

Date	Author	Project No.	Project Name
11.Nov.2005	Alison DeBatt	A169	Ginger Validation

Validation Protocol

Validation of Method for the Identification of Ginger by HPTLC Fingerprint

Approved by study director:

Signature

Date

Accepted by primary lab:

Signature

Date

1. Purpose of method to be validated:

The method for identification of Ginger by HPTLC fingerprint is suitable to identify a given sample of plant material as Ginger (*Zingiber officinale*) based on the presence of gingerols. Adulterants, such as Galangal (*Alpinia officinarum*), show a different profile.

The method may be used to identify an extract or finished product extract as derived from Ginger (*Zingiber officinale*), provided that the material was made from a single herb and is intended to contain the constituent profile seen in Ginger.

2. General acceptance criteria:

The method is valid if:

- A botanically authenticated sample of Ginger (*Zingiber officinale*) 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

Raw materials: 1 g of powdered sample is mixed with 10 mL of methanol, sonicated for 10 min, and then centrifuged or filtered. The supernatant/filtrate is used as test solution.

Dry extracts/finished products: An amount equivalent to 1 g of raw material is extracted with 10 mL of methanol, sonicated for 10 min, and then centrifuged or filtered. 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 solutions: 1 mg each of 6-gingerol and 6-shogaol are individually dissolved in 1 mL of methanol (USP or ChromaDex). Optionally solutions of 8-gingerol and 10-gingerol (ChromaDex) may be prepared the same way.

4.3 Preparation of derivatizing reagent

10 mL of sulfuric acid are carefully added to a mixture of 170 mL ice-cold methanol and 20 mL acetic acid. To that, 1 mL of anisaldehyde is added.

4.4 Stationary phase

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

4.5 Sample application

2 µL of test solution, 2 µL of botanical reference solution, and 2 µL of each chemical reference solution 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.

4.6 Temperature and Humidity

Record the 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: Toluene, ethyl acetate (75: 25) 5 mL (respectively 10 mL) developing solvent in each trough.
Developing distance: 70 mm from lower edge of plate (62 mm from application position).
Drying: 5 min with cold air (hair dryer)

4.8 Derivatization

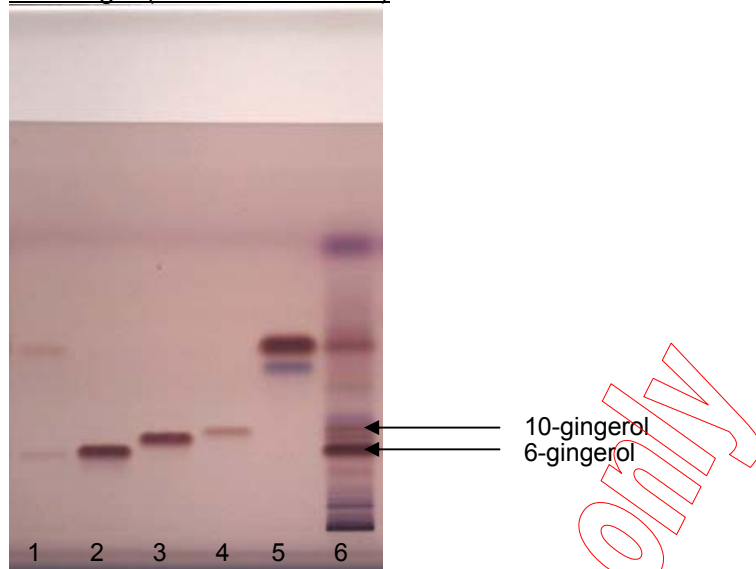
The plate is immersed into the reagent and then dried on a plate heater set to 100°C for 3 minutes.

4.9 Documentation

After derivatization under white light.

4.10 Image of chromatogram

White light (after derivatization)



Track assignment:

- Track 1: USP standard mixture (6-gingerol and 6-shogaol)
- Track 2: 6-gingerol
- Track 3: 8-gingerol
- Track 4: 10-gingerol
- Track 5: 6-shogaol
- Track 6: Ginger BRM (*Zingiber officinale*)

4.11 Evaluation of results:

The reference substances 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol appear as brown zones at $R_F = 0.23, 0.26, 0.28,$ and 0.51 (ΔR_F max 0.05) respectively. The test solution shows zones corresponding in color and position to those of the reference substances. The zones of 6-gingerol and 6-shogaol are most prominent. There are several brown or violet zones located below the zone due to 6-gingerol. Other violet zones may be present above the position of 6-shogaol. No dark blue zone is seen just above 6-shogaol (this zone is found in galangal, an adulterant of ginger).

4.12. System suitability test

The result obtained in the test is suitable for evaluation if the following requirements are met: The two zones due to 6-gingerol and 10-gingerol are clearly separated (see arrows in Figure above). A zone due to 8-gingerol may or may not be seen in between.

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality / Purity
Methanol	EMD	HPLC
Toluene	Spectrum	HPLC
Ethyl acetate	Spectrum	HPLC
Acetic acid	EMD	ACS
Sulfuric Acid	Spectrum	ACS
<i>p</i> -anisaldehyde	Acros	none

5.1.2 Samples and Reference materials

Botanical Reference Material

Name	Source / Batch	Authentication
<i>Zingiber officinale</i>	Removed - proprietary information	Yes

Additional samples

Name	Source / Batch	Authentication
<i>Zingiber officinale</i>	Removed - proprietary information	Yes
<i>Zingiber officinale</i> (powdered extract)		Yes
Ground Ginger spice		No
Ginger power		No
Fresh ginger root		No
Frozen ginger		No
<i>Zingiber officinale roscoe</i> (XRM)		Yes
<i>Zingiber officinale roscoe</i> (BRM)		Yes

Adulterants

Name	Source / Batch	Authentication
Galangal	Removed - proprietary information	No
Alpinia Galangal		Yes

Processed materials

Name	Source / Batch
<i>Zingiber officinale</i> tablets	Removed - proprietary information
<i>Zingiber officinale</i> liquid extract	
<i>Zingiber officinale</i> extract oil	

Standards (marker compounds, chemical references)

Name	Source / Batch
6-shogaol	ChromaDex / 19211-135
6-gingerol	ChromaDex / 07164-125
8-gingerol	ChromaDex / 07163-119
10-gingerol	ChromaDex /
Ginger standard mixture	USP / FOE129

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
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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
Flat bottom chamber	CAMAG	N/a
ADC2	CAMAG	1120410
TLC Plate Heater III	CAMAG	040838
Immersion Device III	CAMAG	031216
Digistore 2	CAMAG	070721
Mill	Ika Works	03193040
Ultrasonic Bath	Fisher Scientific	9493699
Analytical balance	Sartorius	70805243

5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.4.0
VideoScan	CAMAG	1.02.00

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. 2 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. 2 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 2 µL of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 2 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 Ginger (*Zingiber officinale*) 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 Ginger (*Zingiber officinale*) 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 Ginger (*Zingiber officinale*) if the fingerprints of adulterants (Galangal, *Alpinia officinarum*) are significantly different from those of the BRM of Ginger (*Zingiber officinale*) 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 Ginger (*Zingiber officinale*) 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 2 μ 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 and the chemical reference(s). A Flat Bottom Chamber and a Twin-Trough Chamber are compared.

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 only the BRM and the chemical reference(s). 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 only one BRM and the chemical reference(s). 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
30.Nov.2005	Alison DeBatt	A169	Ginger Validation

Validation Results

Validation of Method for the Identification of Ginger by HPTLC Fingerprint

5.2 Stability

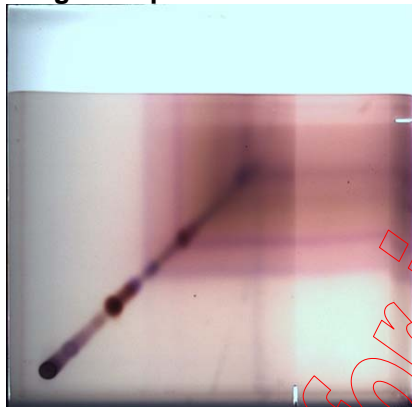
5.2.1 Stability of analyte during chromatography

Temperature recorded 24°C Humidity recorded 55%RH

Results:

No zones are located off of the diagonal, therefore, the sample is considered stable during chromatography.

Image and plate ID: A169-051110-2D-Anis



Accepted: YES

Date: November 10, 2005

Signature:

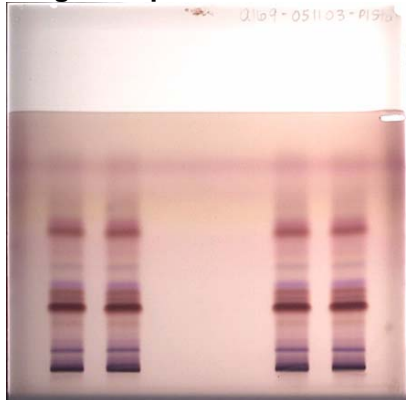
5.2.2 Stability of analyte in solution and on the plate

Temperature recorded 23°C Humidity recorded 43%RH

Results:

No differences are seen in zone intensity and there are no zones that appear or disappear; therefore, the sample is considered stable for at least 3 hours in solution and on the plate.

Image and plate ID: A169-051003-Plate Stability



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: November 3, 2005

Signature:

5.2.3 Stability of derivatization/result

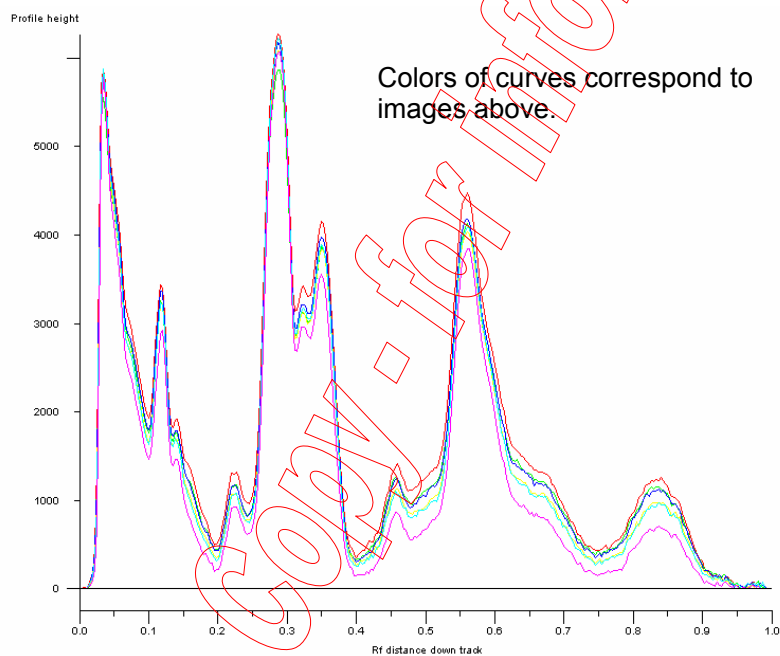
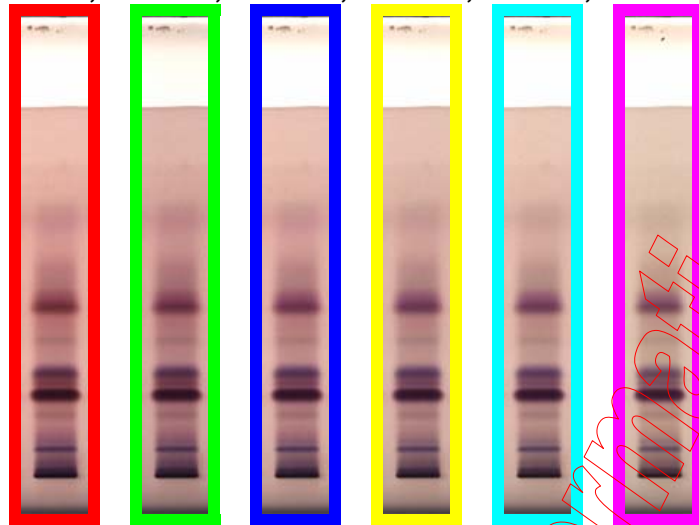
Temperature recorded 24°C Humidity recorded 56%RH

Results:

Zone intensity decreases slightly over time, as does the background color, but there are no zones that appear or disappear; therefore the image is considered stable for at least 1 hour.

Images and plate ID A169-051110-ImageStability-Anis / Densitograms:

2 min, 5 min, 10 min, 20 min, 30 min, 1h



Accepted: Yes

Date: November 10, 2005

Signature:

5.3 Specificity

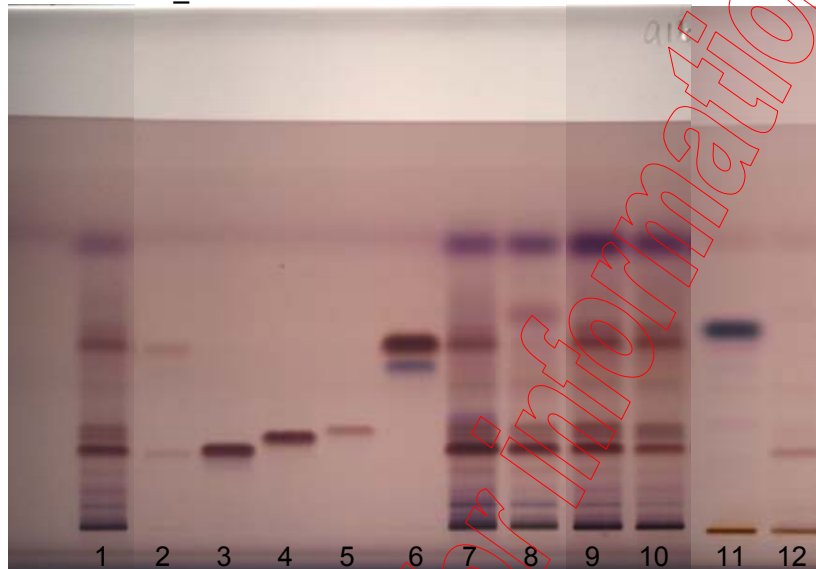
5.3.1 Identification of Ginger samples by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded 24°C Humidity recorded 54%RH

Results:

The method is specific for the identification of ginger. The fingerprints of all but three samples are similar to the fingerprint of the BRM. None of the samples shows the violet zone above 10-gingerol seen in the BRM on track 7. The sample on track 8 fails due to absence of the 6-shogaol zone. The fresh ginger sample on track 12 shows only a very faint zone for 6-gingerol and fails. The sample on track 11 is not ginger: It presents none of the standards and shows a bluish zone just above the position of 6-shogaol (see also below, 5.3.2) indicative of an adulteration with galangal.

Image and plate ID: Tracks 1-10 taken from A169-051024-001, tracks 10 and 12 taken from A169-051012_06



Track assignment

- Track 1: *Zingiber officinale roscoe*
- Track 2: USP standard mixture
- Track 3: 6-gingerol
- Track 4: 8-gingerol
- Track 5: 10-gingerol
- Track 6: 6-shogaol
- Track 7: *Zingiber officinale* (BRM)
- Track 8: *Zingiber officinale* (dried root) **failed**
- Track 9: ginger spice
- Track 10: ginger spice
- Track 11: Frozen ginger
- Track 12: Fresh ginger

System suitability test passed: YES, NO for samples on tracks 8, 11, and 12.

Accepted: YES

Date: October 24, 2005

Signature:

5.3.2 Detection of adulteration

Temperature recorded 24°C Humidity recorded 53%RH

Results:

The method is specific for ginger, and the known adulterant (Galangal, *Alpinia officinarum*) show a different fingerprint when compared to the BRM.

Images and plate ID: A169-051130-001



Track assignment

- Track 1: 6-gingerol
- Track 2: 8-gingerol
- Track 3: 10-gingerol
- Track 4: 6-shogaol
- Track 5: *Zingiber officinale* (BRM)
- Track 6: Galangal
- Track 7: Galangal

System suitability test passed: YES Accepted: YES

Date: November 30, 2005 **Signature:**

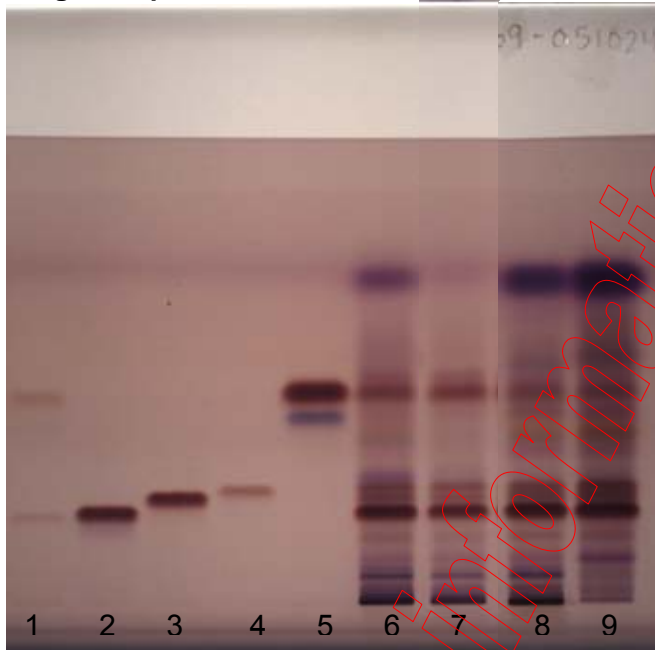
5.3.3 Identification of processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded 24°C Humidity recorded 54%RH

Results:

The method is specific for the identification of processed ginger products. The gingerols are well separated and 6-shogaol is seen in all products. There are minor additional zones seen in most samples.

Image and plate ID: All sections of image taken from plate A169-051024-001



Track assignment

Track 1: USP standard mixture

Track 2: 6-gingerol

Track 3: 8-gingerol

Track 4: 10-gingerol

Track 5: 6-shogaol

Track 6: *Zingiber officinalis* (BRM)

Track 7: ginger tablets

Track 8: ginger tincture

Track 9: ginger extract oil

System suitability test passed: YES Accepted: YES

Date: October 24, 2005

Signature:

5.4 Repeatability

Temperature recorded 24°C Humidity recorded 43%RH

Results:

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

Images and plate ID:

Day One Plate One - A169-051102-001

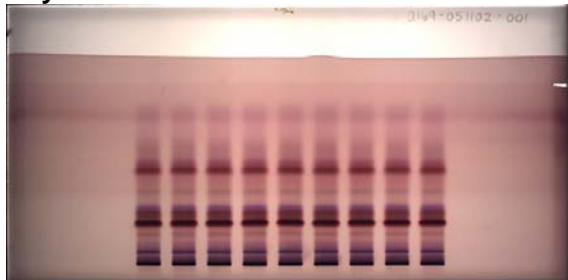


Plate Two - A169-051102-002

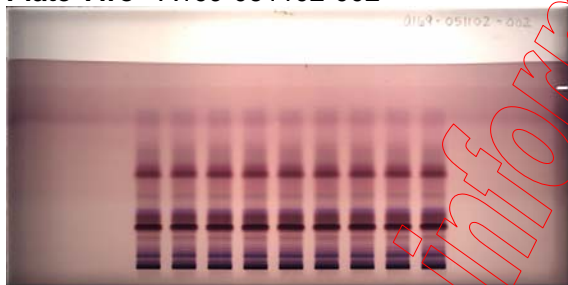
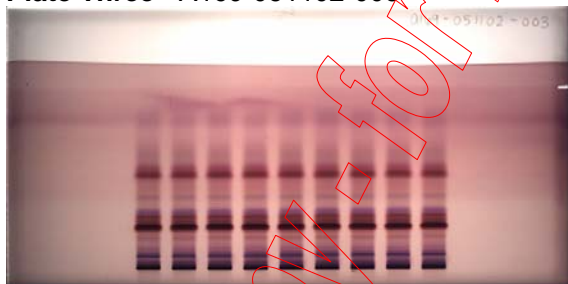


Plate Three - A169-051102-003



R_F	A169-051102-001	A169-051102-002	A169-051102-003	ΔR_F
6-gingerol	0.23	0.23	0.23	0
10-gingerol	0.29	0.29	0.28	0.01
6-shogaol	0.53	0.52	0.51	0.02

System suitability test passed: YES

Accepted: YES

Date: November 2, 2005

Signature:

5.5 Intermediate precision

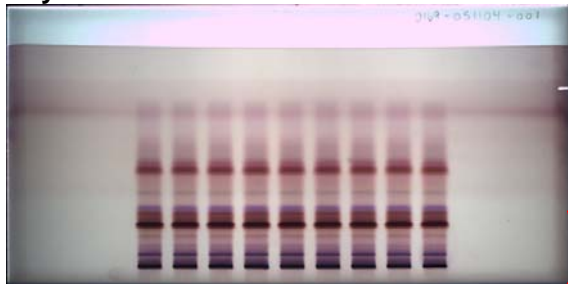
Temperature recorded 24°C Humidity recorded 48%RH

Results:

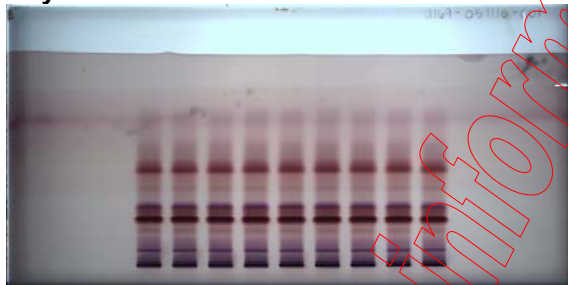
All chromatograms are similar with respect to number, position, color, and intensity. No disturbances are seen.

Images and plate ID:

Day Two – A169-051104-001



Day Three – A169-051110-001



R_F	A169-051102-001	A169-051104-001	A169-051110-001	ΔR_F
6-gingerol	0.23	0.23	0.25	0.02
10-gingerol	0.29	0.30	0.31	0.02
6-shogaol	0.53	0.54	0.54	0.01

System suitability test passed: YES Accepted: YES

Date: November 10, 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 46%RH

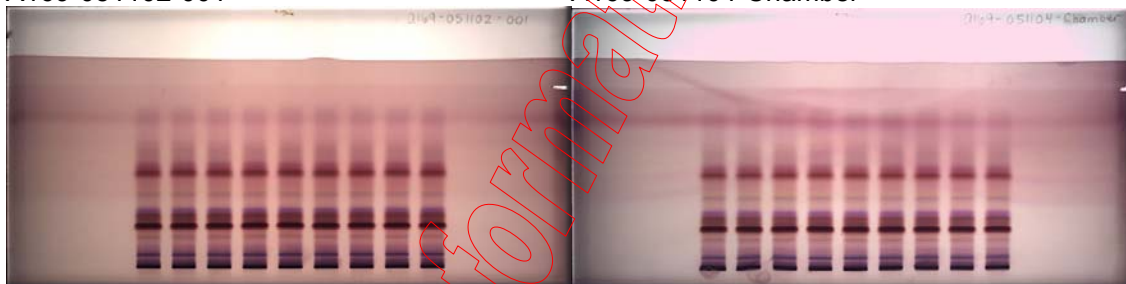
Results:

No significant difference is seen when the plate is developed in a Flat Bottom Chamber, and similar R_F values are observed.

Images and plate ID:

Twin Trough Chamber
A169-051102-001

Flat Bottom Chamber
A169-051104-Chamber



R_F	Twin Trough Chamber	Flat Bottom Chamber	ΔR_F
6-gingerol	0.23	0.22	0.01
10-gingerol	0.29	0.28	0.01
6-shogaol	0.53	0.51	0.02

System suitability test passed: YES Accepted: YES

Date: November 4, 2005 **Signature:**

5.7.2 Developing distance

Temperature recorded 24°C Humidity recorded 45%RH

Results:

No significant difference is seen when the plate is developed to 80 mm and similar Rf values are observed.

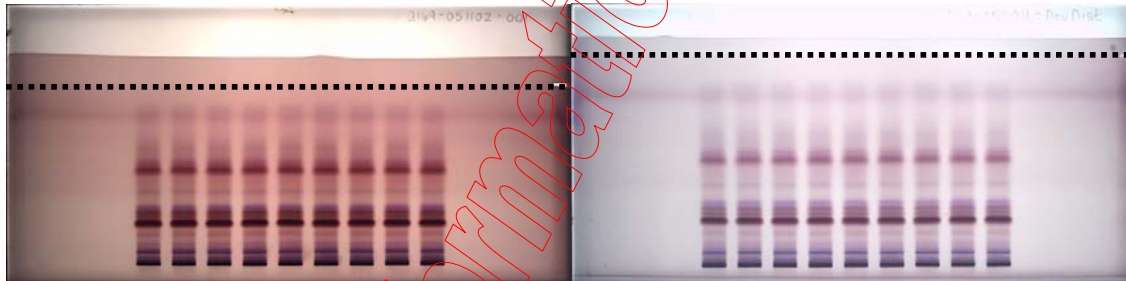
Images and plate ID:

A169-051102-001

70 mm

A169-051104-DevDist

80 mm



R_F	70 mm	80 mm	ΔR_F
6-gingerol	0.23	0.21	0.02
10-gingerol	0.29	0.27	0.02
6-shogaol	0.53	0.50	0.03

System suitability test passed: YES Accepted: YES

Date: November 4, 2005 Signature:

5.7.3 Relative humidity

Temperature recorded 23°C

Results:

9%RH: molecular sieve: There is no separation between 8- and 10-gingerol. The zones are diffuse.

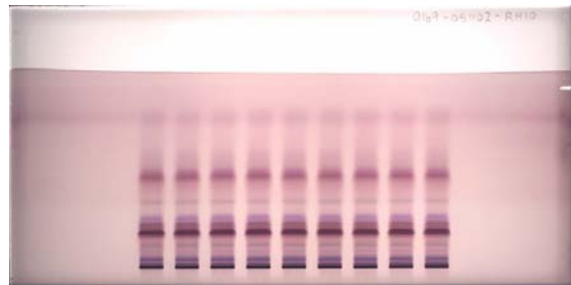
38%RH: magnesium chloride: The separation of 8- and 10-gingerol is improved, the zones are sharper than those at 9%RH.

43%RH: ambient humidity in laboratory: Separation of 6-, 8-, and 10-gingerol is achieved.

69%RH: sodium chloride: Separation of 6-, 8-, and 10-gingerol is improved but 10-gingerol co-elutes with a purple zone above.

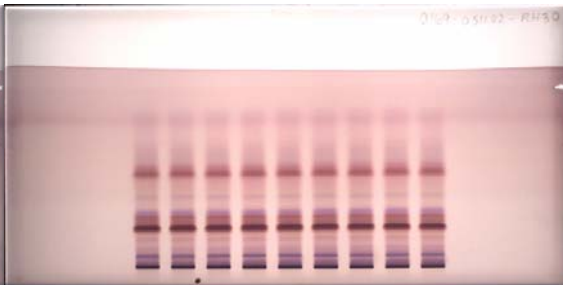
Images:

9% RH



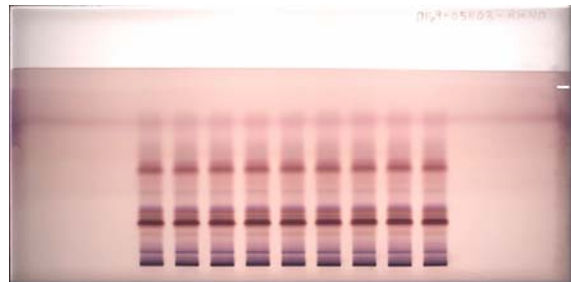
A169-051102-RH10

38%RH



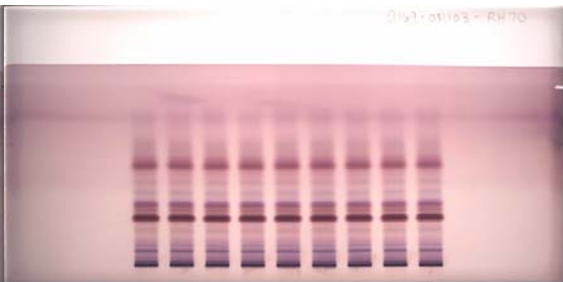
A169-051102-RH30

43%RH



A169-051102-RH70

69%RH



A169-051102-RH40

R_F	9% RH	38%RH	43%RH	69%RH
6-gingerol	0.19	0.22	0.23	0.26
10-gingerol	unknown	0.28	0.30	0.33
6-shogaol	0.51	0.52	0.53	0.55

