

Validation of Method for Identification of Eleuthero by HPTLC Fingerprint

1. Purpose of method to be validated

The method for the identification of Eleuthero by HPTLC fingerprint is suitable to identify a given sample of plant material as Eleuthero root (*Eleutherococcus senticosus* syn. *Acanthopanax senticosus*) based on the separation of Eleutherosides E, B, and E1 (lignan fingerprint).

Adulterants, such as *Periploca sepium*, show a different profile and can be recognized.

The method is also applicable for identification of extracts and finished products as derived from Eleuthero root (*Eleutherococcus senticosus*), provided that the material was made from a single herb and intended to contain the constituent profile seen in Eleuthero.

2. General acceptance criteria

The method is valid if:

- A botanically authenticated sample of *Eleutherococcus senticosus* 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).

3. Personnel

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4. Description of method to be validated

4.1 Preparation of test solutions

1 g of milled root (or enough product equivalent to that amount) is sonicated for 10 min with 5 mL ethanol-water 1:1. The solution is centrifuged and the supernatant is used as test solution.

Liquid samples are used neat.

4.2 Preparation of reference solutions

Botanical reference solution: as 4.1

Chemical reference solutions: 1 mg of each eleutheroside (B, E, E1) is dissolved in 1.5 mL ethanol-water 1:1.

4.3 Preparation of derivatizing reagent

Sulfuric acid reagent: 20 mL sulfuric acid are carefully added to 180 ml ice-cold methanol.

4.4 Stationary phase

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

4.5 Sample application

10 µL of test solution(s) and of botanical reference solution and 2 µL of chemical reference solutions are applied each as 8 mm bands, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate. The application volume of neat liquid samples can be reduced to 2-5 µL.

4.6 Temperature and Humidity

Record temperature and humidity in the laboratory.

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: Chloroform, methanol, water (70:30:4), 5 mL (respectively 10 mL) developing solvent per trough.
Developing distance: 70 mm from lower edge of plate (62 mm from application position)
Drying: 5 min with cold air (hair dryer)

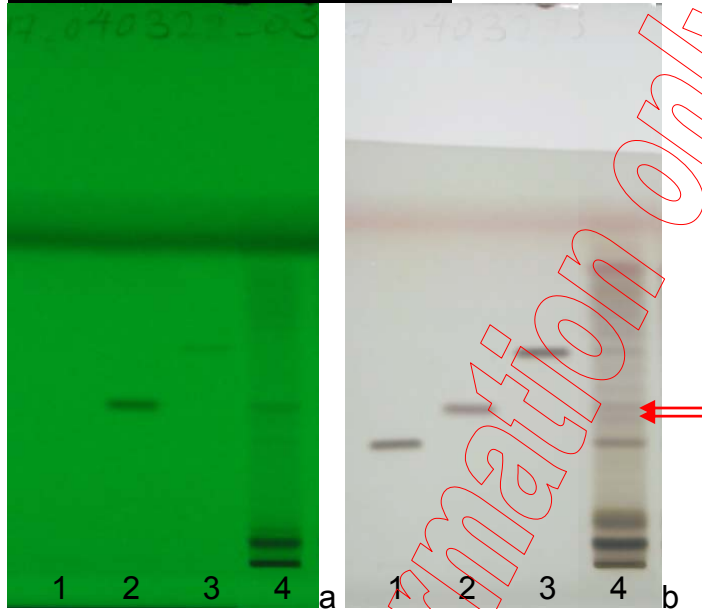
4.8 Derivatization

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

4.9 Documentation

- a) Prior to derivatization under UV 254 nm
- b) After derivatization under white light (reflection and transmission)

4.10 Images of chromatograms



1. Eleutheroside E
2. Eleutheroside B
3. Eleutheroside E1
4. Eleuthero root

4.11 Evaluation of results:

UV 254 nm

The reference substance eleutheroside B (Lane 2, $R_f=0.45$) appears as a dark band. A fainter band for eleutheroside E1 is also seen (Lane 3, $R_f=0.60$). Eleutheroside E (Lane 1) is not seen using this method of detection. A band matching eleutheroside B is seen in the sample. A dark band just above the application position is also seen in the sample.

White light

The reference substance eleutheroside E (Lane 1, $R_f=0.34$), eleutheroside B (Lane 2, $R_f=0.45$), and eleutheroside E1 (Lane 3, $R_f=0.60$) appear as brown bands. The sample shows bands corresponding to eleutherosides E, B, and E1, two very strong bands near the application, and reddish zones near the front. Additional brownish-purple zones are seen.

4.12 System suitability test:

The result obtained in the test is suitable for evaluation if the following requirement is met. After derivatization the fingerprint of the test solution shows a zone corresponding to eleutheroside B and another zone directly below. These two zones appear as two distinct bands, see arrows in Fig. 4.10

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality
Ethanol	Merck	p.a
Methanol	Merck and Acros	p.a
Chloroform	Merck	p.a
Sulfuric acid 95-97%	Merck	p.a
Water	In house demineralization (Ion exchange)	--

5.1.2 Samples and Reference materials

Botanical reference material

Sample Name	Source / Lot	Authentication
Eleuthero Root, shredded	Removed - proprietary information	yes

Additional samples

Sample Name	Source	Authentication
Siberian Ginseng Whole Root*	Removed - proprietary information	yes
Siberian Ginseng Root Bark*		yes
Siberian Ginseng Inner Root*		yes
Siberian Ginseng Whole Root		yes
Siberian Ginseng Inner Root		yes
Siberian Ginseng Whole Root		yes
Siberian Ginseng Whole Root		No
Siberian Ginseng Whole Root		No
Siberian Ginseng Whole Root		No
Siberian Ginseng Root slices		No

* Different parts of the same root

Adulterants

Sample Name	Source	Authentication
Periploca sepium Root Bark	Removed - proprietary information	No
Periploca sepium Root Bark		Yes

Processed materials

Name	Source	Notes
Powdered Eleuthero Extract	Removed - proprietary information	USP reference standard, 2 bottles of same lot.
Siberian ginseng tincture		No ratio given, water/alcohol
Siberian ginseng tincture		Dry herb menstrum ratio 2:1, 32-34% organic dry grain alcohol, custom wildcrafted eleutherococcus.

Siberian ginseng tincture	Removed - proprietary information	40% alcohol/water
Siberian ginseng tablet		Also contains licorice root
Siberian ginseng tea bag		No data
Siberian ginseng capsule		5:1 concentration root extract powder
Siberian ginseng tincture		No data
Siberian ginseng tincture		30-35% grain alcohol, water, vegetable glycerin

Standards (marker compounds, chemical references)

Name	Source
Eleutheroside B	ChromaDex 05060-101
Eleutheroside E	ChromaDex 05065-101
Eleutheroside E1	ChromaDex 05064-101

5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB291342, OB464935
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB302430, OB475743

5.1.4 Instruments

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	061104
DigiStore using Canon G5 camera	CAMAG	070705
TTC 20x10 cm	CAMAG	n. a.
TTC 10x10 cm	CAMAG	n. a.
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

5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.2.6-1.3.3
VideoScan	CAMAG	1.02.00

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. 10 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 10 μ L of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 10 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.

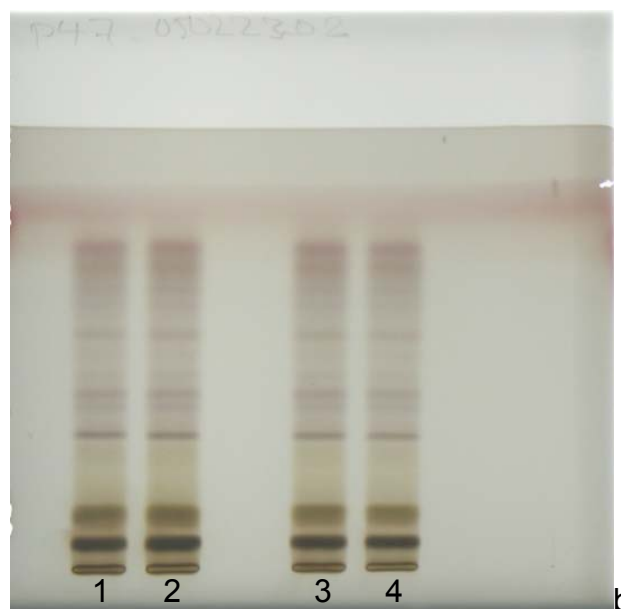
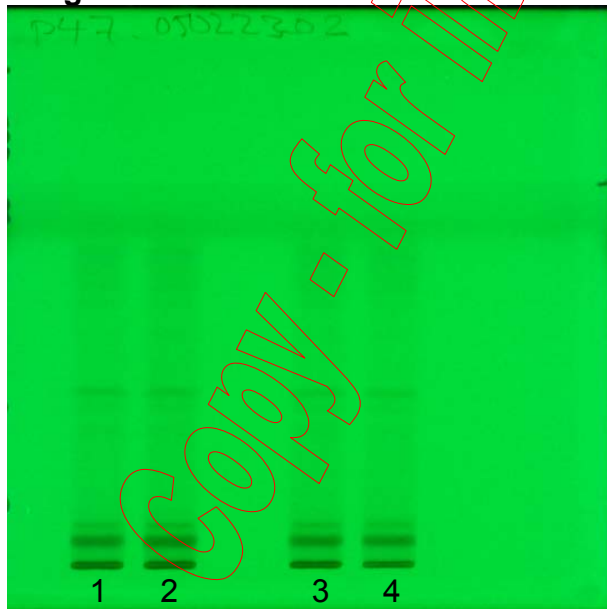
Acceptance criteria:

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

Results:

No difference is seen in any of the chromatograms. The sample is stable on the plate and in solution for at least 3 hours.

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. 10 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. The plate is now turned 90° to the right and developed a second time according to section 4.6 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 zone is located aside of the diagonal. The sample (Eleuthero root) is 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 4.3-4.8. After documentation under white light (4.9), the plate is observed for 1 hour. An image is taken after 5, 10, 30 min, and 1h. The images are compared visually and with the help of video-densitometry.

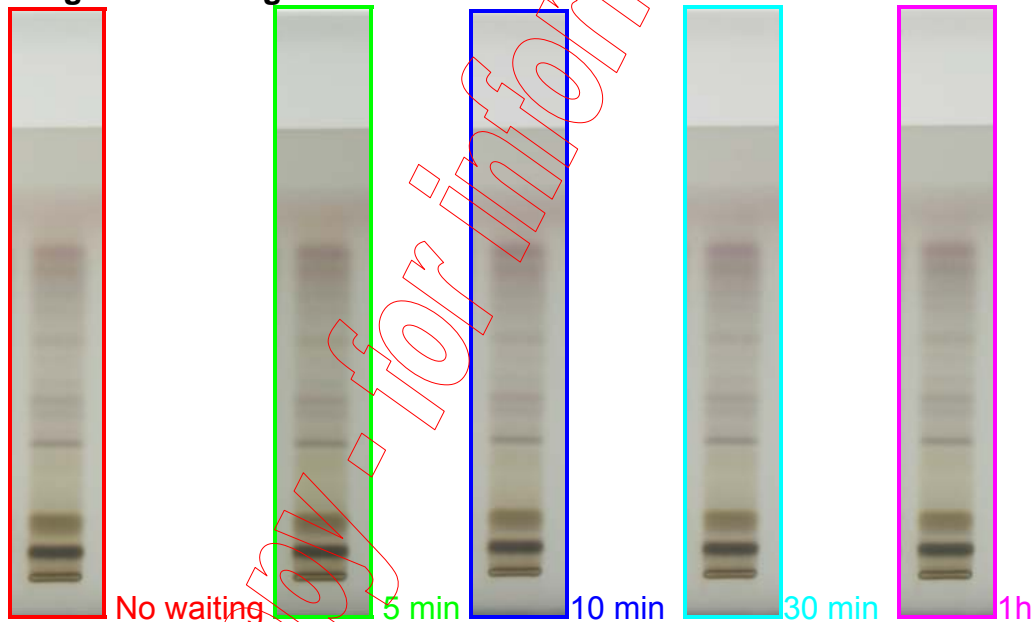
Acceptance criteria:

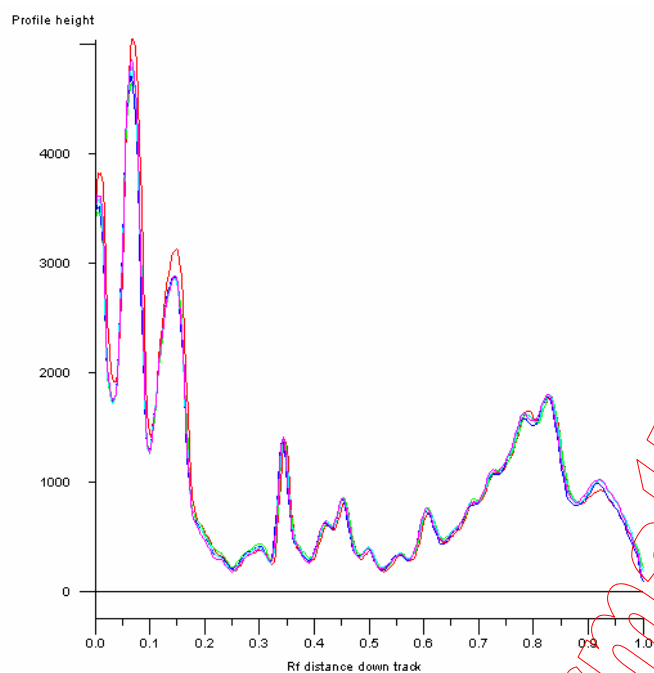
The derivatization yields a stable result, if there is no significant change in the image within 30 min.

Results:

The intensity of zones diminishes slightly in the first 5 min, but no zone disappears. No change between 5 min and 1h.

Images / Densitograms:





Colors of chromatograms correspond to images above.

Accepted: YES

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5.3 Specificity

5.3.1 Identification of Eleuthero root samples 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 of *Eleutherococcus senticosus* 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 4.7) 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 *Eleutherococcus* 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:

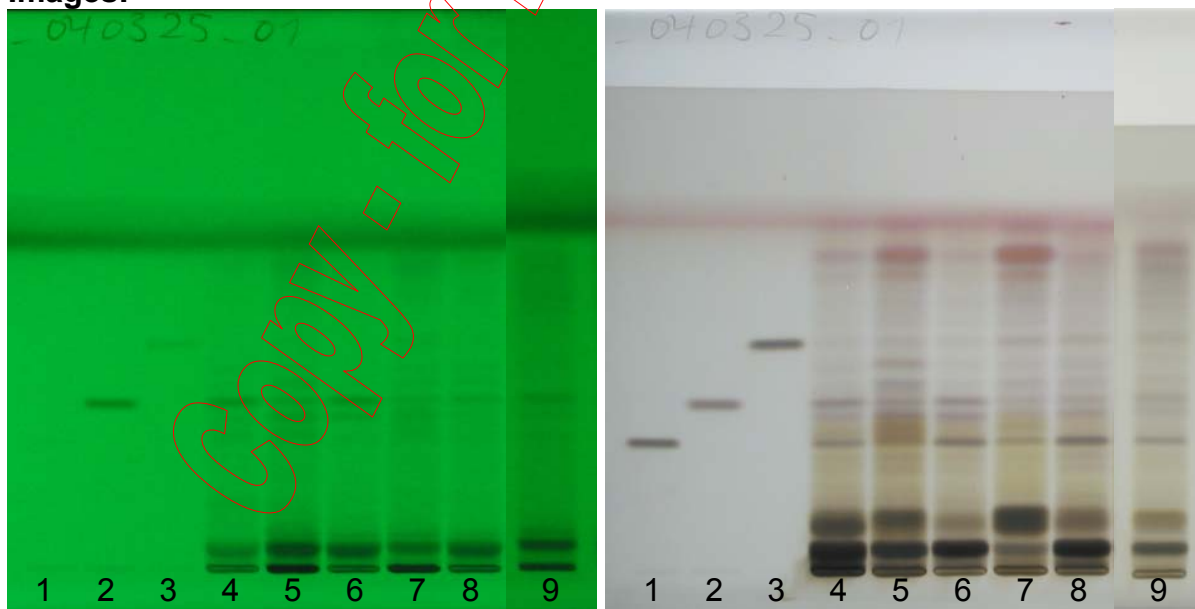
Image 1

All raw material samples show a band corresponding to Eleutheroside B under UV 254 nm and bands corresponding to Eleutheroside E, B, and E1 after derivatization. Strong differences in the relative content of eleutherosides and other unknown compounds are seen between all samples. However, the fingerprints of all samples show a similar sequence of bands and can be identified as that of Eleuthero root.

Image 2

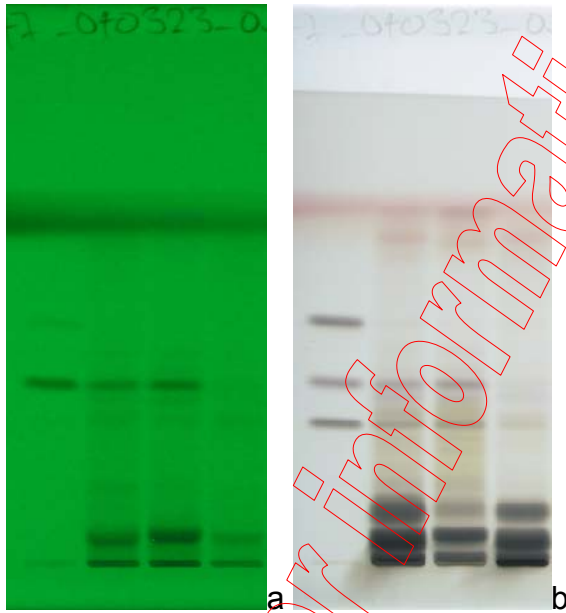
The different root parts show varying amounts of the compounds. The inner root (without bark) shows only very faint bands for the eleutherosides, but strong bands near the application position.

Images:



1. Eleutheroside E

2. Eleutheroside B
3. Eleutheroside E1
4. Siberian Ginseng Whole Root
5. Siberian Ginseng Whole Root
6. Siberian Ginseng Whole Root
7. Siberian Ginseng Whole Root
8. Siberian Ginseng Whole Root
9. Eleuthero root (BRM) [Track 4 from Image 4.10]



1. Eleutheroside E, B, and E1 (ascending)
 2. Siberian Ginseng Whole Root*, [same as Track 4 of previous image]
 3. Siberian Ginseng Root Bark only*
 4. Siberian Ginseng Inner Root only*
- * Different parts of the same root

Accepted: **YES**

5.3.2 Detection of adulteration

Description of experiment:

Authenticated adulterants are prepared according to section 4.1. The BRM of *Eleutherococcus senticosus* 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 4.7) 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 *Eleutherococcus senticosus* if the fingerprints of the adulterants are significantly different from those of the BRM with respect to number, position, color, and intensity of bands.

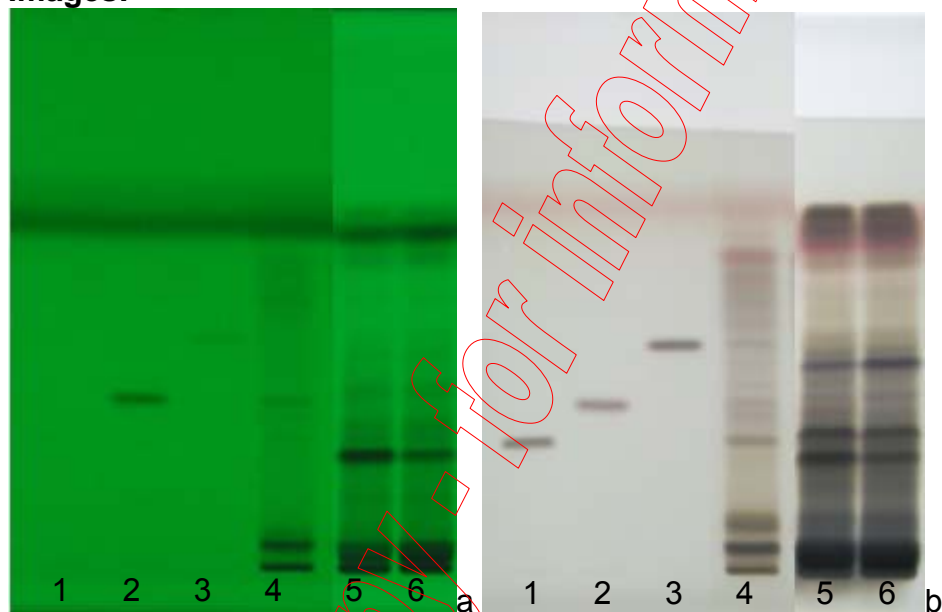
Results:

UV 254 nm: The adulterants on Lanes 5 and 6 show a strong band at $R_f=0.3$.

White light: The adulterants on Lanes 5 and 6 show a black band at $R_f=0.3$, a strong bluish band at $R_f=0.58$, a red and two brown bands near the front, and bands close to the position* of the reference substances eleutheroside E (strong), B (faint) E1 (faint).

*Eleutherosides are not contained in *Periploca*, the bands seen in both samples are due to different compounds migrating to the same position.

Images:



1. Eleutheroside E
2. Eleutheroside B
3. Eleutheroside E1
4. Eleuthero root
5. *Periploca sepium*
6. *Periploca sepium*

Accepted: YES

5.3.3 Identification of processed materials and finished products

Description of experiment:

Samples of extracts and/or finished products are prepared according to section 4.1. The BRM of *Eleutherococcus* 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 4.7) 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:

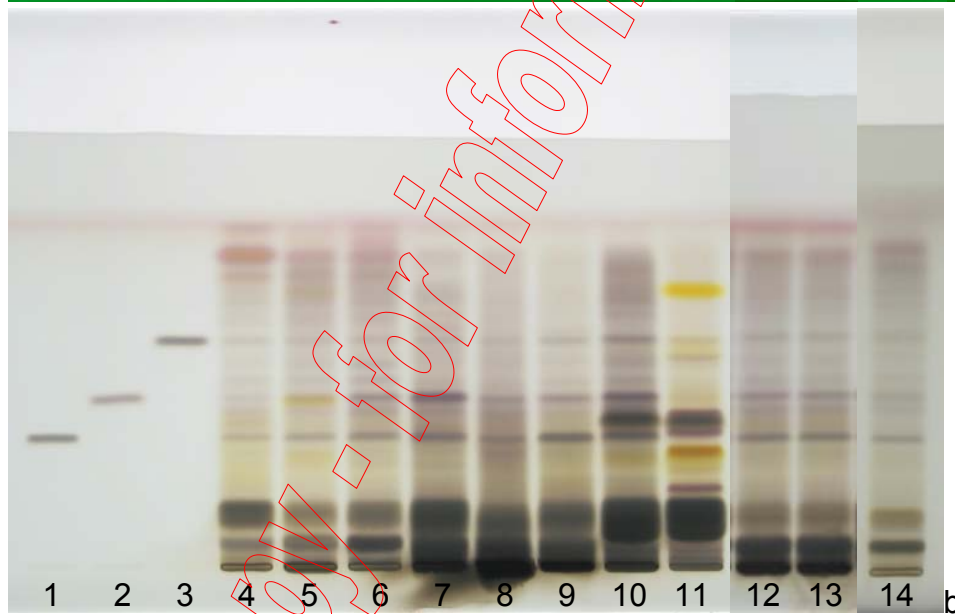
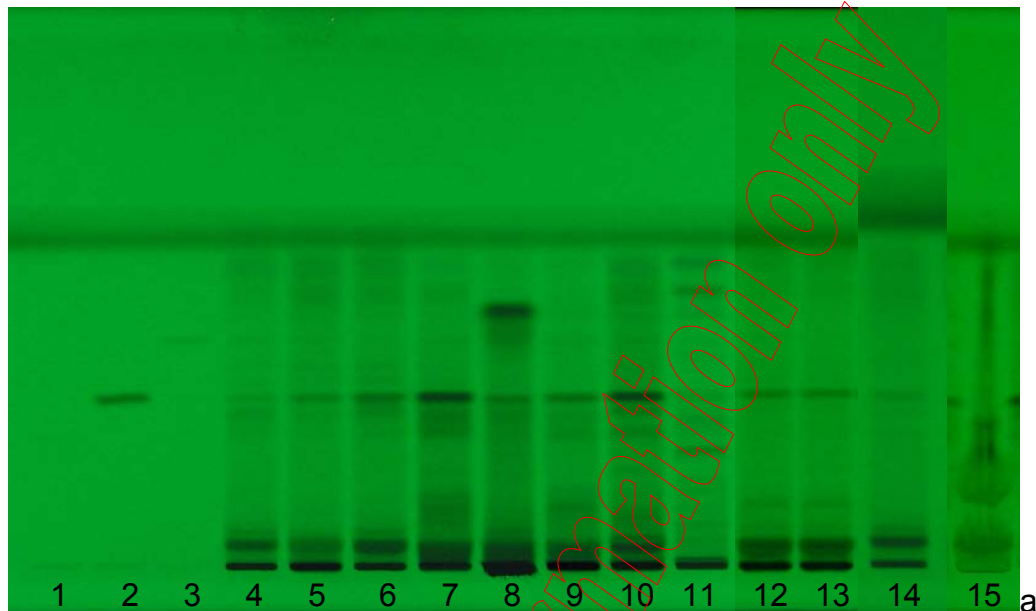
The method is suitable for the identification of processed materials of *Eleutherococcus senticosus* if the fingerprints of the samples are similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands matching the profile of the BRM.

Results:

UV 254 nm: All samples show a dark band corresponding to eleutheroside B. The tincture on track 8 shows an additional strong band at $R_f=0.7$. The tincture on track 11 shows several dark bands over the entire R_f range, but none near the application position. The sample containing glycerin (track 15) is not suitable for use in this test and was not derivatized.

White light: All samples (except sample on track 11) show bands corresponding to the three eleutherosides, and one or two very strong bands near the application. The Licorice contained in the combination product on track 5 doesn't affect the detection of Eleuthero, but there is a yellow band co-eluting with Eleutheroside B. The tincture on track 11 shows only faint bands corresponding to eleutheroside B and E1. Additional yellow and purple zones are seen in this product (probably due to a combination with other plant materials). The band seen close to the position of eleutheroside E is of a different color. This product can not be identified clearly as Eleuthero. Sample on track 10 shows an unknown strong band at $R_f=0.4$. Most of the extracted materials show a more distinct chromatogram than the raw roots.

Images:



1. Eleutheroside E
2. Eleutheroside B
3. Eleutheroside E1
4. Siberian Ginseng Whole Root
5. Tablet (+ Licorice)
6. Tea bag
7. Capsule with dry extract
8. Tincture
9. Tincture
10. Tincture
11. Tincture
12. USP Standard Extract
13. USP Standard Extract

- 14. Eleuthero root (BRM) [Track 4 from Image 4.10]
- 15. Tincture, vegetable glycerin (only image a)

Accepted: YES

Products on tracks 4-10 and 12-13 pass the test, samples on tracks 11 and 15 fail, track 14 is the BRM.

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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 10 μ L of each reference solution are applied according to section 4.5. The plates are chromatographed (section 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 the three eleutherosides 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.

Rf	P47_050223_03	P47_050223_04	P47_050223_05	Δ Rf
Eleutheroside E	0.34	0.36	0.36	0.02
Eleutheroside B	0.45	0.47	0.47	0.02
Eleutheroside E1	0.61	0.63	0.63	0.02

Images (only detection b):



P47_050223_03



P47_050223_04



P47_050223_05

Accepted: YES

5.5 Intermediate precision

Description of experiment:

Repeat the experiment described under 5.4 on 2 other days, with the following modifications. Only one portion of the BRM is prepared according to section 4.2. Onto one 10x10 cm plate, three aliquots of 10 μ L of the solution are applied.

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

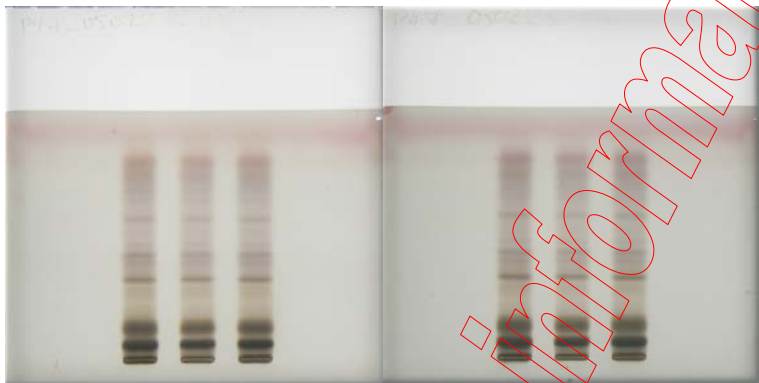
All chromatograms look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen.

Rf	P47_050223_03 (Comparison)	P47_050224_01	P47_050225_01	Δ Rf
Eleutheroside E	0.34	0.33	0.34	0.01
Eleutheroside B	0.45	0.43	0.45	0.02
Eleutheroside E1	0.61	0.59	0.60	0.02

Images (only detection b):



P47_050223_03 (Comparison)



P47_050224_01

P47_050225_01

Accepted: YES

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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 three 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 in obtained in section 5.4 (<0.05 if using plate from the same manufacturer, <0.07 for plates of different manufacturers).

Results:

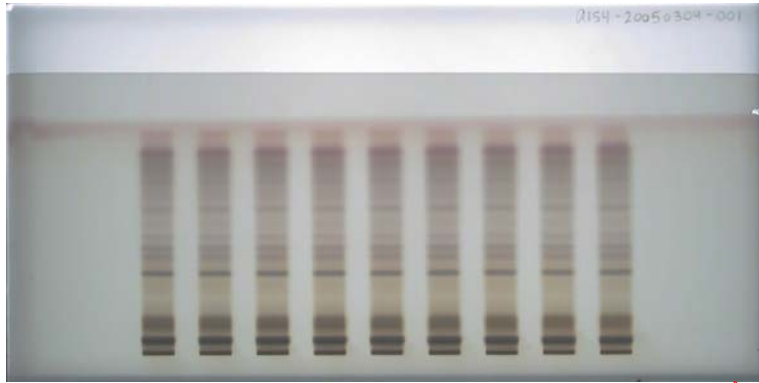
All chromatograms developed by the confirmatory lab look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen. The color/brightness of the image differs slightly from the image made in the primary lab. This could be due to different documentation devices and settings.

Rf	A154-20050309-001	A154-20050309-002	A154-20050309-003	ΔRf	P47_050223_03 (Comparison)	ΔRf
Eleutheroside E	0.34	0.33	0.35	0.02	0.34	0.01
Eleutheroside B	0.45	0.45	0.46	0.01	0.45	0.01
Eleutheroside E1	0.61	0.61	0.62	0.01	0.61	0.01

Images (only detection b):



A154-20050309-001



A154-20050309-002



A154-20050309-003



P47_050223_03 (Comparison)

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. Instead of a Twin Trough Chamber a Flat Bottom Chamber of comparable size is used.

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 Flat Bottom Chamber must be excluded.

Results:

No significant difference is seen when the plate is developed in a Flat Bottom Chamber. Slightly lower Rf values are observed.

Rf	Image 4.10	P47_050225_02	ΔRf
Eleutheroside E	0.34	0.33	0.01
Eleutheroside B	0.45	0.43	0.02
Eleutheroside E1	0.60	0.58	0.02

Images (only detection b):

Twin Trough Chamber Flat Bottom Chamber
(Image of section 4.10)



Accepted: YES

5.7.2 Developing distance

Description of experiment:

The method is executed according to section 4 using only the BRM.
The developing distance is increased to 75 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. Slightly lower Rf values are observed.

Rf	Image 4.10	P47_050223_07	ΔRf
Eleutheroside E	0.34	0.33	0.01
Eleutheroside B	0.45	0.44	0.01
Eleutheroside E1	0.60	0.58	0.02

Images (only detection b):

70 mm

(Image of section 4.10)



75 mm (developing distances measured from lower edge of plate)



Accepted: YES

5.7.3 Waiting times

Because the sample is stable on the plate, in solution, and during chromatography, and the derivatization is not critical, this experiment was not performed in this example. For details see section 5.2.1-5.2.3.

5.7.4 Relative humidity

Description of experiment:

Six plates are prepared according to section 4 using only one BRM. Prior to chromatography (4.6), the plates are conditioned over salt solutions and/or sulfuric acid for adjusting different relative humidity. Relative humidity covering a range of about 20-60% relative humidity should be tested.

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

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:

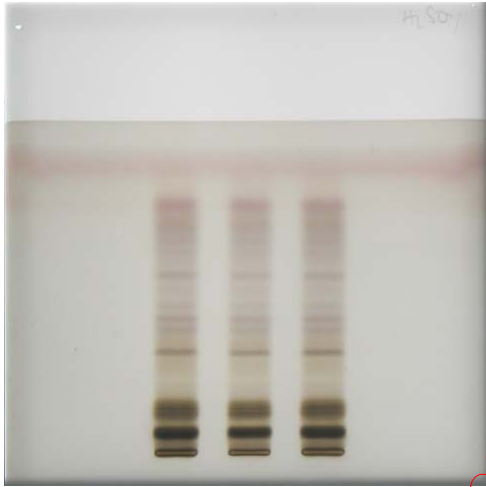
Only small variations in R_f values at low-medium rel. humidity (16-47%). Band broadening and separation difference are seen at high humidity (>58%). The chromatogram should not be developed without humidity control when the surrounding relative humidity exceeds 50%.

R _f	16%	28%	37%	47%	58%	75%
Eleutheroside E	0.33	0.34	0.34	0.33	0.36	0.44
Eleutheroside B	0.43	0.45	0.45	0.43	0.43*	0.57**
Eleutheroside E1	0.57	0.6	0.6	0.59	0.6	0.66

*overlap **very faint

Images (only detection b):

P47_050225_04 (16%)



P47_050225_01 (28%)



P47_050422_02 (37%)



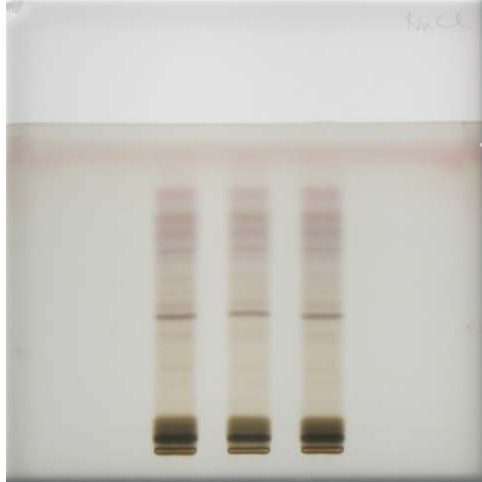
P47_050422_03 (47%)

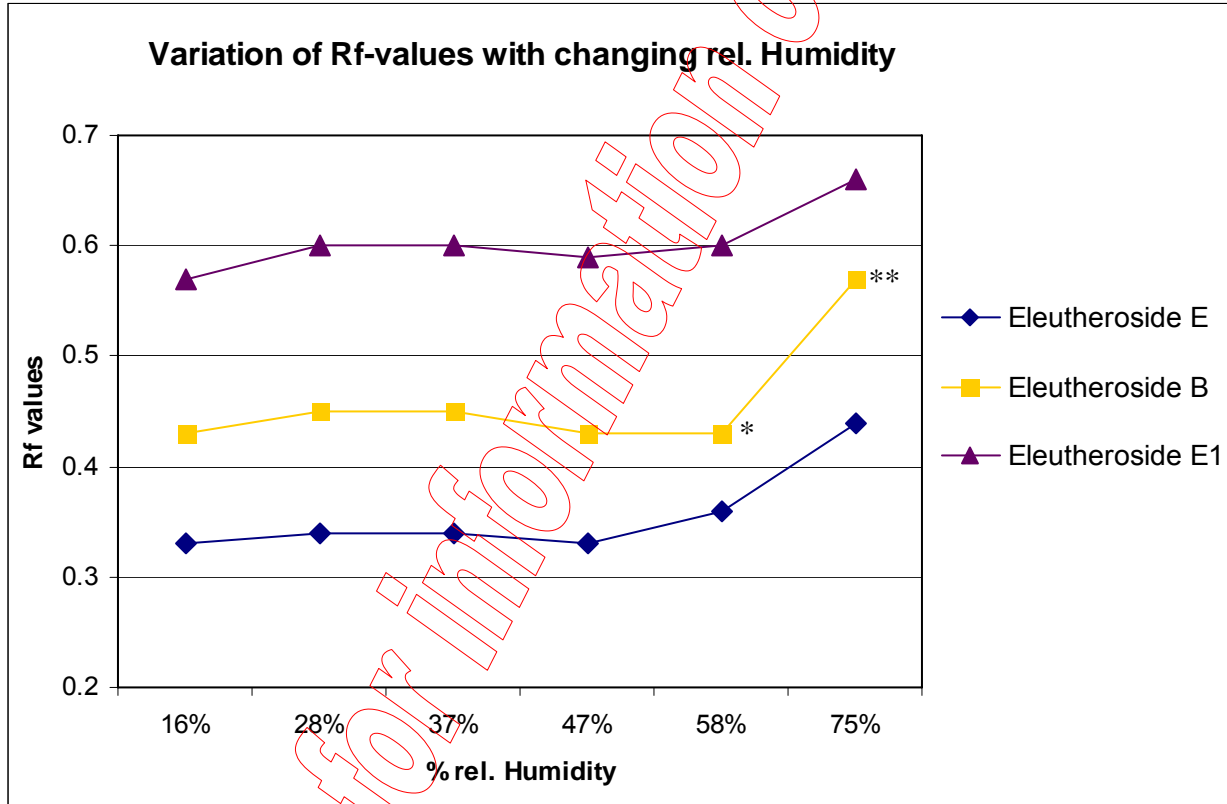


P47_050225_05 (58%)



P47_050225_06 (75%)





*overlap **very faint

6. Conclusions, Approvals, and Signatures

6.1 Conclusions of primary lab

Date:

Analyst of primary lab:

6.2 Conclusions of substantiating lab

Date:

Analyst of substantiating lab:

6.3. Final approval of study director

Date:

Study director:

Copy - for information only