illegible] satisfies the separation requirement stated in the individual monograph.

Procurement of chemical reference substances required for these SSTs may:

- add significant cost to routine analyses if laboratories are applying many different HPTLC methods,
- be time consuming if laboratories have to develop and validate different sets of SSTs.

Loquat leaf – 01/2025:2978



- separation within a specific region of the chromatogram
- specific developing solvent
- specific derivatization step

SST: Reference solutions (a) and (b)
R: Reference solutions (c) and (d)
R¼: Reference solutions (e) and (f)
R2: Reference solution (g)
TSA1-4: Test solutions from different batches of Eriobotrya (50 mg/mL)
TSB1-4: Test solutions from different batches of Eriobotrya, (5 mg/mL)

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

- A more practicable solution is needed for qualifying HPTLC results in routine analysis!

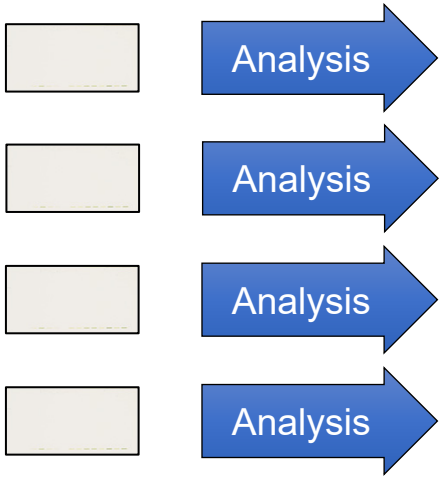
A different and more holistic approach is desired:

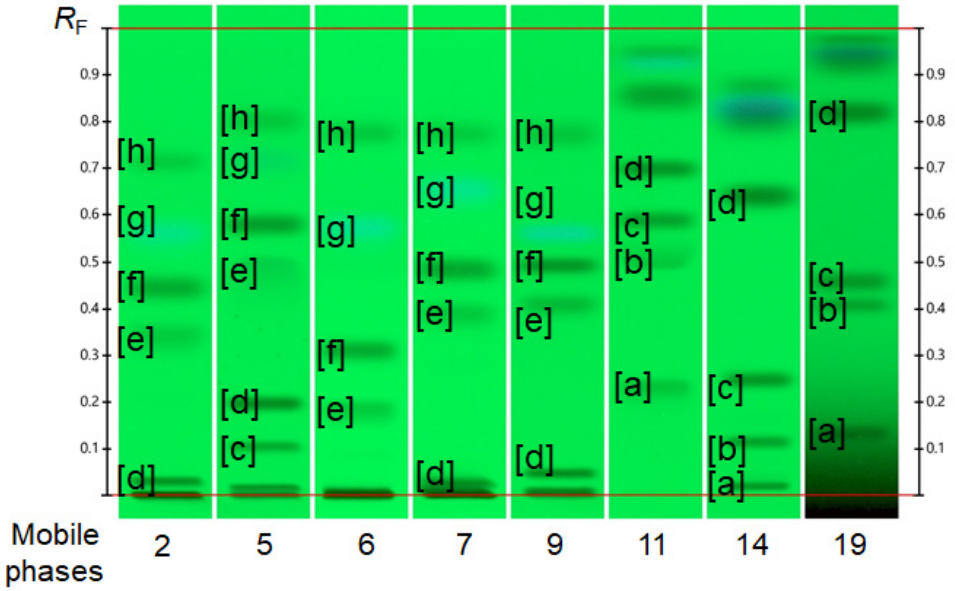
- An “universal” mixture of substances that qualifies the entire R_F range of an HPTLC chromatogram and can be used for different HPTLC methods

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

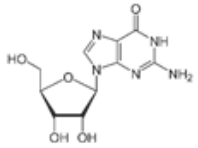
One mixture



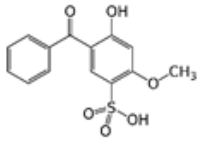


- ✓ cover a wide polarity range
- ✓ achieve an even distribution of at least 3 – 4 substances throughout the entire chromatogram

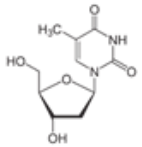
[a] Guanosine



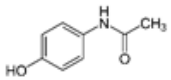
[b] Sulisobenzone



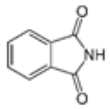
[c] Thymidine



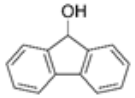
[d] Paracetamol



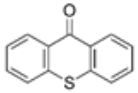
[e] Phthalimide



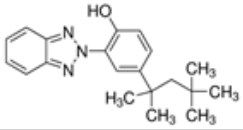
[f] 9-Hydroxyfluorene



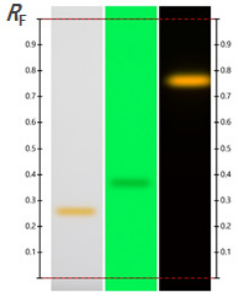
[g] Thioxanthone



[h] 2-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol

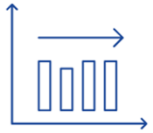


✓ Detectable without derivatization



✓ Minimal hazard

✓ Stable in solution for at least two months



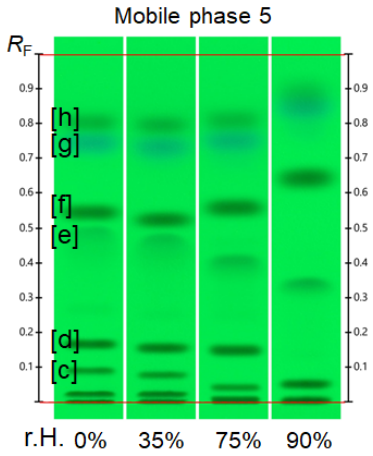
✓ Easily available

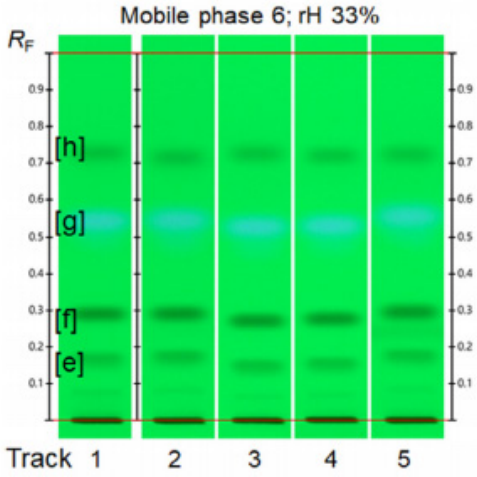
✓ Inexpensive (< 50 CHF/g)

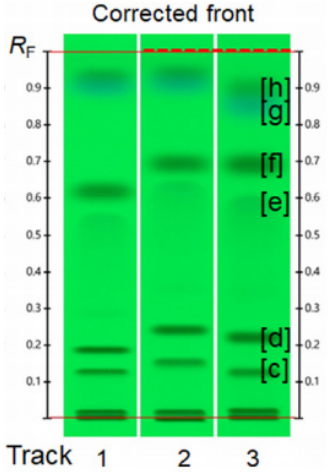
CAMAG 60 YEARS

Response characteristics

Capacity of the UHM to detect changes in chromatographic conditions:







- ✓ General influence of humidity on R_F values can be well detected
- ✓ General influence of mobile phase composition (± 10%) on R_F values can be well detected
- ✓ General influence of the saturation (with and without) on R_F values can be well detected

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

We have initiated a collaborative trial to evaluate the UHM.
Data from four laboratories using different levels of instrumentation (manual and automated) have been received. The initial results showed:

- Using the same developing solvent, the R_F values are similar, with $\Delta R_F < 0.02$.
- For one batch of UHM, the R_F values were consistent with those from other laboratories, but an additional zone was detected.
  - ➢ It appears that the UHM batch sent was not compliant

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

01

02

03

04

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

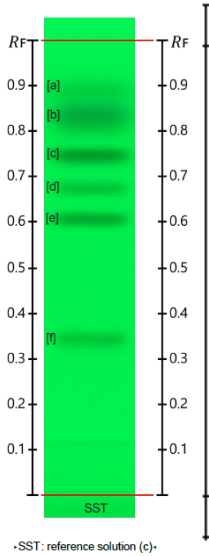
01

02

03

04

Urticae radix (Nettle root)
in Pharmeuropa xxxx:2538



‣SST: reference solution (c)‣

‣Top of the plate‣

‣[a] A quenching zone‣
‣[b] A quenching zone‣
‣[c] A quenching zone‣
‣[d] A quenching zone‣
‣_____‣
‣[e] A quenching zone‣

‣[f] A quenching zone‣
‣_____‣

‣Reference solution (c)‣

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

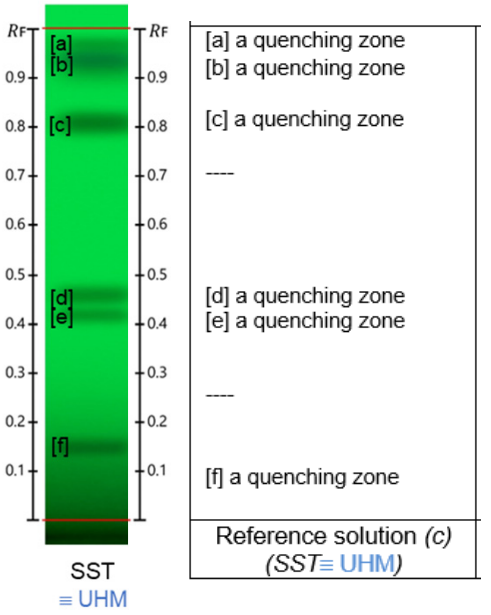
01

02

03

04

Uncaria tomentosa cortex
planned for Pharmeuropa 37.1



Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

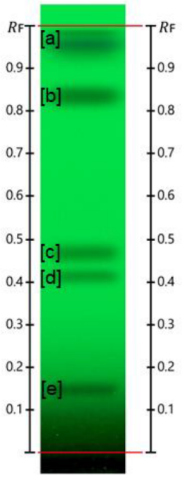
01

02

03

04

Peppermint leaf dry extract
In progress



Top of the plate
[a] Phthalimide, 9-Hydroxyfluorene, Thioxanthone, Octrizole a quenching zone
[b] Paracetamol: a quenching zone
----
[c] Thymidine: a quenching zone
[d] Sulisobenzone: a quenching zone
_____
[e] Guanosine: a quenching zone
Reference solution (c)

Recent development

UNIVERSAL HPTLC MIXTURE (UHM) FOR SYSTEM SUITABILITY TESTING

01

02

03

04

All monographs, currently under evaluation

Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC

- Based on the new concepts and approaches developed since 2018, a revision of general chapter 2.8.25 has been proposed in Pharmeuropa for comments until 31 December 2024.

EUROPEAN PHARMACOPOEIA 9.0 — 2.8.25. HPTLC of herbal drugs and herbal drug preparations

m = mass of the herbal drug used to prepare the test solution, in grams;
V_1 = volume of dilution, in millilitres;
V_t = aliquot taken for immunoaffinity clean-up, in millilitres;
V_2 = volume in which the residue is taken up, in millilitres;
C = measured ochratoxin A concentration of the test solution, in nanograms per millilitre.

Appendix: Decontamination procedures for laboratory glassware

Rinse glassware with *methanol R* and decontaminate by immersion in *strong sodium hypochlorite solution R* for at least 2 h, then wash thoroughly with water.

04/2010:20823

2.8.23. MICROSCOPIC EXAMINATION OF HERBAL DRUGS

The microscopic examination of herbal drugs is carried out on the powdered drug (355) (*2.9.12*) unless otherwise prescribed in the monograph.

Chloral hydrate solution R is the most commonly prescribed reagent. However, certain features are not visible or not easily seen after mounting in this reagent. In this case, other reagents are used, for example, a 50 per cent *V/V* solution of *glycerol R*, which makes it possible to visualise starch granules. It may also be necessary to prescribe specific reagents in 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 are used to identify the presence of lignin in cells or tissues, *ruthenium red solution R*, which is used to show the presence of mucilage in cells or *glycerol R* used to show the presence of starch and inulin.

Examination under polarised light (between crossed nicol prisms) is used to identify starch granules (black cross phenomenon), calcium oxalate crystals (refringence) or lignified structures.

MOUNTING IN CHLORAL HYDRATE SOLUTION

Place 2-3 drops of *chloral hydrate solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling on a hot plate or a micro gas burner. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using 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.

Chloral hydrate tends to crystallise as long needles. To avoid this, proceed as follows: after heating, remove the cover slip; to the preparation add 1 drop of a 10 per cent *V/V* mixture of *chloral hydrate solution R* in *glycerol R*; place a clean cover slip on the preparation; examine under a microscope.

MOUNTING IN A 50 PER CENT *V/V* SOLUTION OF GLYCEROL

Place 2 drops of a 50 per cent *V/V* solution of *glycerol R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Examine under a microscope.

MOUNTING IN A 10 PER CENT *V/V* ALCOHOLIC SOLUTION OF PHLOROGLUCINOL AND HYDROCHLORIC ACID

Place a very small quantity of the powdered drug on a glass microscope slide. Add 1-2 drops of a 10 per cent *V/V* alcoholic solution of *phloroglucinol R*. Mix and allow the solvent to evaporate almost completely. Add 1-2 drops of *hydrochloric acid R* and cover the preparation with a cover slip. Examine immediately under a microscope. The red colour indicates the presence of lignin.

MOUNTING IN LACTIC REAGENT

Place 2-3 drops of *lactic reagent R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Lignified structures stain bright yellow; structures containing cellulose remain colourless. Starch granules stain more or less violet; certain secretions (e.g., essential oils, resins, oleoresins) stain orange and cork stains red.

MOUNTING IN RUTHENIUM RED SOLUTION

Place 2 drops of *ruthenium red solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. After about 1 minute, allow a drop of *distilled water R* to be taken up between the slide and the cover slip. Examine under a microscope. The mucilage stains violet red.

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

2. Methods of analysis — 2. Methods of analysis

2.8.25. HPTLC of herbal drugs and herbal drug preparations — EUROPEAN PHARMACOPOEIA 9.0

EQUIPMENT

The equipment used for qualitative HPTLC typically consists of:
- glass plates, as described above, usually 20 × 10 cm in size;
- devices suitable for the application of specified volumes of solutions as bands and allowing control of the dimensions and position of application;
- a device suitable for conditioning the stationary phase at the prescribed relative humidity;
- a suitable chromatographic tank (for example, a twin trough chamber);
- a device suitable for the reproducible drying of the developed plate;
- devices suitable for the application of reagents to, and heating of, the plate as part of the derivatisation procedure;
- a system suitable for the electronic documentation of chromatograms under 254 nm UV, 366 nm UV and white light.

NOTE: normal thin-layer chromatographic methods using glass plates or sheets coated with particles of 5-40 µm or HPTLC aluminium-backed sheets may be used, provided that the results obtained fulfil the general system suitability criteria that the bands develop perpendicular to the lower edge of the plate and the solvent front is parallel to the upper edge of the plate, and satisfy the system-specific suitability test stated in the individual monograph.

METHOD

Preparation of test solution. Unless otherwise stated in the individual monograph, the test solution is usually prepared as follows.

For dry herbal drugs or dry herbal extracts, mix 0.5 g of the powdered herbal drug or 0.1 g of the dry herbal extract with 5 mL of *methanol R* and sonicate for 15 min; filter or centrifuge and use the filtrate or supernatant as the test solution.

For essential oils, dissolve 50 µL of the essential oil 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, reference solutions are usually prepared as follows. Prepare a 1 mg/mL solution of suitable reagent(s) or reference standard(s) in *methanol R* or, for essential oils, in *toluene R*. Prepare a second reference solution (diluted reference solution) by mixing 1 volume of this solution and 3 volumes of the same solvent. Both solutions are used as intensity references.

Intensity marker. Use one or more of the substances in the reference solution and in the diluted reference solution as intensity marker(s) for the evaluation of the chromatogram.

Preparation of system-specific suitability solution. Prepare the solution as stated in the individual monograph.

Sample application and plate layout. Unless otherwise stated in the individual monograph, samples are applied as narrow bands of 8 mm in length at a distance of 8 mm from the lower edge of the plate. The centre 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 (centre to centre) is 11 mm. A maximum of 15 tracks are applied onto a standard plate. If no electronic solvent front detection device is used, the development distance is marked with a pencil close to the right or left edge of the plate.

Conditioning of the plate. Following sample application and unless otherwise stated in the individual monograph, expose the plate to air with a suitable relative humidity obtained using a saturated solution of *magnesium chloride R* (for example, by allowing the plate to stand in a closed chamber containing such a solution for 1 h or by using preconditioned air).

Preparation of the tank and development of the plate. Unless otherwise stated in the individual monograph, the chromatographic separation is performed in a saturated tank. Where a twin trough chamber is used, place a piece of filter paper in the rear trough. Load the tank with a sufficient quantity of mobile phase to wet the filter paper completely and achieve a level of 5 mm in both troughs. With the lid closed, leave the tank for 20 min for saturation. Introduce the plate in a vertical position into the front trough of the tank so that the coating layer faces the filter paper. When the mobile phase has reached 70 mm, remove the plate from the tank and dry in a vertical position in a stream of air at room temperature. Other tank configurations and developing distances may be specified in an individual monograph.

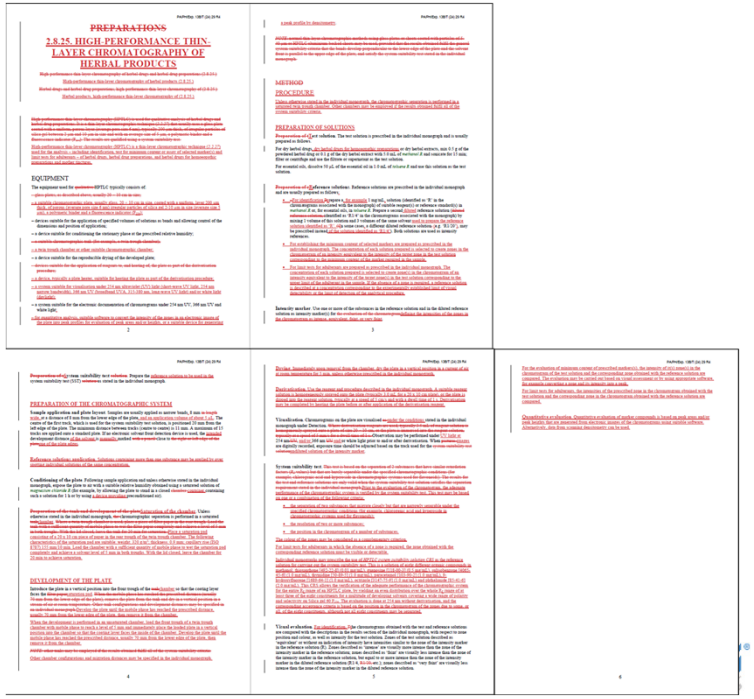
NOTE: other tanks may be employed if the results obtained fulfil all of the system suitability criteria.

Visualisation. Chromatograms on the plate are visualised as stated in the individual monograph. Where derivatisation reagents are used, typically 3.5 mL of reagent solution is homogenously sprayed onto a plate of size 20 × 10 cm, or the plate is immersed into the reagent solution, typically at a speed of 5 mm/s for a dwell time of 1 s. Observation may be performed under 254 nm UV, 366 nm UV or white light prior to and/or after derivatisation. When pictures are digitally recorded, exposure time should be adjusted based on the track with the system-specific suitability solution.

System-specific suitability test. This 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 (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 requirement stated in the individual monograph.

Visual evaluation. The chromatograms obtained with the test and reference solutions are compared against the description, under the results section in the individual monograph, with respect to zone position and colour, as well as intensity for the test solution. Zones of the test solution described in the results table without a descriptor have intensities similar to the zone of the intensity marker in the reference solution. Zones described as 'intense' are visually more intense than the zone of the intensity marker in the reference solution; zones described as 'very faint' are visually less intense than the zone of the intensity marker in the diluted reference solution.

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



PREPARATIONS

2.8.25. HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF HERBAL PRODUCTS

PROCEDURE

METHOD

PREPARATION OF SOLUTIONS

EQUIPMENT

PREPARATION OF THE CHROMATOGRAPHIC SYSTEM

DEVELOPMENT OF THE PLATE

Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC

***Title**: the scope is widened by referring to herbal products, instead of herbal drugs and herbal drug preparations, hence including herbal drugs for homoeopathic preparations and mother tinctures.*

***Equipment**: greater details are given on suitable equipment for performing HPTLC analysis.*

***Preparation of solutions**: additional information is given on minimum content of selected markers, and for limit tests for adulterants.*

***Preparation of the chromatographic system**: application of reference solutions containing more than one substance may, alternatively, be carried out by over spotting individual solutions of the same concentration.*

***Saturation of the chamber**: a description of how to saturate chambers is included.*

***Development of the plate**: a description of how the development in an unsaturated chamber is performed is included.*

Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC

***System suitability test**: the basis for developing system suitability tests is not restricted to the separation of only two substances that migrate closely but that are narrowly separable under the specified chromatographic conditions. Individual monographs may prescribe the use of HPTLC system suitability solution CRS as the reference solution for carrying out the system suitability test. This is a solution of eight different organic compounds dissolved in methanol. This CRS allows the verification of the adequate performance of the chromatographic system for the entire R_F range of an HPTLC plate, by yielding an even distribution over the whole R_F range of at least three of the eight constituents for a multitude of developing solvents covering a wide range of polarity and selectivity on Silica gel 60 F_{254}. The evaluation is done at 254 nm without derivatisation, and the corresponding acceptance criteria is based on the position in the chromatogram of the zones due to some, or all, of the eight constituents, although not all eight constituents may be separated.*

Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC

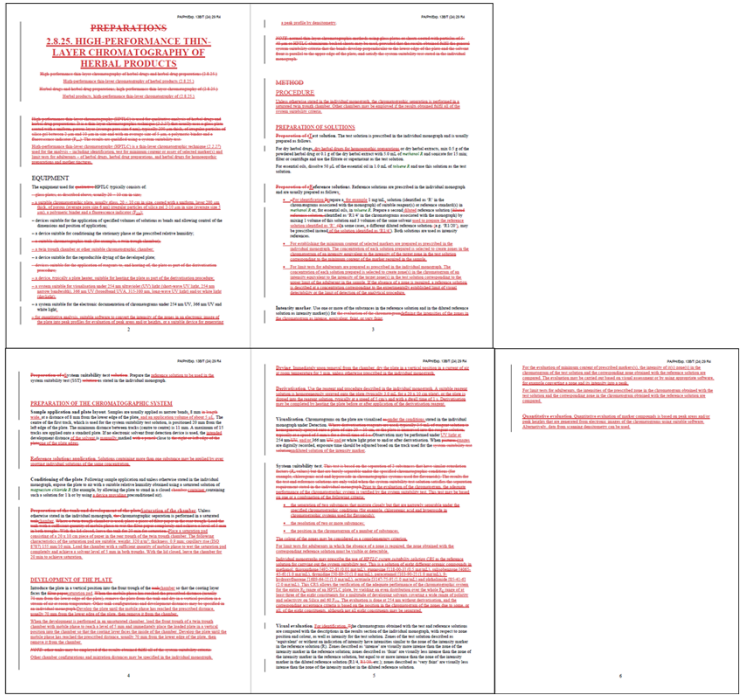
***Visual evaluation**: additional information is given on carrying out the visual evaluation of HPTLC plates for the evaluation of minimum content of prescribed markers(s), and for limit tests for adulterants.*

> *The evaluation may be carried out based on visual assessment or by using appropriate software, for example converting a zone and its intensity into a peak*

***Quantitative evaluation**: a new section on quantitative evaluation is introduced, hence extending the scope of this chapter for the use of HPTLC in quantitative analysis. Performing quantitative analysis by thin layer chromatography is already foreseen in chapter 2.2.27.*

> *Quantitative evaluation of marker compounds is based on peak areas and/or peak heights that are generated from electronic images of the chromatograms using suitable software. Alternatively, data from scanning densitometry can be used*

Revised Chapter 2.8.25 - New Concepts and Approaches in HPTLC



Published for comments until
31 December 2024

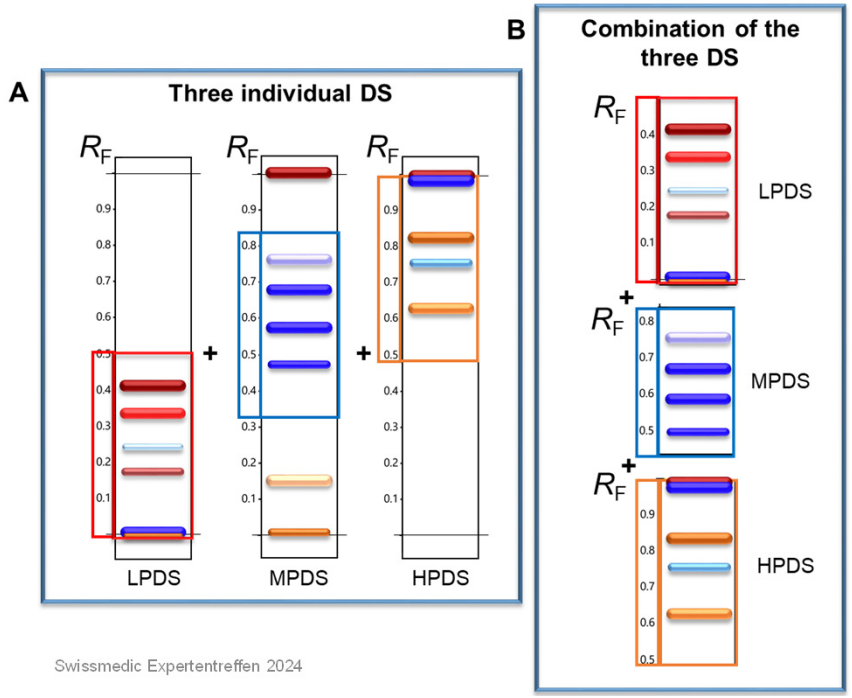
Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications

For a non-targeted screening approach:

- Limited separation due to the short developing distance
- Broadening the selectivity and polarity ranges due to the isocratic developing solvents

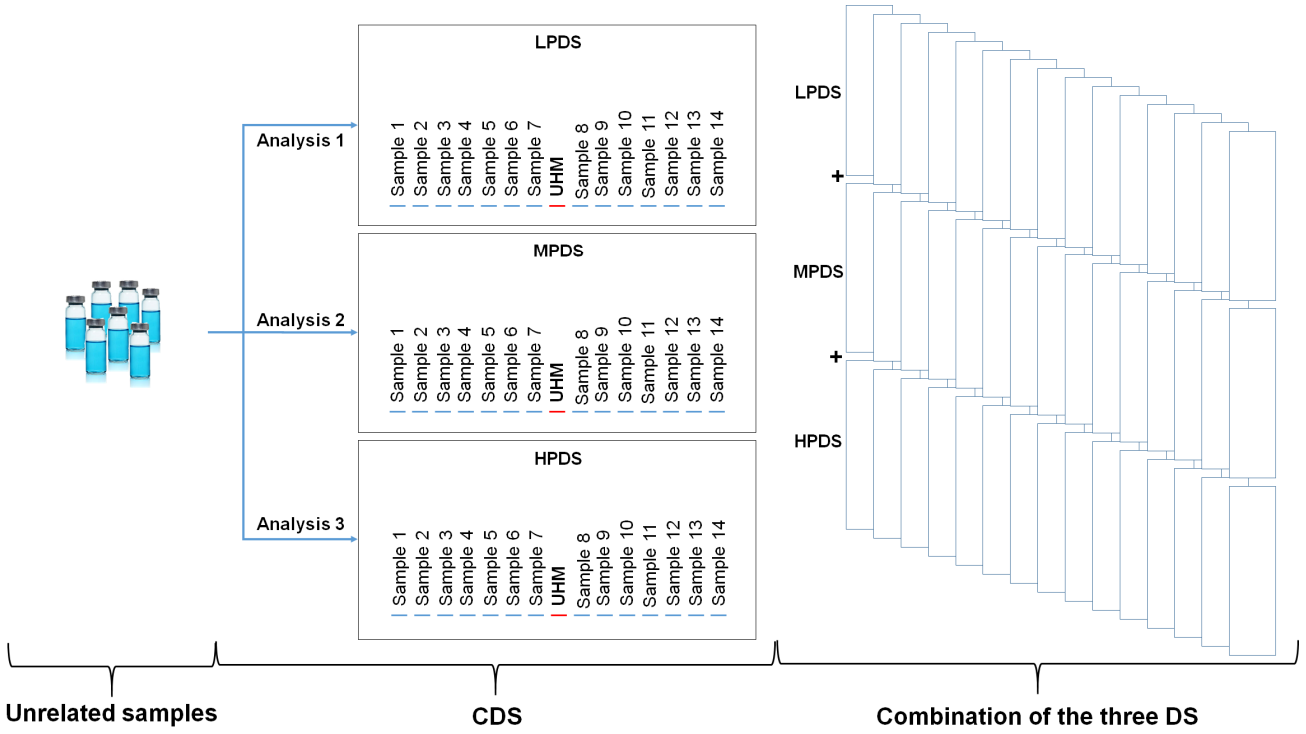
Combination of three DS

→ harmonization of methods, fewer solvents and standards in QC lab



Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications

One set of **3 D**eveloping **S**olvents, optimized for polarity and selectivity, for **ANY** sample

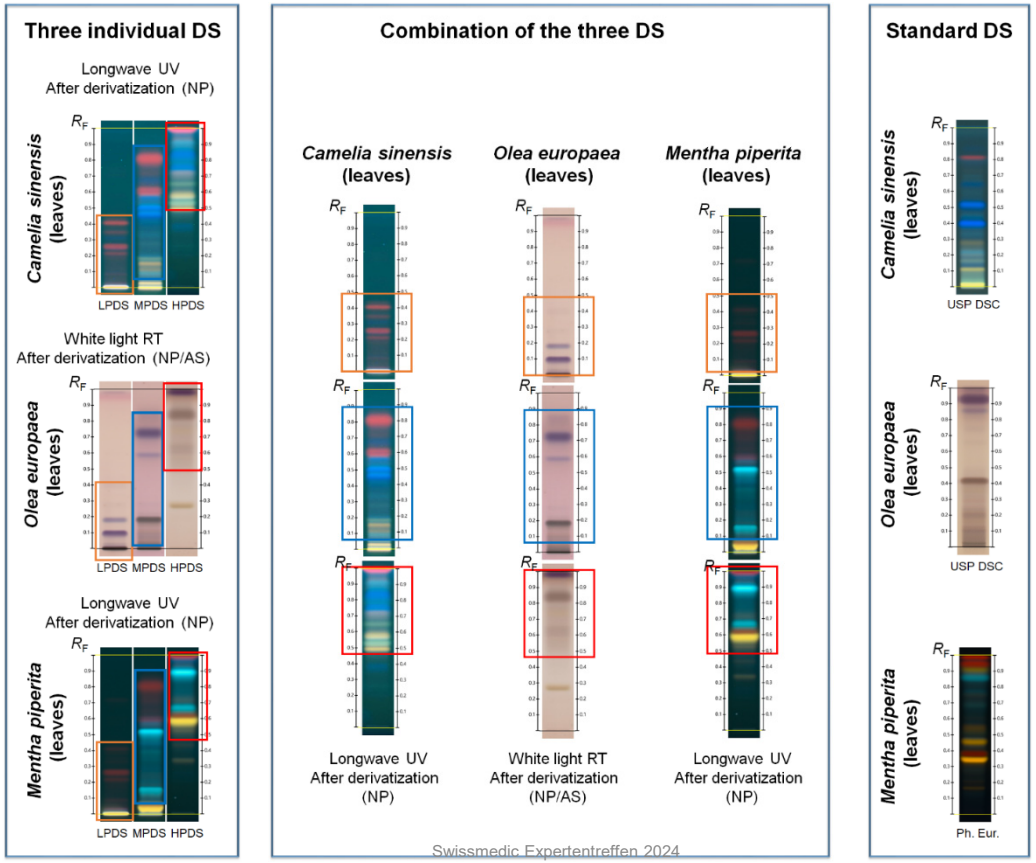


CDS requires 3 analyses instead of one ☹, **but:**

- Any kind of (herbal) samples can be combined on all plates
- Improved separation → more information
- Simplification and standardization
- Little maintenance
- Can be fully automated → cost efficiency

Do et.al.: Complementary developing solvents for simpler and more powerful routine analysis by HPTLC; https://doi.org/10.1007/s00764-001885-1

Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications

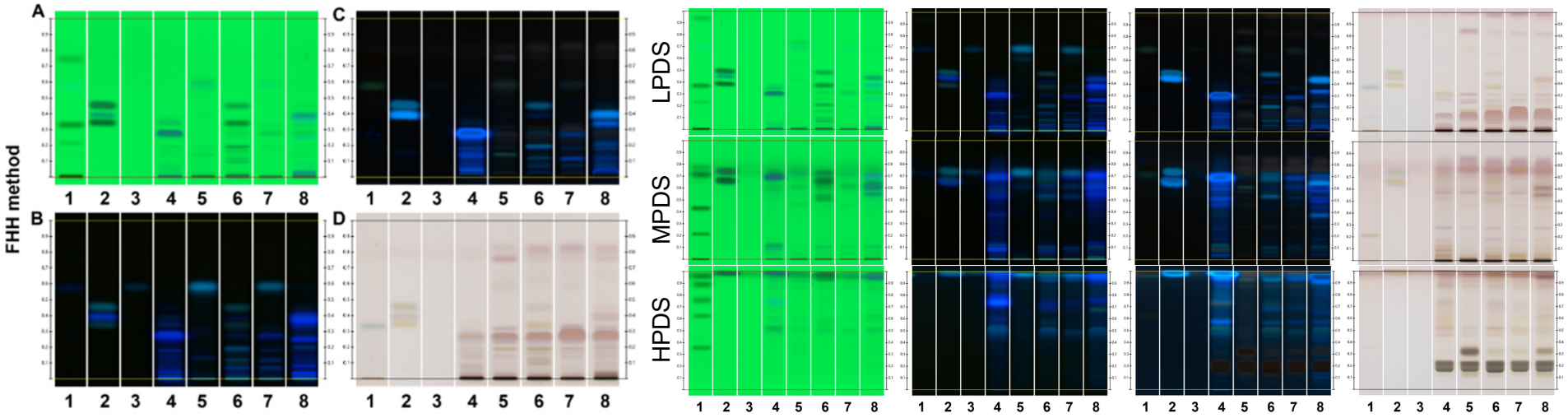


CAMAG 60 YEARS

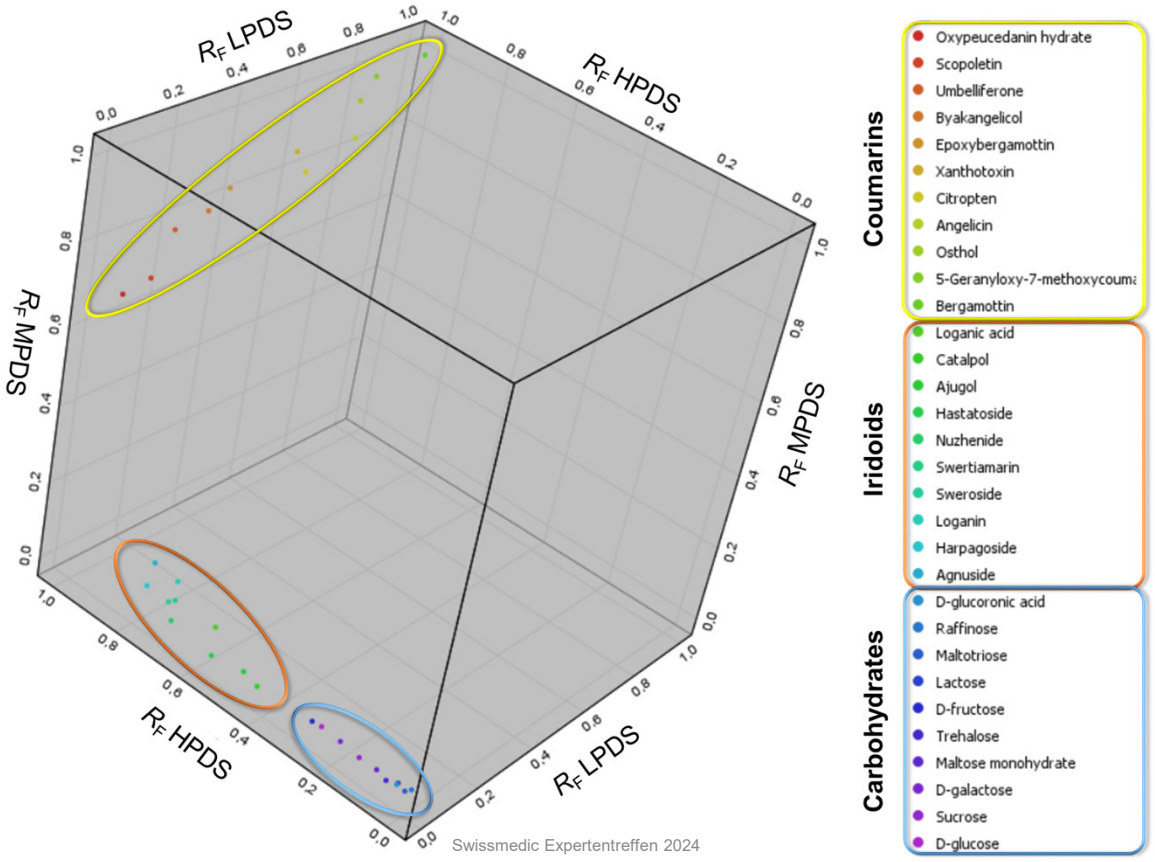
Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications

Identification of *Angelica* species

1: UHM; 2: imperatorin, osthole, isoimperatorin (with incr. R_F) 3: z-ligustilide; 4: *A. gigas;* 5: *A. sinensis*; 6: *A. dahurica* ; 7: *A. acutiloba* ; 8: *A. pubescens*

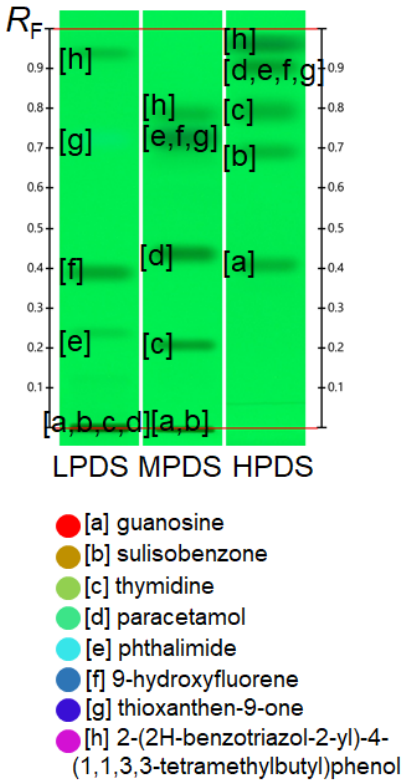


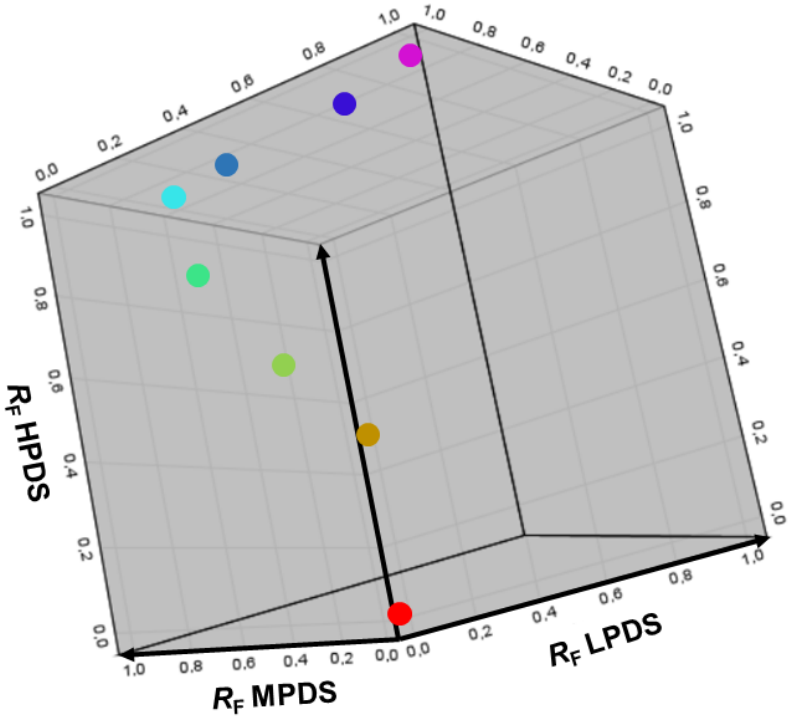
Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications



CAMAG 60 YEARS

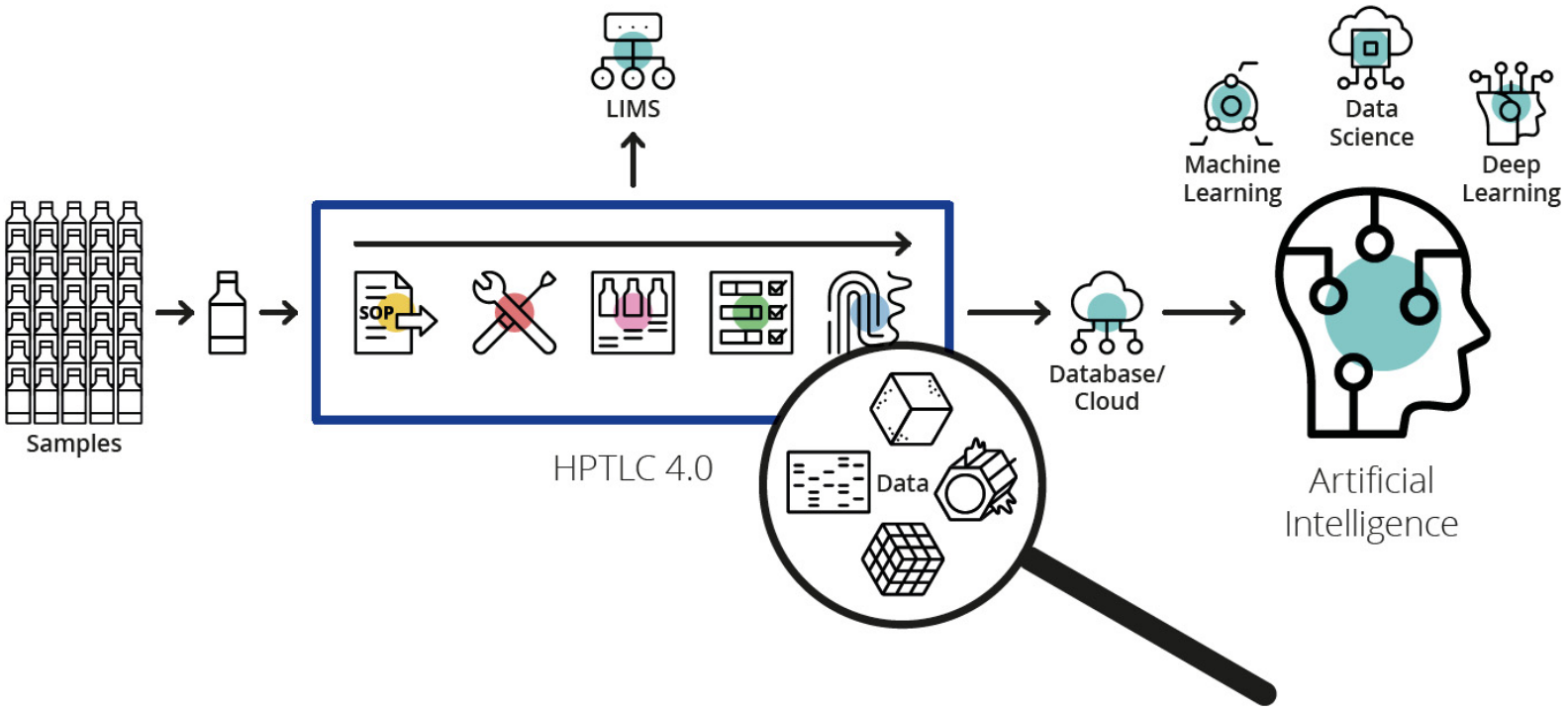
Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications



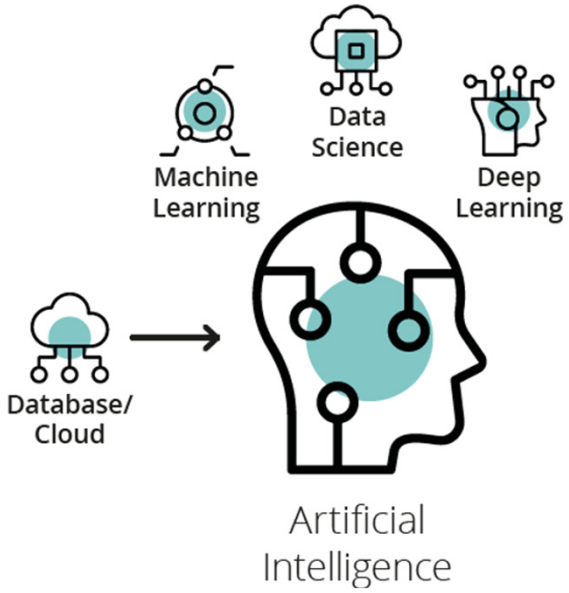


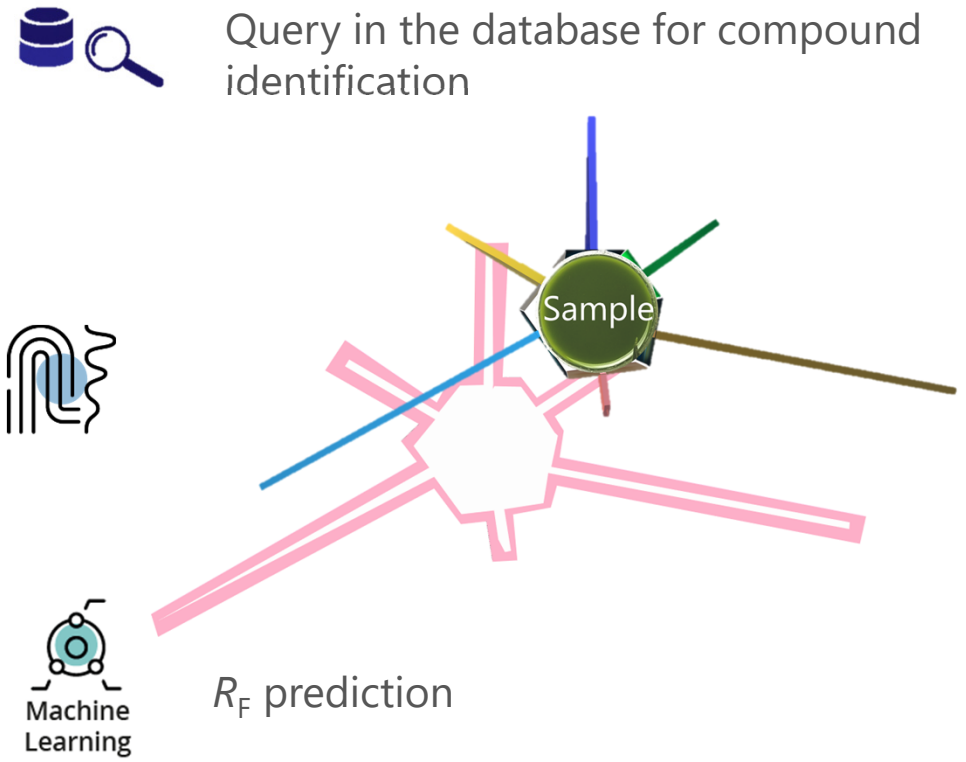
- Separation of the UHM with the CDS and the 3D plot of its components based on the R_F values obtained with the CDS
- **UHM is suitable as system suitability test for each DS of the CDS**
- All components are well distributed in the 3D plot

Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications



Complementary Developing Solvents (CDS) - Unsupervised Analysis and Cross-Disciplinary Applications





Thank you

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