

Date	Author	Project No.	Project Name
03.Oct.2005	Alison DeBatt	A175	Feverfew

## Validation Protocol

### Validation of Method for the Identification of Feverfew by HPTLC Fingerprint

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Approved by study director:

\_\_\_\_\_  
Signature

\_\_\_\_\_  
Date

Accepted by primary lab:

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Signature

\_\_\_\_\_  
Date

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

The method for the identification of Feverfew (herb) by HPTLC fingerprint is suitable to identify a given sample of plant material as Feverfew (*Tanacetum parthenium*) based on its fingerprint, and using parthenolide as a reference.

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

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

The method is valid if:

- A botanically authenticated sample of Feverfew 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**

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

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#### **3.3 Analyst of confirmatory lab**

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

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

1 g of milled plant material (or enough product equivalent to that amount) is sonicated for 10 min with 10 mL of methanol. The solution is centrifuged or filtered and the supernatant / filtrate is used as test solution.

Liquid samples are diluted with the same solvent (as on the label) to obtain a solution with the same concentration as that of a test solution from raw material.

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

Botanical reference solution: as 4.1.

Chemical reference solution(s): Dissolve 1 mg of parthenolide in 1 mL of methanol.

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

Anisaldehyde Sulfuric acid Reagent: 10 mL of sulfuric acid are carefully added to an ice-cold mixture of 170 mL of methanol and 20 mL of acetic acid. To this solution 1 mL of anisaldehyde is added.

##### **4.4 Stationary phase**

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F<sub>254</sub> (Merck).

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

5 µL of test solution(s) and of botanical reference solution and 5 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 adjusted.

##### **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:	Cyclohexane, ethyl acetate (1:1), 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)

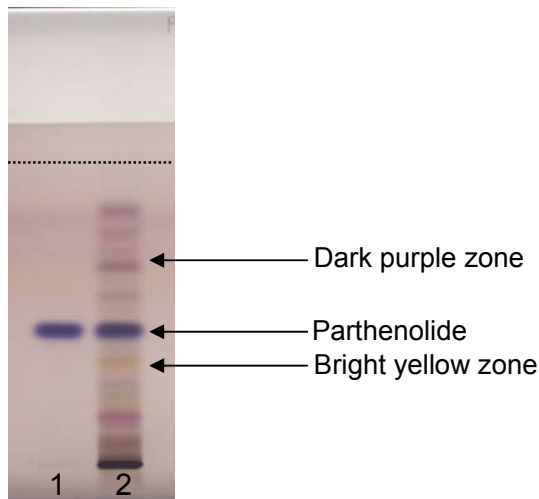
##### **4.8 Derivatization**

The plate is immersed in the reagent for 1 second and then heated at 100°C for 3 minutes.

##### **4.9 Documentation (only necessary images)**

After derivatization under white light (reflection and transmission) within 10 min after the completion of derivatization.

##### **4.10 Images of chromatograms**



**Track assignment:**

- 1: Parthenolide
- 2: Feverfew (BRM)

**4.11 Evaluation of results**

The chemical reference standard parthenolide (track 1) appears as a dark blue zone at  $R_f=0.43$ . The major zone in the sample corresponds in color and position to that of parthenolide. There is a dark purple zone at  $R_f=0.63$  and a bright yellow at  $R_f=0.33$ . Several additional zones are seen as well but no blue zone is seen below parthenolide at  $R_f=0.3-0.4$ .

**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 parthenolide at  $R_f=0.43 (+/- 0.05)$ .

**5. Validation**

**5.1 Materials**

**5.1.1 Chemicals and solvents**

Name	Manufacturer	Quality / Purity
Methanol	EMD	HPLC
Ethyl acetate	Fisher Scientific	HPLC
Cyclohexane	Fisher Scientific	HPLC
Acetic Acid	EMD	ACS
p-anisaldehyde	Acros	99+%
Sulfuric Acid	Spectrum	ACS

**5.1.2 Samples and Reference materials**

**Botanical Reference Material**

Name	Source / Batch	Authentication
Feverfew whole plant mixed BRM ( <i>Tanacetum parthenium</i> )	Removed - proprietary information	Yes

### Additional samples

Name	Source / Batch	Authentication
Feverfew whole plant mixed	Removed - proprietary information	Yes
Feverfew flowers		Yes
Feverfew leaves		Yes
Feverfew stems		Yes
<i>Tanacetum parthenium</i> , Heptinstall feverfew		-
<i>Tanacetum parthenium</i> USDA Germany		-
<i>Tanacetum parthenium</i> , German feverfew		-
<i>Tanacetum parthenium</i> , USDA Germany		-
<i>Tanacetum parthenium</i> , Heptinstall feverfew		-

### Adulterants

Name	Source / Batch	Authentication
<i>Matricariae flos</i>	Removed - proprietary information	Yes
<i>Chamomillae romanae flos</i>		Yes
Mexican feverfew ( <i>Tanacetum parthenium</i> )		Yes
<i>Tanacetum parthenium</i> , Mexican feverfew		-

### Processed materials

Name	Source / Batch
Feverfew powdered extract 0.7% parthenolide	Removed - proprietary information
Feverfew hydro alcohol extract (1:5), 68-74% alcohol from dried herb	
Feverfew hydro alcohol extract (1:5), 55-60% alcohol, yielding 1% parthenolide from dried herb	
Feverfew hydro alcohol extract (undefined strength), 40-50% alcohol from dried herb	
Feverfew dry extract .5%	
Feverfew freeze dried flower/leaf, Capsules 0.1-0.2% parthenolide	

### Standards (marker compounds, chemical references)

Name	Source / Batch
Parthenolide	Chromadex 19225-755

#### 5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	Unknown
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB412660

#### 5.1.4 Instruments

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	070616
TTC 20x10 cm	CAMAG	n/a

TTC 10x10 cm	CAMAG	n/a
ADC2	CAMAG	1120410
TLC Plate Heater III	CAMAG	040838
Immersion Device III	CAMAG	031216
Digistore 2	CAMAG	070721
Mill KB5/10	IKA	03193040
Ultrasonic Bath	Fischer Scientific	9493699
Balance	Sartorius	70805243

#### 5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.4.0
VideoScan	CAMAG	1.02.00

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## **5.2 Stability**

### **5.2.1 Stability of analyte during chromatography**

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

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

#### **Acceptance criteria:**

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

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

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

A portion of the BRM is extracted according to section 4.2. 5 µL 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 5 µL of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 5 µL 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.

### **5.2.3 Stability of derivatization/result**

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

The botanical reference solution (4.2) is chromatographed according to section 4.4-4.8. After documentation under white light (4.9), the plate is observed for 1 hour. An image is taken after 2, 5, 10, 20, 30 min, and about 1h. The images are compared visually and with the help of video-densitometry.

#### **Acceptance criteria:**

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

## **5.3 Specificity**

### **5.3.1 Identification of Feverfew 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 and the chemical references are prepared according to section 4.2. All samples are applied onto the same plate according to section 4.5. Following chromatography (section 4.6-4.7) and derivatization (section 4.8) the plate is documented (section 4.9) and the results compared to those shown in section 4.10.

#### **Acceptance criteria:**

The method is specific if the fingerprints obtained with the test solutions representing Feverfew 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. Any authenticated sample must pass; the other samples may pass or fail.

### **5.3.2 Detection of adulteration**

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

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

#### **Acceptance criteria:**

The method is specific for Feverfew if the fingerprints of adulterants (Mexican feverfew, Chamomile, and Roman Chamomile) are significantly different from those of the BRM of Feverfew with respect to number, position, color, and intensity of bands.

### **5.3.3 Identification of processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.**

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

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

#### **Acceptance criteria:**

The method is specific if the fingerprints obtained with the test solutions representing Feverfew 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. Each sample may pass or fail.

### **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 5  $\mu$ L of each reference solution are applied according to section 4.5. The plates are chromatographed (section 4.6-4.7) subsequently using the same chamber but fresh portions of the developing solvent and fresh filter paper. The plates are derivatized and documented according to 4.8-4.9.

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

#### **Acceptance criteria:**

The repeatability of the method is acceptable if:

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

### **5.5 Intermediate precision**

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

Repeat the experiment described under 5.4 on 2 other days, one plate per day only.

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

#### **Acceptance criteria:**

The intermediate precision of the method is acceptable if:

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

### **5.6 Reproducibility**



**Description of experiment:**

The secondary 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  $R_F$  values for each of the three zones on the three plates don't vary more than 0.02.

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

**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 with 40 mL of developing solvent.

**Acceptance criteria:**

The fingerprints obtained in both chambers are similar with respect to number, position, color, and intensity of zones. The  $R_F$  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.

**5.7.2 Developing distance**

**Description of experiment:**

The method is executed according to section 4 using 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  $R_F$  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.

**5.7.3 Relative humidity**

**Description of experiment:**

Four to six plates are prepared according to section 4 using the BRM. Prior to chromatography (4.7), the plates are conditioned over salt solutions or using a molecular sieve for adjusting different relative humidity (in ADC2). Relative humidity covering a range of about 5-70%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.

Date	Analyst	Project No.	Project Name
16.Nov.2005	Alison DeBatt	A175	Feverfew

## Validation Results

### Validation of Method for the Identification of Feverfew by HPTLC Fingerprint

#### 5.2 Stability

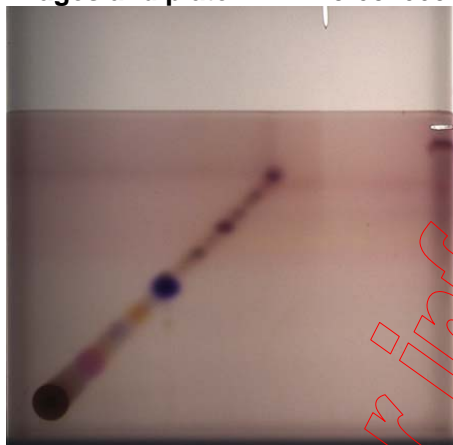
##### 5.2.1 Stability of analyte during chromatography

Temperature recorded: 24°C, Humidity recorded: 55%RH

#### Results:

No zones are located outside of the diagonal; therefore the sample is stable during chromatography.

Images and plate ID: A175-051003-2D



Accepted: YES

Date: 03.Oct.2005

Signature:

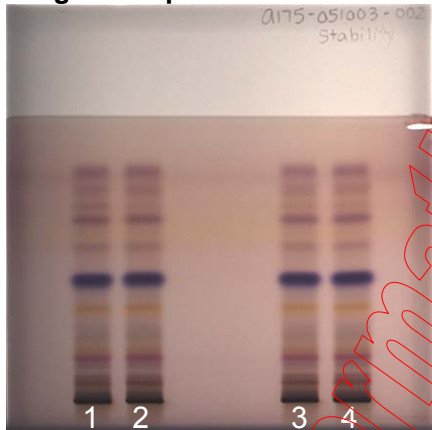
### 5.2.2 Stability of analyte in solution and on the plate

Temperature recorded: 24°C, Humidity recorded: 55%RH

#### Results:

No differences are seen between the tracks; therefore the sample is considered stable for at least 3 hours in solution and on the plate.

Images and plate ID: A175-051003-SolutionStability



#### Track assignment:

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

Date: 03.Oct.2005

Signature:

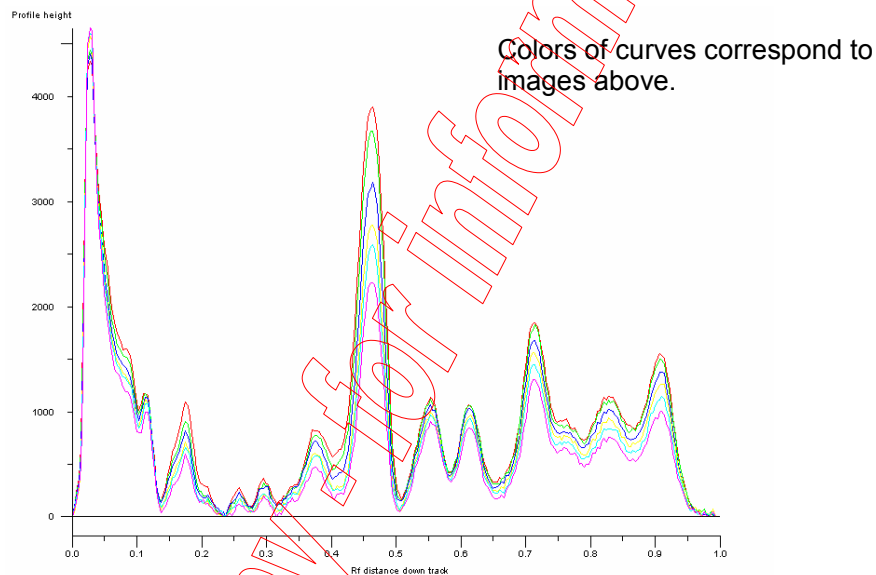
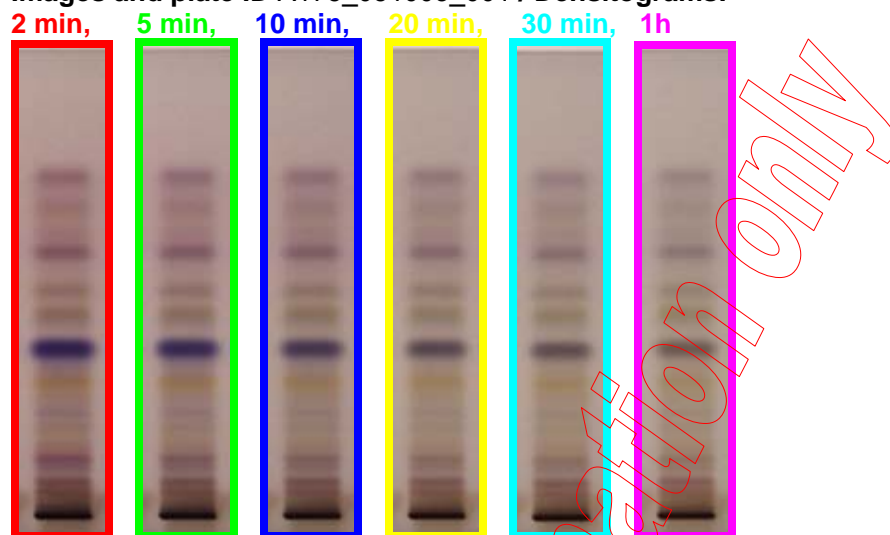
### 5.2.3 Stability of derivatization/result

Temperature recorded: 24°C, Humidity recorded: 55%RH

#### Results:

The intensity of the zones decreases over time, but no fractions appear or disappear. Images should be taken within the first 10 minutes after derivatization. After that time, parthenolide begins to turn to gray.

#### Images and plate ID A175\_051003\_001 / Densitograms:



**Accepted:** YES, with the following limitation: the plate should be documented within 10 min after derivatization. **Date:** 03.Oct.2005 **Signature:**

### **5.3 Specificity**

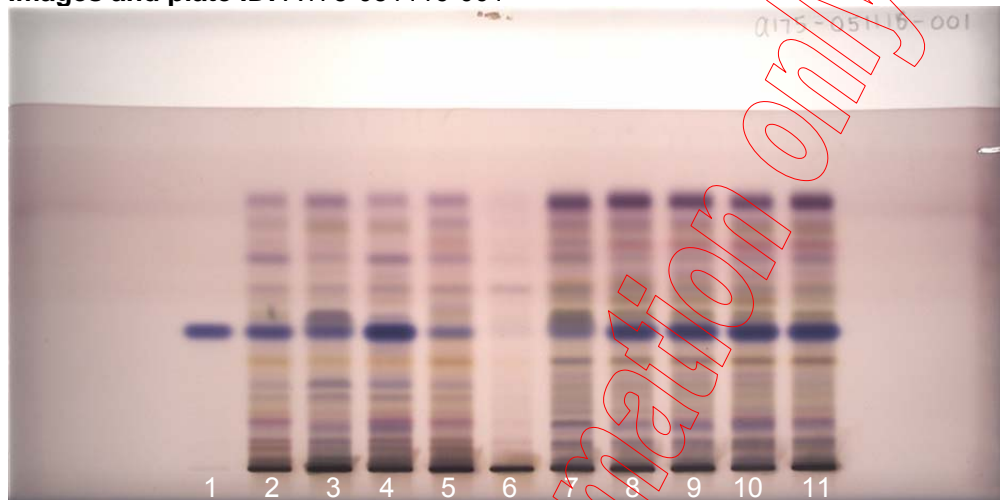
#### **5.3.1 Identification of Feverfew samples and processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.**

Temperature recorded: 25°C, Humidity recorded: 60%RH

#### **Results:**

The stems of Feverfew (track 6) don't show any major zones. Parthenolide is the major zone in all other samples. All other samples show a profile similar to the BRM. Sample on track 7 show a low content of parthenolide. The different parts of the plant show varying amounts of the compounds. The flowers (track 4) have the highest amount of parthenolide. Some samples show various minor zones, which are not seen in the BRM.

Images and plate ID: A175-051116-001



#### **Track assignment**

- 1: Parthenolide
- 2: Feverfew BRM
- 3: Feverfew
- 4: Feverfew flowers
- 5: Feverfew leaves
- 6: Feverfew stems
- 7-11: Feverfew

**System suitability test passed: YES Accepted: YES Date: 16.Nov.2005 Signature:**

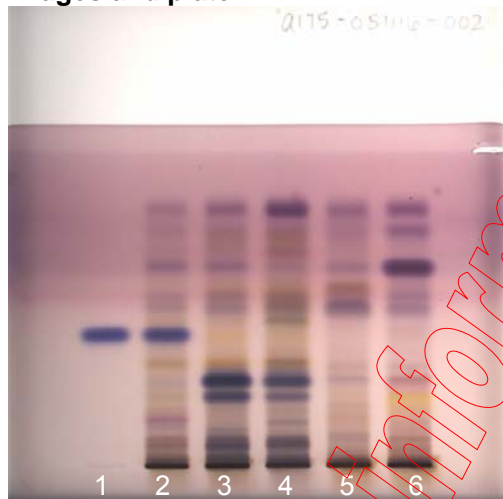
### 5.3.2 Detection of adulteration

Temperature recorded: 25°C, Humidity recorded: 60%RH

#### Results:

None of the adulterants show a fingerprint similar to Feverfew. Parthenolide is absent in all samples.

#### Images and plate ID:



A175-05116-002

#### Track assignment

- 1: Parthenolide
- 2: Feverfew BRM
- 3, 4: Mexican feverfew
- 5: Chamomile (*Matricariae flos*)
- 6: Roman Chamomile (*Chamomillae romanae flos*)

**System suitability test passed: YES Accepted: YES Date: 16.Nov.2005 Signature:**

### 5.3.3 Identification of processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded: 25°C, Humidity recorded: 60%RH

#### Results:

There is a band of different intensity in all samples corresponding in color and position to that of parthenolide. The samples on tracks 5, 6, and 7 shows one to two blue zones at  $R_f=0.3-0.4$ , which are characteristic for Mexican Feverfew, this might indicate a mixture of both species. The sample on track 7 is not compatible with the evaluated method.

#### Images and plate ID:



#### Track assignment

- 1: Parthenolide
- 2: Feverfew BRM
- 3: Feverfew – powdered extract
- 4: Feverfew – dry extract
- 5: Feverfew – freeze dried flowers
- 6: Feverfew – alcohol extract
- 7: Feverfew – alcohol extract

**System suitability test passed:** YES      **Accepted:** YES, No for samples on tracks 5, 6, and 7.

**Date:** 16.Nov.2005

**Signature:**

**5.4 Repeatability**

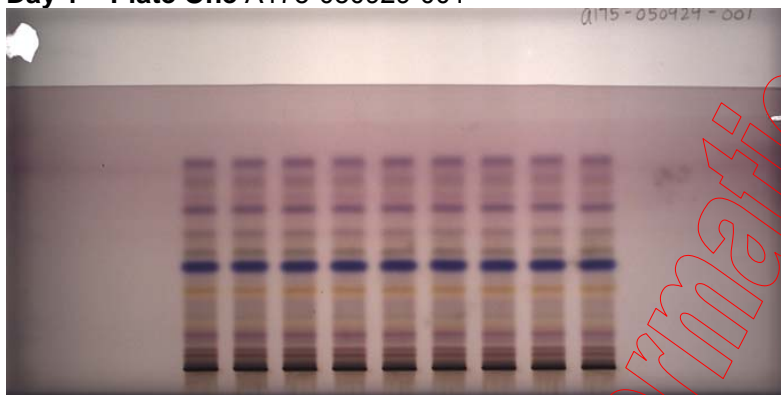
Temperature recorded: 24°C, Humidity recorded: 51%RH

**Results:**

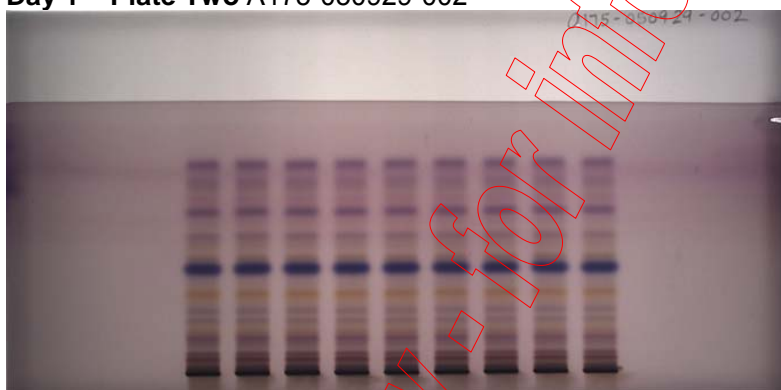
All chromatograms are similar with respect to number, position, color, and intensity of zones. No disturbances are seen and the  $R_F$  values are within the required range.

**Images and plates ID:**

**Day 1 – Plate One** A175-050929-001



**Day 1 – Plate Two** A175-050929-002



**Day 1 – Plate Three** A175-050929-003





$R_F$	Plate A175-050929-001	Plate A175-050929-002	Plate A175-050929-003	$\Delta R_F$
Bright yellow fraction	0.31	0.31	0.31	0.0
Parthenolide	0.41	0.41	0.41	0.0
Dark purple fraction	0.63	0.63	0.63	0.0

**System suitability test passed: YES Accepted: YES Date: 03.Oct.2003 Signature:**

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**5.5 Intermediate precision**

Day 2: Temperature recorded: 24°C,  
 Day 3: Temperature recorded: 24°C,

Humidity recorded: 51%RH  
 Humidity recorded: 55%RH

**Results:**

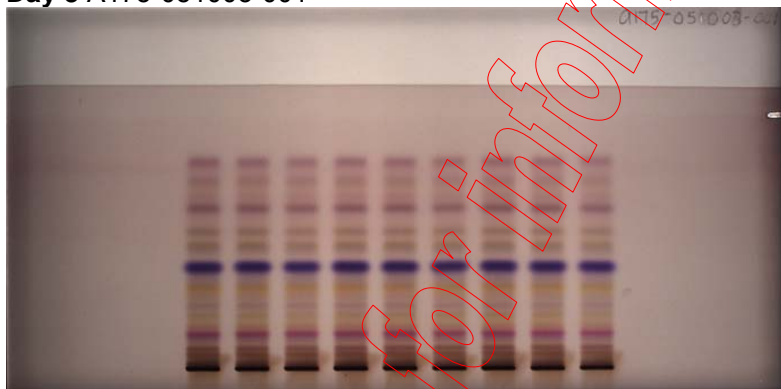
All chromatograms are similar with respect to number, position, color, and intensity. No disturbances are seen and the  $R_F$  values are all within the required range.

**Images and plates ID:**

**Day 2** A175-050930-001



**Day 3** A175-051003-001



$R_F$	Plate A175-050929-001	Plate A175-050930-001	Plate A175-051003-001	$\Delta R_F$
Bright yellow fraction	0.31	0.32	0.32	0.01
Parthenolide	0.41	0.42	0.41	0.01
Dark purple fraction	0.63	0.64	0.63	0.01

**System suitability test passed: YES Accepted: YES Date: 03.Oct.2005 Signature:**

## 5.6 Reproducibility

### Results:

See FO 70.002.05b "Checklist for secondary lab".

## 5.7. Robustness

### 5.7.1 Chamber type

Temperature recorded: 24°C,

Humidity recorded: 51%RH

### Results:

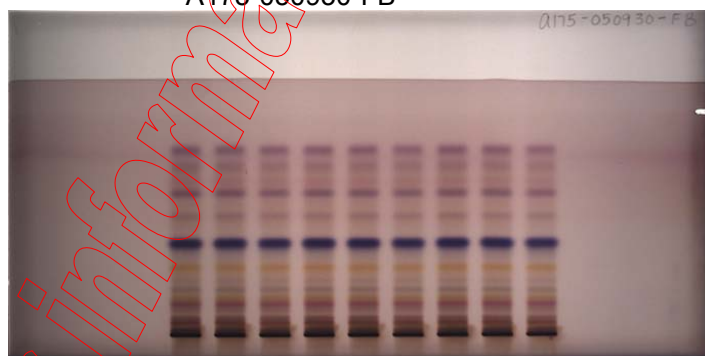
No significant difference is seen when the plate is developed in a Flat Bottom Chamber.

### Images and plate ID:

Twin Trough Chamber  
(Image of section 4.10)



Flat Bottom Chamber  
A175-050930-FB



$R_F$	Twin Trough Chamber	Flat Bottom Chamber	$\Delta R_F$
Bright yellow fraction	0.33	0.31	0.02
Parthenolide	0.43	0.41	0.02
Dark purple fraction	0.63	0.64	0.01

**System suitability test passed: YES Accepted: YES Date: 03.Oct.2005 Signature:**

### 5.7.2 Developing distance

Temperature recorded: 24°C,

Humidity recorded: 51%RH

#### Results:

No significant difference is seen when the developing distance is increased to 80 mm.

#### Images and plate ID:

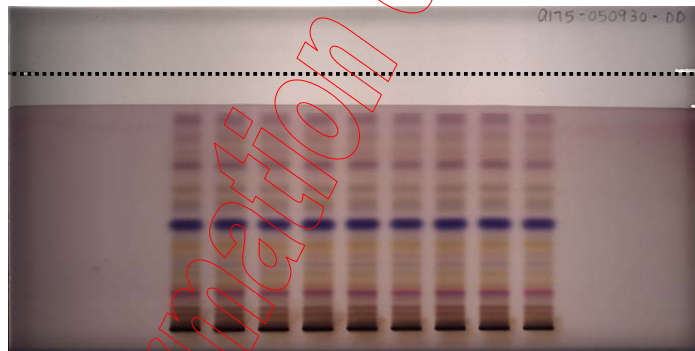
70 mm

(Image of section 4.10)



80 mm

A175-050930-DD



$R_F$	70 mm	80 mm	$\Delta R_F$
Bright yellow fraction	0.33	0.32	0.01
Parthenolide	0.43	0.41	0.02
Dark purple fraction	0.63	0.63	0.0

**System suitability test passed: YES Accepted: YES Date: 03.Oct.2005 Signature:**

### 5.7.3 Relative humidity

Temperature recorded: 24°C

#### Results:

Molecular sieve: 2.9%RH

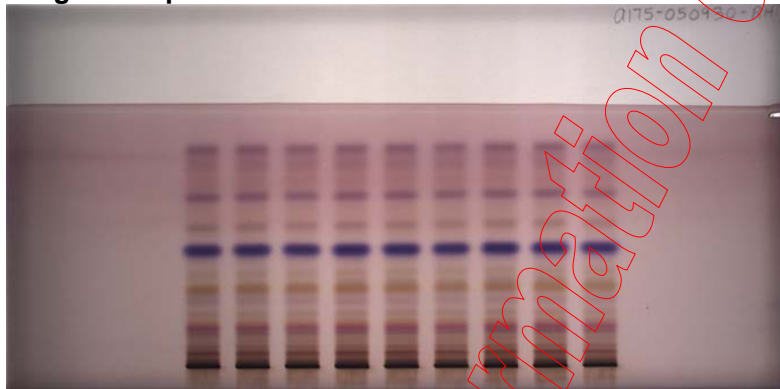
Magnesium chloride: 37%RH

Sodium chloride: 70%RH

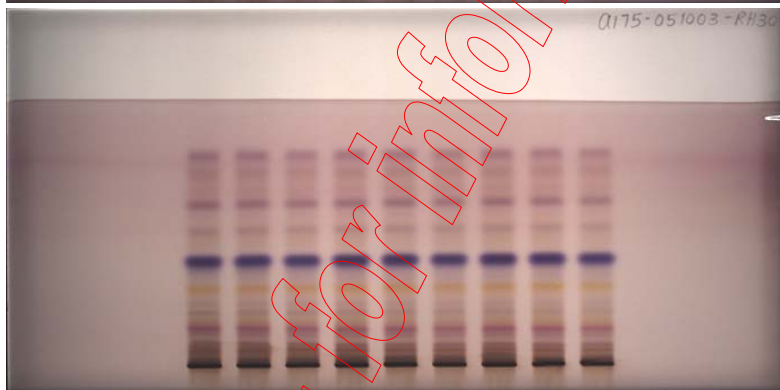
Ambient humidity: 50%RH

The relative humidity does not affect the result significantly. The system suitability test passes for all plates.

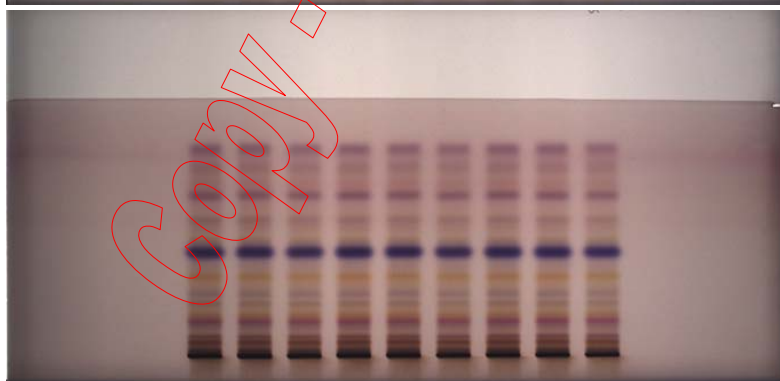
#### Images and plates ID:



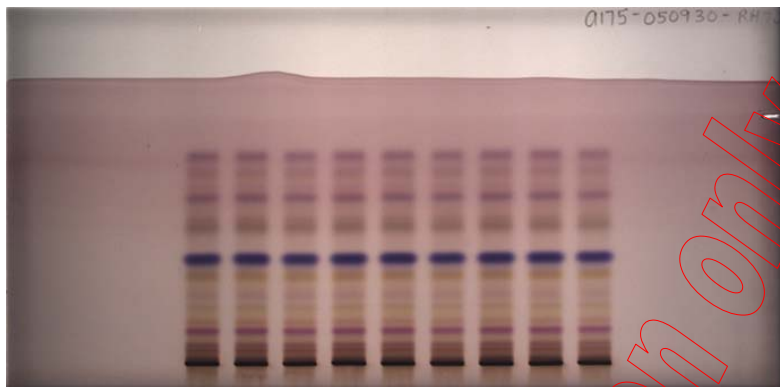
A175-050930 2.9% RH



A175-050930 37% RH



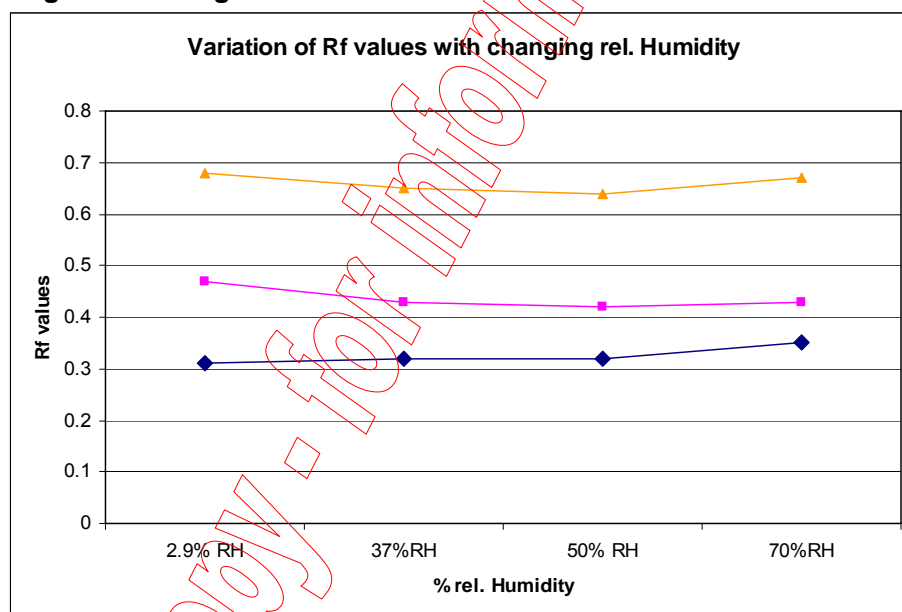
A175-050930 50% RH



A175-050930 70% RH

$R_F$	2.9% RH	37%RH	50% RH	70%RH
Bright yellow fraction	0.31	0.32	0.32	0.35
Parthenolide	0.47	0.43	0.42	0.43
Dark purple fraction	0.68	0.65	0.64	0.67

Diagram showing  $R_F$  value as function of %RH:



System suitability test passed: YES Accepted: YES Date: 03.Oct.2005 Signature:

**Comments**

The method works as expected.  
10 min after the derivatization the color of the parthenolide changes.

**Suggested changes**

The plate should be documented within 10 min after derivatization.

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Completed / Printed

Date:

Signed:

Date of review:

Name:

Title:

Signature:

Copy - for information only