An alternative and simplified approach to identification and test for minimum content of TCM herbal drugs

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ABSTRACT

Following a decision of the European Pharmacopoeia (Ph. Eur.) Commission, the Traditional Chinese Medicines (TCM) Working Party started a pilot phase to examine the suitability of a high-performance thin-layer chromatography (HPTLC) minimum content test as an alternative to the classical assay in TCM monographs. This approach was evaluated with two TCM herbal drugs: Fritillaria thunbergii bulbs (FTB) and Corydalis rhizome (CYR).

Firstly, the existing HPTLC methods were optimised for both drugs. The new methods were applied to the evaluation of multiple samples, and acceptance criteria for the identification, following Ph. Eur. chapter 2.8.25. High-performance thin-layer chromatography of herbal drugs and herbal drug preparations, were set. The HPTLC test for minimum content of markers was then developed and validated. In this test, the intensity of the marker zone in the fingerprint of the sample is compared to the corresponding zone in the reference solution, which has a concentration giving an intensity equivalent to the acceptance criterion. This test gives a pass or fail result rather than a content and can be performed visually (on the images) or by software (using peak profiles from images; PPI).

Reproducibility of the HPTLC methods was evaluated in a collaborative trial including six laboratories. In summary, results for FTB from five laboratories were in agreement. The remaining laboratory did not pass the identification of the samples. For CYR, all laboratories presented the same results for identification. In the test for minimum content, one borderline sample passed in four laboratories and failed in two. All laboratories reached similar conclusions for the other seven samples.

The HPTLC methods proposed offer a simplified approach to evaluating identity and minimum content of TCM drugs in a single analysis.

KEYWORDS

HPTLC, *Fritillaria thunbergii* bulbs, Corydalis rhizome, minimum content test, TCM.

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1. INTRODUCTION

The European Pharmacopoeia (Ph. Eur.) plays an important role in the quality control of herbal drugs in Europe and beyond. The 10th Edition (including Supplement 10.5) contains 346 monographs for herbal drugs and herbal drug preparations [1]. Of these, 78 are devoted to the quality of traditional Chinese medicine (TCM) drugs. In order to evaluate the quality of herbal drugs and preparations, monographs contain a suite of tests, used for the identification of the herbal drug or preparation, detection of impurities and content of a constituent or a group of constituents.

The constituents used for quality control of herbal drugs are classified in three categories: compounds with known therapeutic activity, active markers and analytical markers. The first two groups have an accepted role or contribution to the clinical efficacy of the herbal drug. Quantification of these constituents is considered relevant in the assessment of the quality of herbal drugs. However, for most herbal drugs, due to limited knowledge on the role of their constituents in their clinical efficacy, a constituent or group of constituents is used as analytical marker(s). The quantification of an analytical marker can help in the control of the manufacturing process of a herbal drug and the resulting medicinal product. In many cases, the determined content of analytical marker(s) is not indicative of the suitability of the herbal drug for the intended use and guarantees neither the quality nor the stability of the drug. Nevertheless, the usual practice is that, wherever possible, an assay shall be described in monographs on herbal drugs and preparations and this assay is very often based on chromatographic methods, such as LC or GC, using reference standards with assigned content.

Usually, one analytical marker is determined quantitatively in these assays and the Definition section of the monograph describes a minimum content. However, as an analytical marker is not responsible for the therapeutic activity, there is an ongoing discussion amongst experts whether less sophisticated methods could fulfil the same quality control objectives while simultaneously enabling the control of several markers and therefore providing a more holistic approach. This concept has already been described in the literature as “comprehensive HPTLC fingerprinting” [2, 3] and has the advantage that an identification test and determination of analytical marker(s) can be performed within one test. Furthermore, with the modernisation of the monographs in the Ph. Eur., the new general chapter on HPTLC (2.8.25) acknowledges the quantitative aspects of the HPTLC image with the inclusion of intensity markers.

Following a decision of the Ph. Eur. Commission at its 157th session in November 2016, the TCM Working Party (WP) started a pilot phase to examine the suitability of a semi-quantitative HPTLC approach as an alternative to the classical assay in monographs on TCMs which are not covered by a marketing authorisation in Europe. The idea was to offer a simpler test for minimum content that can be performed in parallel with identification. This approach was tested in two case studies, chosen by the experts of the TCM WP: Fritillaria thunbergii bulbs (FTB) and Corydalis rhizome (CYR).

The results of the pilot phase are described in this paper.

2. MATERIAL AND METHODS

2.1. Samples, reagents and instruments

Papaverine hydrochloride (95 % pure), yohimbine hydrochloride (95 % pure), peimine (98.8 % pure), peiminine (99.5 % pure), isofraxidin (95 % pure), scopoletin (95 % pure), corydaline (95 % pure), tetrahydropalmatine (95 % pure) and all samples of FTB and CYR were provided by the European Directorate for the Quality of Medicines and HealthCare (EDQM) (Strasbourg, France). The purity of the reagents was determined by LC by the providers, except for scopoletin, which was determined by TLC. Other standards presented in Figures 5 and 12 were obtained from Sigma Aldrich.

HPTLC instruments from CAMAG (Muttenz, Switzerland) were used, including: Automatic TLC Sampler (ATS 4), Automatic Development Chamber (ADC 2) with humidity control, Plate Heater 3, TLC Visualizer 2 and Immersion Device 3. These instruments were also used by the

laboratories participating in the collaborative trial, where available. Solvents (≥ 95 % pure) and reagents were purchased from Roth (Karlsruhe, Germany), Acros (Gent, Belgium), Fisher Scientific (Hampton, United States) and Merck (Darmstadt, Germany). Silica gel 60 F254 HPTLC glass plates (20 × 10 cm) were manufactured by Merck (Darmstadt, Germany).

2.2. Sample preparation

2.2.1. *Fritillaria thunbergii bulb*

In a closed centrifuge tube, 0.5 g of the powdered herbal drug was macerated for 30 min with 2.5 mL of concentrated ammonia (min. 30 %). Then 12.5 mL of methanol was added, the tube was closed again and the mixture was shaken for 20 min. Following centrifugation for 5 min at 2660 relative centrifugal force (RCF), the supernatant was used as the test solution.

Other sample preparation methods investigated are shown in Table 1.

Table 1 – *Description of the sample preparation methods tested with pooled sample of Fritillaria thunbergii bulb*

Method	Name	Description
1	HPLC method	In a 250 mL round-bottomed flask, 1.0 g of the powdered herbal drug was macerated for 30 min with 2 mL of concentrated ammonia (25–35 %). Then, 20 mL of a mixture of dichloromethane and methanol (4:1) was accurately added, and the mixture was heated under reflux on a water bath at 60 °C for 45 min. After cooling, the mixture was filtered over sodium sulfate into a 20 mL volumetric flask. The round-bottomed flask was rinsed twice, the rinsings were transferred to the volumetric flask and the volume was filled up to the mark with the solvent mixture. 10 mL of the filtrate was evaporated to dryness and the residue was dissolved in 2 mL methanol.
2	Sonication 10’	In a stoppered centrifuge tube, 0.5 g of the powdered herbal drug was macerated for 30 min with 2.5 mL of concentrated ammonia (25–35 %). Then, 12.5 mL of a mixture of dichloromethane and methanol (1:1) was added, the tube was again stoppered and the mixture was sonicated for 10 min. The test solution was centrifuged at 2660 RCF and the supernatant was used. For quantification, 1 mL of the supernatant was diluted with 3 mL of methanol.
3	Shaking 20’ MeOH	In a stoppered centrifuge tube, 0.5 g of the powdered herbal drug was macerated for 30 min with 2.5 mL of concentrated ammonia (25–35 %). Then, 12.5 mL of methanol was added, the tube was again stoppered, and the mixture was shaken for 20 min. The test solution was centrifuged at 2660 RCF and the supernatant was used. For quantification, 1 mL of the supernatant was diluted with 3 mL of methanol.
4	Original HPTLC	In a stoppered centrifuge tube, 0.5 g of the powdered herbal drug was macerated for 30 min with 0.5 mL of concentrated ammonia (25–35 %). Then, 2.5 mL of a mixture of dichloromethane and methanol (1:1) was added, the tube was again stoppered and the mixture was sonicated for 10 min. The test solution was centrifuged at 2660 RCF and the supernatant was used.

2.2.2. *Corydalis rhizome*

In a closed centrifuge tube, 0.5 g of the powdered herbal drug was sonicated for 15 min with 10 mL of 50 % aqueous ethanol. Then, following centrifugation for 5 min at 2660 RCF, 1.0 mL of the clear supernatant was transferred into a 10 mL volumetric flask and diluted to 10 mL with 50 % aqueous ethanol.

2.3. High-performance thin-layer chromatography (HPTLC)

General HPTLC parameters for plate layout, sample application, conditioning of the plate, plate development and visualisation were in accordance with Ph. Eur. general chapter 2.8.25 [4]. The specific parameters are described in Tables 2 and 3.

Table 2 – *Parameters for HPTLC analysis of Fritillaria thunbergii bulb*

Stationary phase	20 × 10 cm glass plates silica gel 60 F_{254} (2–10 µm) (Merck)
Ref. solution a	Papaverine at 0.5 mg/mL in methanol
Ref. solution b	Yohimbine at 0.1 mg/mL in methanol
Ref. solution c	Peimine at 40 µg/mL in methanol
Ref. solution d	Peiminine at 40 µg/mL in methanol
Ref. solution e	Peimine at 10 µg/mL in methanol
Ref. solution f	Peiminine at 10 µg/mL in methanol
Ref. solution g	Peimine at 20 µg/mL in methanol
Ref. solution h	Peiminine at 6.7 µg/mL in methanol
Application	5 µL of test and reference solutions, quantitative mode
Developing solvent	Toluene, acetone, diethylamine (9:9:1.2, v/v/v)
Development	Unsaturated chamber (deviating from HPTLC standard conditions), 10 min conditioning at 33 % relative humidity (with $MgCl_2$), to 70 mm from the lower edge of the plate, room temperature (22 ± 5 °C)
Detection prior to derivatisation	UV 254 nm, UV 366 nm, and white light
Derivatisation reagent	10 % sulfuric acid in methanol: to 180 mL of cold methanol, 20 mL of sulfuric acid was added. The mixture was allowed to cool to room temperature before use.
Derivatisation	Plates were dipped (speed: 1, dwell time: 0) into the derivatisation reagent, dried for 1 min in a stream of cold air and then heated at 120 °C for 5 min.
Detection after derivatisation	Images were recorded immediately after derivatisation, under UV 366 nm and white light.

Table 3 – *Parameters for HPTLC analysis of Corydalis rhizome*

Stationary phase	20 × 10 cm glass plates silica gel 60 F_{254} (2–10 µm) (Merck)
Ref. solution a	Isofraxidin at 0.5 mg/mL in methanol
Ref. solution b	Scopoletin at 0.5 mg/mL in methanol
Ref. solution c	Corydaline at 12.5 µg/mL in methanol
Ref. solution d	Tetrahydropalmatine at 12.5 µg/mL in methanol
Ref. solution e	Corydaline at 3.125 µg/mL in methanol
Ref. solution f	Tetrahydropalmatine at 3.125 µg/mL in methanol
Ref. solution g	Corydaline at 2 µg/mL in methanol
Ref. solution h	Tetrahydropalmatine at 3 µg/mL in methanol
Application	2 µL of test and reference solutions, quantitative mode
Developing solvent	Toluene, anhydrous tert-butyl methyl ether, isopropanol (8:2:0.2, v/v/v)
Development	Unsaturated chamber (deviating from HPTLC standard conditions), 10 min conditioning at 33 % relative humidity (with $MgCl_2$), to 70 mm from the lower edge of the plate, room temperature (22 ± 5 °C)
Detection prior to derivatisation	UV 254 nm, UV 366 nm and white light
Derivatisation reagent	Iodine vapour: a glass chamber was saturated for 30 min with 1 g of iodine, spread evenly.
Derivatisation	The plate was exposed to iodine vapour for 3 min. The excess of iodine absorbed on the plate was removed in a stream of cold air for 10 min.
Detection after derivatisation	Images were taken immediately after derivatisation, under UV 366 nm and white light

2.4. Generation of peak profiles from images (PPI)

visionCATS software (CAMAG, Switzerland) was used to generate PPI (fingerprints) by calculating the luminance L = (⅓ R) + (⅓ G) + (⅓ B) from the averaged signals of the red (R), green (G) and blue (B) channels of each pixel line of the track. L in fluorescence mode, respectively L-1 in absorption mode, is plotted as a function of the RF value [5].

3. RESULTS AND DISCUSSION

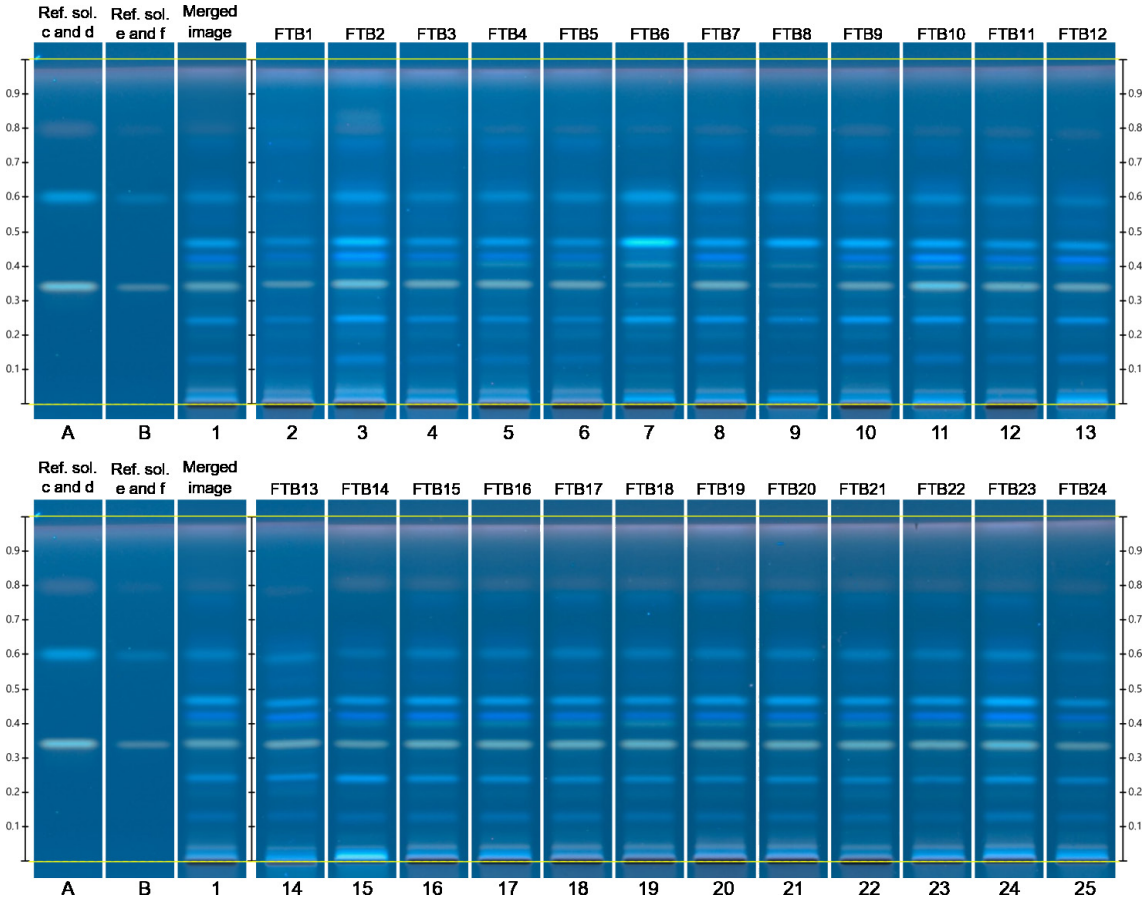
3.1. *Fritillaria thunbergii* bulb (FTB)

The dried bulb of *Fritillaria thunbergii* Miq. (FTB) is used in TCM for treating cough and airway inflammatory diseases [6]. According to Li *et al.* [7], the alkaloids peimine (verticine) and peiminine (verticinone) are the major constituents of Fritillaria and are primarily responsible for the activity of the drug. Therefore, identification C of the first draft Ph. Eur. monograph elaborated by the TCM WP (confidential document) describes alkaloids as markers, and in the monograph for Fritillariae Thunbergii Bulbus of the Chinese Pharmacopoeia [8] an assay for peimine and peiminine is prescribed. Before starting the work presented here, the sample preparation and HPTLC methods of the Ph. Eur. first draft were optimised to improve repeatability (data not shown).

3.1.1. Identification, intensity marker, system suitability test (SST) and specificity of the method

The optimised method, as described in the experimental section, was used to evaluate 24 samples of FTB. Fingerprints are shown in Figure 1 together with the chromatograms of the reference solutions (tracks A to D) and, as test solution, an average fingerprint generated through electronic merging of the individual fingerprints of all FTB samples (track 1). The eight main zones of the test solution are labelled alphabetically on the chromatogram and described in a table as acceptance criteria for identification. Under UV 366 nm after derivatisation a sample should contain a blue fluorescent zone at the position of peiminine [e], a blue fluorescent zone [f], a blue fluorescent zone [g] barely separated from a faint green fluorescent zone [h], a greenish fluorescent zone at the position of peimine [j], one blue zone [k] and a faint blue zone [l].

Figure 1 – *Fingerprints of all Fritillaria thunbergii bulb (FTB) evaluated samples (FTB1-24) in UV 366 nm after derivatisation. Track 1 – Electronically merged image of all fingerprints*

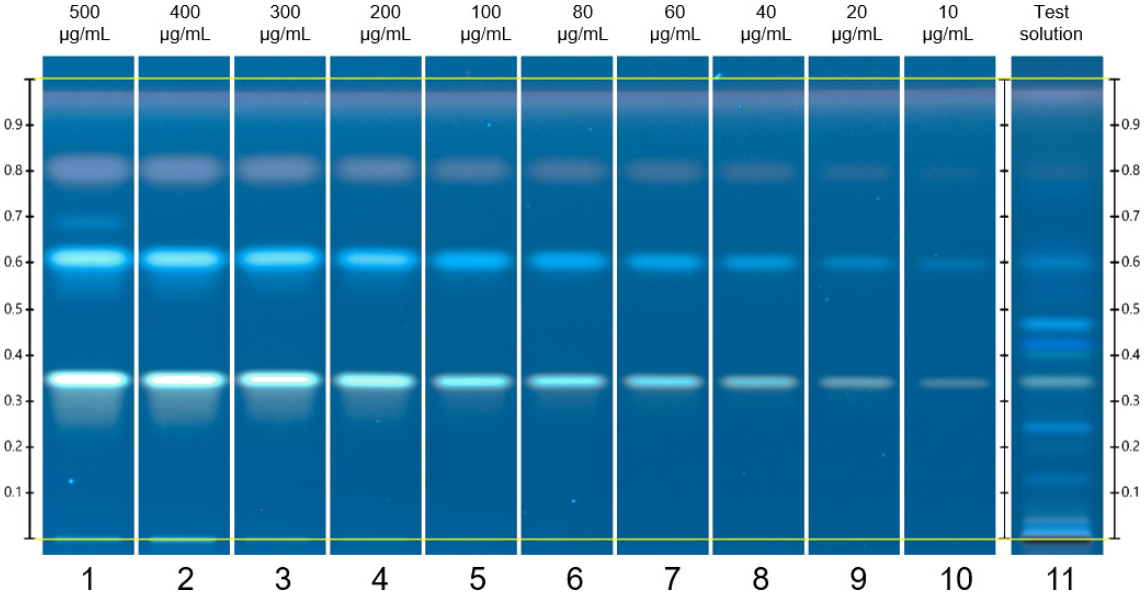


INTENSITY MARKER

In order to improve the description of the intensity of the zones in the chromatogram, the concept of intensity markers adopted in the HPTLC general chapter *2.8.25* [4] was explored. According to this chapter, solutions of one or two substances are prepared in two concentrations, one that resembles the intensity of the main zone(s) in the fingerprint of the test solution (R) and another with the same standard(s) diluted 4-fold (R/4). To describe the intensity of the zones in the fingerprints, their intensities are visually compared with the intensity of the zones in solutions (R) and (R/4). Zones that are more intense than solution (R) are described as intense zones, while those of similar intensity to solution (R) are described as equivalent zones or have no indication of intensity. Zones that show intensity between solutions (R) and (R/4) are referred to as faint, and those less intense than solution (R/4) are described as very faint.

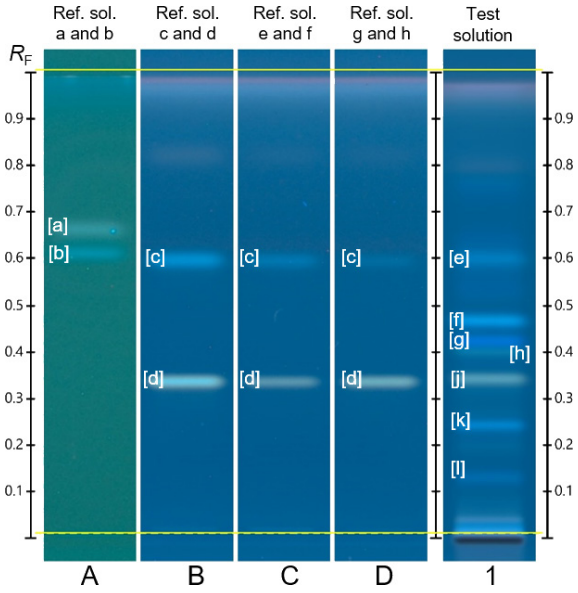
For FTB, reference solutions of peimine and peiminine were used as intensity markers. First, they were prepared at ten different concentrations, between 500 µg/mL and 10 µg/mL (Figure 2). The solutions at a concentration of 40 µg/mL were suitable as first intensity markers (reference solutions c and d) and their 4-fold dilutions (10 µg/mL) as diluted intensity markers (reference solutions e and f) (Figure 3). Peimine is used as intensity marker for the corresponding zones [j] and [h], and peiminine for all the other zones.

Figure 2 – *Fingerprints of peimine (green zone at RF 0.34) and peiminine (blue zone at RF 0.60), prepared at 10 different concentrations (tracks 1–10) and an electronically merged image of all FTB fingerprints, in UV 366 nm after derivatisation*



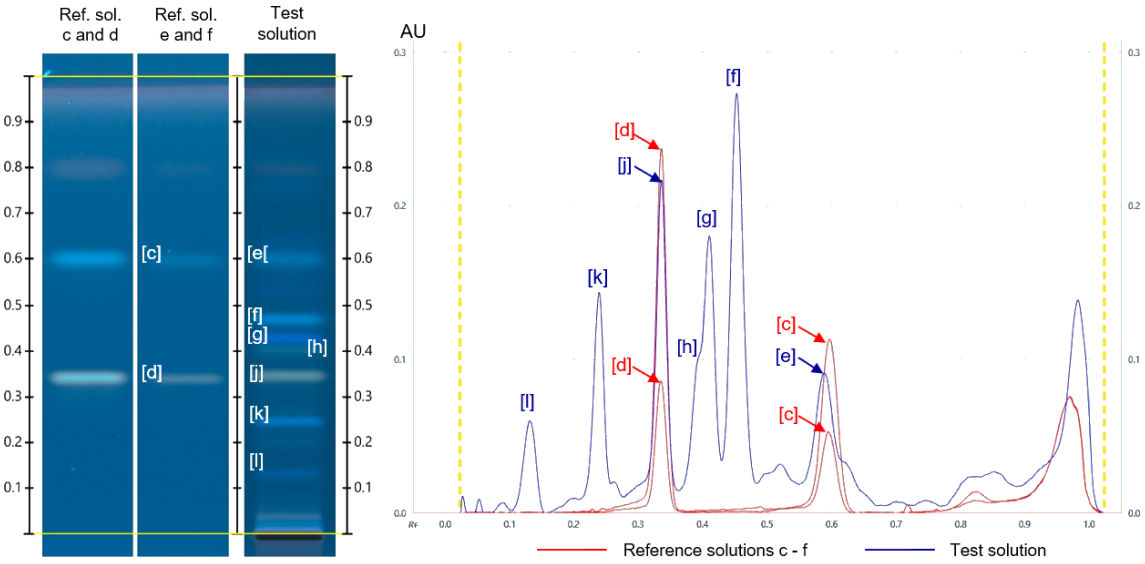
A more objective description of the intensity is possible using peak profiles from images (PPI). In this approach, the height of the peaks can be directly compared using suitable software. An example of the fingerprint for one FTB sample is shown in Figure 4. Peaks [j] and [h] are compared to peimine as intensity marker [d] and would be described as faint. The other peaks are compared to peiminine as intensity marker [c] and, consequently, peaks corresponding to the zones [f], [g] and [k] would be described as intense zones, whereas peaks [e] and [l] would be described as faint zones.

Figure 3 – *HPTLC chromatograms and table description for visual identification and test for the minimum content of peimine and peiminine in Fritillaria thunbergii bulb (FTB). Track A: SST; Tracks B to D: reference solutions. Track 1: FTB test solution (average fingerprint). Tracks B to D and 1 normalized over peiminine in track B. Intensity markers: peimine for zones [h] and [j], peiminine for all the other zones*



Top of the plate		
[a] Papaverine: a green zone [b] Yohimbine: a blue zone	---	[c] Peiminine: a blue fluorescent zone
	---	[d] Peimine: a greenish fluorescent zone
		[e] A faint to equivalent blue zone (peiminine)
		[f] A blue fluorescent zone
		[g] A blue fluorescent zone
		[h] A very faint to faint greenish zone
		[j] A greenish fluorescent zone (peimine)
		[k] A blue zone
		[l] A faint to equivalent blue zone
Reference solutions (a) and (b)	Reference solutions (c) and (d)	Test solution

Figure 4 – *Chromatograms and PPI of reference and test solutions. Peimine (zone [d]) and peiminine (zone [c]) are used as intensity markers for zones [h] and [j] and all the other zones, respectively*



SYSTEM SUITABILITY TEST (SST)

Besides intensity markers, general chapter *2.8.25* [4] also requires the use of an SST to evaluate the quality of the chromatographic elution. According to this chapter, the SST consists of two substances on the same track, which, after chromatography, are barely separable. Different separation patterns can indicate problems in the chromatography. For FTB, 28 standards were tested (Figure 5). Only 12 alkaloids migrated between RF 0.2 and 0.8. Those that showed similar RF values were then combined. While no suitable combination was obtained after derivatisation, three combinations were proposed for evaluation prior to derivatisation under UV 254 and 366 nm (Figure 6). Quinine and boldine showed good separation, but after derivatisation the intensity of the zone due to quinine interfered with the detection of other zones on the plate. The combination of brucine and theobromine was tested in a collaborative trial, including six laboratories, and some participants had difficulties completely dissolving theobromine, which affected the detectability of this standard. Therefore, papaverine and yohimbine were finally selected as SST standards. Detection at UV 366 nm was chosen for its higher sensitivity, particularly for yohimbine. The concentration of the standards was adjusted to 0.5 mg/mL for papaverine and 0.1 mg/mL for yohimbine as shown in Figure 3.

Figure 5 – *Fingerprints of the reference solutions at 1 mg/mL from secondary metabolites of different classes (e.g. flavonoids, saponins, sugars and alkaloids), analysed for the selection of a system suitability test (SST) for Fritillaria thunbergii bulb HPTLC method*

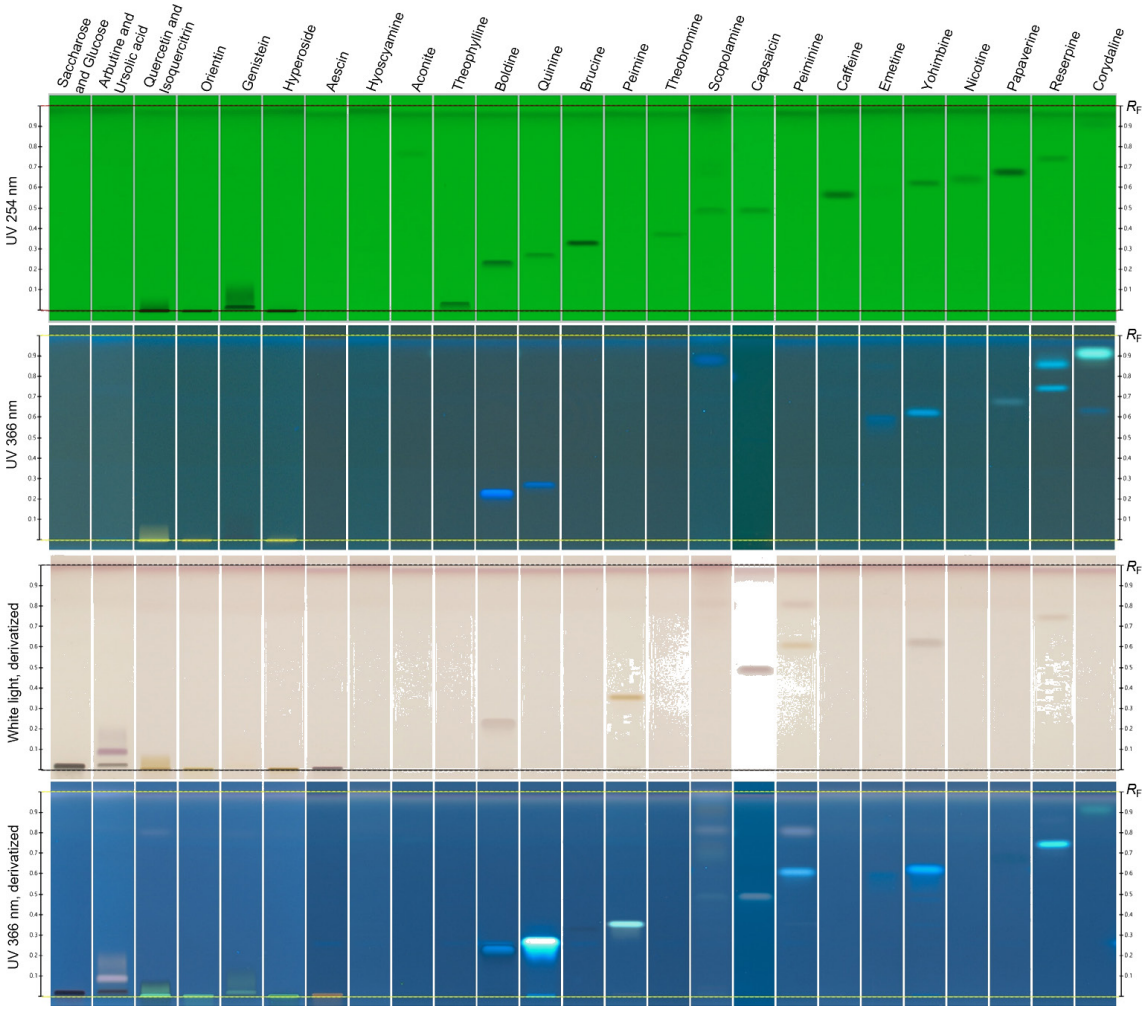
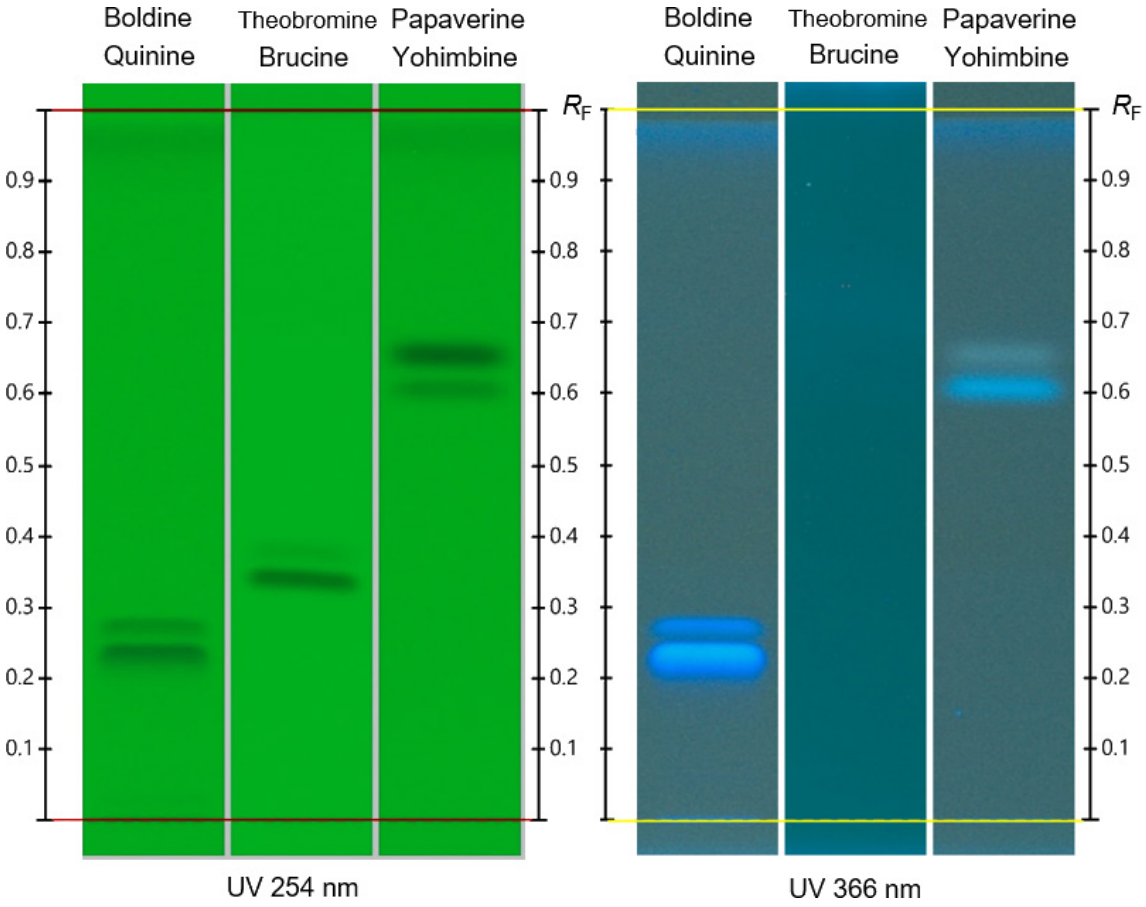


Figure 6 – *Images of the possible SST, in UV 254 and 366 nm prior to derivatisation*



3.1.2. Development of the test for minimum content based on PPI

For FTB, the HPTLC test for minimum content was developed based on the LC assay of the Chinese pharmacopoeia [8]. In the first part of the HPTLC investigation, peimine and peiminine were quantified in nine authenticated samples (FTB1–9). The minimum content of both markers was established. Then, the optimised HPTLC method was validated with a new set of samples (FTB 10-24), and results were compared to data obtained by LC.

ESTABLISHING MINIMUM CONTENT OF PEIMINE AND PEIMININE

The contents of peimine and peiminine were established prior to method optimisation, using the HPTLC method from the draft monograph (confidential document) and diluted test solutions, prepared according to method 2 in Table 1. A calibration curve using peak areas from PPI for peimine was used to calculate both peimine and peiminine concentrations in samples FTB1-9. First, the linear working range was evaluated. Linearity was achieved with five concentrations ranging from 2 to 8 µg/mL (4-16 ng/application). To fit the test solutions into the linear working range, they were further diluted 4-fold.

The content of peimine in samples FTB1-9 varied between 0.01 % and 0.10 % (0.06 % on average), with 67 % of the samples having a content ≥ 0.06 %. The content of peiminine in these samples varied between 0.01 % and 0.05 % (0.02 % on average), with 89 % of the samples showing a percentage ≥ 0.02 %. Results are shown in Table 4. Based on these results, it was agreed to propose 0.06 % and 0.02 % as minimum contents for peimine and peiminine, respectively. These limits include 56 % of the samples analysed.

Table 4 – *Contents of peimine and peiminine in Fritillaria thunbergii bulb samples (FTB1–9), calculated with the original HPTLC method. Detection mode: UV 366 nm after derivatisation*

Sample	Peimine UV 366 nm (PPI) %	Peiminine UV 366 nm (PPI) %
FTB1	0.08	0.02
FTB2	0.10	0.03
FTB3	0.07	0.02
FTB4	0.08	0.02
FTB5	0.07	0.01
FTB6	0.01	0.05
FTB7	0.08	0.02
FTB8	0.01	0.02
FTB9	0.03	0.04
Agreed minimum content	0.06	0.02

VALIDATION OF THE HPTLC METHOD IN COMPARISON TO LC ASSAY

To validate the new HPTLC method against the existing LC assay method elaborated and validated by the experts of the Ph. Eur. TCM WP (confidential document), the content of peimine and peiminine was assessed in 15 new samples (FTB10-24). To improve accuracy of the HPTLC determination, a separate calibration curve for peiminine, ranging from 2-10 µg/mL (4-20 ng/application), was prepared (Figure 7), whereas for peimine, the calibration curve described in section 3.1.2.1 was used. The sample preparation for LC is described in Table 1 (method 1). For HPTLC, the same sample preparation was used, but with an additional 25-fold dilution in order to fit in the linear range. Quantifications were performed on the PPI by peak area for peimine and peak height for peiminine, due to a lack of baseline separation from the neighbouring peak. HPTLC results were compared to LC data provided by an expert of the TCM WP (confidential document).

Figure 7 – *Calibration curves of peimine and peiminine. Detection mode: UV 366 nm after derivatisation*

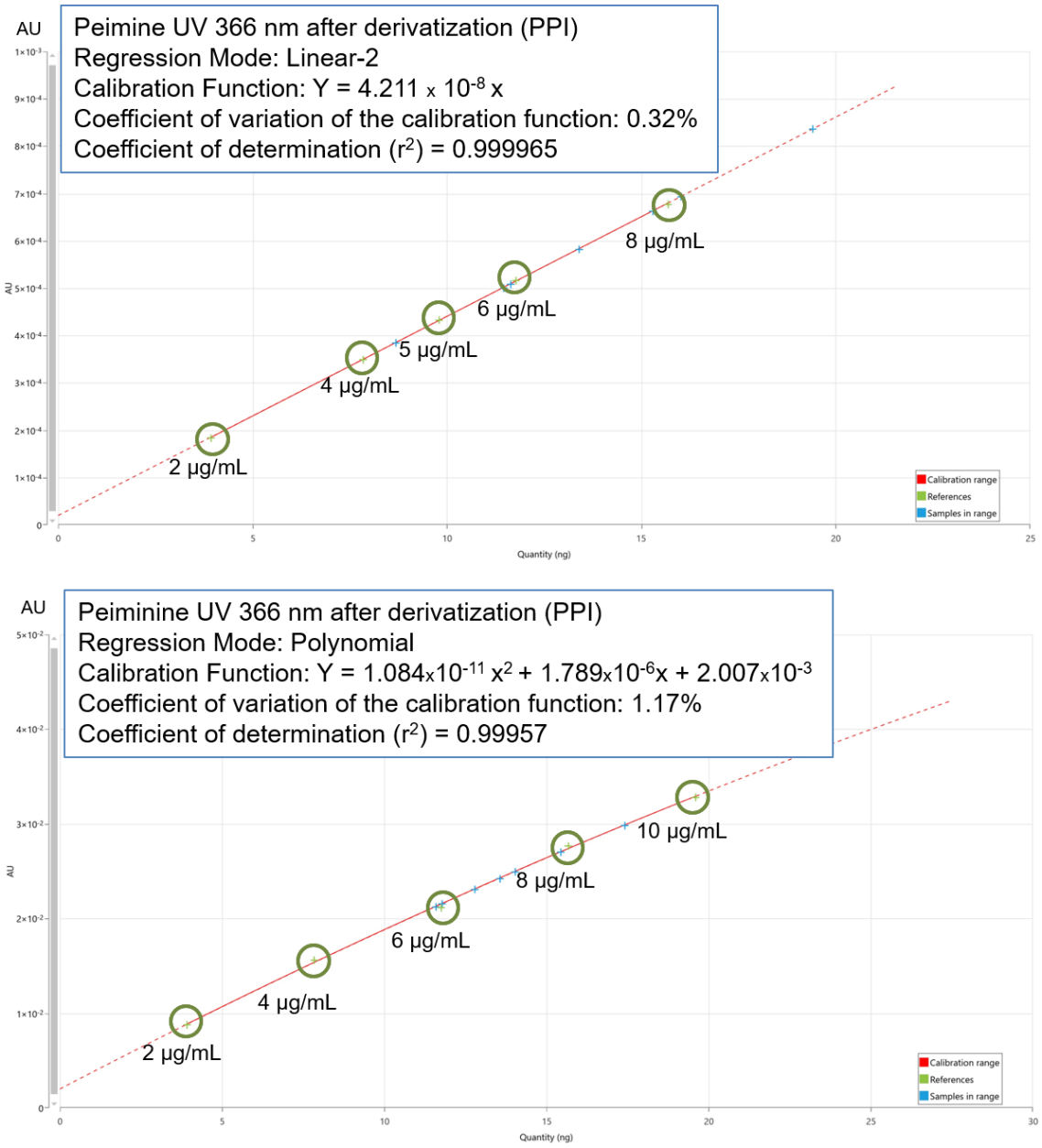
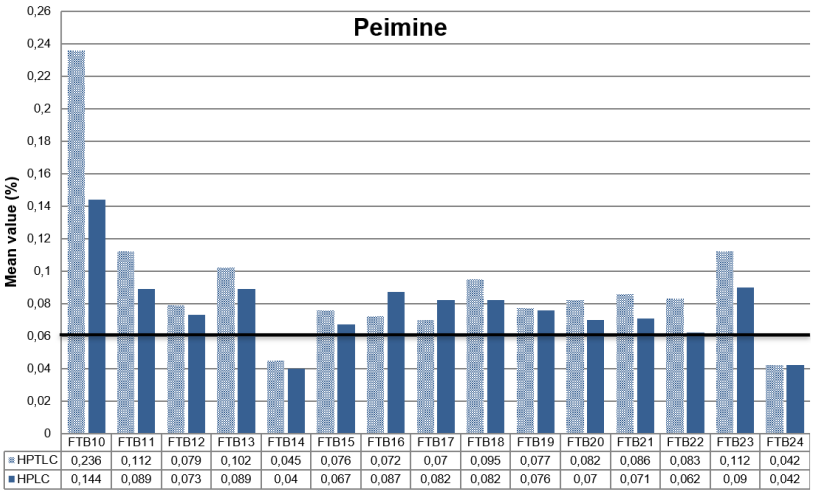
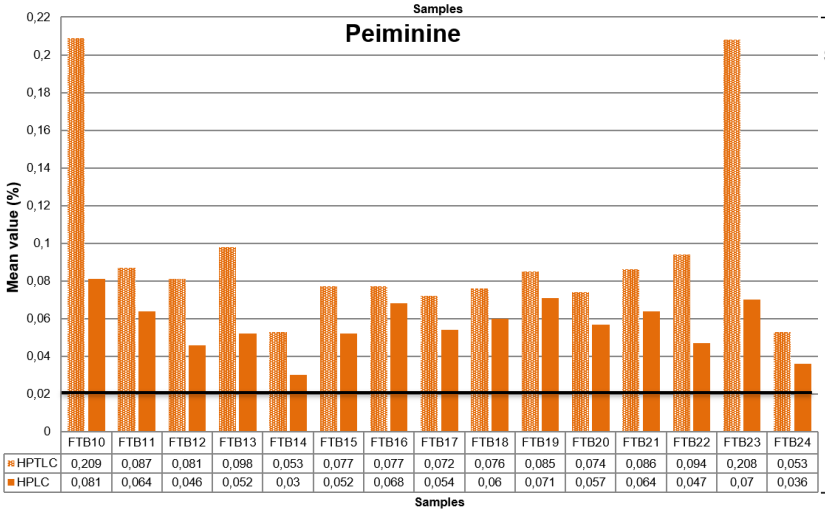


Figure 8 – *Comparison of the contents of peimine and peiminine by HPTLC and LC. Black lines: minimum content of peimine (0.06 %) and peiminine (0.02 %).*



Samples	HPTLC values %	HPLC values %	Ratio HPTLC/ HPLC
FTB10	0.236	0.144	1.641
FTB11	0.112	0.089	1.261
FTB12	0.079	0.073	1.083
FTB13	0.102	0.089	1.149
FTB14	0.045	0.040	1.131
FTB15	0.076	0.067	1.130
FTB16	0.072	0.087	0.823
FTB17	0.070	0.082	0.858
FTB18	0.095	0.082	1.154
FTB19	0.077	0.076	1.017
FTB20	0.082	0.070	1.166
FTB21	0.086	0.071	1.214
FTB22	0.083	0.062	1.339
FTB23	0.112	0.090	1.248
FTB24	0.042	0.042	0.996
	Average		1.112



Samples	HPTLC values %	HPLC values %	Ratio HPTLC/ HPLC
FTB10	0.209	0.081	2.574
FTB11	0.087	0.064	1.361
FTB12	0.081	0.046	1.755
FTB13	0.098	0.052	1.888
FTB14	0.053	0.030	1.750
FTB15	0.077	0.052	1.488
FTB16	0.077	0.068	1.138
FTB17	0.072	0.054	1.329
FTB18	0.076	0.060	1.275
FTB19	0.085	0.071	1.200
FTB20	0.074	0.057	1.299
FTB21	0.086	0.064	1.343
FTB22	0.094	0.047	2.005
FTB23	0.208	0.070	2.971
FTB24	0.053	0.036	1.481
	Average		1.486

In Figure 8, HPTLC results are, on average, 1.1 and 1.5-fold greater than those of LC for peimine and peiminine, respectively. Nevertheless, both techniques led to same conclusion of the test for minimum content for peimine (13 samples passed) and peiminine (all samples passed).

3.1.3. Collaborative trial: organisation

The reproducibility of the new HPTLC method was evaluated in a collaborative trial involving six laboratories using a common set of six samples (FTB 25-30) and reference solutions. Reference solutions (a) to (f) and test solutions for identification were prepared according to the experimental section. Brucine and theobromine were used as SST reagents.

The test for minimum content was performed visually and by PPI using single concentrations of peimine and peiminine equivalent to 0.06 % and 0.02 %, respectively, in the herbal drug (reference solutions (g) and (h)).

The test performed by PPI (software) involved an additional set of test and reference solutions at a 4-fold dilution ensuring determination within the linear range. Due to the low intensity of the zones this set is not suitable for visual evaluation.

The participating laboratories evaluated whether the SST, intensity markers and samples met the acceptance criteria below:

- Evaluation 1, SST: The quenching zones due to theobromine [a] and brucine [b] are barely separable and are seen at RF ~ 0.36 and 0.32, respectively.
- Evaluation 1, intensity markers: A blue zone due to peiminine [c] and a greenish zone due to peimine [d] are seen at RF ~ 0.59 and 0.34, respectively. The ΔRF between experimental and theoretical RF should not be greater than 0.05.
- Evaluation 2, identification: Fingerprint of test solution should contain zones [e] to [k], with colours and intensities similar to those described in the table of Figure 3. Reference solutions (c) to (f) are used.
- Evaluation 3, minimum content (visual evaluation): under UV 366 nm after derivatisation, the intensity of the zones due to peimine and peiminine in the fingerprint of the test solution is not less than that obtained with reference solutions g and h, respectively.
- Evaluation 4, minimum content (PPI): In the PPI of the image under UV 366 nm after derivatisation (non-diluted and diluted), peak heights of peimine and peiminine recorded for the test solution are equal to or greater than those recorded for reference solutions.

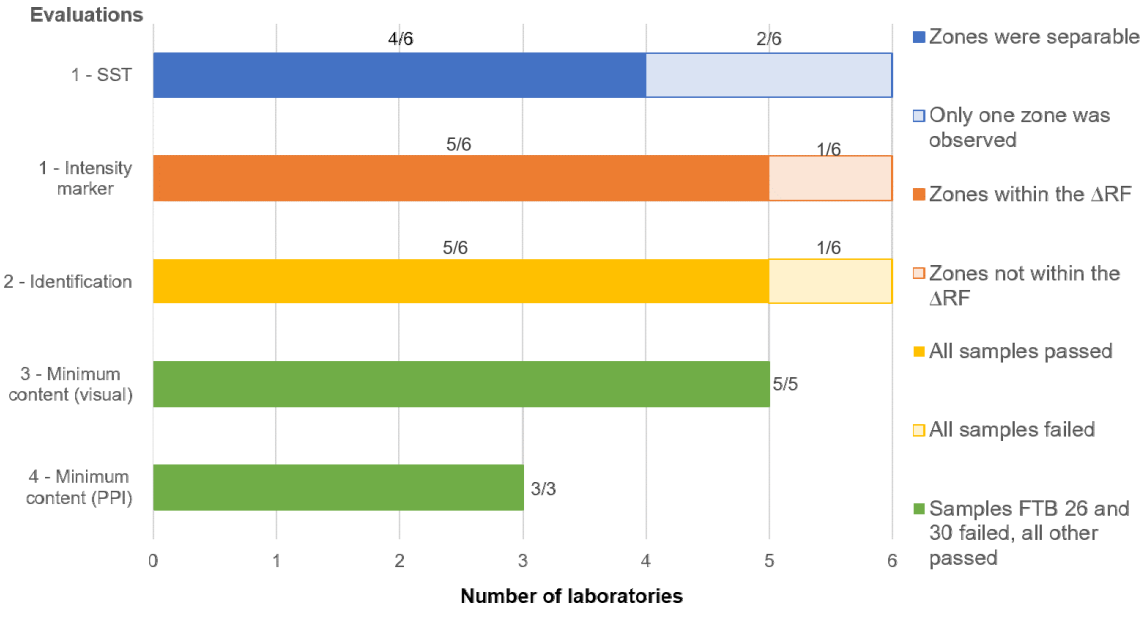
3.1.4. Collaborative trial: results

Results of the collaborative trial are presented in Table 5 and Figure 9. The first SST (brucine and theobromine) passed in four laboratories, but problems with solubility and detection of theobromine were observed. This could be the reason for the observation of only one zone in two laboratories, causing the SST to fail. Therefore, the new SST-2 (yohimbine and papaverine) was proposed, positively tested in several laboratories, and implemented in the method proposed for the monograph. Results from all laboratories were compliant regarding the position of the intensity markers, except for one that showed a ΔRF greater than 0.05 for both markers.

For the identification (evaluation 2), all laboratories reported the seven zones with colours matching those of the description table. One laboratory (4) obtained universally faint fingerprints. The reason for this is not clear. Because all samples failed the ID test, the results of the test for minimum content from Laboratory 4 were not considered.

In the visual test for minimum content (evaluation 3), five laboratories passed samples FTB 25 and 27–29. Samples FTB 26 and 30 were reported as having a lower content of peimine. The same result was observed in the test for minimum content based on evaluation of the PPI performed by three participants. Because results were similar, the experts of the TCM WP adopted the non-diluted set for this test.

Figure 9 – *Results of the collaborative trial for FTB with 6 participant laboratories.*



In summary, five laboratories came to uniform pass/fail decisions which were in line with those based on the LC assay. Based on this study, the draft monograph under elaboration was updated and the final version published in Pharmeuropa.

Table 5 – *Summary of the results of the collaborative trial for Fritillaria thunbergii bulb HPTLC method, from six laboratories*

Evaluation 1: SST and intensity markers

	Laboratory	1	2	3	4	5	6
SST	Ref. solution a (theobromine)	Pass	Pass	Pass	Fail (no separation)	Pass	Fail (no separation)
	Ref. solution b (brucine)	Pass	Pass	Pass	Fail (no separation)	Pass	Fail (no separation)
SST2	Ref. solution a (papaverine)	Pass	n.a.	n.a.	n.a.	Pass	n.a.
	Ref. solution b (yohimbine)	Pass	n.a.	n.a.	n.a.	Pass	n.a.
IM[a]	Ref. solution c and e (peimine)	Fail (deviating R_F values)	Pass	Pass	Pass	Pass	Pass
	Ref. solution d and f (peiminine)	Fail (deviating R_F values)	Pass	Pass	Pass	Pass	Pass

[a] Intensity marker.

Evaluation 2: Identity

Laboratory	1	2	3	4	5	6
FTB 25	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass
FTB 26	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass
FTB 27	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass
FTB 28	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass
FTB 29	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass
FTB 30	Pass	Pass	Pass	Fail (faint fingerprint)	Pass	Pass

Evaluation 3: Minimum content, visual evaluation

Laboratory	1	2	3	4	5	6
FTB 25	Pass	Pass	Pass	n.a.	Pass	Pass
FTB 26	Fail (peimine)	Fail (peimine)	Fail (peimine)	n.a.	Fail (peimine)	Fail (peimine)
FTB 27	Pass	Pass	Pass	n.a.	Pass	Pass
FTB 28	Pass	Pass	Pass	n.a.	Pass	Pass
FTB 29	Pass	Pass	Pass	n.a.	Pass	Pass
FTB 30	Fail (peimine)	Fail (peimine)	Fail (peimine)	n.a.	Fail (peimine)	Fail (peimine)

Evaluation 4: Minimum content, PPI (diluted test and reference solutions)

Laboratory	1	2	3	4	5	6
FTB 25	n.a.	Pass	Pass	n.a.	Pass	n.a.
FTB 26	n.a.	Fail (peimine)	Fail (peimine)	n.a.	Fail (peimine)	n.a.
FTB 27	n.a.	Pass	Pass	n.a.	Pass	n.a.
FTB 28	n.a.	Pass	Pass	n.a.	Pass	n.a.
FTB 29	n.a.	Pass	Pass	n.a.	Pass	n.a.
FTB 30	n.a.	Fail (peimine)	Fail (peimine)	n.a.	Fail (peimine)	n.a.

3.2. Corydalis rhizome (CYR)

The dried rhizome of Corydalis yanhusuo (Y.H. Chou and Chun C. Hsu) W.T. Wang ex Z.Y. Su and C.Y. Wu (CYR) has been used traditionally to promote blood circulation and as an analgesic. CYR contains isoquinoline alkaloids, such as tetrahydropalmatine (THP), corydaline, isocorypalmine, stylopine, columbamine and coptisine. Some studies demonstrated a relationship between I-THP and the analgesic effect [9]. Due to its importance for the biological effect of the herbal drug, the chemical identification and assay of analytical markers in the current Ph. Eur. monograph 2976 [10] focus on the isoquinoline alkaloid profile. Before starting the work described here, the sample preparation and HPTLC method were optimised to improve repeatability (data not shown).

3.2.1. Identification of multiple samples, SST and intensity marker

The optimised method was used to evaluate 35 samples of Corydalis (CYR1-35, Figure 10). Figure 11 shows the chromatograms of the reference solutions (tracks A to D) and an average fingerprint generated through electronic merging of the individual fingerprints of all CYR samples (track 1). The four main zones observed in the test solutions are labelled alphabetically and described in a table as acceptance criteria for identification. Under UV 366 nm after derivatisation, the test solution shows a faint to equivalent green fluorescent zone [e], a faint to equivalent blue fluorescent zone [f], a very faint green fluorescent zone [g], a faint to equivalent green fluorescent zone [h], and an intense fluorescent green zone at the application position [j].

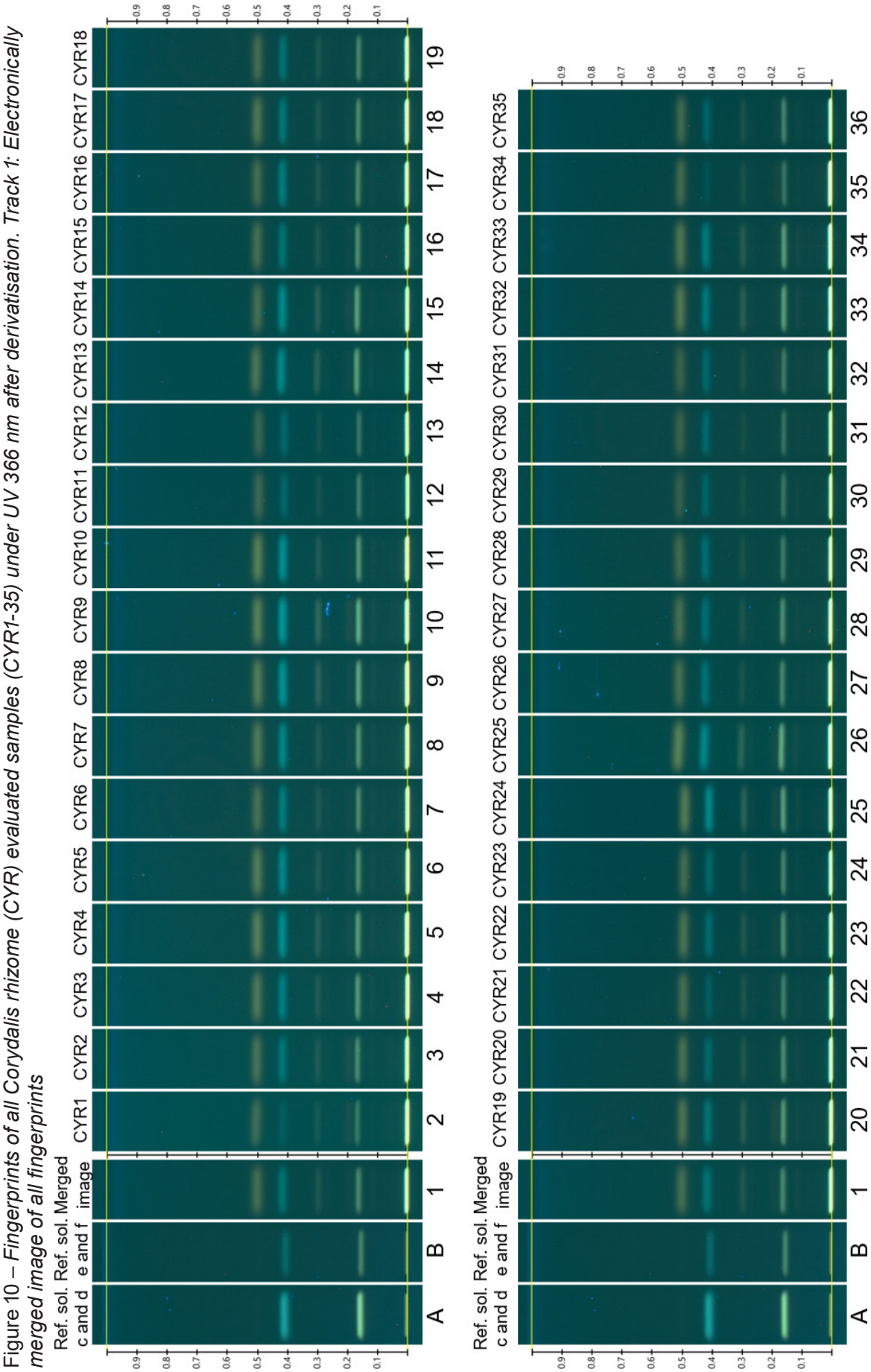
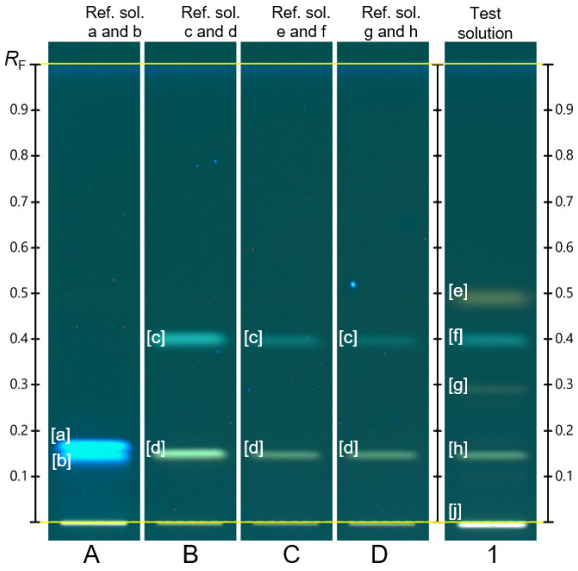


Figure 10 – *Fingerprints of all Corydalis rhizome (CYR) evaluated samples (CYR1-35) under UV 366 nm after derivatisation. Track 1: Electronically merged image of all fingerprints*

Figure 11 – *HPTLC chromatogram and acceptance criteria for visual identification and test for minimum content of corydaline and tetrahydropalmatine (THP) in corydalis rhizome (CYR). Track A: SST; Tracks B to D: reference solutions. Track 1: CYR test solution (average fingerprint). Tracks B-D and 1 are normalised over track B. Intensity markers: corydaline for zone [f], THP for all the other zones.*



Top of the plate		
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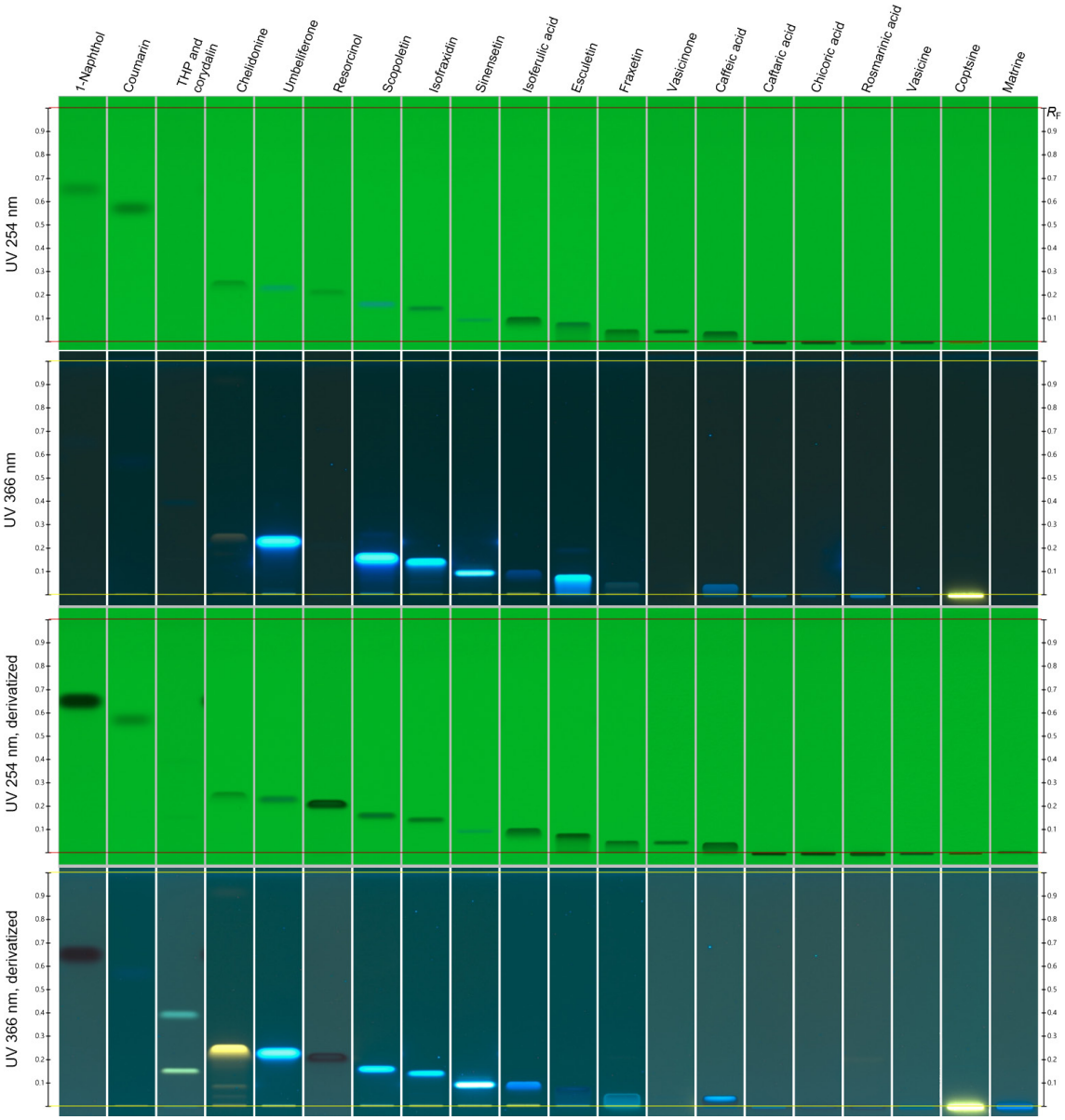
		[e] A faint to equivalent green fluorescent zone
	[c] Corydaline: a blue fluorescent zone	[f] A faint to equivalent blue fluorescent zone
---	---	[g] A very faint green zone ---
[a] Scopoletin: a blue fluorescent zone		
[b] Isofraxidin: a blue fluorescent zone	[d] Tetrohydropalmatine: a greenish fluorescent zone	[h] A faint to equivalent green fluorescent zone
		[j] An intense fluorescent green zone
Reference solutions (a) and (b)	Reference solutions (c) and (d)	Test solution

SST AND INTENSITY MARKERS

To select an SST for CYR, 21 substances were tested with the mobile phase described in the experimental section (Figure 12). Mainly coumarins and some alkaloids migrated between RF 0.1 and 0.3. The most suitable pair was isofraxidin and scopoletin detected under UV 366 nm after derivatisation. Concentrations were adjusted to 0.5 mg/mL (Reference solutions (a) and (b), track A, Figure 11).

To set intensity markers, the same process as for FTB was followed. Reference solutions of tetrahydropalmatine and corydaline at 12.5 µg/mL were used as reference solutions (c) and (d). These solutions were diluted 4-fold to yield a concentration of 3.125 µg/mL for reference solutions (e) and (f).

Figure 12 – *Fingerprints of the reference solutions at 1 mg/mL from secondary metabolites of different classes (e.g. coumarins, alkaloids, hydroxycinnamic acids and flavonoids), analysed for the selection of the system suitability test (SST) for the Corydalis rhizome HPTLC method.*



3.2.2. Development of the test for minimum content based on PPI

The HPTLC test for the minimum content of CYR was developed based on the existing method from the Ph. Eur. [10], in which the contents of corydaline and THP are quantified by LC and combined. With HPTLC, minimum contents for each marker were individually established. As for FTB, first the contents were assessed in two authenticated samples (CYR1 and 2), and a minimum content was agreed upon. In the second part, the linear working ranges for both substances were investigated. The concentration of test solutions was adjusted to the linear range of both standards.

ESTABLISHING MINIMUM CONTENT OF CORYDALINE AND THP

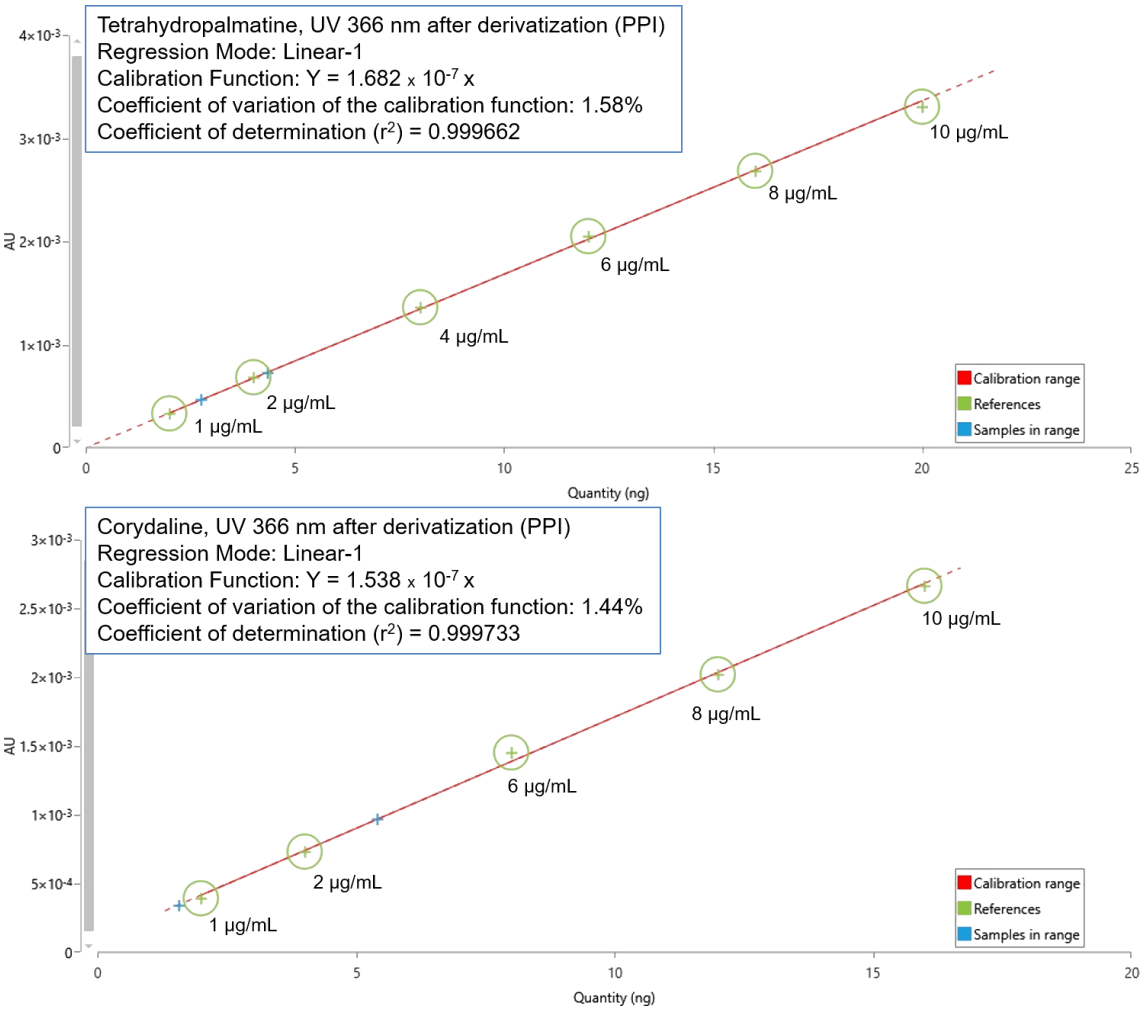
The minimum content of corydaline and THP were established prior to method optimisation, using the HPTLC method from the current monograph on Corydalis rhizome [10]. The concentration of all solutions was 10-fold higher. This part of the work was performed by the EDQM

laboratory. First, the contents of both markers were quantified in samples CYR1 and 2, prepared in triplicate and using calibration curves with five levels of concentration. The calibration data were obtained from PPI under UV 366 nm after derivatisation. The amounts of corydaline were 0.04 % and 0.09 % and THP were 0.06 % and 0.08 % in samples CYR1 and 2, respectively. For the minimum content, it was agreed to use the lowest content of corydaline and THP in these two samples: 0.04 % and 0.06 %, respectively.

ESTABLISHING A LINEAR WORKING RANGE FOR TEST SOLUTIONS

In order to achieve a more accurate quantification by single level calibration, used in the test for minimum content, the linear working range for corydaline and THP in the test solutions was evaluated using the optimised HPTLC method. Calculations were based on peak areas from PPI under UV 366 nm after derivatisation. Linearity was achieved for five concentration levels ranging from 1 to 10 µg/mL (2-20 ng/application) for both markers. Fitting the test solutions into the linear working range required a 10-fold dilution of the test solution described in the current monograph (to a concentration equivalent to 5 mg/mL of the herbal drug). Calibration curves are shown in Figure 13.

Figure 13 – *Calibration curves of tetrahydropalmatine and corydaline. Detection mode: UV 366 nm after derivatisation*



3.2.3. Collaborative trial: organisation and results

As for FTB, a collaborative trial was performed by six laboratories. Eight samples of CYR (CYR36–43), chemical compounds for reference solutions and a Standard Operating Procedure (SOP) were distributed to the participants.

The following acceptance criteria were used to pass/fail samples and reference solutions:

- Evaluation 1, SST: Two blue fluorescent zones due to the standards scopoletin [a] and isofraxidin [b] are barely separable and are seen at RF ~ 0.16 and 0.14, respectively.
- Evaluation 1, intensity markers: A blue zone due to corydaline [c] and a green zone due to THP [d] are seen at RF ~ 0.40 and 0.16, respectively in the tracks corresponding to the reference solutions (c)+(d) and (e)+(f). The ΔRF between experimental and theoretical RF should not be greater than 0.05.
- Evaluation 2, identification: The fingerprint of the test solution should contain zones [e] to [j], with colours and intensities similar to those described in the table of Figure 11. Reference solutions c-f are used.
- Evaluation 3, minimum content (visual evaluation): The intensity of the zones due to corydaline and THP in the fingerprint of the test solution is not less than that obtained with reference solutions (g) and (h), respectively.
- Evaluation 4, minimum content (PPI evaluation): The PPI of the image under UV 366 nm after derivatisation was used. The peak heights of corydaline and THP, recorded for the test solution, are equal to or greater than those recorded for reference solutions (g) and (h).

The results of the collaborative trial are presented in Table 6 and Figure 14. The RF values for the SST and intensity markers were compliant with the acceptance criteria in all laboratories (evaluation 1). All eight samples were compliant with the table description, regarding the number of zones, their colours, and intensities (evaluation 2).

For the test of minimum content based on visual evaluation (evaluation 3), all six laboratories passed samples CYR38–43 and failed sample CYR 36. Two laboratories failed sample CYR37 due to the low intensity of either THP (Laboratory 2) or both markers (Laboratory 1). All other participants considered this sample to pass. The same pattern was observed for the test for minimum content based on PPI (evaluation 4): Laboratories 1 and 2 found sample CYR37 to have a lower content of THP. All other samples had similar results to evaluation 3.

In general, all laboratories participating in the trial came to uniform pass/fail decisions for seven out of the eight samples in all evaluations. Discrepancies were observed only for CYR37 showing a borderline situation, particularly for THP. In this case, it is recommended to repeat the test twice (3 test in total) to confirm the results.

These samples were also assayed by LC. The results of the collaborative trial showed that there was good agreement between results obtained by HPTLC and LC, with the exception of one sample (CYR37) whose THP content failed by HPTLC in two labs and passed by LC. This might be explained by the fact that the content of the marker in the sample was borderline.

Figure 14 – *Results of the collaborative trial for CYR with 6 participant laboratories*

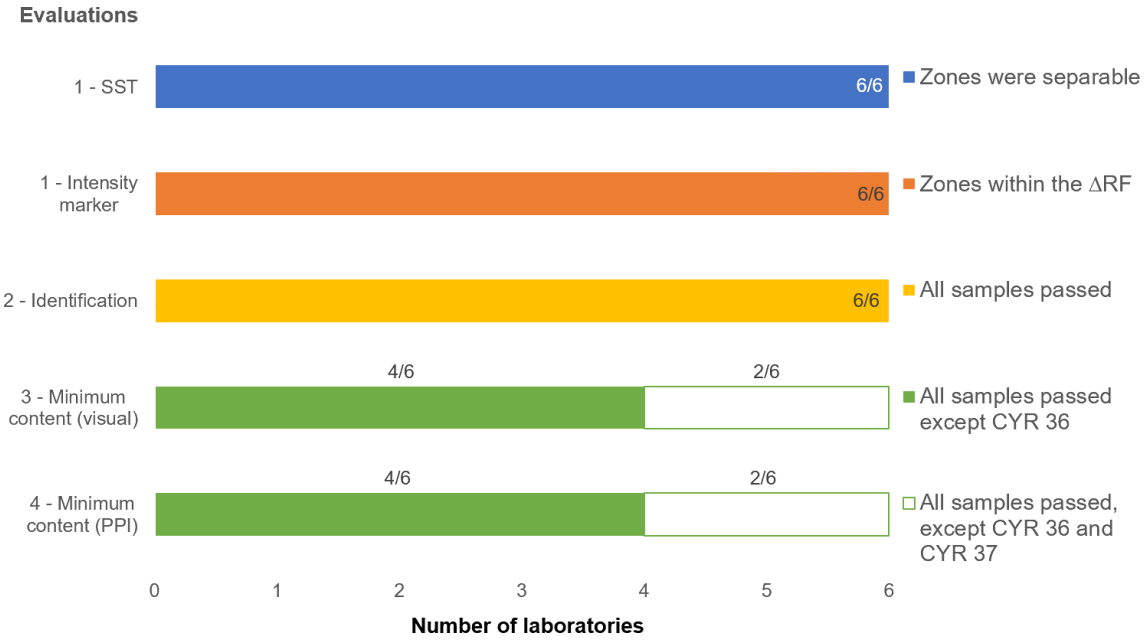


Table 6 – *Summary of the results of the collaborative trial for Corydalis rhizome (CYR) HPTLC method, from six laboratories*

Evaluation 1: SST and intensity markers

	Laboratory	1	2	3	4	5	6
SST	Ref. solution a (scopoletin)	Pass	Pass	Pass	Pass	Pass	Pass
	Ref. solution b (isofraxidin)	Pass	Pass	Pass	Pass	Pass	Pass
IM[a]	Ref. solution c and e (corydaline)	Pass	Pass	Pass	Pass	Pass	Pass
	Ref. solution d and f (THP)	Pass	Pass	Pass	Pass	Pass	Pass

[a] Intensity marker.

Evaluation 2: Identity

Laboratory	1	2	3	4	5	6
CYR36	Pass	Pass	Pass	Pass	Pass	Pass
CYR37	Pass	Pass	Pass	Pass	Pass	Pass
CYR38	Pass	Pass	Pass	Pass	Pass	Pass
CYR39	Pass	Pass	Pass	Pass	Pass	Pass
CYR40	Pass	Pass	Pass	Pass	Pass	Pass
CYR41	Pass	Pass	Pass	Pass	Pass	Pass
CYR42	Pass	Pass	Pass	Pass	Pass	Pass
CYR43	Pass	Pass	Pass	Pass	Pass	Pass

Evaluation 3: Minimum content, visual evaluation

Laboratory	1	2	3	4	5	6
CYR36	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)
CYR37	Fail (corydaline and THP)	Fail (THP)	Pass	Pass	Pass	Pass
CYR38	Pass	Pass	Pass	Pass	Pass	Pass
CYR39	Pass	Pass	Pass	Pass	Pass	Pass
CYR40	Pass	Pass	Pass	Pass	Pass	Pass
CYR41	Pass	Pass	Pass	Pass	Pass	Pass
CYR42	Pass	Pass	Pass	Pass	Pass	Pass
CYR43	Pass	Pass	Pass	Pass	Pass	Pass

Evaluation 4: Minimum content, PPI

Laboratory	1	2	3	4	5	6
CYR36	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)	Fail (corydaline and THP)
CYR37	Fail (THP)	Fail (THP)	Pass	Pass	Pass	Pass
CYR38	Pass	Pass	Pass	Pass	Pass	Pass
CYR39	Pass	Pass	Pass	Pass	Pass	Pass
CYR40	Pass	Pass	Pass	Pass	Pass	Pass
CYR41	Pass	Pass	Pass	Pass	Pass	Pass
CYR42	Pass	Pass	Pass	Pass	Pass	Pass
CYR43	Pass	Pass	Pass	Pass	Pass	Pass

3.3. Discussion

During the initial evaluations, several reproducibility problems were encountered for the existing (HP)TLC methods from pharmacopoeias. This is because most of these methods have never been optimised and fully validated to achieve reproducible quantitative results in different laboratories. Therefore, new HPTLC methods were established based on the investigation and optimisation of different parameters, standardisation and validation of the methodologies. After this step, multiple samples were evaluated with the optimised methods and acceptance criteria for the identification were set. Reference solutions, used as SSTs and intensity markers, were established according to chapter *2.8.25*.

This work shows how to use PPI for the analysis and description of the intensities of the zones in the identification of herbal drugs. The intensity (height) of the peaks in the reference solutions (undiluted and 4-fold diluted) and the test solutions are compared, providing a more objective method of evaluation than visual observation.

The innovative part of this work demonstrated how the HPTLC method for identification can be used for the test for minimum content of markers in herbal drugs. First, the actual contents of the markers were quantitatively determined in samples against a five-level calibration curve. Then the minimum content for each substance was established based on the outcome of this test. For simplicity of routine use, the HPTLC test for minimum content features only one reference solution, prepared at a concentration within the linear range and equivalent to the minimum content in the sample. This approach is suitable for software evaluation through PPI, as well as for visual evaluation.

The reproducibility of the HPTLC methods for the test for minimum content were evaluated and found to be suitable in collaborative trials.

An interim report was presented to the Ph. Eur. Commission during its 159th session and the Ph. Eur. Commission encouraged the TCM WP to continue the work. Finally, the Ph. Eur. Commission accepted the conclusions of the pilot phase unanimously at the 163rd session in March 2019.

4. CONCLUSIONS

The presented HPTLC methods offer a simplified and alternative approach to the verification of the quality of two TCM drugs by eliminating the need for an LC-based assay. The new HPTLC methods combine identification and the test for minimum content in a single analysis. The methods proposed to the Ph. Eur. are suitable for any type of laboratory: those that perform manual HPTLC and laboratories equipped with HPTLC instruments and software. This gives laboratories with a low budget a better chance to test compliance with the pharmacopoeia. After the introduction of the intensity marker to describe the intensity of fingerprints, the (visual) assessment of the minimum content of a single marker against a chemical reference represents an essential step towards a comprehensive use of HPTLC fingerprints. Further studies will show if this concept can be extended to a multi-marker analysis that enables a more holistic approach for the quality control of herbal drugs.

5. ACKNOWLEDGEMENTS

The authors wish to thank the following experts and their teams, who participated in both HPTLC collaborative trials: Prof. Dr Rudolf Bauer and Saskia Hofer, Institute of Pharmaceutical Sciences, Karl-Franzens-Universität, Graz; Prof. Dr Pierre Duez and Mr. Claudio Palmieri, Université de Mons, Belgium; Dr Matthias Weber, EDQM laboratory; Dr Catherine Lenihan, British Pharmacopoeia Commission MHRA.

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