

Validation of Method for Identification of Licorice by HPTLC Fingerprint

1. Purpose of method to be validated:

The identification method for Licorice by HPTLC fingerprint is suitable to identify a given sample of plant material as Licorice (*Glycyrrhiza glabra* Linne or *Glycyrrhiza uralensis* Fischer).

The method may be used to identify an extract, or finished product as derived from Licorice (*Glycyrrhiza glabra* Linne or *Glycyrrhiza uralensis* Fischer), provided that the material was made from a single herb and intended to contain the constituent profile seen in Licorice.

2. General acceptance criteria:

The method is valid if:

- A botanically authenticated sample of *Glycyrrhiza glabra* Linne or *Glycyrrhiza uralensis* Fischer 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

3.1 Study director

Name: Kerri LeVanseler
Company: Institute for Nutraceutical Advancement
Address: 789 Dixboro Road
City: Ann Arbor, Michigan 48105
Phone: 734 827 6815
Fax: 734 827 6832
E-mail: levanseler@nsf.org

3.2 Reviewer

Name: Eike Reich
Company: CAMAG
Address: Sonnenmattstrasse 11
City: Muttenz, CH 4132
Phone: (41) 61 467 3434
Fax: (41) 61 461 0702
E-mail: eike.reich@camag.com

3.3 Analyst of primary lab

Name: Anne Schibli
Company: CAMAG
Address: Sonnenmattstrasse 11
City: Muttenz, CH 4132
Phone: (41) 61 467 3434
Fax: (41) 61 461 0702
E-mail: anne.schibli@camag.com

3.4 Analyst of confirmatory lab

Name: Alison DeBatt
Company: CAMAG Scientific Inc.
Address: 515 Cornelius Harnett Drive
City: Wilmington NC 28401
Phone: 910 343 1830
Fax: 910 343 1834
E-mail: tlc@camagusa.com

4. Description of method to be validated

4.1 Preparation of test solutions

0.5 g of milled root (or enough product equivalent to that amount) are sonicated for 10 min with 10 mL ethanol-water 70:30. The solution is centrifuged and the supernatant is used as test solution.

4.2 Preparation of reference solutions

Botanical reference solution: 0.5 g of botanically authenticated and milled root (BRM, botanical reference material) are sonicated for 10 min with 10 mL ethanol-water 70:30. The solution is centrifuged and the supernatant is used as reference solution.

Chemical reference solution: 1 mg ammonium glycyrrhizate is dissolved in 10 mL ethanol-water 70:30.

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

2 µL of test solution, 2 µL of reference solution, and 10 µL of standard 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.

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:	Ethyl acetate, formic acid, acetic acid, water (15:1:1:2); 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

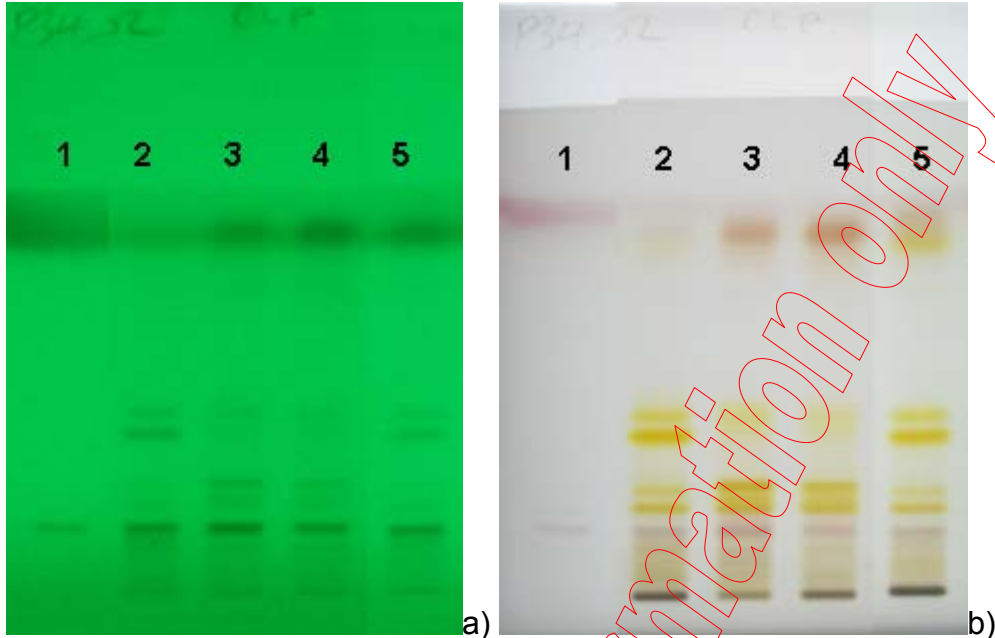
The plate is immersed into reagent for 1 s, then heated at 100°C for 10 min.

4.9 Documentation

a) Prior to derivatization under UV 254 nm (not shown in all examples)

b) After derivatization under white light

4.10 Images of chromatograms



- 1: Ammonium glycyrrhizate
- 2: Licorice root, milled (*Glycyrrhiza glabra*)
- 3: *Glycyrrhiza glabra* AUZ
- 4: *Glycyrrhiza glabra* AUZ
- 5: *Glycyrrhiza uralensis*

All samples included in the images are authenticated botanical reference materials (BRM).

4.11 Evaluation of results:

UV 254 nm

The chromatogram of the standard (track 1) shows a quenching zone around $R_f=0.2$. All BRMs show a quenching zone at the same position. Four additional quenching zones are seen at middle R_f in the BRMs. The pattern of BRMs on tracks 2 and 5 are similar and so are those on tracks 3 and 4.

White light

The chromatogram of the standard (track 1) shows a violet zone around $R_f=0.2$. All BRMs show a violet zone at the same position. Four yellow zones are seen at middle R_f in the BRMs. The intensity of the yellow zones at middle R_f varies in the samples. Additional zones are seen near the front and between application position and ammonium glycyrrhizate. The pattern of BRMs on tracks 2 and 5 are similar and so are those on tracks 3 and 4.

G. glabra and *G. uralensis* don't show any significant difference in their fingerprints. Two types of samples are seen, which differ in the intensity of the zones in the middle of the R_f region and the presence of zones near the front.

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

Copy - for information only

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality
Ethanol	Merck	p.a
Methanol	Merck	p.a
Ethyl acetate	Merck	p.a
Glacial acetic acid 99%	Merck	p.a
Formic acid 98-100%	Merck	p.a
Sulfuric acid 98%	Merck	p.a
Water	In house	--

5.1.2 Samples and Reference materials

Botanical reference material

Sample Name	Source / Lot	Authentication
Licorice root, milled (<i>Glycyrrhiza glabra</i>)	Removed - proprietary information	Yes
<i>Glycyrrhiza glabra</i> AUIZ		Yes
<i>Glycyrrhiza glabra</i> AUIZ		Yes
<i>Glycyrrhiza uralensis</i>		Yes

Additional samples

Name	Source	Authentication
<i>Glycyrrhiza uralensis</i> Fisch.	Removed - proprietary information	No
<i>Glycyrrhiza glabra</i>		No
Commercial Licorice Slices 2 mm x 3-4 cm		No

Processed materials

Name	Source	Authentication
Licorice root capsules, 450 mg	Removed - proprietary information	No
DGL De-glycyrrhinated Licorice extract Lozenge 400 mg		No
Licorice + herbal blended extract (liquid)		No

Standards (marker compounds, chemical references)

Name	Source
Ammonium glycyrrhizate (=glycyrrhizic acid)	Roth, 23236047

5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB 291342, OB 302430
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB 464935, OB 345031

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.

Image(s):

White light after derivatization



G. glabra

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:

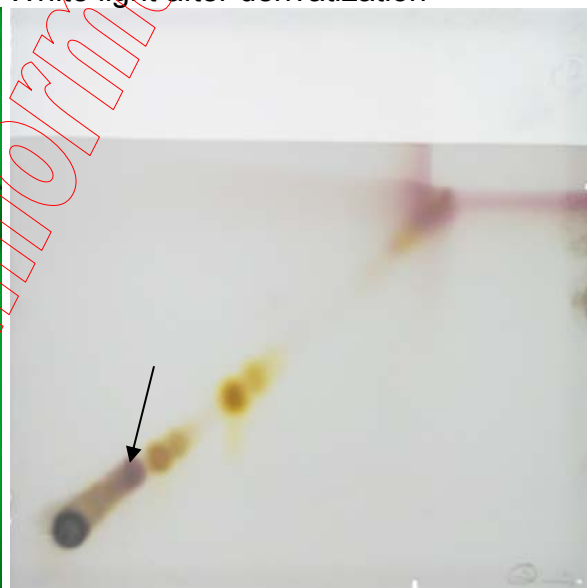
No zone is located aside of the diagonal. The sample (Licorice root, milled *Glycyrrhiza glabra*) is stable during chromatography. The arrows in the images mark the position of ammonium glycyrrhizate.

Image(s):

UV 254 nm prior to derivatization



White light after derivatization



Accepted: YES

5.2.3 Stability of derivatization/result

Description of experiment:

One botanical reference solution (4.2) is chromatographed according to section 4. After documentation under white light, the plate is observed for 30 more minutes. An image is taken after 2, 5, 10, 15, 20 and 30 min. The images are compared visually and with the help of video-densitometry.

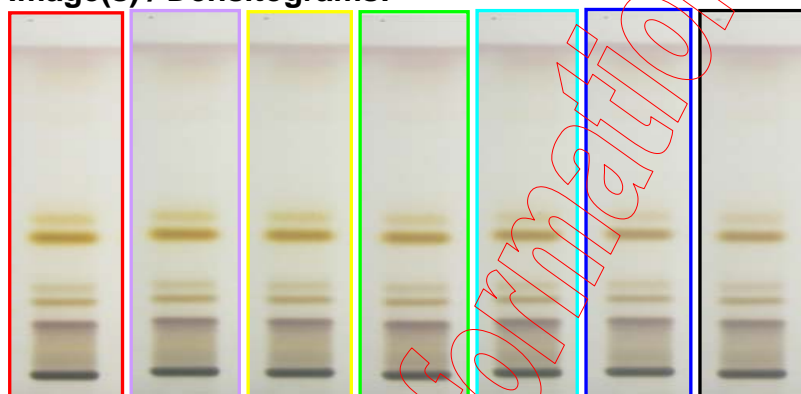
Acceptance criteria:

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

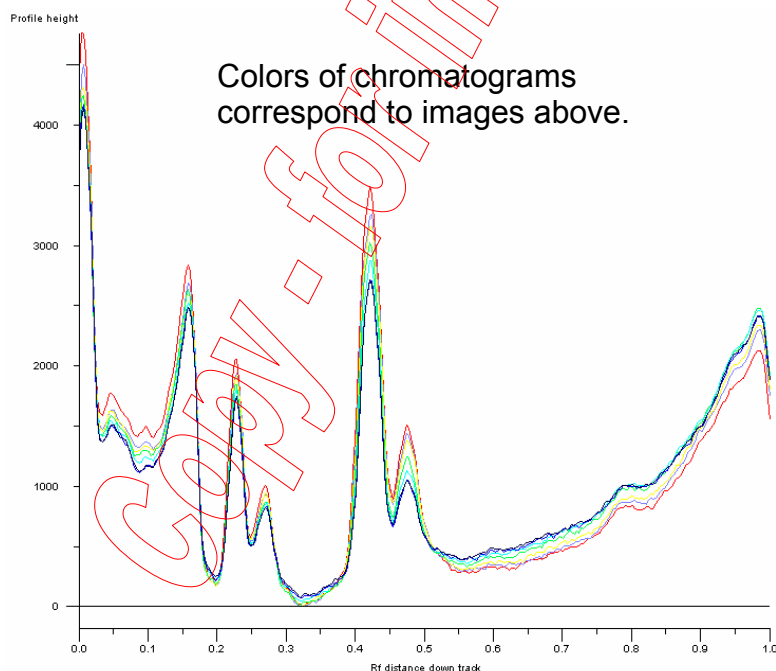
Results:

The color and intensity of zones changes slightly over time, but no zone disappears. No change between 20 and 30 min.

Image(s) / Densitograms:



No waiting, 5 min, 5 min, 10 min, 15 min, 20 min, 30 min



Accepted: YES

5.3 Specificity

5.3.1 Identification of Licorice samples, processed materials and finished products by comparison to Botanical Reference Materials (BRM) and chemical reference

Description of experiment:

Test solutions are prepared according to section 4.1. The BRMs of *G. glabra* and *G. uralensis* are prepared according to section 4.2 (botanical reference solutions). All samples are applied onto the same plate according to section 4.5. Following chromatography (sec.4.7) and derivatization (sec.4.8) the plates are documented (sec.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 licorice are similar to that shown in section 4.10 of the method with respect to number, position, color, and intensity of bands matching either of the two types of BRMs and samples of other identity yield different fingerprints.

Note: In this test the individual samples can either pass or fail, however, authenticated samples must pass.

Results:

The method is specific. All raw material samples and the capsules meet the acceptance criteria. The DGL-lozenges and the blended liquid extract don't.

Image(s):

Image prior to derivatization under UV 254 nm

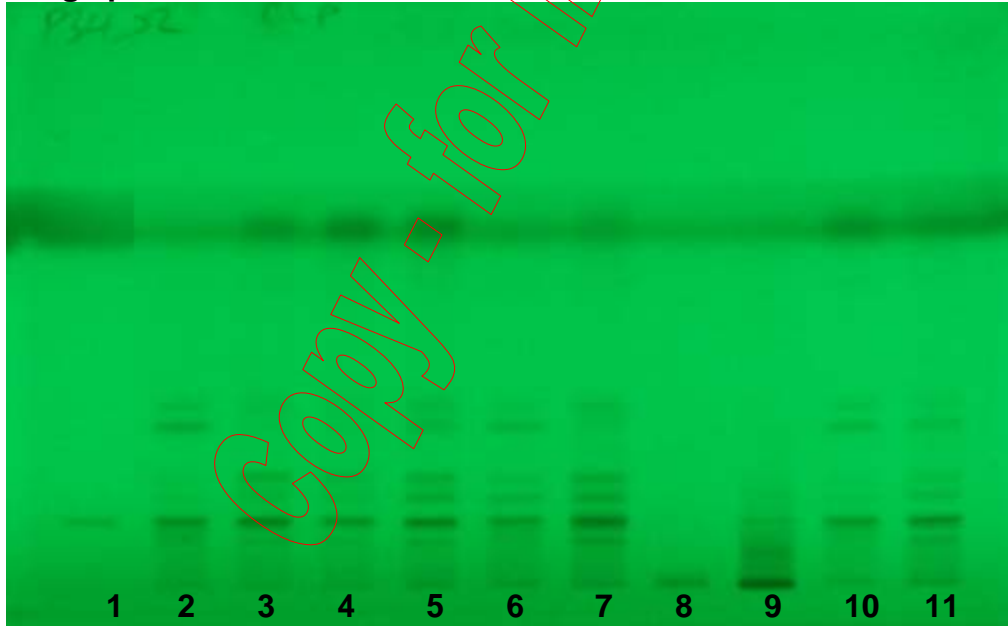


Image after derivatization under white light



- 1: Ammonium glycyrrhizate
 - 2: Licorice root, milled (*Glycyrrhiza glabra*), Phytolab, 2701303 *
 - 3: *Glycyrrhiza glabra* AUIZ, Received by INA 08/03 *
 - 4: *Glycyrrhiza glabra* AUIZ, Botanical Liaisons, AV12 *
 - 5: *Glycyrrhiza glabra*, China, Nikyang Entr.
 - 6: Commercial Licorice Slices 2 mm x 3-4 cm, China, Nikyang Entr.
 - 7: Licorice root capsules, 450 mg, Received by INA 07/03
 - 8: DGL De-glycyrrhinated Licorice extract Lozenge 400 mg, Received by INA 07/03
 - 9: Licorice + herbal blended extract (liquid), Received by INA 07/03
 - 10: *Glycyrrhiza uralensis*, Received by INA 10/03, TCM Collection No 435 *
 - 11: *Glycyrrhiza uralensis* Fisch., China Nikyang Entr.
- *BRM

Accepted: YES

Samples on tracks 5, 6, 7, and 11 pass; samples on tracks 8 and 9 fail.

5.3.2 Detection of adulteration

Description of experiment:

Authenticated adulterants are prepared according to section 4.1. The BRM of *G. glabra* and *G. uralensis* are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (sec.4.7) and derivatization (sec.4.8) the plates are documented (sec.4.9) and the results compared to those shown in section 4.10.

Acceptance criteria:

The method is specific for *G. glabra* and *G. uralensis* if the fingerprints of the adulterants are significantly different from those of the BRMs with respect to number, position, color, and intensity of bands **and** the acceptance criteria of section 5.3.1 are met.

Results: Test does not apply, no adulterants are known.

Copy - for information only

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 4.7) 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):





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 2 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**

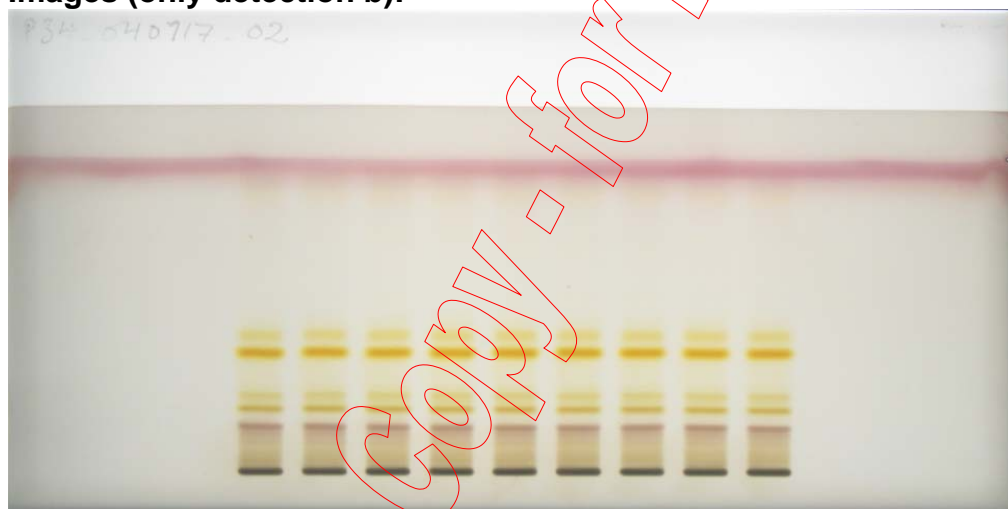
The average Rf values for each of the three zones on the three plates don't vary more than 0.05.

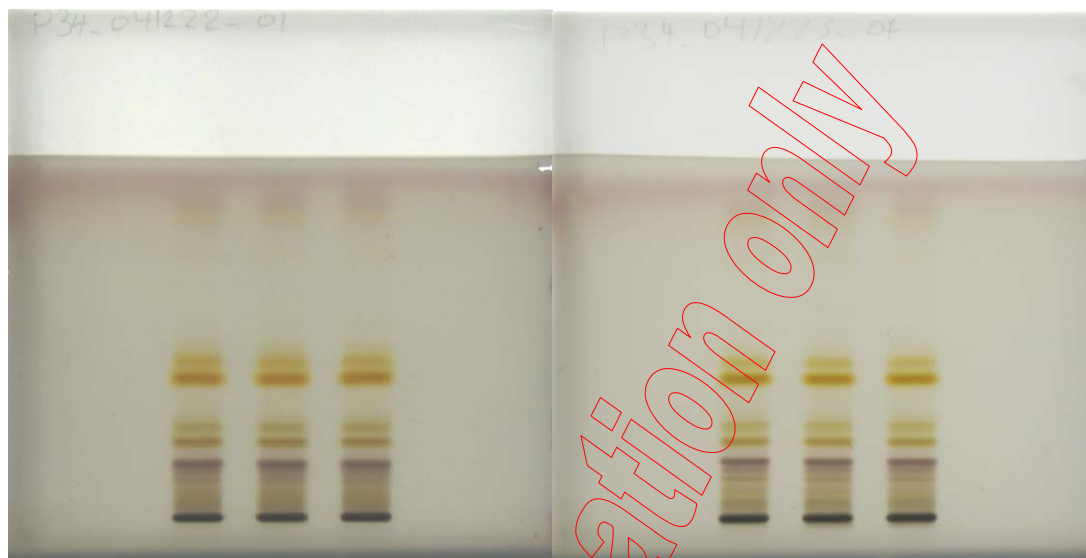
Results:

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):





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 three zones on the three plates don't vary more than 0.02.

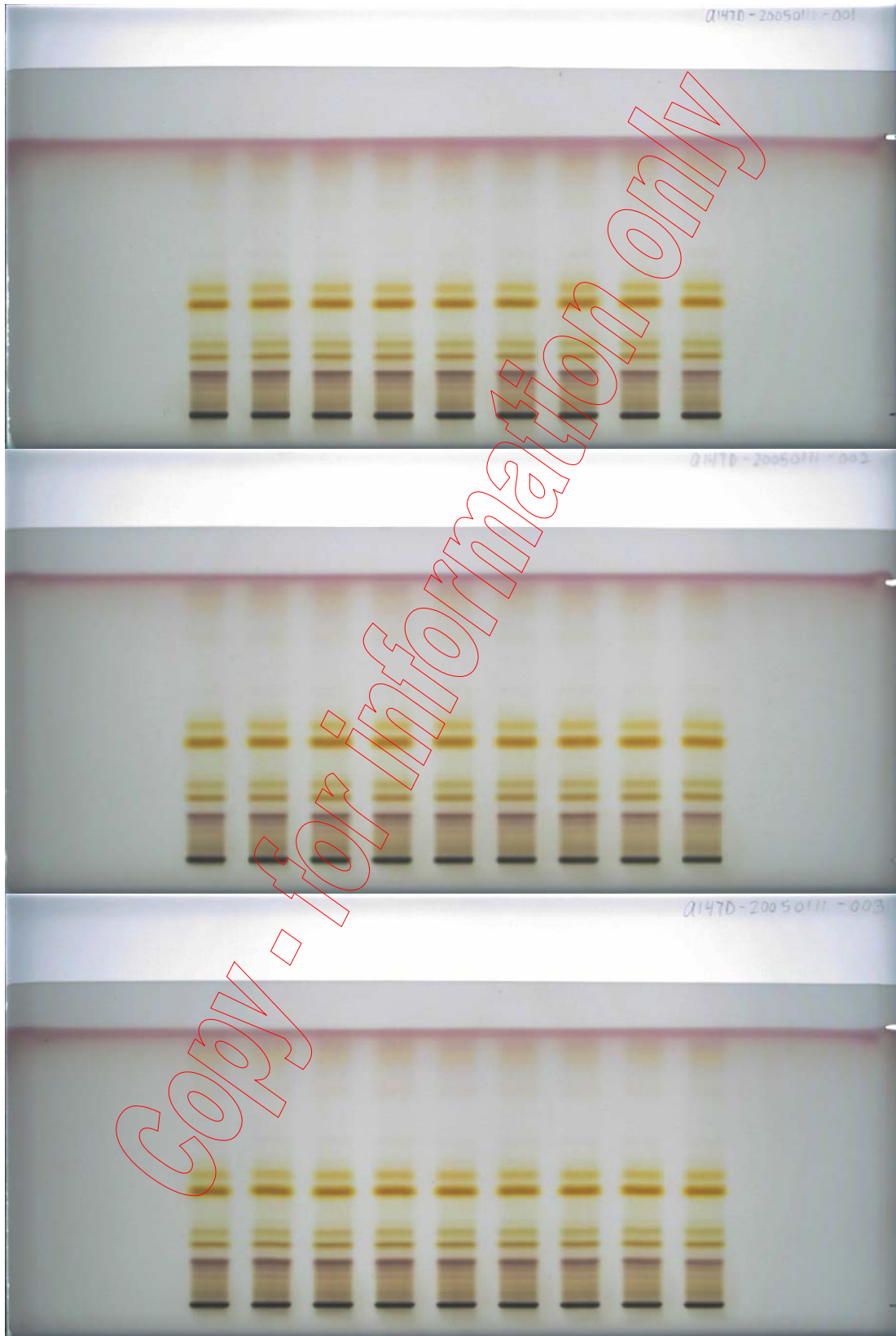
The reproducibility is acceptable if the Rf obtained in this test are not significantly different from those in obtained in section 5.4 (<0.05 if using plate from the same manufacturer, <0.07 for plates of different manufacturers).

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	Δ Rf	P47_050223_03 (Comparison)	Δ Rf
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):



Accepted: YES

5.7. Robustness

5.7.1 Chamber type

Description of experiment:

The method is executed according to section 4 using only one 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 difference is seen when the plate is developed in a Flat Bottom Chamber

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

Image(s):

Twin Trough Chamber



Flat Bottom Chamber



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 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. 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

Image(s):

70 mm



80 mm



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:

Four 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% rH should be tested.

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

Acceptance criteria:

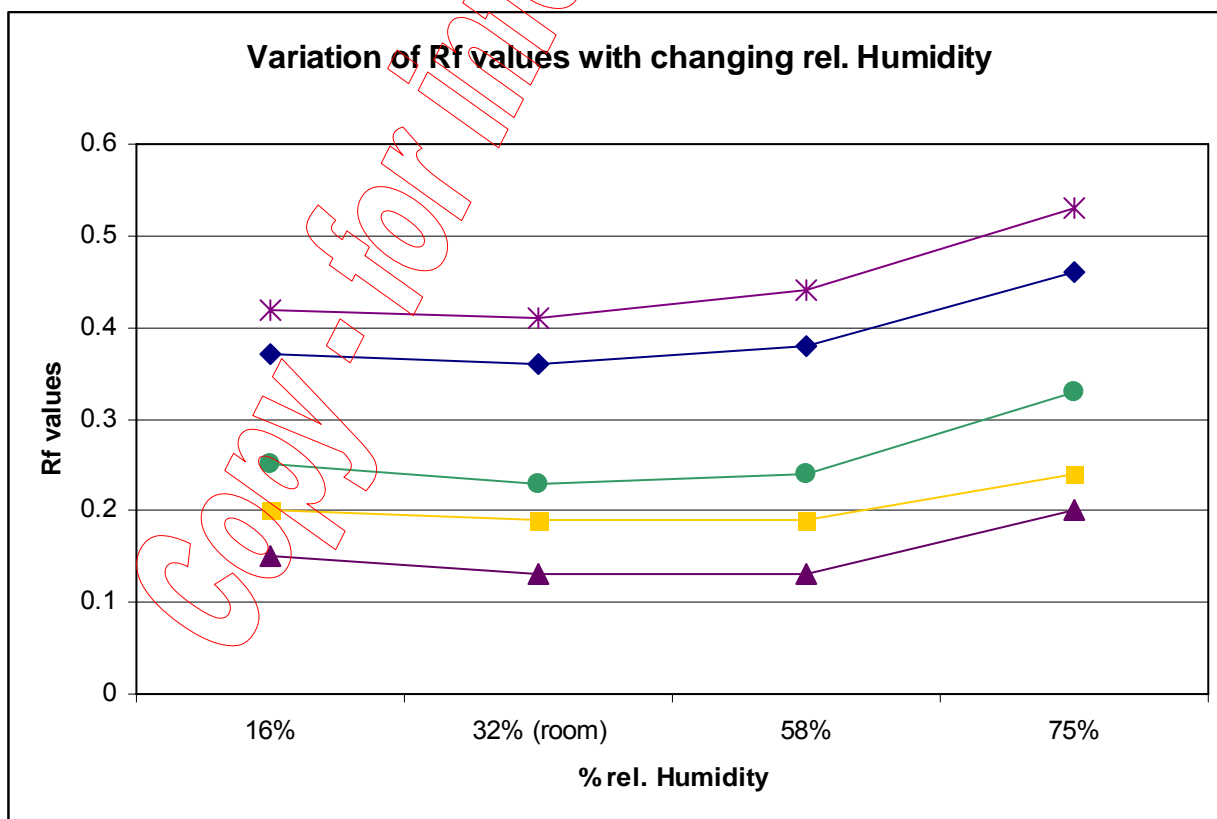
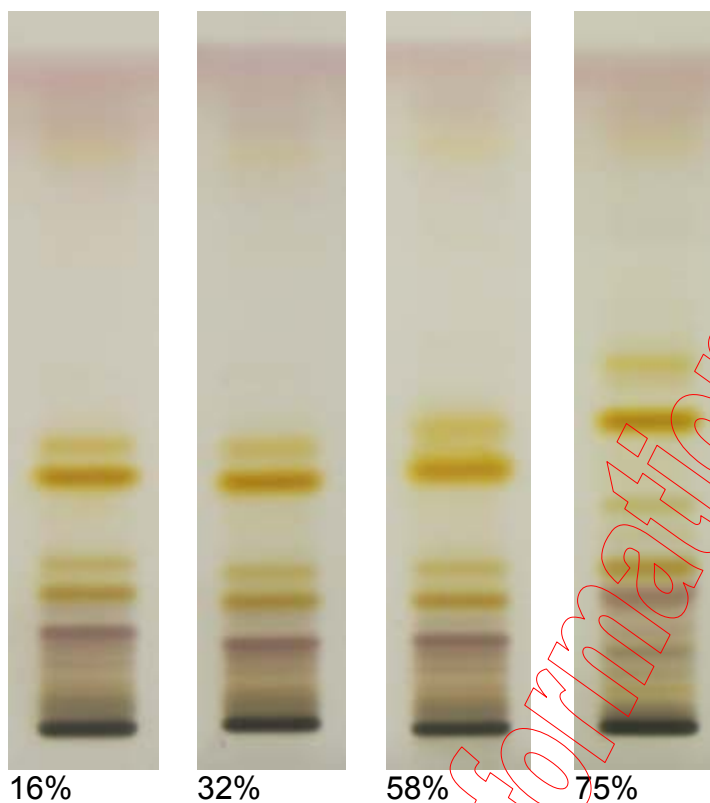
The fingerprints obtained under different relative humidity are similar with respect to number, position, color, and intensity. In this case the relative humidity does not affect the result. In the case of differences of the results, the method may require the control of relative humidity.

Results:

Only small variations in Rf values at low-medium rel. humidity (16-58%). Band broadening and separation difference at high humidity (75%). The chromatogram should not be developed without humidity control when the surrounding relative humidity exceeds 60%.

Rf	16%	32%	58%	75%
Eleutheroside E	0.33		0.36	0.44
Eleutheroside B	0.43		0.43*	0.57**
Eleutheroside E1	0.57		0.6	0.66

Images:



Accepted: YES

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 Conclusions and approval of reviewer

Date:

Reviewer:

6.4. Final approval of study director

Date:

Study director:

Copy - for information only