

## **Validation of Method for the Identification of Ginseng Species by HPTLC Fingerprint**

### **1. Purpose of method to be validated:**

The method for the identification of Ginseng species by HPTLC fingerprint is suitable to identify a given sample of plant material as Ginseng root of one of the species *Panax ginseng*, *P. quinquefolium*, or *P. notoginseng* (syn. *P. pseudoginseng*) based on its ginsenoside fingerprint. The three species yield characteristic fingerprints, which allow a specific identification with certainty.

### **2. General acceptance criteria:**

The method is valid if:

- A botanically authenticated sample of *Panax ginseng*, *P. quinquefolium*, and/or *P. notoginseng* 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).

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### **3. Personnel**

#### **3.1 Study director**

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#### **3.2 Analyst of primary lab**

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#### **3.3 Analyst of confirmatory lab (also performed 5.2 Stability, 5.4 Repeatability, 5.5 Intermediate precision, 5.7.1 Chamber type, and 5.7.2 Developing distance for primary lab)**

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#### **4. Description of method**

##### **4.1 Preparation of test solutions**

1 g of powdered raw material is mixed with 10 mL of absolute ethanol, sonicated for 10 min, and centrifuged. The supernatant is used as test solution.

##### **4.2. Preparation of reference solutions**

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

Chemical reference solutions: 1 mg of each ginsenoside (Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rh1, Rh2), pseudoginsenoside F11, panaxadiol, and panaxatriol is dissolved in each 5 mL methanol.

##### **4.3. Preparation of derivatizing reagent**

Sulfuric acid reagent: 20 mL of 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 F<sub>254</sub> (Merck).

##### **4.5 Sample application**

10 µL of test solution and 5 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 (filter paper, wetted with developing solvent, in trough opposite to the plate)
Developing solvent:	Chloroform, ethyl acetate, methanol, water (15:40:22:9), 5 mL (respectively 10 mL) developing solvent per trough.
Developing distance:	80 mm from lower edge of plate (72 mm from application position)
Drying:	5 min with cold air (hair dryer)

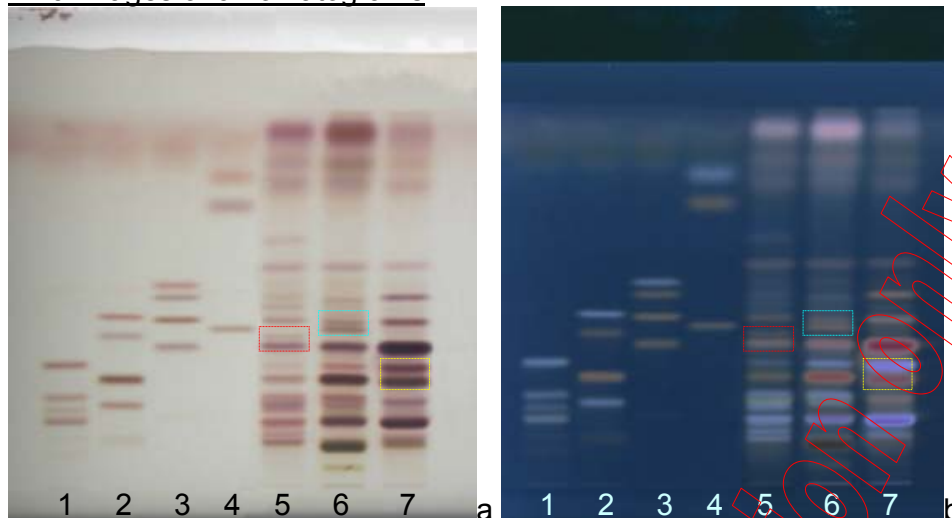
##### **4.8 Derivatization**

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

##### **4.9 Documentation**

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

#### 4.10 Images of chromatograms



- 1: Ginsenosides Rb1, Rb2, Rc, Rd  
2: Ginsenosides Rb3, Re, Rf, Rg3  
3: Ginsenosides Rg1, Rg2, Rh1, Rh2  
4: Pseudoginsenoside F11, panaxatriol, panaxadiol  
5: *Panax ginseng* (Asian ginseng)  
6: *Panax quinquefolium* (American ginseng), cultivated  
7: *Panax notoginseng* (syn. *Panax pseudoginseng*)

#### 4.11 Evaluation of results:

##### 4.11.1 After derivatization under white light

All standards on tracks 1-4 appear as brownish bands. Bands of different intensities matching ginsenoside Rb1, Rc, Rd, Re, Rg1, and Rg2 are seen in all Ginseng samples (ginsenoside Rc is not seen in *P. notoginseng*).

- A band corresponding to ginsenoside Rf is seen in *P. ginseng* only.
- Pseudoginsenoside F11 is only detected in *P. quinquefolium*.
- *P. notoginseng* shows an intense band about the position of ginsenoside Rh1 (probably notoginsenoside R1). *P. ginseng* and *P. quinquefolium* have only a weak zone below this point.
- In *P. ginseng*, the intensity of all bands is similar, whereas *P. quinquefolium* and *P. notoginseng* show prominent bands for ginsenosides Rb1, Re, and Rg1.

Additional brown to green bands are seen above ginsenoside Rh2 and below Rb1 in the samples.

##### 4.11.2 After derivatization under UV 366 nm

A similar picture is seen, but better differentiation of the compounds can be made due to the coloration of the zones. Ginsenosides Rb1, Rb2, Rc, Rd, Rb3, Rg3, and Rh2 are blue (panaxadiol derivatives), and ginsenosides Re, Rf, Rg1, Rg2, Rh1, and Pseudoginsenoside F11 are brownish (panaxatriol derivatives).

#### 4.12 System suitability test:

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

In *P. ginseng*, the ginsenosides Rg1 and Rf must be seen as two distinct zones (red box in Image 4.10).

In *P. quinquefolium*, pseudoginsenoside F11 and ginsenoside Rg2 must be seen as two distinct zones (blue box in Image 4.10).

In *P. notoginseng*, the ginsenosides Rd and Re must be seen as two distinct zones (yellow box in Image 4.10).

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## **5. Validation**

### **5.1 Materials**

#### **5.1.1 Chemicals and solvents**

<b>Name</b>	<b>Manufacturer</b>	<b>Quality</b>
Ethanol	Merck	p.a
Methanol	Acros	p.a
Chloroform	Acros	p.a
Ethyl acetate	Merck	p.a
Sulfuric acid	Merck	p.a
Water	In house	--

#### **5.1.2 Samples and Reference materials**

##### Botanical reference material

<i>Sample Name</i>	<i>Source / Lot</i>	<i>Authentication</i>
<i>Panax ginseng</i> white body 6 yr old	AHP #433A	Yes
<i>Panax quinquefolium</i> 5 yr old root whole dried @ 40°C	AHP #443	Yes
<i>Panax pseudoginseng</i> black (Notoginseng)	AHP 175	Yes

##### Additional samples

<i>Sample Name</i>	<i>Source / Lot</i>	<i>Authentication</i>
<i>Panax quinquefolium</i> leaf from 4 yr old root	AHP #449	Yes
<i>Panax quinquefolium</i> root tails	AHP #459	Yes
<i>Panax quinquefolium</i> wild 7-10 yr old root whole	AHP #354	Yes
<i>Panax quinquefolium</i> wild 12 yr old root whole	AHP #64	Yes
<i>Panax ginseng</i> Shih Chu body #10	AHP #474	Yes
<i>Panax ginseng</i> Kirin Head/Body #1	AHP #469	Yes
<i>Panax ginseng</i> white whole root	AHP #460	Yes
<i>Panax notoginseng</i>	CAMAG	Yes

#### Adulterants

Sample Name	Source / Lot	Authentication
<i>Eleutherococcus senticosus</i> (Siberian ginseng)	CAMAG	Yes

#### Standards (marker compounds, chemical references)

Name	Source
Ginsenoside Rb1	ChromaDex 07190-903
Ginsenoside Rb2	ChromaDex 07195-812
Ginsenoside Rb3	ChromaDex 07197-215
Ginsenoside Rc	ChromaDex 07201-206
Ginsenoside Rd	ChromaDex 07206-381
Ginsenoside Re	ChromaDex 07211-518
Ginsenoside Rf	ChromaDex 07216-508
Ginsenoside Rg1	ChromaDex 07221-110
Ginsenoside Rg2	ChromaDex 07222-801
Ginsenoside Rg3	ChromaDex 07223-920
Ginsenoside Rh1	ChromaDex 07228-106
Ginsenoside Rh2	ChromaDex 07229-016
Pseudoginsenoside F11	ChromaDex 16345-126
Panaxadiol	ChromaDex 16053-013
Panaxatriol	ChromaDex 16054-016

#### 5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB 347651
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB 526793

#### 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.
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	1.3.3-1.3.4
VideoScan	CAMAG	1.02.00

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## **5.2 Stability** (performed by confirmatory lab)

### **5.2.1 Stability of analyte in solution and on the plate**

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

#### **Description of experiment:**

One portion of each BRM is extracted according to section 4.2. 10  $\mu$ L of this solution are applied onto a 20x10 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  $\mu$ L of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 10  $\mu$ 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 between the four chromatograms of each species. The samples of the three Ginseng species are stable on the plate and in solution for at least 3 hours.

#### **Images:**

*Panax ginseng* *P. quinquefolium* *P. notoginseng* *Panax ginseng* *P. quinquefolium* *P. notoginseng*



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 Ginseng species.

#### Description of experiment:

One portion of each BRM is extracted according to section 4.2. 10  $\mu$ 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:

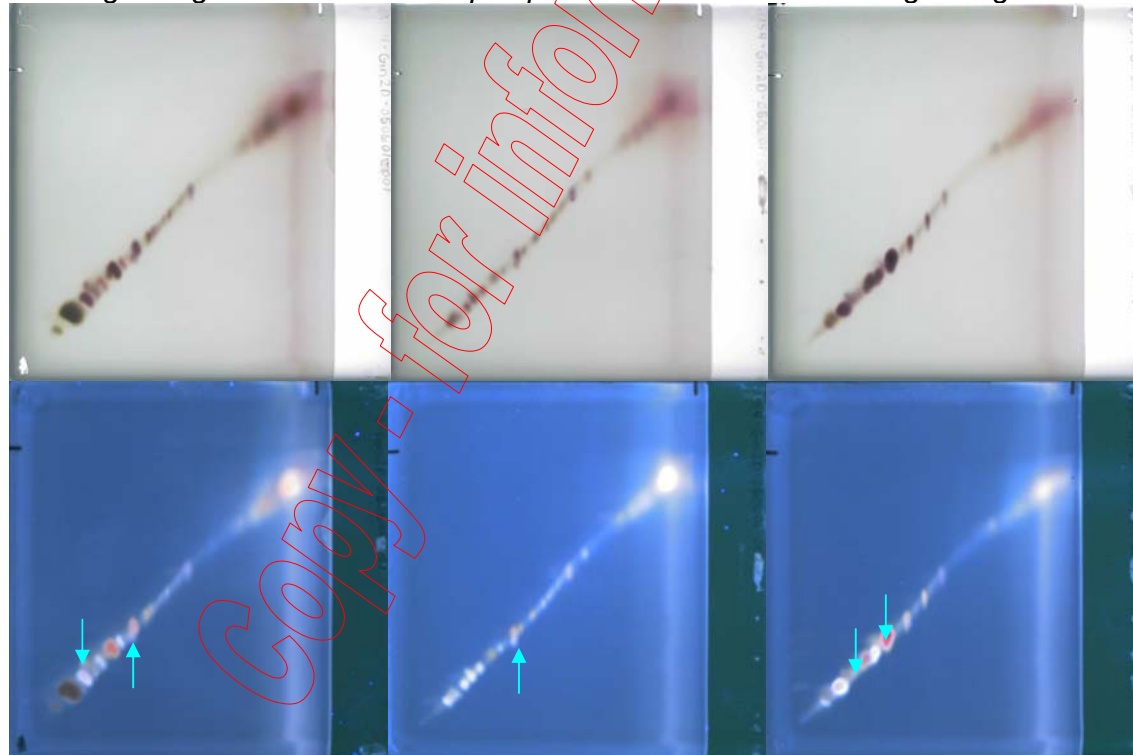
Very few minor spots are located off the diagonal (see images below). The samples of the three Ginseng species are regarded as stable during chromatography.

#### Images:

*Panax ginseng*

*P. quinquefolium*

*P. notoginseng*



**Accepted: YES, with the following limitation: two faint zones in the fingerprints of *P. ginseng* and *P. notoginseng* and one faint zone in the fingerprint of *P. quinquefolium* may be due to artifacts.**

### 5.2.3 Stability of derivatization/result

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

#### Description of experiment:

One portion of each BRM is extracted according to section 4.2. 10  $\mu$ L are applied and 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, 20, 30 min, and 1h. The images are compared visually and with the help of video-densitometry.

#### Acceptance criteria:

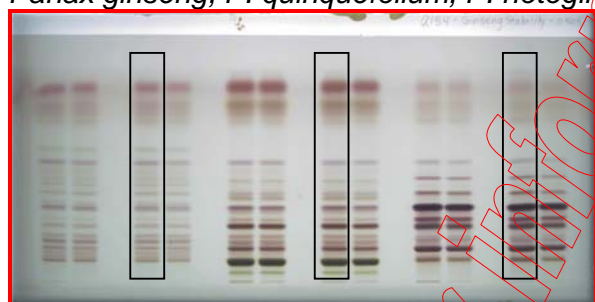
The derivatization yields a stable result, if there is no significant change in the image within 30 min.

#### Results:

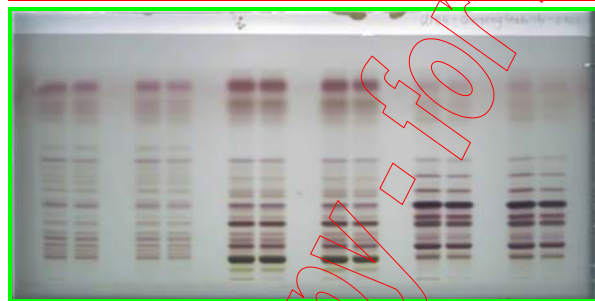
The plate obtained in experiment 5.2.1 was used.  
The overall intensity of zones decreases slightly with time, but no zone disappears.

#### Images / Densitograms:

*Panax ginseng*, *P. quinquefolium*, *P. notoginseng*



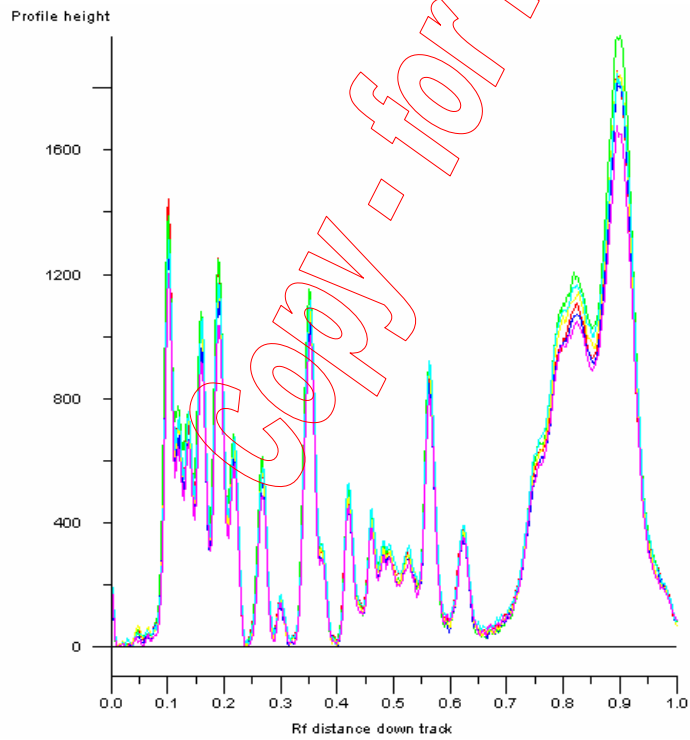
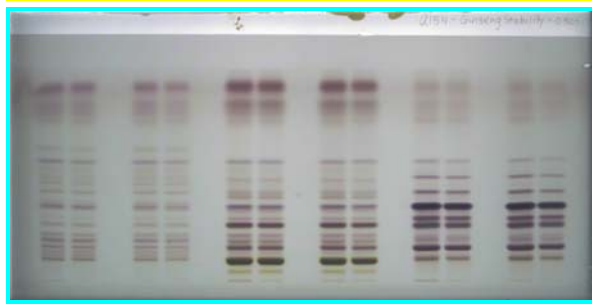
No waiting

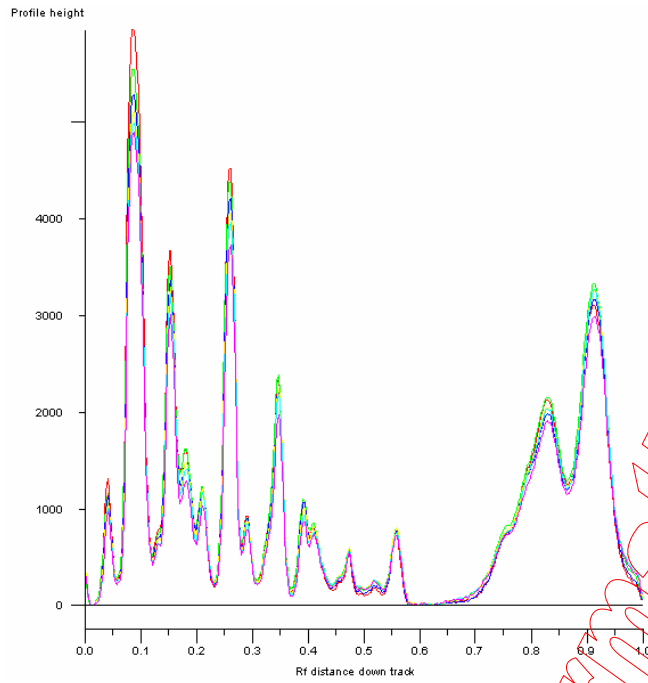


5 min

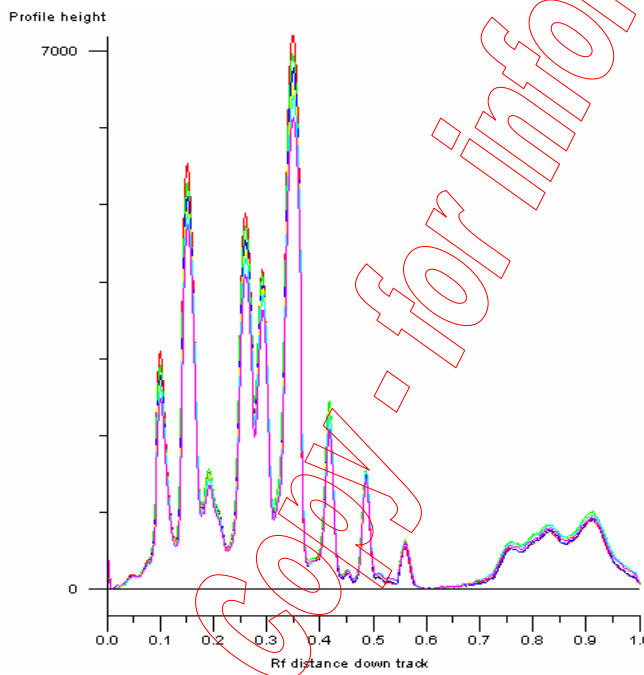


10 min





*P. quinquefolium*



*P. notoginseng*

**Accepted: YES**

### **5.3 Specificity**

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

##### **Description of experiment:**

Test solutions of various Ginseng samples and of one adulterant (*Eleutherococcus senticosus*) are prepared according to section 4.1. The BRMs of *Panax ginseng*, *P. quinquefolium*, and *P. notoginseng* are prepared according to section 4.2 (botanical reference solution). 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 *Panax ginseng*, *P. quinquefolium*, and *P. notoginseng* if the fingerprints obtained with the test solutions representing Ginseng are similar to only one of the fingerprints shown in section 4.10 with respect to number, position, color, and intensity of bands matching the chromatogram of the corresponding BRM **and** samples of other identity yield different fingerprints.

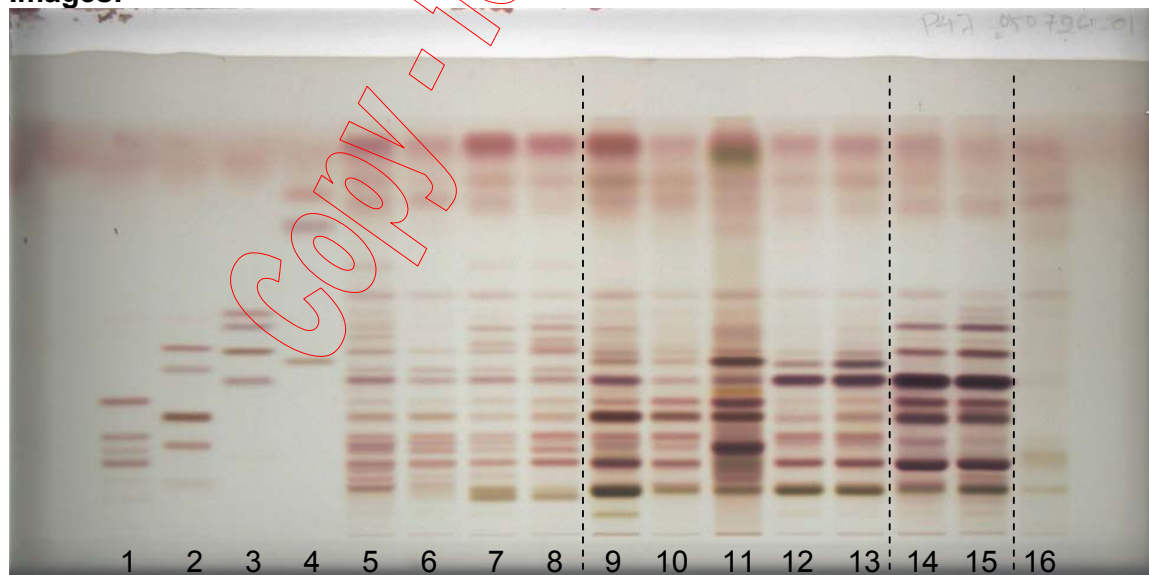
Furthermore, the method is specific if the fingerprint of the adulterant is significantly different from those of any Ginseng BRM with respect to number, position, color, and intensity of bands.

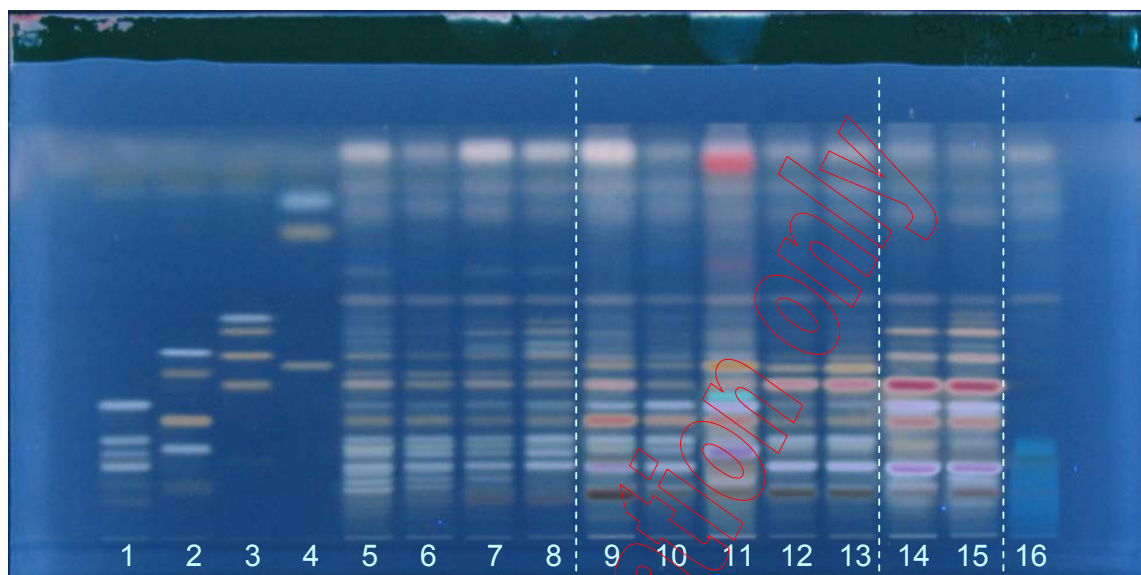
**Note:** In this test the individual samples can either pass or fail, however, authenticated samples must pass.

##### **Results:**

The method is specific. All raw material samples meet the acceptance criteria and the fingerprint of the adulterant (*Eleutherococcus senticosus*) is significantly different.

##### **Images:**





- 1: Ginsenosides Rb1, Rb2, Rc, Rd
- 2: Ginsenosides Rb3, Re, Rf, Rg3
- 3: Ginsenosides Rg1, Rg2, Rh1, Rh2
- 4: Pseudoginsenoside F11, panaxatriol, panaxadiol
- 5\*: White *Panax ginseng* (Asian ginseng)
- 6: White *Panax ginseng* (Asian ginseng)
- 7: Kirin *Panax ginseng* (Red Asian ginseng)
- 8: Shih Chu *Panax ginseng* (Red Asian ginseng)
- 9\*: cultivated *Panax quinquefolium* (American ginseng)
- 10: cultivated *Panax quinquefolium* (American ginseng), root tails only
- 11: leaves of *Panax quinquefolium* (American ginseng)
- 12-13: wild *Panax quinquefolium* (American ginseng)
- 14\*-15: *Panax notoginseng* (syn. *Panax pseudoginseng*)
- 16: Adulterant *Eleutherococcus senticosus* (Siberian ginseng)

\*BRMs

**Accepted: YES**

**5.4 Repeatability** (performed by confirmatory lab)

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

One portion of each BRM is individually prepared according to section 4.2. Onto three 20x10 cm plates, three aliquots of 10  $\mu$ 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 three prominent zones (ginsenosides Rb1, Re, and Rg1) are determined for across 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 and the system suitability test (4.12) 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:**

*Panax ginseng*, *P. quinquefolium*, *P. notoginseng* (3 sets, alternating)



A154-050531-01



A154-050531-02





A154-050531-03

Rf-values	A154-050531-01	A154-050531-02	A154-050531-03	$\Delta R_f$
Rg1	0.35	0.36	0.36	0.01
Re	0.26	0.26	0.27	0.01
Rb1	0.15	0.16	0.16	0.01

**Accepted: YES**

**5.5 Intermediate precision** (performed by confirmatory lab)

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

**Description of experiment:**

Repeat the experiment described under 5.4 on 2 other days. Onto one 20x10 cm plate, three aliquots of 10  $\mu$ L of the solution are applied.

The Rf values of three prominent zones 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:

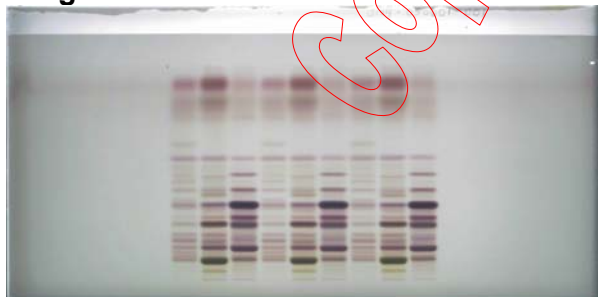
All fingerprints on each plate are identical with respect to number, position, color, and intensity. Across each plate the zones – due to the same compounds – form parallel lines with no disturbance (waves or curves) **and**

The average Rf values for each of the three zones on the three plates don't vary more than 0.05 and the system suitability test (4.12) 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:**



A154-050601-01



A154-050606-01

Rf-values	A154-050531-01 (Comparison)	A154-050601-01	A154-050606-01	$\Delta R_f$
Rg1	0.35	0.35	0.35	0.0
Re	0.26	0.26	0.26	0.0
Rb1	0.15	0.15	0.16	0.01

**Accepted: YES**

**5.6 Reproducibility** (performed by primary lab)

The same procedure is performed in parallel for each Ginseng 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 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) 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 documentation devices and settings.

**Images:**



Plate P47\_050726\_03



Plate P47\_050726\_04



Plate P47\_050726\_05

Rf-values	Plate P47_050726_03	Plate P47_050726_04	Plate P47_050726_05	$\Delta Rf$	A154-050531-01 (Comparison)	$\Delta Rf$
Rg1	0.38	0.38	0.36	0.02	0.35	0.03
Re	0.28	0.29	0.27	0.02	0.26	0.03
Rb1	0.16	0.18	0.16	0.02	0.15	0.03

**Accepted: YES**

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**5.7. Robustness**

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

**5.7.1 Chamber type** (performed by confirmatory lab)

**Description of experiment:**

The method is executed according to section 4 using the BRMs. Instead of a Twin Trough Chamber a Horizontal Chamber of comparable size is used (saturated mode). **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 ( $<0.05$ ) and the system suitability test (4.12) is met. In the case of differences between the results the use of a Horizontal Chamber must be excluded.

**Results:**

The Rf values vary far more than the acceptance criteria. The use of a Horizontal Chamber is not permitted.

**Images:**

Twin Trough Chamber



Horizontal Chamber



Rf-values	TTC (A154-050531-01)	HDC	$\Delta Rf$
Rg1	0.35	0.42	0.07
Re	0.26	0.33	0.07
Rb1	0.15	0.23	0.08

**Accepted: No. The use of a Horizontal Chamber is not permitted.**

### 5.7.2 Developing distance (performed by confirmatory lab)

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

#### Description of experiment:

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

The developing distance is decreased to 70 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 R<sub>f</sub> values obtained in this test are not significantly different from those described in section 4.10 (<0.05) and the system suitability test (4.12) is met. In the case of differences between the results, the developing distance of less than 80 mm yields invalid results.

#### Results:

The R<sub>f</sub> values are not affected by the reduced developing distance. However, the original developing distance of 80 mm gives the impression of a better separation. The system suitability test is met.

#### Images:

70 mm

80mm



R <sub>f</sub> -values	70 mm	80 mm (A154-050531-01)	ΔR <sub>f</sub>
R <sub>g</sub> 1	0.35	0.35	0.0
R <sub>e</sub>	0.27	0.26	0.01
R <sub>b</sub> 1	0.16	0.15	0.01

**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 in this example. For details see section 5.2.1-5.2.3.

#### 5.7.4 Relative humidity

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

##### Description of experiment:

Five plates are prepared according to section 4 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), 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.

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

Molecular sieve: 1.5%RH

Magnesium chloride: 35%RH

Potassium thiocyanate: 47%RH

Sodium chloride: 68%RH

Ambient humidity: 40%RH

##### Acceptance criteria:

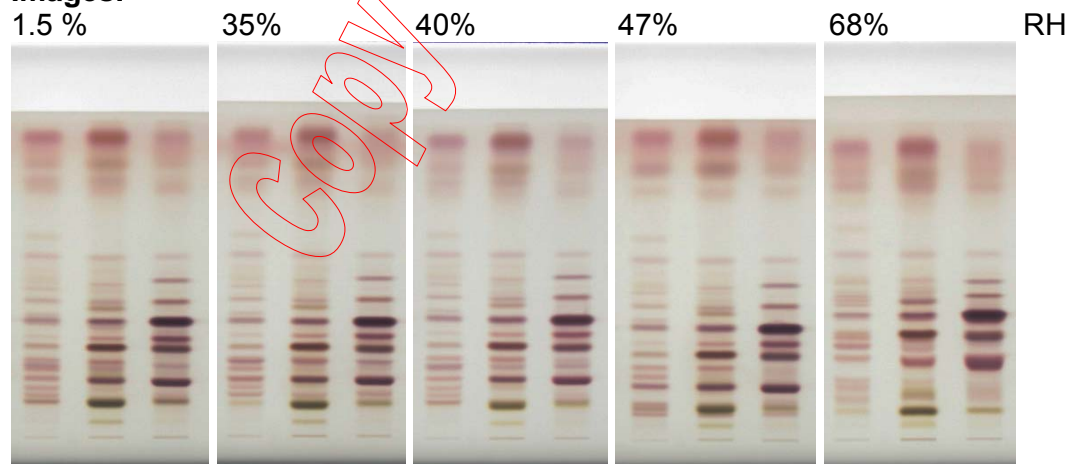
The fingerprints obtained under different relative humidity are similar with respect to number, position, color, and intensity, and the system suitability test (4.12) is met. In this case the relative humidity does not affect the result. In the case of differences of the results, the method may require the control of relative humidity.

##### Results:

Only small variations in R<sub>f</sub> values at low-medium rel. humidity (1.5-47%) are observed. The lowest zones of the chromatograms developed with 47%RH are less resolved. However, this is not relevant for the identification or discrimination of any Ginseng species. The system suitability test is met for chromatograms between 1.5% and 47%RH.

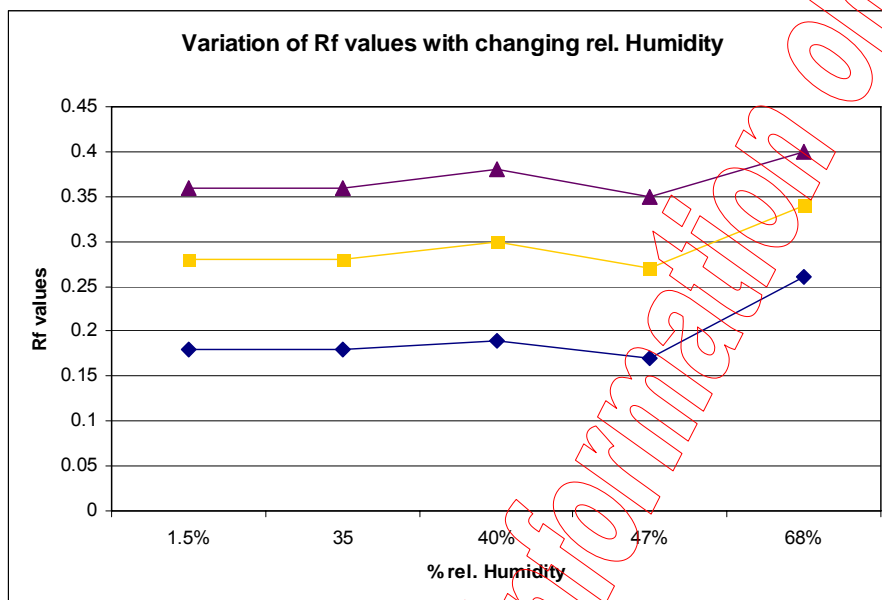
Band broadening and decrease of separation power are seen at high humidity (>68%). The chromatogram should not be developed without humidity control when the surrounding relative humidity exceeds 50%.

##### Images:



Left: *Panax ginseng*, Middle: *P. quinquefolium*, Right: *P. notoginseng*

Rf-values	1.5%RH	35%RH	40%RH	47%RH	68%RH
Rg1	0.18	0.18	0.19	0.17	0.26
Re	0.28	0.28	0.30	0.27	0.34
Rb1	0.36	0.36	0.38	0.35	0.40



**Accepted: YES**

**6. Conclusions, Approvals, and Signatures**

**6.1 Conclusions of primary lab**

Date: The method is valid  
 August 10, 2005

Analyst of primary lab:  
 Signature removed

**6.2 Conclusions of substantiating lab**

Date:

Analyst of substantiating lab:  
 Signature removed

**6.3. Final approval of study director**

Date: The method is valid  
 August 10, 2005

Study director:  
 Signature removed