

**Validation of Method for the Identification of Roots of Echinacea
Species by HPTLC Fingerprint
Alkylamide and Phenylpropanoid fingerprints**

1. Purpose of method to be validated:

The method for the identification of roots of Echinacea species by HPTLC fingerprint is suitable to identify a given sample of plant material as Echinacea root of one of the species *Echinacea purpurea*, *E. angustifolia*, or *E. pallida* based on its alkylamide and phenylpropanoid fingerprint. Each of the three species yields characteristic fingerprints, which allow identification with certainty if both profiles are evaluated.

Adulterations, such as *Echinacea atrorubens*, *Liatris punctata*, and *Parthenium integrifolium* show a different profile.

2. General acceptance criteria:

The method is valid if:

- Each botanically authenticated sample of *Echinacea purpurea*, *E. angustifolia*, and *E. pallida* yields a fingerprint which is similar to that shown in section 4.10 a/b 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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3.3 Analyst of confirmatory lab

(also performed 5.2.1 Stability of analyte in solution and on the plate, and 5.5 Intermediate precision for primary lab)

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4a. Description of method for analysis of alkylamides

4.1a Preparation of test solutions

1 g powdered raw material is mixed with 10 mL dichloromethane, sonicated for 10 min, and centrifuged. The supernatant is used as reference solution.

4.2a Preparation of reference solutions

Botanical reference solution: 1 g powdered raw material is mixed with 10 mL dichloromethane, sonicated for 10 min, and centrifuged. The supernatant is used as reference solution.

Chemical reference solutions: 1 mg 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide and 1 mg β -sitosterol are individually dissolved in 5 mL methanol each.

4.3a Preparation of derivatizing reagent

170 mL methanol is placed in a 200 mL glass bottle and cooled it down in a water-ice cubes-salt bath or in a freezer. To the ice-cold methanol 20 mL of acetic acid and 10 mL of sulfuric acid are carefully added and mixed. After the mixture cooled down to room temperature, 1 mL anisaldehyde is added.

4.4a Stationary phase

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

4.5a Sample application

5 μ L of test solution and 2 μ L of standards 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.

4.6a Temperature and Humidity

Record temperature and humidity in the laboratory.

4.7a Chromatography

Chamber type:	10x10 cm (or 20x10 cm) Twin Trough Chamber
Configuration:	Saturated for 20 min (filter paper, wetted with developing solvent, in trough opposite to the plate)
Developing solvent:	Toluene, ethyl acetate, cyclohexane, formic acid (80:20:10:3), 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)

4.8a Derivatization

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

4.9a Documentation

The derivatized plate is documented using illumination with white light (reflection and transmission).

4.10a Results



- 1: 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide
- 2: β -sitosterol
- 3: *E. purpurea* root
- 4: *E. angustifolia* root
- 5: *E. pallida* root

4.11a Description of results

The chemical reference standards 2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide (DAI) and β -sitosterol show a blue violet zone at R_f 0.31 and 0.38 respectively. BRMs of each species show a prominent violet zone corresponding to in color and position to β -sitosterol and another violet zone at R_f 0.47 which is particularly strong for *E. purpurea* root and weak for *E. angustifolia* root and *E. pallida* root. Close to the solvent front a broad pink violet zone is seen.

- *E. purpurea* root and *E. angustifolia* root must show a prominent zone corresponding in color and position to DAI. This zone is absent in *E. pallida* root.
- *E. angustifolia* root shows a characteristic yellow zone below DAI. (**NOTE:** This zone turns reddish pink when the plate is heated at 100°C for more than 10 min!)
- *E. pallida* root shows two brownish zones below the position of DAI and a characteristic broad olive green/yellow double zone at R_f 0.60. *E. purpurea* root and *E. angustifolia* root show only faint zones at this position.

4.12a System suitability test

The result obtained in the test is suitable for evaluation if the following requirements are met:

E. purpurea root: The zone corresponding to β -sitosterol is clearly separated from the zones above and below.

E. angustifolia root: the zone of DAI and the yellow zone directly below are clearly separated.

E. pallida root: The zone corresponding to β -sitosterol is clearly separated.

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4b. Description of method for analysis of phenylpropanoids

4.1b Preparation of test solutions

1 g powdered raw material is mixed with 10 mL methanol, sonicated for 10 min, and centrifuged. The supernatant is used as reference solution.

4.2b Preparation of reference solutions

Botanical reference solution: 1 g powdered raw material is mixed with 10 mL dichloromethane, sonicated for 10 min, and centrifuged. The supernatant is used as reference solution.

Chemical reference solutions: 2 mg echinacoside, 2 mg cynarin, 1 mg chlorogenic acid, 1 mg caffeic acid, 0.5 mg caftaric acid, and 0.5 mg cichoric acid are dissolved in 10 mL methanol each.

4.3b Preparation of derivatizing reagent

In 200 mL glass bottle 1 g diphenylborinic acid aminoethylester (Natural Product Reagent; NP) are dissolved in 200 mL ethyl acetate.

4.4b Stationary phase

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

4.5b Sample application

5 µL of test solution and 2 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.

4.6b Temperature and Humidity

Record temperature and humidity in the laboratory.

4.7b Chromatography

Chamber type:	10x10 cm (or 20x10 cm) Twin Trough Chamber
Configuration:	Saturated for 20 min (filter paper, wetted with developing solvent, in trough opposite to the plate)
Developing solvent:	Ethyl acetate, ethylmethyl ketone, water, formic acid (50:30:10:10), 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)

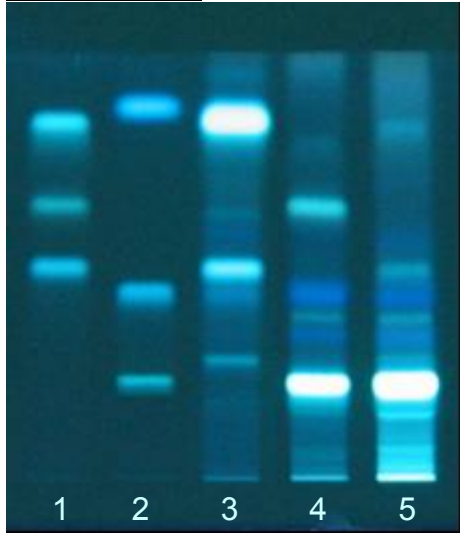
4.8b Derivatization

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

4.9b Documentation

The derivatized plate is documented using illumination with UV 366 nm light.

4.10b Results



- 1: Caftaric acid, cynarin, cichoric acid (with increasing Rf)
- 2: Echinacoside, chlorogenic acid, caffeic acid (with increasing Rf)
- 3: *E. purpurea* root
- 4: *E. angustifolia* root
- 5: *E. pallida* root

4.11b Description of results

The chemical reference substances give blue-white fluorescing zones in the following order (increasing Rf): Echinacoside (0.22), chlorogenic acid (0.44), caftaric acid (0.50), cynarin (0.64), cichoric acid (0.84), caffeic acid (0.87).

- *E. purpurea* root shows a prominent zone corresponding to caftaric acid and very strong zone corresponding to cichoric acid. Other weak zones may be present but **NO** zones are seen at the positions of echinacoside and cynarin!
- *E. angustifolia* root shows a very strong zone corresponding to echinacoside and a prominent zone corresponding to cynarin. A weak zone at or slightly below the position of cichoric acid can be present. Several weak zones may be present between the positions of echinacoside and cynarin but **NO** zone is seen at the position of caftaric acid.
- *E. pallida* root shows a very strong zone corresponding to echinacoside. Weak zones corresponding to caftaric acid and cichoric acid are present. Several weak zones are seen above and below the position of echinacoside but **NO** zone is seen at the position of cynarin.

4.12b System suitability test

The result obtained in the test is suitable for evaluation if the following requirements are met:

E. purpurea root: the position of the zone corresponding caftaric acid must be at Rf 0.5.

E. angustifolia root and *E. pallida* root: the position of the zone corresponding echinacoside must be at Rf 0.2. Rf values may vary by +/- 0.05.

Optional: The chemical reference standards chlorogenic acid and caftaric acid are clearly separated.

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5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality
Acetic acid	Merck	p.a
Anisaldehyde	Fluka	98%
Cyclohexane	Merck	p.a
Dichloromethane	Merck	p.a
Diphenylborinic acid aminoethylester	Roth	98%
Ethyl acetate	Merck	p.a
Ethylmethyl ketone	Merck	p.a
Formic acid 98-100%	Merck	p.a
Methanol	Merck	p.a
Sulfuric acid	Merck	p.a
Toluene	Merck	p.a
Water	In house	demineralized

5.1.2 Samples and Reference materials

Botanical reference material

Sample Name	Source / Lot	Authentication
<i>Echinacea purpurea</i> root	Removed - proprietary information	Yes
<i>Echinacea angustifolia</i> root		Yes
<i>Echinacea pallida</i> root		Yes

Additional samples

Sample Name	Source / Lot	Authentication
<i>Echinacea purpurea</i> dried whole root	Removed - proprietary information	No
<i>Echinacea purpurea</i> dried cut root		No
<i>Echinacea purpurea</i> dried whole root		No
<i>Echinacea purpurea</i> dried whole root		No
<i>Echinacea angustifolia</i> dried whole root		Yes
<i>Echinacea angustifolia</i> dried whole root		No
<i>Echinacea angustifolia</i> dried whole root		No
<i>Echinacea pallida</i> dried whole root		Yes

<i>Echinacea pallida</i> root		Yes
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Adulterants

Sample Name	Source / Lot	Authentication
<i>Echinacea atrorubens</i> dried whole root	Removed - proprietary information	Yes
<i>Liatris punctata</i> dried whole root		Yes
<i>Parthenium integrifolium</i> dried whole root		Yes

Standards (marker compounds, chemical references)

Name	Source
β-Sitosterol	Fluka 423759/1 64501
2E,4E,8Z,10E/Z-dodecatetraenoic acid isobutylamide	Phytochem 04.263-304
Echinacoside	ChromaDex 01-05020-101
Cynarin	ChromaDex 02-03990-722
Cichoric acid	ChromaDex 00-03640-300
Chlorogenic acid	ChromaDex 01-03455-203
Caftaric acid	ChromaDex 01-03028-301
Caffeic acid	Fluka 353773/1 41198

5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB464935
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB134830, OB526793

5.1.4 Instruments

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	061104
VideoStore using Hitachi HVC20 video camera	CAMAG	--
DigiStore using Canon G5 camera	CAMAG	070705
TTC 20x10 cm	CAMAG	n. a.
TTC 10x10 cm	CAMAG	n. a.
ADC 2 with humidity control	CAMAG	120425
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
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WinCATS	CAMAG	1.2.6-1.3.4
VideoScan	CAMAG	1.02.00

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5.2 Stability

5.2.1 Stability of analyte in solution and on the plate (performed by secondary lab)

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

One portion of each BRM is extracted according to sections 4.2 a/b. 5 µL of this solution are applied onto a 20x10 cm plate according to sections 4.4 a/b and 4.5 a/b. The samples and the plate with the applied samples (wrapped in aluminum foil) are set aside. After 3 hours another portion each of the same BRMs are extracted according to section 4.2 a/b. Two times 5 µL of this solution are applied according to section 4.5 a/b next to the first sample on the set-aside plate, followed by 5 µL of the set-aside samples (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 a/b to 4.9 a/b.

Acceptance criteria:

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

Results:

Alkylamides: No difference is seen in between the four chromatograms of each species. The samples of the three Echinacea species are stable on the plate and in solution for at least 3 hours.

Phenylpropanoids: No difference is seen in between the four chromatograms of each species. The samples of the three Echinacea species are stable on the plate and in solution for at least 3 hours.

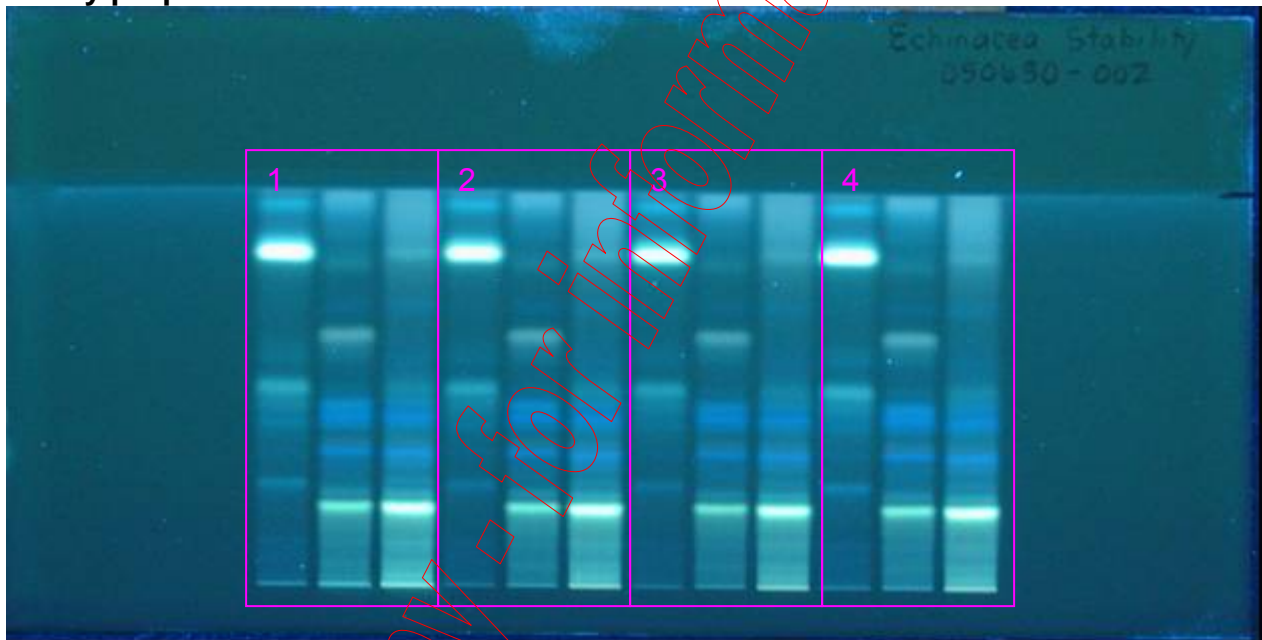
Images:

4 sets of *E. purpurea*, *E. angustifolia*, *E. pallida* (alternating)

Alkylamides



Phenylpropanoids



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

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

One portion of each BRM is extracted according to section 4.2 a/b. 5 µL are applied as spot at the lower right corner of a 10x10 cm plate (10 mm from each edge). The plate is developed and dried according to section 4.6 a/b. The plate is now turned 90° to the right and developed a second time according to section 4.6 a/b with a fresh portion of developing solvent.

The plate is derivatized and documented according to section 4.8 a/b and 4.9 a/b.

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:

Very few minor spots are located off the diagonal (see images below). The samples of the three Echinacea species are regarded as stable during chromatography in both systems.

Images:

Alkylamides

E. purpurea,

E. angustifolia,

E. pallida

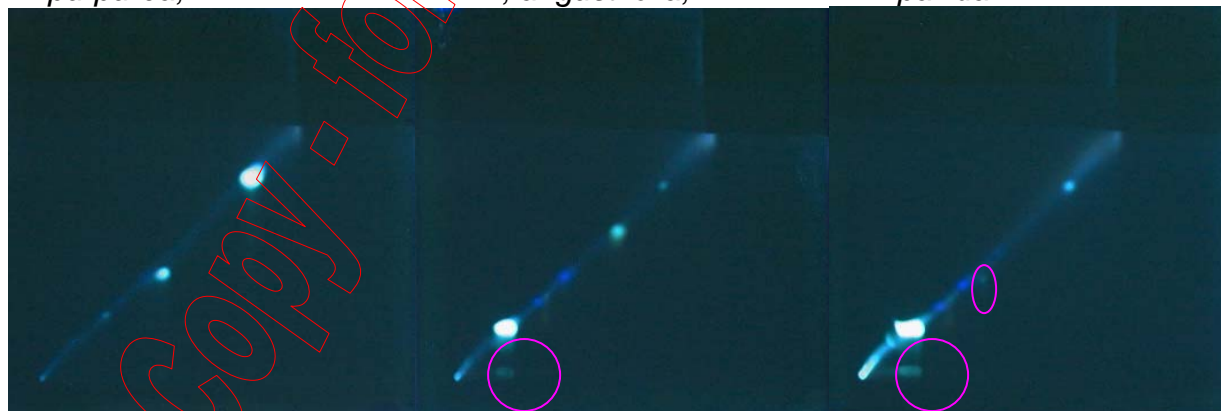


Phenylpropanoids

E. purpurea,

E. angustifolia,

E. pallida



Accepted: YES with the following limitation: the circled zones probably represent degradation products or may be due to local overloading. Neither of those zones interferes with the identification of the species.

5.2.3 Stability of derivatization/result

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

One portion of each BRM is extracted according to section 4.2 a/b. 5 µL are applied and chromatographed according to section 4.3 a/b to 4.8 a/b. After documentation under white light (4.9 a) the plate is observed for 30 min. An image is taken after 4, 6, 8, 10, 12, 20, and 30 min. The images are compared visually and with the help of video-densitometry. After documentation under UV 366 nm (4.9 b) the plate is observed for 30 min. An image is taken after 1, 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.

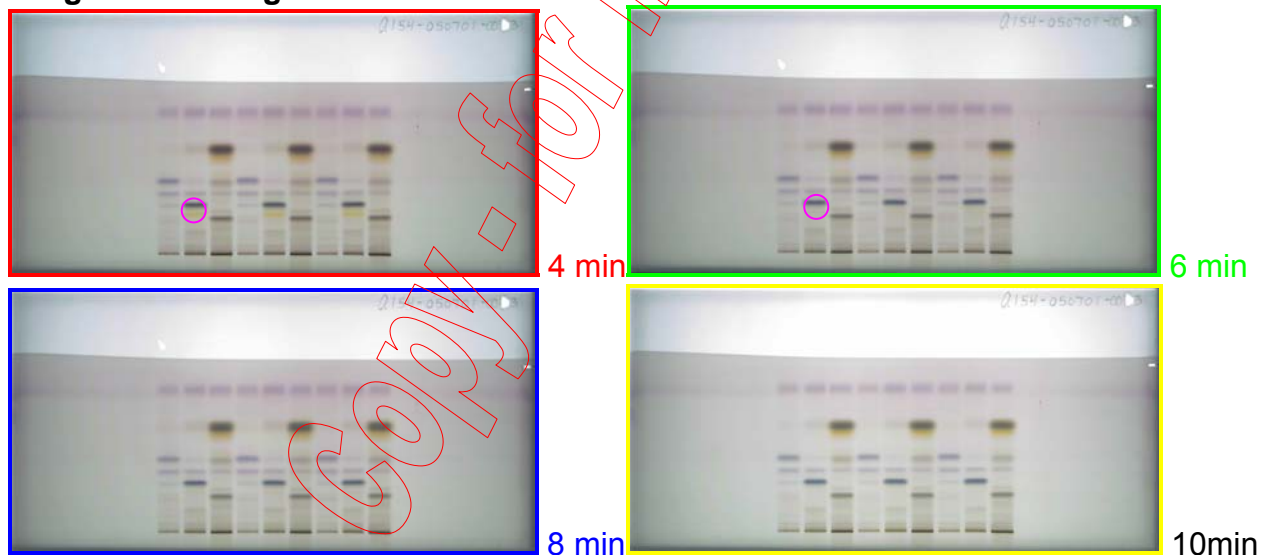
a) Alkylamides

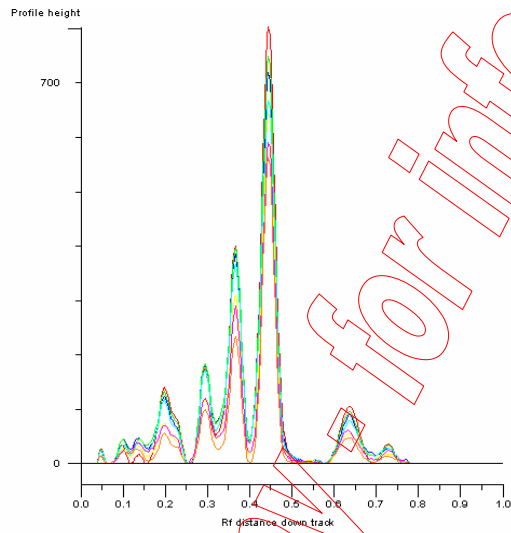
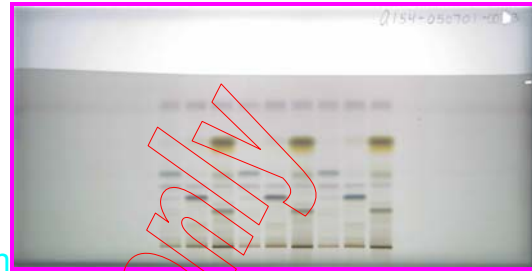
Results:

The plates obtained in experiment 5.4 and 5.5 were used.

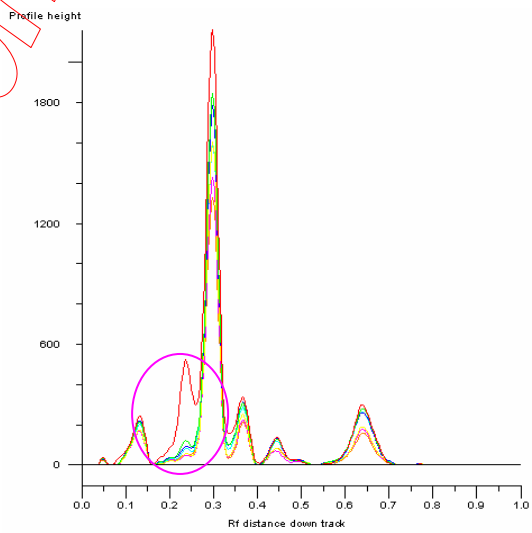
The overall intensity of zones changes slightly with time. Some zones change the color with time. The yellow zone in the fingerprint of *E. angustifolia* (marked with a pink circle) disappears between 4 and 6 min.

Images / Densitograms:

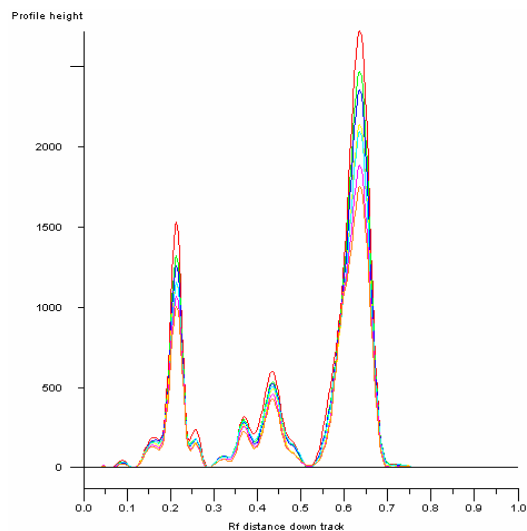




E. purpurea



E. angustifolia



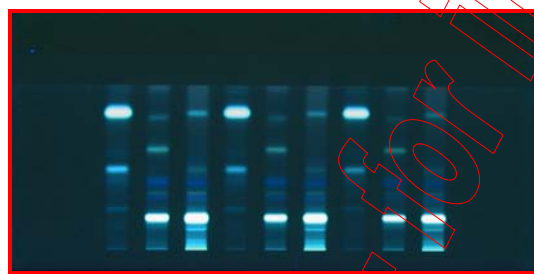
E. pallida

Accepted: Yes, with the following limitation: Identification of *E. angustifolia* requires documentation within 4 min after the completion of derivatization.

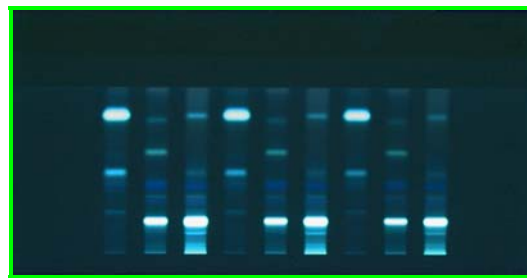
b) Phenylpropanoids

Results: No change is observed over 30 min

Images / Densitograms:



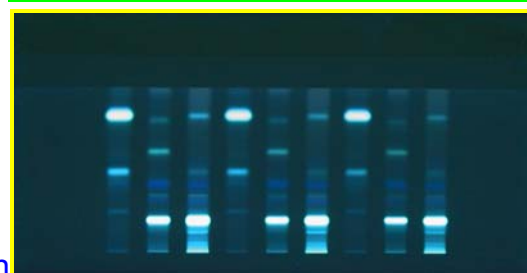
1 min



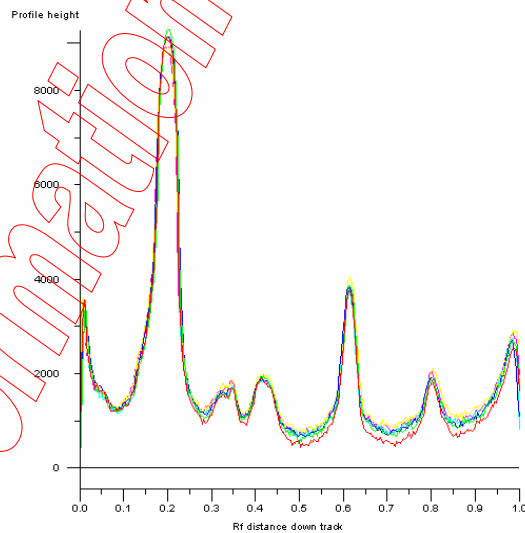
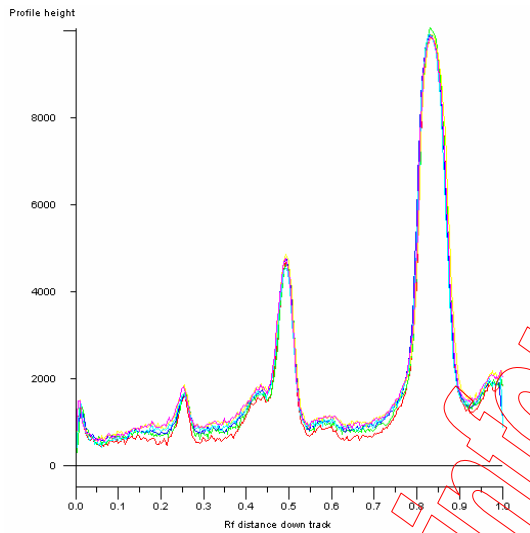
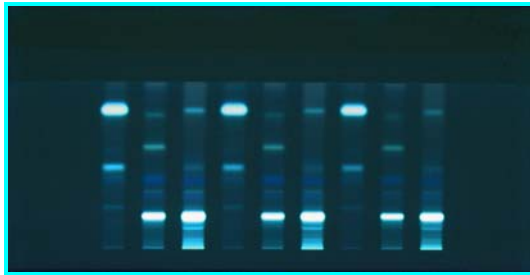
5 min



10min

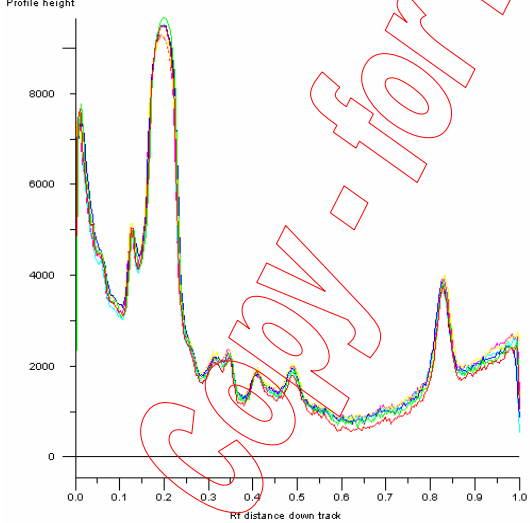


15min



E. purpurea

E. angustifolia



E. pallida

Accepted: YES

5.3 Specificity

5.3.1 Identification of Echinacea root samples by comparison to botanical reference materials (BRM) and detection of adulteration

Description of experiment:

Test solutions of various Echinacea samples and of three adulterants (*E. atrorubens*, *Liatrix punctata*, *Parthenium integrifolium*) are prepared according to section 4.1 a/b. All samples are applied onto the same plate according to section 4.5 a/b. Following chromatography (section 4.7 a/b) and derivatization (section 4.8 a/b) the plate is documented (section 4.9 a/b) and the results compared to those shown in section 4.10 a/b. **NOTE:** These experiments were performed during method development and peer verification in 2002. Developing distance was only 60 mm. According to section 5.7.2 this change is acceptable.

Acceptance criteria:

The method is specific for *E. purpurea*, *E. angustifolia*, *E. pallida* if the fingerprints obtained with the test solutions representing Echinacea are similar to only one of the fingerprint shown in section 4.10 a/b with respect to number, position, color, and intensity of bands matching only the chromatogram of the corresponding BRM and samples of other identity yield different fingerprints. Furthermore, the method is specific if the fingerprints of the adulterants are significantly different from those of any Echinacea BRM with respect to number, position, color, and intensity of bands.

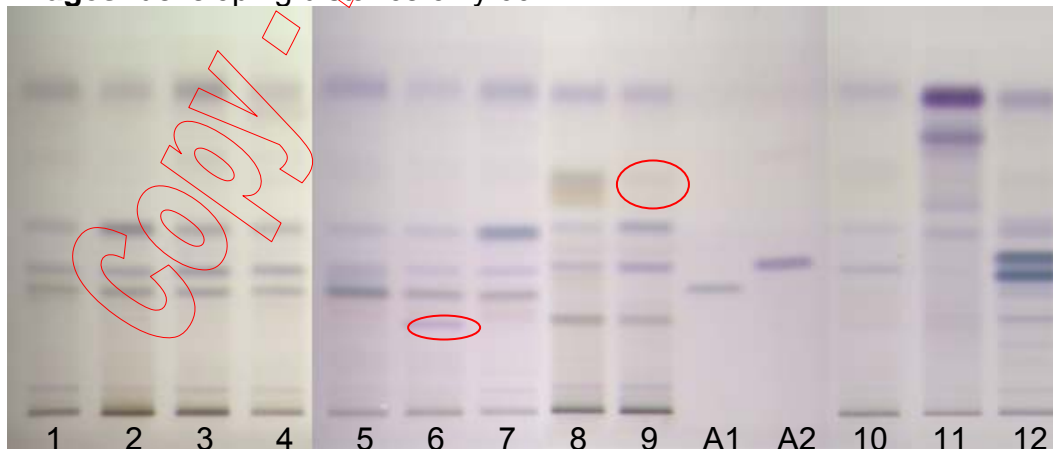
Note: In this test the individual samples can either pass or fail, however, authenticated samples must pass.

a) Alkylamides

Results:

The method is specific. All but 2 Echinacea samples meet the acceptance criteria. Sample 6 shows a not permitted zone and sample 9 misses a required zone. The fingerprints of the adulterants (*E. atrorubens*, *Liatrix punctata*, *Parthenium integrifolium*) are significantly different.

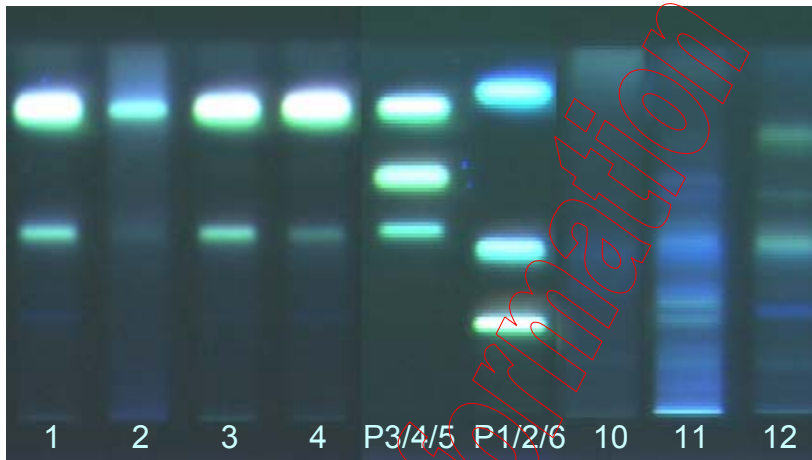
Images: developing distance only 60 mm



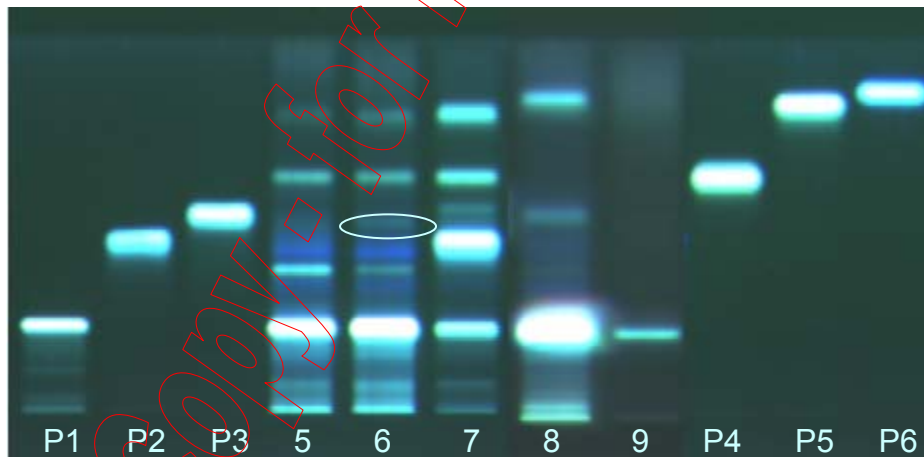
1-4: *E. purpurea* root, 5-7: *E. angustifolia* root, 8-9: *E. pallida* root, A1: DAI, A2: β -sitosterol, 10: *E. atrorubens*, 11: *Liatris punctata*, 12: *Parthenium integrifolium*

b) Phenylpropanoids

Results: The method is specific. All but 3 Echinacea samples meet the acceptance criteria. Sample 6 (*E. angustifolia*) shows traces of caftaric acid. The echinacoside zone in sample 9 (*E. pallida*) is too weak and caftaric acid and cichoric acid are missing. Sample 7 shows not permitted zones. The fingerprints of the adulterants (*E. atrorubens*, *Liatris punctata*, *Parthenium integrifolium*) are significantly different.



1-4: *E. purpurea* root, 10: *E. atrorubens*, 11: *Liatris punctata*, 12: *Parthenium integrifolium*, P3/4/5: caftaric acid, cynarin, cichoric acid (with increasing Rf), P1/2/6: echinacoside, chlorogenic acid, caffeic acid (with increasing Rf)



P1: echinacoside, P2: chlorogenic acid, P3: caftaric acid
5-7: *E. angustifolia* root, 8-9: *E. pallida* root
P4: cynarin, P5: cichoric acid, P6: caffeic acid

Accepted: YES

5.4 Repeatability

The same procedure is performed in parallel for each Echinacea species.

One portion of each BRM is individually prepared according to section 4.2 a/b. Onto three 20x10 cm plates, three aliquots of 5 μ L of each reference solution are applied according to section 4.5 a/b. The plates are chromatographed (section 4.7 a/b) 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 a/b and 4.9 a/b.

The results across each plate and from plate to plate are evaluated. The average Rf values of a) two (DAI, β -sitosterol) respectively b) four prominent zones (echinacoside, cynarin, caftaric acid, cichoric acid) are determined for each track on each plate.

Acceptance criteria:

The repeatability of the method is acceptable if:

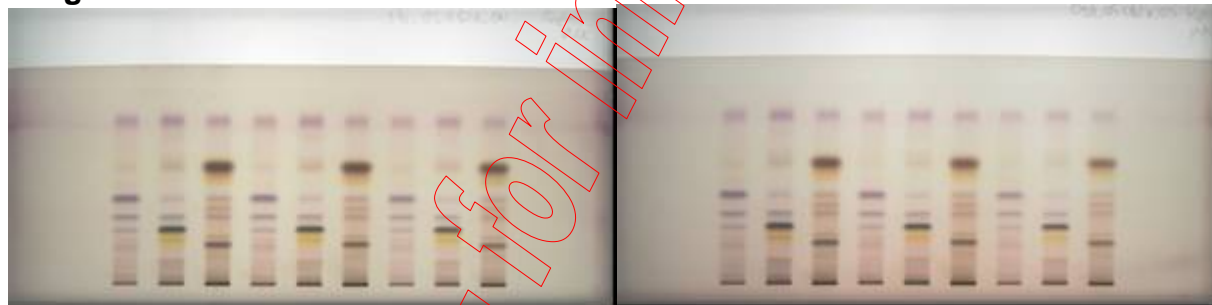
All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and**

The Rf values for each of the specified zones on the three plates don't vary more than 0.02 and the system suitability test (4.12 a/b) is met.

Results:

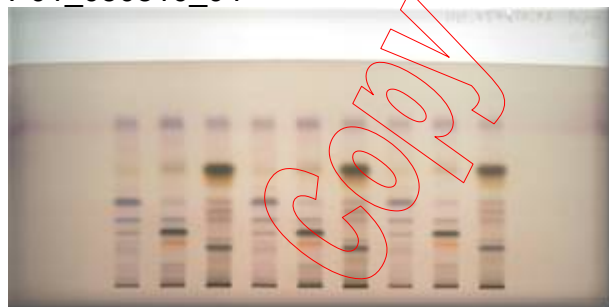
All chromatograms look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen and system suitability test is met.

Images:



P01_050810_04

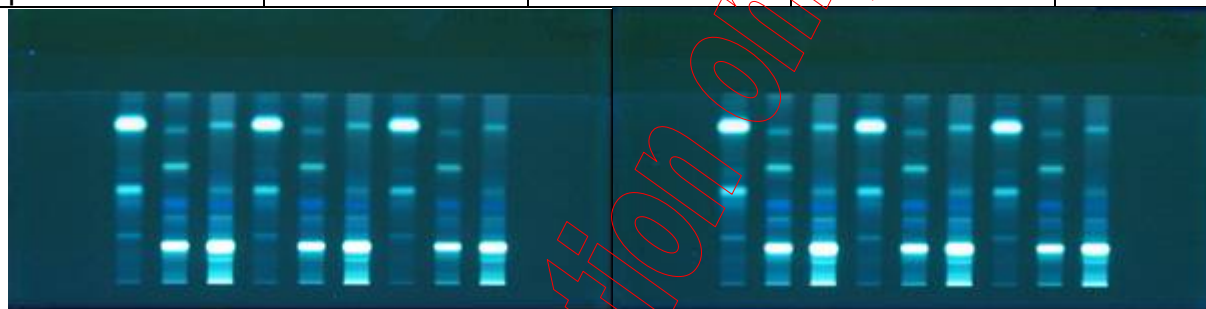
P01_050810_05



P01_050810_06

Rf-values	P01_050810_04	P01_050810_05	P01_050810_06	Δ Rf
DAI (not present)	0.28	0.29	0.29	0.01

in <i>E. pallida</i> !)				
β -sitosterol	0.35	0.36	0.36	0.01



P01_050810_01

P01_050810_02



P01_050810_03

Rf-values	P01_050810_01	P01_050810_02	P01_050810_03	Δ Rf
Echinacoside (in <i>E. angustifolia</i> and <i>E. pallida</i>)	0.19	0.09	0.19	0.0
Cichoric acid (in <i>E. purpurea</i>)	0.82	0.82	0.82	0.0
Cynarin (in <i>E. angustifolia</i>)	0.61	0.61	0.60	0.01
Caftaric acid (in <i>E. purpurea</i>)	0.48	0.49	0.48	0.01

Accepted: YES

5.5 Intermediate precision (performed by confirmatory lab)

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

Repeat the experiment described under 5.4 on 2 other days. Onto one 20x10 cm plate, three aliquots of 5 μ L of the solution are applied.

The Rf values of two respectively four prominent zones are determined for each track on each plate and variations of average Rf values from plate to plate are evaluated including one plate prepared during experiment 5.4.

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 the specified zones on the three plates don't vary more than 0.05 and the system suitability test (4.12 a/b) is met.

Results:

All chromatograms look very similar with respect to number, position, color, and intensity of zones. No disturbances are seen and the system suitability test is met.

Images:



Plate Nr A154-050629-001

Plate Nr A154-050701-001

Rf	A154-050630-001 (Comparison)	A154-050629-001	A154-050701-001	Δ Rf
DAI (not present in <i>E. pallida</i> !)	0.31	0.30	0.30	0.01
β -sitosterol	0.38	0.38	0.38	0.0

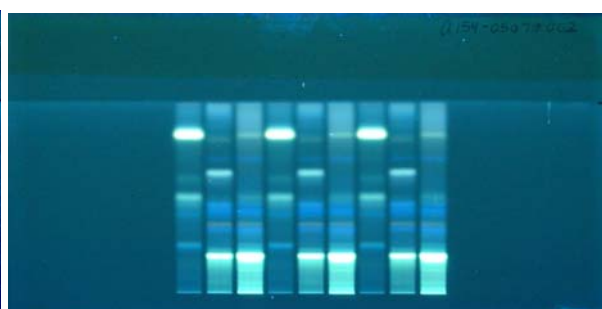
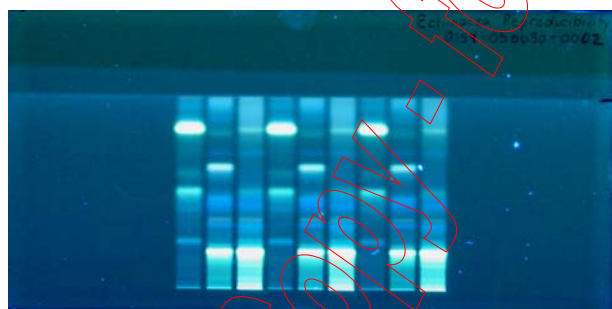


Plate Nr A154-050630-002

Plate Nr A154-050701-002

Rf	A154-050629-004B (Comparison)	A154-050630-002	A154-050701-002	Δ Rf
Echinacoside (in <i>E. angustifolia</i> and <i>E. pallida</i>)	0.19	0.19	0.20	0.01
Cichoric acid (in <i>E. purpurea</i>)	0.82	0.83	0.83	0.01
Cynarin (in <i>E.</i>)	0.62	0.60	0.63	0.03

<i>angustifolia</i>)				
Caftaric acid (in <i>E. purpurea</i>)	0.50	0.51	0.50	0.01

Accepted: YES

5.6 Reproducibility

The same procedure is performed in parallel for each Echinacea species.

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

The reproducibility is acceptable if the Rf values 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) and the system suitability test (4.12) is met.

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 and the system suitability test is met. The color/brightness of the image differs slightly from the image obtained in the primary lab. This could be due to different and settings of the documentation devices.

Images:



Plate Nr A154-050630-001A



Plate Nr A154-050630-001B

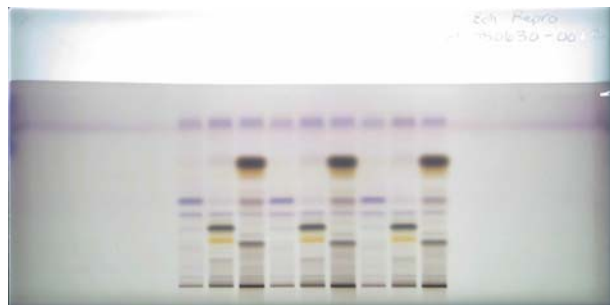
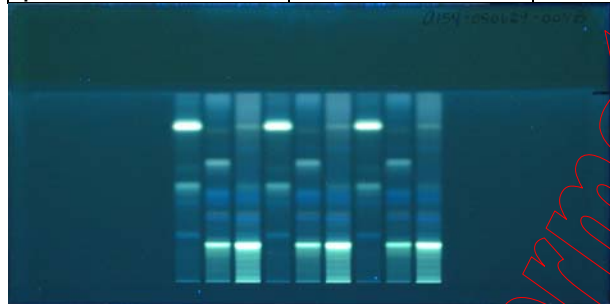
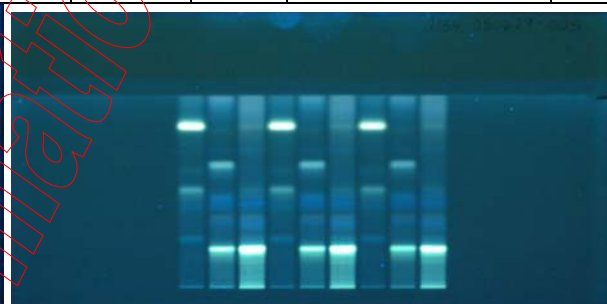


Plate Nr A154-050630-001C

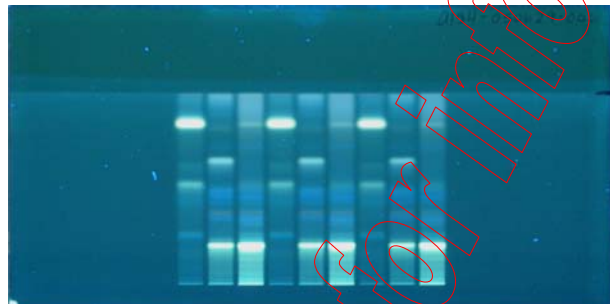
Rf	A154-050630-001A	Nr - 001B	Nr - 001C	ΔRf	P01_050810_04 (Comparison)	ΔRf
DAI (not present in <i>E. pallida</i> !)	0.31	0.30	0.31	0.01	0.28	0.03
β -sitosterol	0.38	0.38	0.38	0.0	0.35	0.03



A154-050629-004B



A154-050629-005



A154-050629-006

Rf	A154-050629-004B	Nr -005	Nr -006	ΔRf	P01_050810_01 (Comparison)	ΔRf
Echinacoside (in <i>E. angustifolia</i> and <i>E. pallida</i>)	0.19	0.21	0.20	0.02	0.19	0.02
Cichoric acid (in <i>E. purpurea</i>)	0.82	0.84	0.84	0.02	0.82	0.02
Cynarin (in <i>E. angustifolia</i>)	0.62	0.64	0.64	0.02	0.61	0.03
Caftaric acid (in <i>E. purpurea</i>)	0.50	0.51	0.52	0.02	0.48	0.04

Accepted: YES

5.7. Robustness

The same procedure is performed in parallel for each Echinacea species.

5.7.1 Chamber type

Not performed.

5.7.2 Developing distance

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

The method is executed according to section 4 a/b using only the BRMs.

The developing distance is decreased to 60 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 a/b (<0.05) and the system suitability test (4.12 a/b) is met. In the case of differences between the results, the developing distance of less than 70 mm yields invalid results.

Results:

The Rf values are not affected by the reduced developing distance.

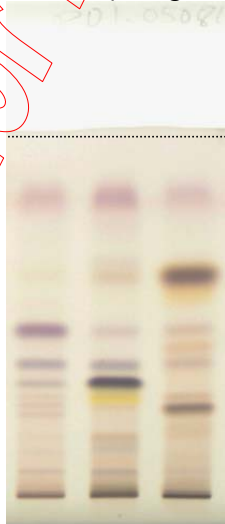
The system suitability test is met.

Images:

60 mm



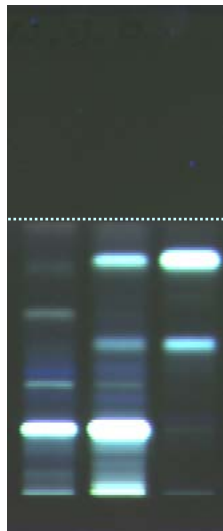
70mm (image 4.10a)



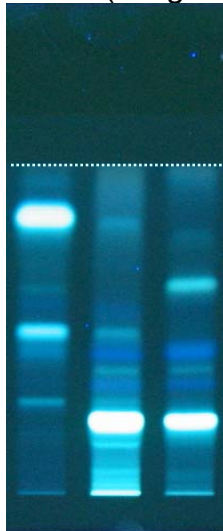
1: *E. purpurea*, 2: *E. angustifolia*, 3: *E. pallida*

Rf	60 mm	70 mm (Image 4.10a)	Δ Rf
DAI (not present in <i>E. pallida</i> !)	0.31	0.31	0.0
β -sitosterol	0.38	0.38	0.0

60 mm



70mm (image 4.10b)



1: *E. purpurea*, 2: *E. angustifolia*, 3: *E. pallida*

Rf	60 mm	70 mm (Image 4.10b)	ΔRf
Echinacoside (in <i>E. angustifolia</i> and <i>E. pallida</i>)	0.24	0.22	0.05
Cichoric acid (in <i>E. purpurea</i>)	0.86	0.84	0.04
Cynarin (in <i>E. angustifolia</i>)	0.66	0.64	0.05
Caftaric acid (in <i>E. purpurea</i>)	0.55	0.50	0.05

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, the experiment was not performed. For details see section 5.2.1-5.2.3.

5.7.4 Relative humidity

The same procedure is performed in parallel for each Echinacea species.

Description of experiment:

Five plates are prepared according to section 4 a/b using only one BRM of each species. The plates are developed in an automatic chamber with humidity control (ADC2, CAMAG). Prior to chromatography (4.6 a/b), the plates are conditioned over salt solutions or molecular sieve during 10 min at different relative humidity. Relative humidity covering a range of about 5-60%RH should be tested.

Molecular sieve: 1.5%RH

Magnesium chloride: 35%RH

Potassium thiocyanate: 47%RH

Sodium chloride: 68%RH

Ambient humidity: 42%RH

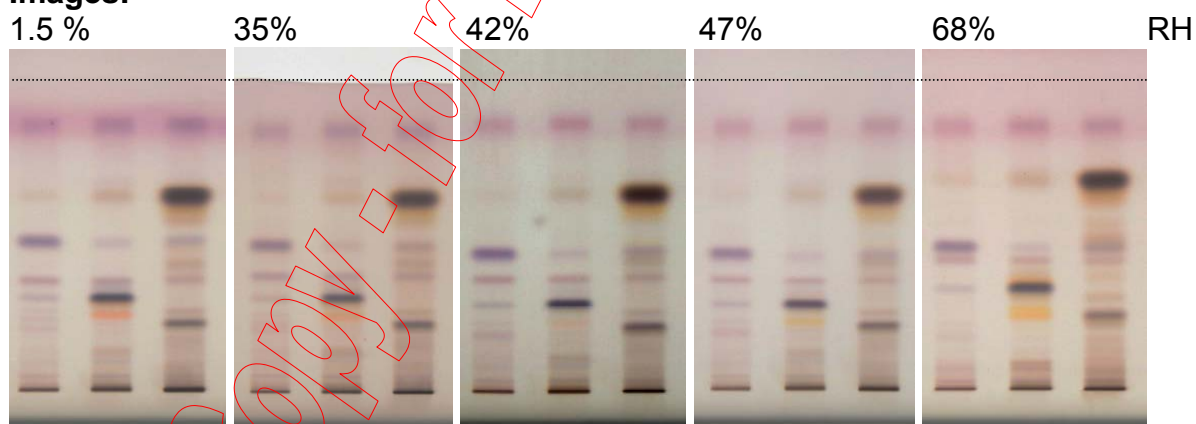
Acceptance criteria:

The fingerprints obtained under different relative humidity are similar with respect to number, position, color, and intensity, and the system suitability test (4.12) is met. 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.

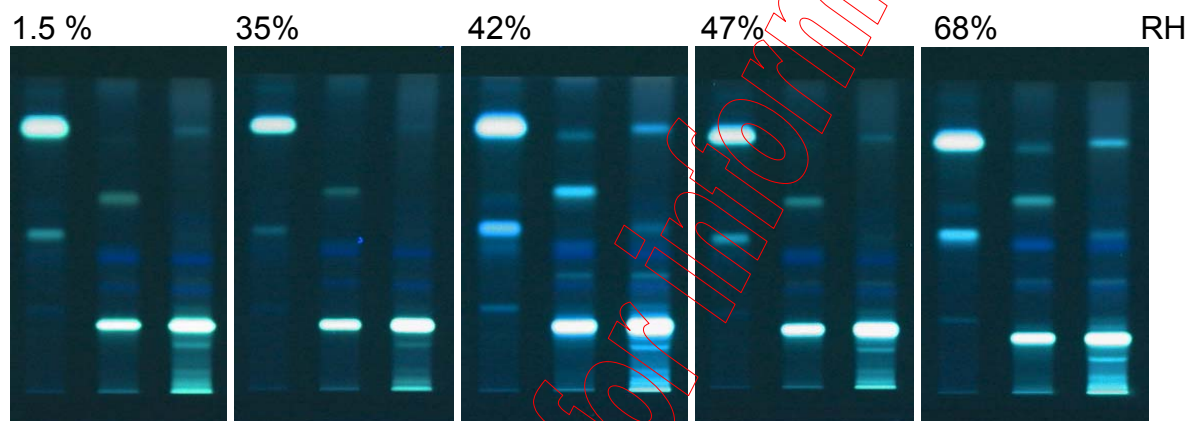
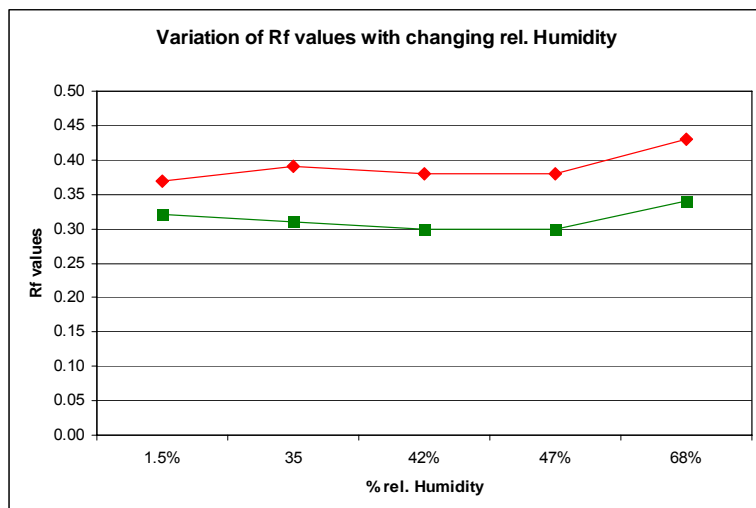
Results:

Only small variations in R_f values are seen. The system suitability test is met for chromatograms.

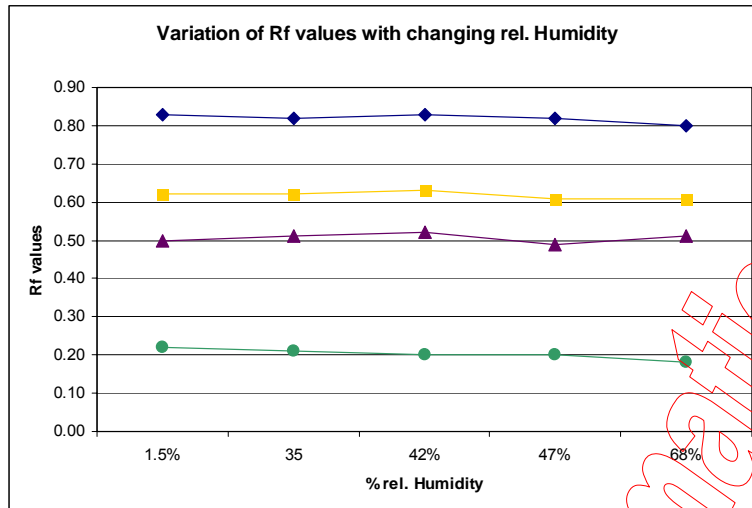
Images:



R _f -values	1.5%RH	35%RH	42%RH	47%RH	68%RH
DAI (not present in <i>E. pallida</i>)	0.37	0.39	0.38	0.38	0.43
β-sitosterol	0.32	0.31	0.30	0.30	0.34



Rf-values	1.5%RH	35%RH	42%RH	47%RH	68%RH
Cichoric acid	0.83	0.82	0.83	0.82	0.80
Cynarine	0.62	0.62	0.63	0.61	0.61
Caftaric acid	0.50	0.51	0.52	0.49	0.51
Echinacoside	0.22	0.21	0.20	0.20	0.18



Accepted: YES

6. Conclusions, Approvals, and Signatures

6.1 Conclusions of primary lab

Both investigated methods are valid for the stated purpose. The documentation step of the alkylamide analysis should be completed within 4 min. *E. purpurea* and *E. angustifolia* can be identified with the phenylpropanoid method alone, but the alkylamide method assures the presence of alkylamides without which the quality of the material is compromised. *E. pallida* can only be identified with certainty using the alkylamide method.

Date: August 12, 2005

Analyst of primary lab: **Signature removed**

6.2 Conclusions of substantiating lab

No problems encountered.

Date: July 1st, 2005

Analyst of substantiating lab: **Signature removed**

6.3. Final approval of study director

The method is valid.

Date: August 12, 2005

Study director: **Signature removed**