

Validation of Method for the Identification of Black Cohosh by HPTLC Fingerprint

1. Purpose of method to be validated:

The method for identification of Black Cohosh by HPTLC fingerprint is suitable to identify a given sample of plant material as Black Cohosh (*Actaea racemosa* or *Cimicifuga racemosa**) based on its triterpene fingerprint and its pattern of plant acids.

Adulterants, such as other Cohosh species, Caulophyllum thalictroides, and Chinese Sheng Ma, show a different profile.

The method may be used to identify an extract as derived from Black Cohosh (*Actaea racemosa*), provided that the material was made from a single herb and is intended to contain the constituent profile seen in Black Cohosh.

2. General acceptance criteria:

The method is valid if:

- A botanically authenticated sample of Black Cohosh (*Actaea racemosa*) yields a fingerprint which is similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands **and**
- All acceptance criteria specified in sections 5.2 to 5.6 are met **and**
- Any deviation from the expected result doesn't exceed those deviations seen under section 5.7 (Robustness).

* The denominations *Actaea* and *Cimicifuga* are interchangeable. We used the plant names as stated when received.

3. Personnel

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4. Description of method

4.1 Preparation of test solutions

Raw materials: 500 mg of each sample are mixed with 10 mL of ethanol-water 5:5, sonicated for 10 min, and then centrifuged. The supernatant is used as test solution.

Extracts: 200 mg are extracted with 10 mL of ethanol-water (5:5), sonicated for 10 min, and then centrifuged. The supernatant is used as test solution.

4.2 Preparation of reference solutions

Botanical reference solution: as 4.1

Chemical reference solutions: 1 mg each of actein, isoferulic acid, caffeic acid, chlorogenic acid is dissolved in 10 mL methanol each.

4.3 Preparation of derivatizing reagent

20 mL of sulfuric acid are carefully added to 180 mL of ice-cooled methanol.

4.4 Stationary phase

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F₂₅₄ (Merck).

4.5 Sample application

2 μ L of test solution, 2 μ L of botanical reference solution, and 2 μ L of each chemical reference solution are applied each as 8 mm band, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate.

4.6 Temperature and Humidity

Record temperature and humidity in the laboratory. Condition the plate to about 5%RH using a suitable device.

4.7 Chromatography

Chamber type:	10x10 cm (or 20x10 cm) Twin Trough Chamber
Configuration:	Saturated for 20 min (wetted filter paper in trough opposite to the plate)
Developing solvent:	Ethyl formate, toluene, formic acid (30:50:20) 5 mL (respectively 10 mL) developing solvent in each trough.
Developing distance:	70 mm from lower edge of plate (62 mm from application position).
Drying:	5 min with cold air (hair dryer)

4.8 Derivatization

The plate is immersed into reagent for 1 s, then heated at 100°C for 5 min.

4.9 Documentation

- Prior to derivatization under UV 366 nm
- After derivatization under white light
- After derivatization under UV 366 nm

4.10 Image of chromatograms



1. Chlorogenic acid
2. Caffeic acid
3. Isoferulic acid
4. Actein
5. *Actaea racemosa* BRM

4.11 Evaluation of results:

a) UV 366 nm

The standards chlorogenic acid (track 1), caffeic acid (track 2), and isoferulic acid (track 3) each show a blue fluorescing band. Actein (track 4) is not seen. The sample shows several blue fluorescing bands of different intensities. The lowest band is slightly above the position of chlorogenic acid. A band is seen just below the position of caffeic acid. A very faint band corresponding to isoferulic acid is seen.

b) White light after derivatiation

The standards chlorogenic acid (track 1) and caffeic acid (track 2) are not well detected. Isoferulic acid (track 3) and actein (track 4) each show a brownish band. The sample shows one brownish band corresponding to actein and several brownish bands below that zone. There may be brownish zones above the band of actein. One brown zone and two broad blue zones are seen above the application position.

c) UV 366 nm after derivatiation

The standards chlorogenic acid (track 1) and caffeic acid (track 2) and isoferulic acid (track 3) each show a blue to green fluorescing band. Actein (track 4) shows a brownish fluorescing band. The sample shows a cluster of alternating brownish and bluish bands, of which one brown band matches the zone obtained for actein in color and position. One dark zone and two bluish-green zones are seen above the application position.

4.12 System suitability test:

After derivatization, actein is detected as a sharp and clearly distinguished zone in the sample under white light as well as under UV 366 nm.

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality
Toluene	Acros	p.a
Ethyl formiate	Merck and Acros	>98%
Formic acid	Acros	99%
Methanol	Acros	p.a
Sulfuric acid 95-97%	Merck	p.a
Ethanol	Merck	p.a
Water	In house	demineralization

5.1.2 Samples and Reference materials

Botanical reference material

Name	Source / Lot	Authentication
<i>Actaea racemosa</i> (Black Cohosh root)	Removed - proprietary information	Yes

Additional samples

Name	Source / Lot	Authentication
<i>Cimicifuga racemosa</i>	Removed - proprietary information	Yes
<i>Cimicifuga racemosa</i> powder		?
<i>Cimicifuga racemosa</i> powder		?
<i>Cimicifuga</i> (wild USA)		No
<i>Cimicifuga</i> (cultivated Germany)		No
<i>Cimicifuga</i> (cultivated Switzerland)		No
<i>Cimicifuga</i> (cultivated Switzerland)		No
<i>Cimicifuga</i> (wild USA)		No
<i>Cimicifugae rhizome conc.</i>		No

Adulterants

Name	Source / Lot	Authentication
<i>Actaea podocarpa</i> (Yellow cohosh root)	Removed - proprietary information	Yes
<i>Actaea podocarpa</i>		No
<i>Actaea podocarpa</i>		Yes
<i>Actaea pachypoda</i> (White)		Yes

Cohosh root)		
<i>Actaea pachypoda</i> (White Cohosh leaves)	Removed - proprietary information	Yes
<i>Actaea rubra</i>		Yes
<i>Actaea pachypoda</i>		Yes
<i>Cimicifuga foetida</i> powder		?
<i>Cimicifuga heracleifolia</i> powder		?
<i>Cimicifuga dahurica</i> / Bei Shengma		Yes
Sheng Ma (<i>Cimicifugae dahuricae</i> rhizoma)		Yes
<i>Cimicifuga americana</i> ?		No
<i>Cimicifuga</i> species unknown		No
<i>Cimicifuga</i> sp unknown		No
<i>Caulophyllum thalictroides</i> (Blue Cohosh root)		Yes
Guang Dong Sheng Ma		No
Guang Shengma (no <i>Cimicifuga</i>)		Yes

Processed materials

Name	Source / Lot
<i>Cimicifuga racemosa</i> extract 2.5%	Removed - proprietary information

Standards (marker compounds, chemical references)

Name	Source
Actein	ChromaDex 00-01355-101
Isoferulic acid	ChromaDex 01-09251-7100
Caffeic acid	Fluka 353773/1 41198
Chlorogenic acid	Merck 1285758

5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB526793

5.1.4 Instruments[†]

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	061104

[†] All plates have been developed in an automatic chamber with humidity control (ADC2) if not otherwise specified.

TTC 20x10 cm	CAMAG	n. a.
TTC 10x10 cm	CAMAG	n. a.
ADC2	CAMAG	120425 and 120424
TLC Plate Heater III	CAMAG	981109
Immersion Device III	CAMAG	090301
Mill KB5/10	IKA	00.183107
Centrifuge EBA21	Hettich	0000799-01-00
Ultrasonic Bath TPC25	Telsonic	2003043
Balance AG245	Mettler-Toledo	1114402254
Conditioning tray	CAMAG	n.a.

5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.3.4 and 1.4.0
VideoScan	CAMAG	1.02.00

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5.2 Stability

5.2.1 Stability of analyte in solution and on the plate

Description of experiment:

A portion of the BRM is extracted according to section 4.2. 2 μ L of this solution are applied onto a 10x10 cm plate according to sections 4.4-4.5. The sample and the plate with the applied sample (wrapped in aluminum foil) are set aside. After 3 hours another portion of the same BRM is extracted according to section 4.2. Two times 2 μ L of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 2 μ L of the set-aside sample (see illustration below).

The 4 samples on the plate represent the following: (A) Sample on the plate for 3 hours prior to chromatography, (B) fresh sample applied immediately prior to chromatography (twice), (C) sample prepared 3 hours prior to chromatography (in solution).

The plate is treated according to sections 4.6 to 4.9.

Acceptance criteria:

The sample is stable for at least 3 hours in solution and 3 hours on the plate prior to chromatography.

Results:

The sample is stable.

Images:



1. Sample on the plate for 3 hours prior to chromatography (A)
2. Fresh sample applied immediately prior to chromatography (B)
3. Sample prepared 3 hours prior to chromatography (in solution) (C)
4. Fresh sample applied immediately prior to chromatography (identical with 2) (B)

Accepted: YES

5.2.2 Stability of analyte during chromatography

Description of experiment:

A portion of the BRM is extracted according to section 4.2. 5 μ L are applied as spot at the lower right corner of a 10x10cm plate (10 mm from each edge). The plate is developed and dried according to section 4.6-4.7. The plate is now turned 90° to the right and developed a second time according to section 4.6-4.7 with a fresh portion of developing solvent. The plate is derivatized and documented according to section 4.8 and 4.9.

Acceptance criteria:

The sample is stable during chromatography if all zones are located on the diagonal connecting the application position with the intersection of the two solvent fronts.

Results:

No major zone is located aside of the diagonal. A secondary front in the chromatogram causes a bent in the diagonal. A few minor zones are located off the diagonal. They suggest some instability respectively irreversible adsorption (arrow)
Nevertheless the sample is regarded as stable during chromatography.

Images:



Accepted: YES

5.2.3 Stability of derivatization/result

Description of experiment:

The botanical reference solution (4.2) is chromatographed according to section 4.4-4.8. After documentation under white light (4.9), the plate is observed for 1 hour. An image is taken after 1, 5, 10, 20, 30 min, and about 1h. The images are compared visually and with the help of video-densitometry.

Acceptance criteria:

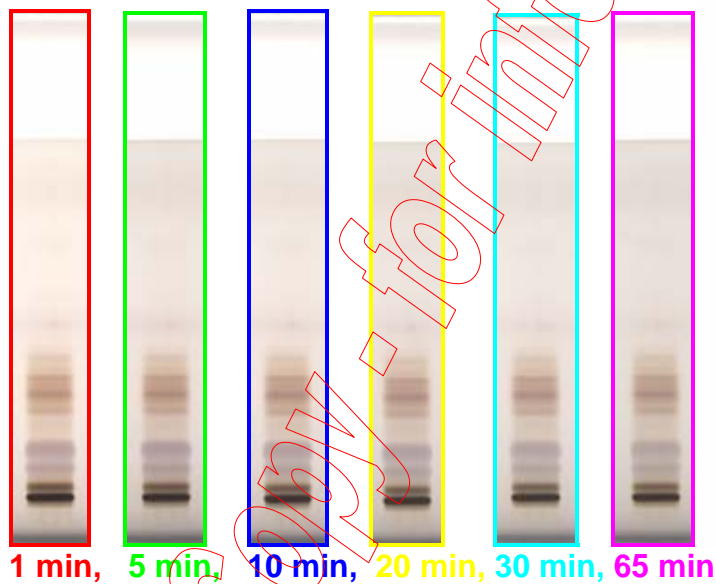
The derivatization yields a stable result, if there is no significant change in the image within 30 min.

Results:

The color of the zones (white light) on the derivatized plate fades slightly over time, but no zone disappears.

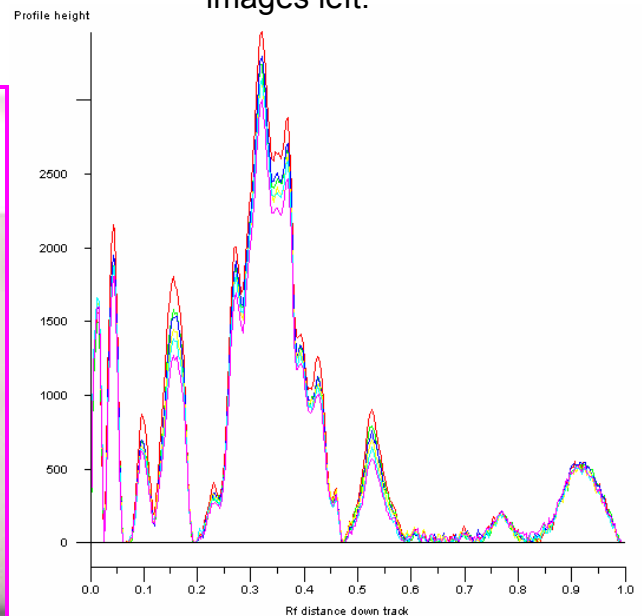
The intensity of the fluorescence (UV 366 nm) increases in the first 10 min and then decreases again. No zone disappears.

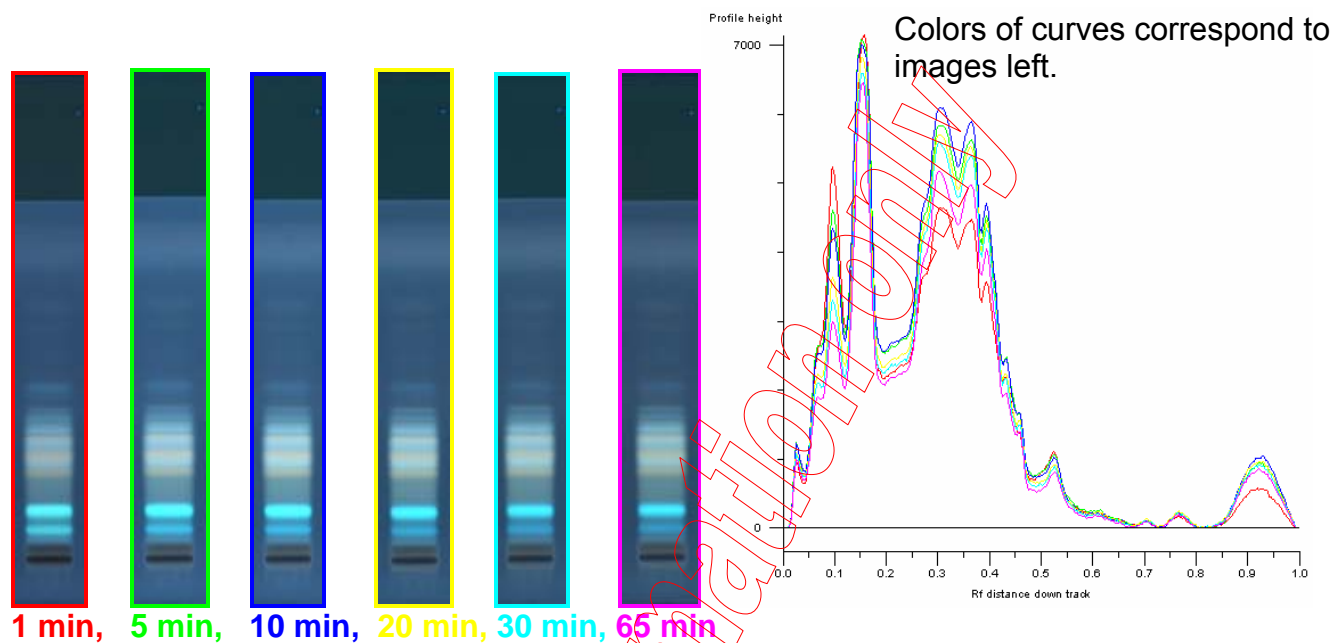
Images / Densitograms:



White light

Colors of curves correspond to images left.





UV 366 nm

Accepted: YES

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5.3 Specificity

5.3.1 Identification of Black Cohosh samples and processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Description of experiment:

Test solutions are prepared according to section 4.1. The BRM and the chemical references are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.6-4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

Acceptance criteria:

The method is specific if the fingerprints obtained with the test solutions representing Black Cohosh are similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands matching the chromatogram of the BRM **and** samples of other identity, if present, yield different fingerprints.

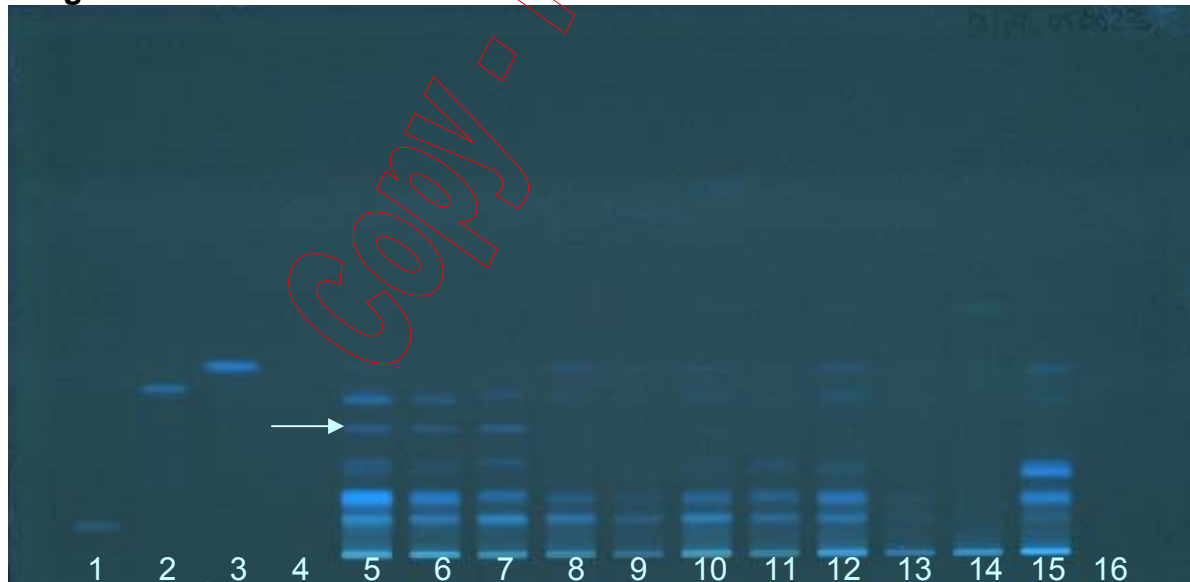
Results:

UV 366 nm: Only samples on tracks 6 and 7 match the BRM on track 5. Samples on tracks 8-12 miss the blue band marked with an arrow and the lower zones are fainter. Sample on track 13 (extract) shows only very faint zones. Sample on track 14 doesn't show any blue zone. The intensity of zones in sample on track 15 is inverted.

After derivatization, white light: Actein is detected in all samples on tracks 5-13. The profile of those samples is similar (samples on track 9, 11, and 13 are faint). Sample on track 13 misses the two blue zones near the application position. Sample on track 14 doesn't show any zone. The profile of zones in sample on track 15 is different, actein is missing.

After derivatization, UV 366 nm: Actein is detected in all samples on tracks 5-13. The profile of those samples is similar (samples on track 9, 11, and 13 are faint and the brown zone at Rf 0.24 is missing or very faint). Sample on track 14 doesn't show any zone. The profile of zones in sample on track 15 is different, actein and other brown zones are missing. Samples on tracks 7, 8, 9, 11 and 14 don't show a brown zone above the application position.

Images:





- | | |
|---|---|
| 1. Chlorogenic acid | 9. <i>Cimicifuga</i> (cultivated Switzerland)* |
| 2. Caffeic acid | 10. <i>Cimicifuga</i> (cultivated Switzerland)* |
| 3. Isoferulic acid | 11. <i>Cimicifuga</i> (wild USA)* |
| 4. Actein | 12. <i>Cimicifugae</i> rhizome conc.* |
| 5. <i>Actaea racemosa</i> BRM | 13. <i>Cimicifuga racemosa</i> extract 2.5%* |
| 6. <i>Cimicifuga racemosa</i> Collected in Watauga Co, NC | 14. <i>Cimicifuga racemosa</i> powder |
| 7. <i>Cimicifuga</i> (wild USA) | 15. <i>Cimicifuga racemosa</i> powder |
| 8. <i>Cimicifuga</i> (cultivated Germany)* | 16. Actein |
- * These samples are about 5 years old.

Accepted: YES

Samples on tracks 14 and 15 don't comply.

Sample 9 is low in content.

The extract (track 13) shows only faint bands (low content).

5.3.2 Detection of adulteration

Description of experiment:

Adulterants are prepared according to section 4.1. The BRM and chemical references are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.6-4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

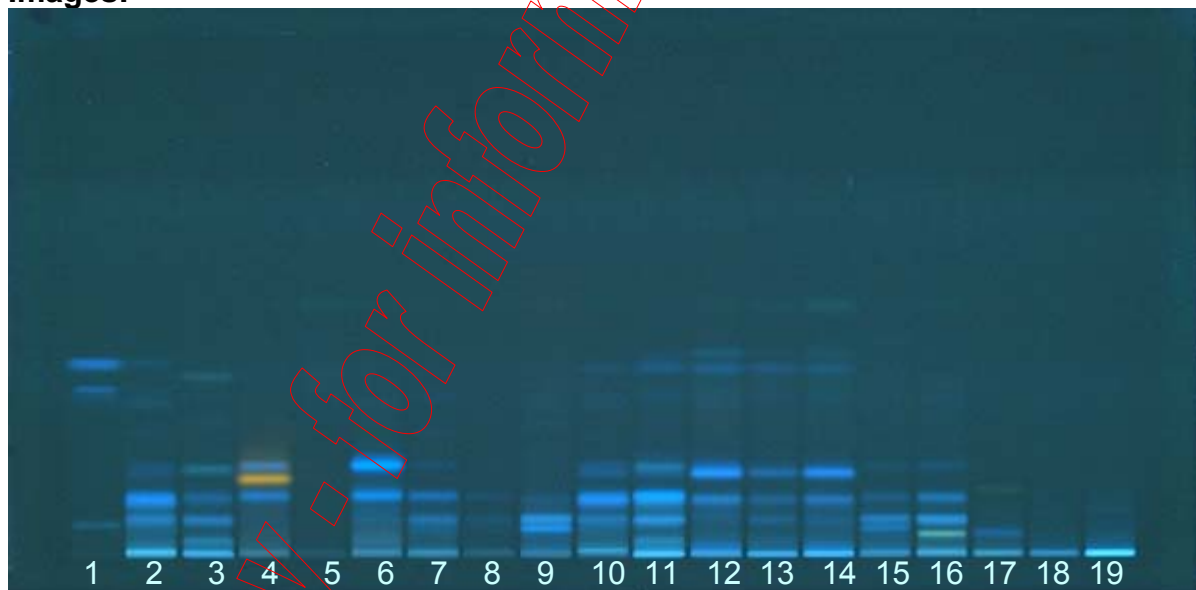
Acceptance criteria:

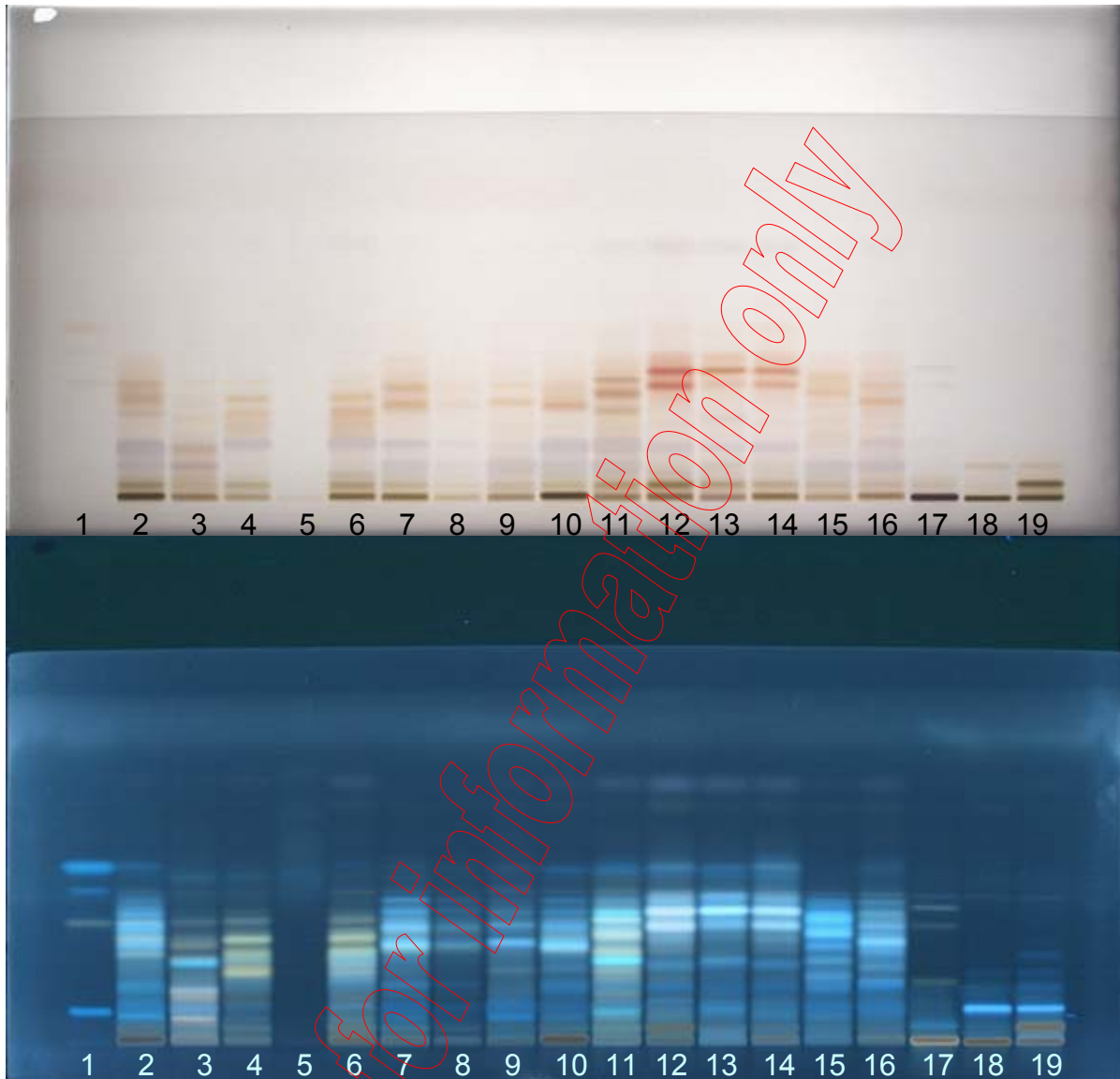
The method is specific for Black Cohosh (*Actaea racemosa*) if the fingerprints of other Cohoshes and adulterants (*Actaea* species, *Caulophyllum thalictroides*, Chinese Sheng Ma) are significantly different from those of the BRM with respect to number, position, color, and intensity of bands.

Results:

No other *Actaea* species shows the same fingerprint as *A. racemosa*. Neither do the other adulterants.

Images:





1. Chlorogenic acid, actein, caffeic acid, and isoferulic acid (increasing Rf values)
 2. *Actaea racemosa* BRM
 3. *Cimicifuga americana**? = *Actaea podocarpa*
 4. *Actaea podocarpa* (Yellow cohosh root)
 5. *Actaea podocarpa* (authenticated)
 6. *Actaea podocarpa*
 7. *Actaea pachypoda*
 8. *Actaea pachypoda*
 9. *Actaea pachypoda* (leaves)
 10. *Actaea rubra*
 11. *Cimicifuga foetida* powder
 12. *Cimicifuga dahurica*
 13. *Cimicifuga dahurica* / Bei Shengma
 14. *Cimicifuga heracleifolia* powder
 15. *Cimicifuga* sp unknown*
 16. *Cimicifuga* sp unknown*
 17. *Caulophyllum thalictroides* (Blue Cohosh root)
 18. Guang Shengma (no *Cimicifuga*)
 19. Guang Dong Sheng Ma
- * These samples are about 5 years old.

Accepted: YES

Sample 5 (authenticated *Actaea podocarpa*) doesn't pass (shows no zone)

Sample 3 is probably not *Cimicifuga americana* (syn. *Actaea podocarpa*).

Note: sample on track 14 (*Cimicifuga heracleifolia*) looks like *Cimicifuga dahurica* (tracks 12 and 13).

5.4 Repeatability

Description of experiment:

Three portions of the BRM are individually prepared according to section 4.2. Onto three 20x10 cm plates, three aliquots of 2 μ L of each reference solution are applied according to section 4.5. The plates are chromatographed (section 4.6-4.7) subsequently using the same chamber but fresh portions of the developing solvent and fresh filter paper. The plates are derivatized and documented according to 4.8-4.9.

The results across each plate and from plate to plate are evaluated. The average Rf values of three zones are determined for each track on each plate.

Acceptance criteria:

The repeatability of the method is acceptable if:

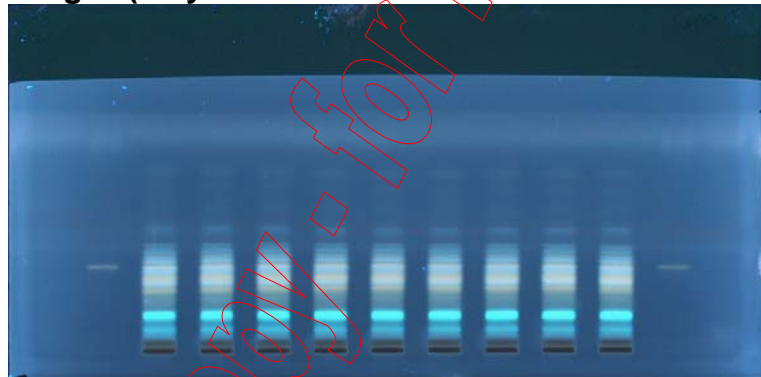
All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and** the Rf values for each of the three zones on the three plates don't vary more than 0.02.

Results:

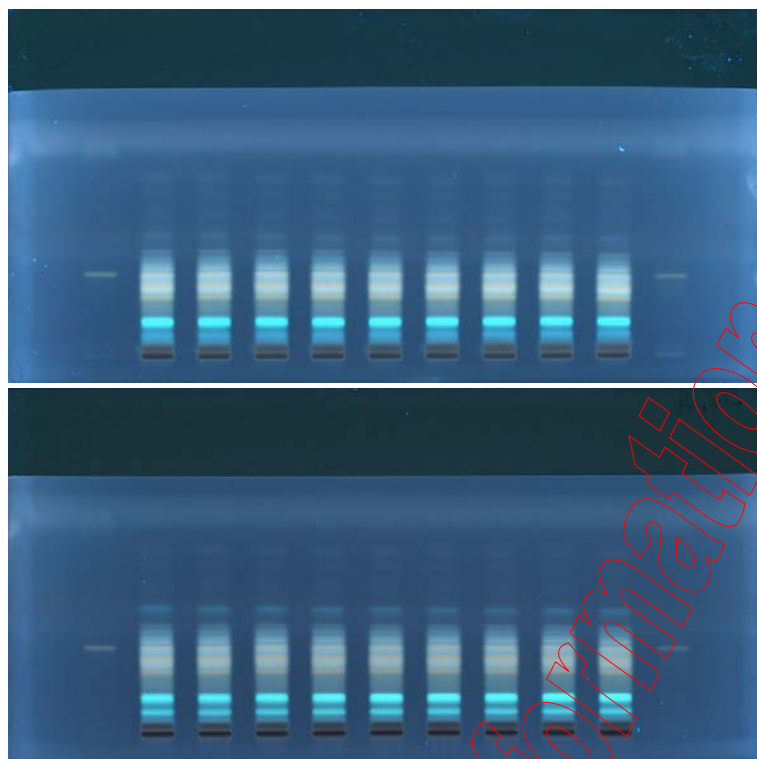
All chromatograms look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen.

Note: the chemical reference acetin was also included for better interpretation.

Images (only detection under UV 366 nm after derivatization):



P119_050830_01



P119_050830_02

P119_050830_03

Rf	P119_050830_01	P119_050830_02	P119_050830_03	ΔRf
Fraction 1 (Actein)	0.36	0.35	0.35	0.01
Fraction 2	0.30	0.30	0.30	0.0
Fraction 3	0.25	0.25	0.25	0.0

Accepted: YES

5.5 Intermediate precision

Description of experiment:

Repeat the experiment described under 5.4 on 2 other days.

The average Rf values of the three zones are determined for each track on each plate (one plate prepared during experiment 5.4, 2 plates on different days) and variations from plate to plate are evaluated.

Acceptance criteria:

The intermediate precision of the method is acceptable if:

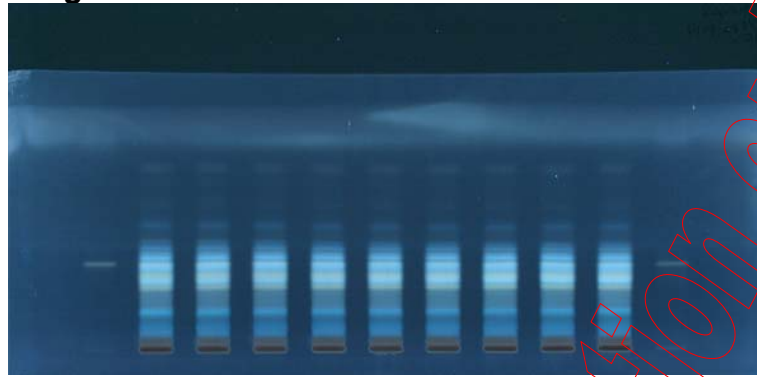
All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and** the average Rf values for each of the three zones on the three plates don't vary more than 0.05.

Results:

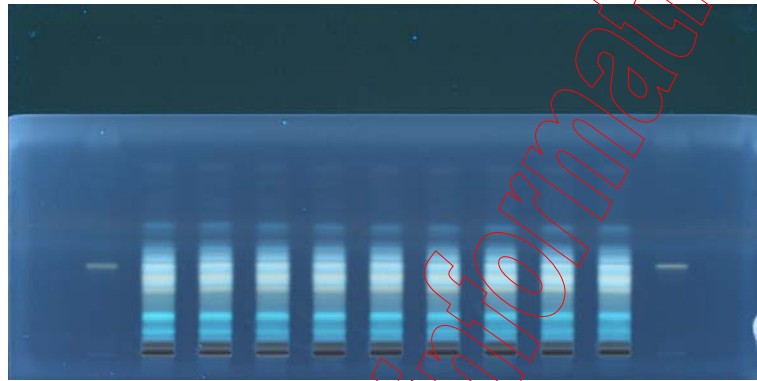
All chromatograms look very similar with respect to number, position, color, and intensity. No disturbances are seen.

Note: the chemical reference actein was also included for better interpretation.

Images:



P119_050831_01



P119_050901_01

Rf	P119_050830_01	P119_050831_01	P119_050901_01	Δ Rf
Fraction 1 (Actein)	0.36	0.34	0.38	0.04
Fraction 2	0.30	0.28	0.33	0.05
Fraction 3	0.25	0.24	0.28	0.04

Accepted: YES

5.6 Reproducibility

Description of experiment:

The confirmatory lab repeats the experiment described under 5.4 (Repeatability).

Acceptance criteria:

The reproducibility of the method is acceptable if:

All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and** the average Rf values for each of the four zones on the three plates don't vary more than 0.02.

The reproducibility is acceptable if the Rf obtained in this test are not significantly different from those obtained in section 5.4 (<0.05 if using plate from the same manufacturer, <0.07 for plates of different manufacturers).

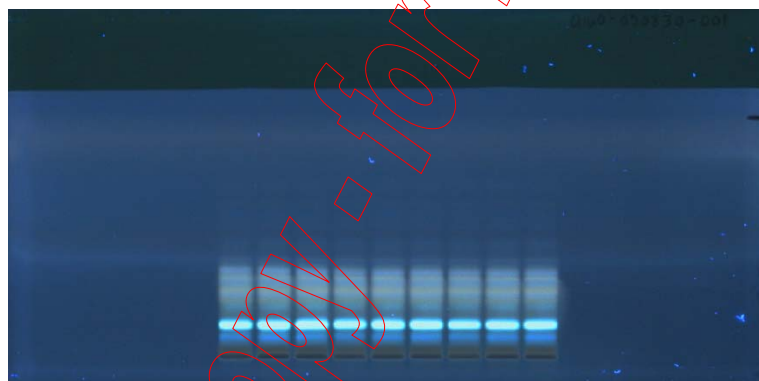
Results:

All chromatograms developed by the confirmatory lab look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen.

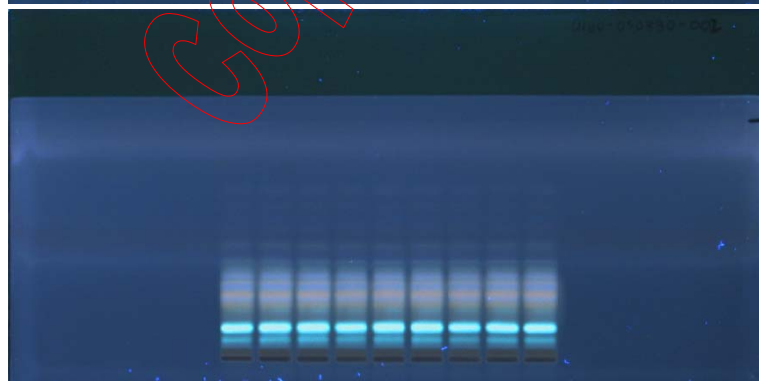
The color/brightness of the image differs slightly from the image made in the primary lab. This could be due to different documentation devices and settings.

Note: the humidity was adjusted by conditioning of the plate over sulfuric acid for 30 min prior to development in a Twin Trough Chamber.

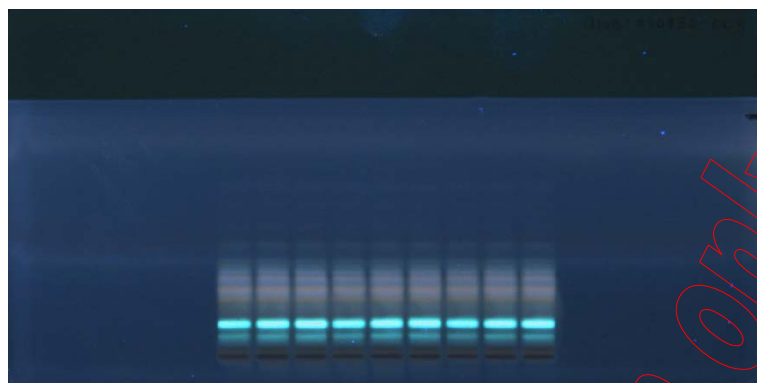
Images:



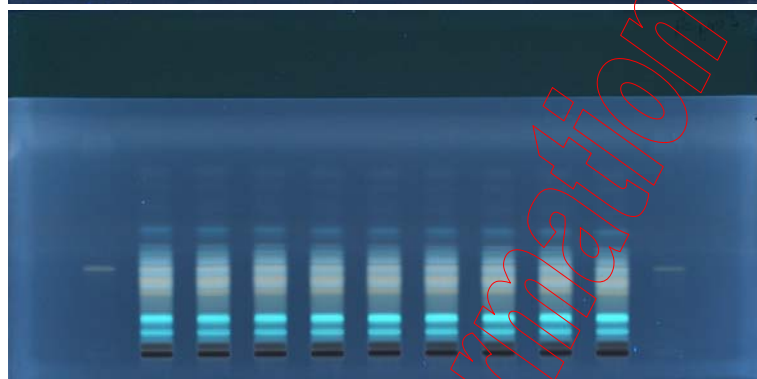
A160-050830-01



A160-050830-02



A160-050830-03



P119_050830_01

Rf	A160-050830-01	A160-050830-02	A160-050830-03	ΔRf	P119_050830_01 (Comparison)	ΔRf
Fraction 1 (Actein)	0.33	0.33	0.33	0.0	0.36	0.03
Fraction 2	0.28	0.28	0.28	0.0	0.30	0.02
Fraction 3	0.23	0.23	0.23	0.0	0.25	0.02

Accepted: YES

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5.7. Robustness

5.7.1 Chamber type

Description of experiment:

The method is executed according to section 4 using the BRM and the chemical reference actein. An automatic chamber with humidity control (ADC2) and a Twin Trough Chamber are compared. The plate is conditioned over sulfuric acid for 30 min prior to development (Twin Trough Chamber).

Acceptance criteria:

The fingerprints obtained in both chambers are similar with respect to number, position, color, and intensity of zones. The Rf values obtained in this test are not significantly different from those described in section 4.10 (<0.05). In the case of differences between the results the use of a Twin Trough Chamber must be excluded.

Results:

No difference is seen when the plate is developed in an automatic chamber (ADC 2) or in a Twin Trough Chamber.

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Images:

ADC2



Twin Trough Chamber



Rf	ADC2 <i>P119_050830_01</i>	Twin Trough Chamber	ΔRf
Fraction 1 (Actein)	0.36	0.34	0.02
Fraction 2	0.30	0.30	0.0
Fraction 3	0.25	0.25	0.0

Accepted: YES

5.7.2 Developing distance

Description of experiment:

The method is executed according to section 4 using only the BRM and the chemical reference actein. The developing distance is increased to 80 mm from the lower edge of plate.

Acceptance criteria:

The fingerprints obtained with different developing distances are similar with respect to number, position, color, and intensity of zones. The Rf values obtained in this test are not significantly different from those described in section 4.10 (<0.05). In the case of differences between the results the developing distance of more than 70 mm yields invalid results.

Results:

The separation is not affected by the increased developing distance

Images:

70 mm



80 mm



Rf	P119_050830_01	P119_050830_04	Δ Rf
Fraction 1 (Actein)	0.36	0.33	0.03
Fraction 2	0.30	0.28	0.02
Fraction 3	0.25	0.24	0.01

Accepted: YES

5.7.3 Waiting times

This experiment was not performed. The samples have to be chromatographed, derivatized, and evaluated without any delay.

5.7.4 Relative humidity

Description of experiment:

Four plates are prepared according to section 4 using only one BRM and the chemical reference actein. Prior to chromatography (4.7), the plates are conditioned over salt solutions or using a molecular sieve for adjusting different relative humidity (in ADC2). Relative humidity covering a range of about 2-50%RH should be tested.

Alternatively, results of plates developed under different relative humidity are compared.

2%: molecular sieve

35 %: magnesium chloride

46%: potassium thiocyanate

50%: ambient humidity in laboratory

Acceptance criteria:

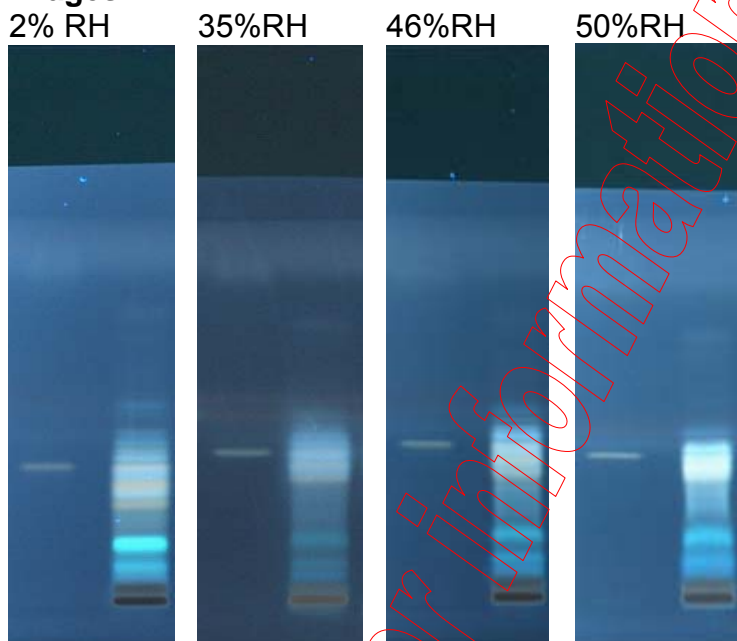
The fingerprints obtained under different relative humidity are similar with respect to number, position, color, and intensity. In this case the relative humidity does not affect the result. In the case of differences of the results, the method may require the control of relative humidity.

Results:

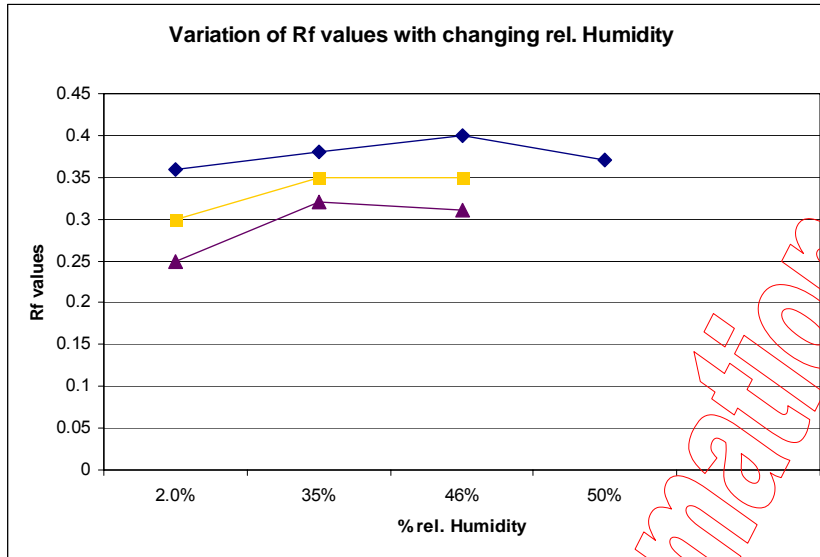
The Rf values of the selected marker compounds vary significantly. The separated zones become more diffuse with increasing humidity. No separation is achieved for the cluster at the position of actein at RH 50%.

It was concluded that the method has to be performed at less than 5% RH.

Images:



Rf	2% RH	35%RH	46%RH	50%RH
Fraction 1 (Actein)	0.36	0.38	0.40	0.37
Fraction 2	0.30	0.35	0.35	-
Fraction 3	0.25	0.32	0.31	-



Accepted: No

6. Conclusions, Approvals, and Signatures

6.1 Conclusions of primary lab

The method is valid if chromatography is performed with plated conditioned at or below 5% RH.

Date:

Analyst of primary lab:

6.2 Conclusions of substantiating lab

The method is valid

Date:

Analyst of substantiating lab:

6.3 Final approval of study director

The method is valid

Date:

Study director: