

Date	Author	Project No.	Project Name
30.Sept.2005	Anne Schibli	P08	Kava

Validation Protocol

Validation of Method for the Identification of Kava 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 Kava by HPTLC fingerprint is suitable to identify a given sample of plant material as Kava (*Piper methysticum*) based on its kavalactone fingerprint.

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

2. General acceptance criteria:

The method is valid if:

- A botanically authenticated sample of Kava 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

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4. Description of method

4.1 Preparation of test solutions

Raw materials: 1 g of powdered sample are 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 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 of each kavalactone (kawain, dihydrokawain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin, available from ChromaDex) is dissolved individually in 2 mL toluene.

4.3 Preparation of derivatizing reagent

10 mL of sulfuric acid are carefully added to an ice-cooled 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

Caffeine-impregnated HPTLC plates are available from Merck (EM-Science) or Macherey&Nagel. The impregnation can also be performed on standard 10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 as follows:

8 g of caffeine are dissolved in 200 mL of dichloromethane. Plates are immersed in the solution for 1 second and allowed to dry at room temperature in a fume hood for 5 minutes. Drying of plates is then completed in an oven for 5 minutes at 80°C.

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 temperature and humidity in the laboratory.

4.7 Chromatography

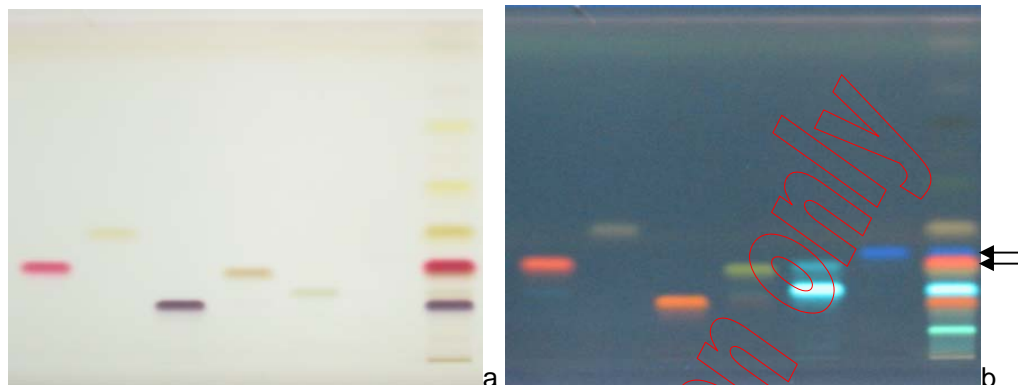
Chamber type: 10x10 cm (or 20x10 cm) Twin Trough Chamber
Configuration: Unsaturated
Developing solvent: tert-butylmethyl ether, n-hexane (7:3), 5 mL (respectively 10 mL) of developing solvent in the front trough.
Developing distance: 70 mm from lower edge of plate (62 mm from application position).
Drying: 5 min with cold air (hair dryer)

4.8 Derivatization

The plate is immersed into reagent for 1 second, then heated to 100°C for 5 minutes.

4.9 Documentation

- a) After derivatization under white light (reflection and transmission) while still hot
- b) After derivatization under UV 366 nm while still hot



Track assignment:

1. Kawain
2. Dihydrokawain
3. Methysticin
4. Dihydromethysticin (yellowish main zone)
5. Yangonin (main zone)
6. Desmethoxyyangonin
7. Kava (*Piper methysticum*) BRM

11. Evaluation of results

White light (after derivatization)

The standards kawain (track 1), dihydrokawain (track 2), methysticin (track 3), dihydro-methysticin (track 4), and yangonin (track 5) show colored bands. Desmethoxyyangonin (track 6) is not detected.

There are bands in the BRM corresponding in color and position to the standards. Kawain and dihydromethysticin are not well resolved and the brown band of the latter is difficult to see in the sample. Additional yellow or violet bands in the upper RF region are seen.

UV 366 nm (after derivatization)

The standards kawain (track 1), dihydrokawain (track 2), methysticin (track 3), dihydro-methysticin (track 4), yangonin (track 5), and desmethoxyyangonin (track 6) show fluorescing bands of characteristic color. Some of the standards used here are not pure and show additional weaker bands due to impurities. There are bands in the BRM corresponding in color and position to the standards. The BRM show additional week bands in the upper RF region, and a greenish-blue band is seen between the application and the band of methysticin.

12. System suitability test

The result obtained in the test is suitable for evaluation if the following requirement is met. After derivatization the zones of kawain and desmethoxyyangonin (black arrows) are separated in the fingerprint of the test solution.

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality / Purity
Methanol	EMD	HPLC
Toluene	Spectrum	HPLC
Butyl-t-methyl ether	Spectrum	HPLC
Hexanes	EMD	HPLC

Acetic acid	EMD	ACS
p-anisaldehyde	Acros	99+%
Sulfuric acid	Spectrum	ACS
Caffeine	Merck	pure (>98.5%)
Dichloromethane	EMScience	HPLC

5.1.2 Samples and Reference materials

Botanical Reference Material

Name	Source / Batch	Authentication
<i>Piper methysticum</i> lateral roots and chips	Removed - proprietary information	Yes

Additional samples

Name	Source / Batch	Authentication
Kava Kava root lateral	Removed - proprietary information	Yes
Kava Kava main root		Yes
Kava Kava main root		Yes
Kava Kava lateral root		Yes
Kava Kava lateral root		Yes
Kava Kava root stump		Yes
Kava Kava root stump		Yes
Kava Kava lateral root		Yes
Kava Kava all root		Yes
Piper methysticum-basal stem		Yes
Piper methysticum-rhizome chips		Yes
Piper methysticum-lateral roots and chips, Vanuatu		Yes
Piper methysticum-lateral root, Vanuatu		Yes
Piper methysticum-peelings from rhizome		Yes
Kava Kava Root (Piper methysticum) [fine rootlets]		Yes
Piper methysticum stem	Yes	

Adulterants

Name	Source / Batch	Authentication
None		

Processed materials

Name	Source / Batch
Piper methysticum-acetone extract (dry)	Removed - proprietary information
Piper methysticum-dry extract, Kaviar 40%	
Piper methysticum-tincture, KavaPure, 150 mg/mL Kavalactone	
Piper methysticum-root paste, Kaviar 80+, solvent free extraction	
1:6 Kava kava rhizome 90%YLD Liquid	
Kava kava Liquid Phyto-Caps 225mg Kavalactones per 3 capsules, 409mg extract equivalent to 3750mg crude herb equivalent.	
Kava "Nakamal" Capules (Piper methysticum fresh	

freeze dried Juice, 425mg/capsule) 15-20% of Kavalactones	
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Standards (marker compounds, chemical references)

Name	Source / Batch
D/L-Kavain	ChromaDex 01-11300-101
Dihydrokavain	ChromaDex 01-04476-101
Methysticin	ChromaDex 01-13860-101
Dihydromethysticin	ChromaDex 01-04477-101
Yangonin	ChromaDex 01-25010-101
Desmethoxyyangonin	ChromaDex 01-04236-101

5.1.3 Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F ₂₅₄	20x10 cm	Merck	OB412660
HPTLC F _{254s} Si 60 Caffeine impregnated	20x10 cm	Merck	45212327 and 740245329
HPTLC Nano-SIL-PAH (Caffeine impregnated)	20x10 cm	Macherey-Nagel	006179

5.1.4 Instruments

Instrument	Manufacturer	Serial Number
Automatic TLC Sampler 4	CAMAG	070616
DigiStore 2	CAMAG	070721
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
Mill KB5/10	IKA	03193040
Ultrasonic Bath	Fischer Scientific	9493699
Balance	Sartorius	70805243

5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.2.5-1.4.0
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 immediately, 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 Kava 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 Kava 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:

No adulterants of Kava are known.

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 Kava 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 TLC plate

Description of experiment:

The method is executed according to section 4 using the plates from different manufacturers and self-impregnated plates.

Different impregnation times will be tested; i.e 1 s (as specified), 0.5s, and 2 s.

Acceptance criteria:

The fingerprints obtained on all plates 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 one plate to the other (<0.07). In the case of differences between the plates, one specific manufacturer has to be specified.

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
16.Nov.2005	Anne Schibli	P08	Kava

Validation Results

Validation of Method for the Identification of Kava by HPTLC Fingerprint

5.2 Stability

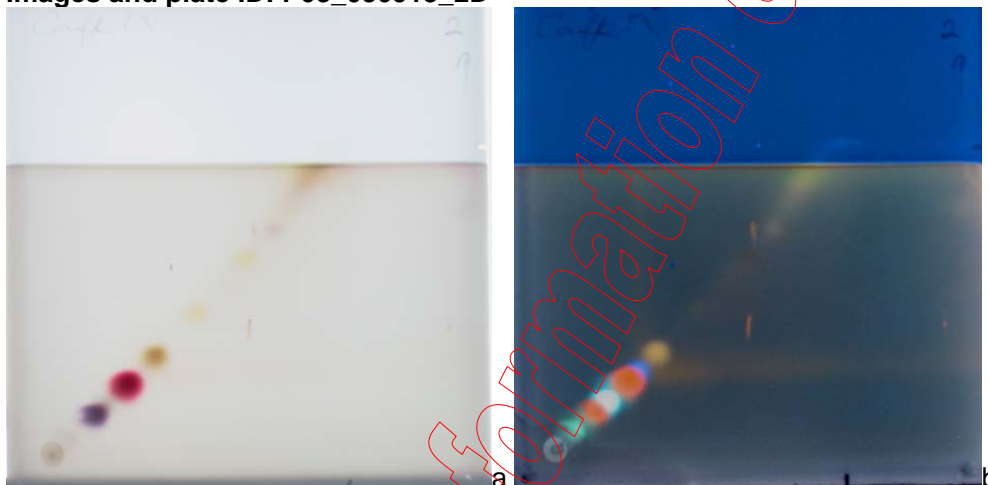
5.2.1 Stability of analyte during chromatography

Temperature recorded: 23°C, Humidity recorded: 40%RH

Results:

No zone is located aside of the diagonal. The sample is stable during chromatography.
[Merck plate]

Images and plate ID: P08_030915_2D



Accepted: Yes

Date: 15.Sept.2005

Signature:

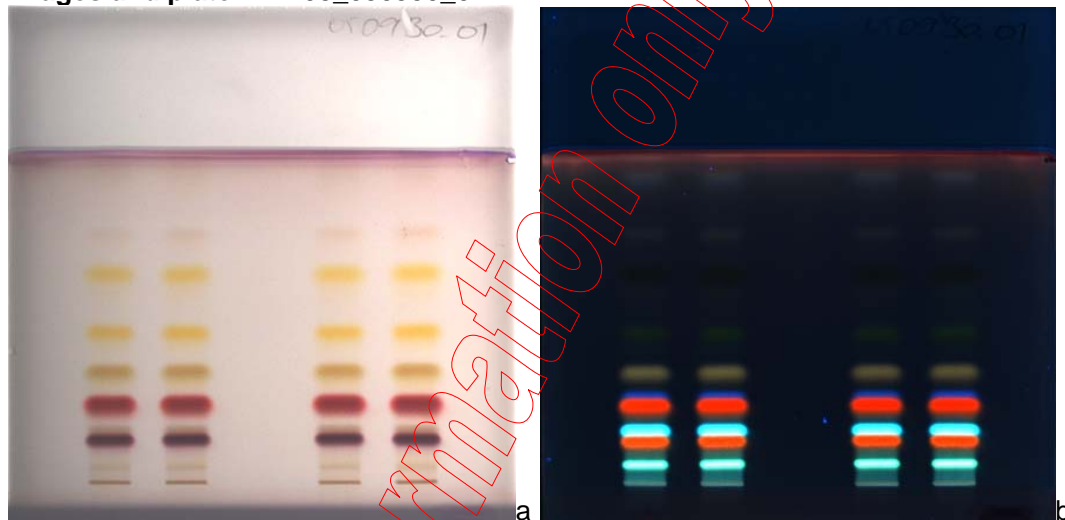
5.2.2 Stability of analyte in solution and on the plate

Temperature recorded: 25°C, Humidity recorded: 50%RH

Results:

No differences are seen between the tracks. The sample is stable for 3 hours in solution and 3 hours on the plate. [Merck plate]

Images and plate ID: P08_050930_01



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: 30.Sept.2005

Signature:

5.2.3 Stability of derivatization/result

Temperature recorded: 25°C, Humidity recorded: 50%RH

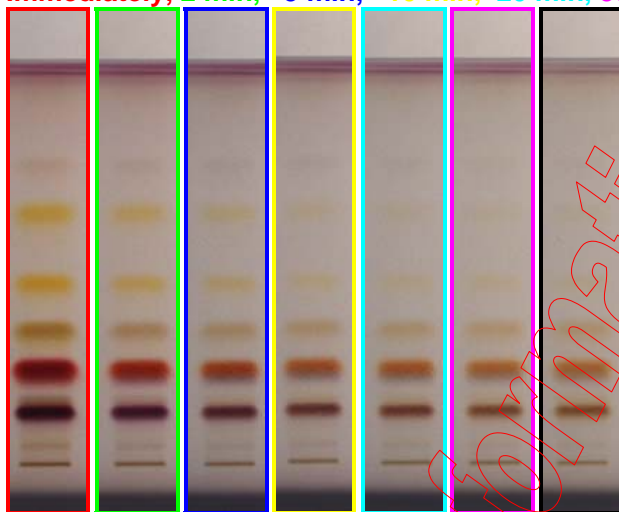
Results:

The result of the derivatized plate is not stable. Significant changes in the coloration of bands are seen within 2 min after derivatization, particularly under white light. The plate should be looked at/documentated immediately after heating, while still hot.

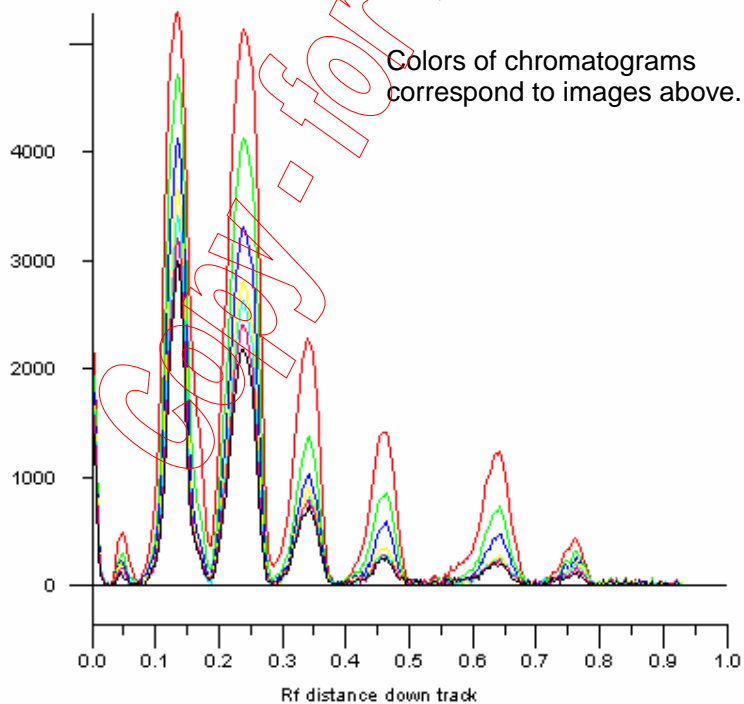
[Merck plate]

Images and plate ID: P08_050930_01 / Densitograms:

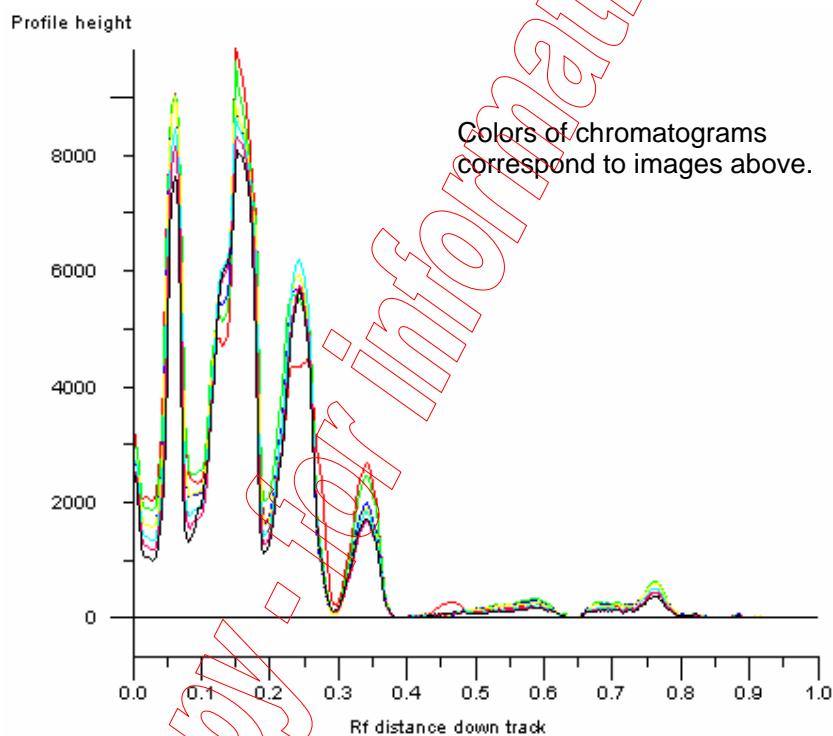
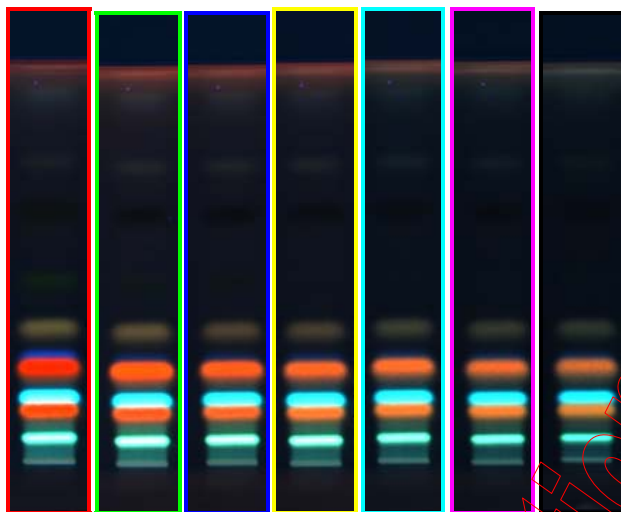
Immediately, 2 min, 5 min, 10 min, 20 min, 30 min, 1h



Profile height



White light



UV 366 nm

Accepted: Yes, with the following restriction: the plate must be documented immediately after derivatization, while still hot. **Date:** 30.Sept.2005 **Signature:**

5.3 Specificity

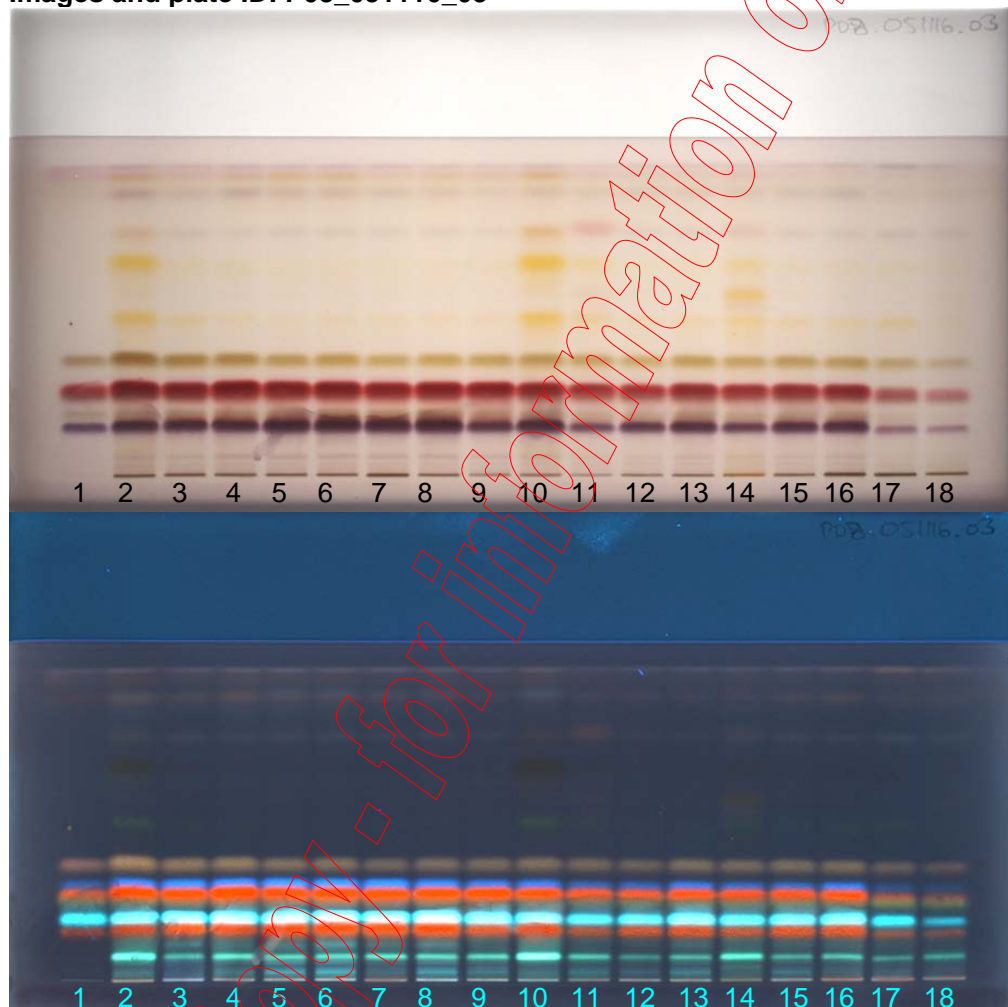
5.3.1 Identification of Kava samples and processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded: 23°C, Humidity recorded: 37%RH

Results:

All samples show a similar fingerprint to that of the BRM. The six kavalactone standards are seen in all samples. The overall variable intensity of zones between the different parts of the rhizome/root varies. The highest content is seen in the BRM, whole roots, and lateral roots. The rhizome and stem show the lowest content.

Images and plate ID: P08_051116_03



Track assignment

- | | |
|---------------------------------|--------------------------------|
| 1. Standard Mix | 10. Kava fine rootlets |
| 2. Kava BRM | 11. Kava rhizome chips |
| 3. Kava all root | 12. Kava main root |
| 4. Kava lateral roots and chips | 13. Kava main root |
| 5. Kava lateral root | 14. Kava peelings from rhizome |
| 6. Kava lateral root | 15. Kava stump |
| 7. Kava lateral root | 16. Kava stump |
| 8. Kava lateral root | 17. Kava stem |
| 9. Kava lateral root | 18. Kava basal stem |

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

5.3.2 Detection of adulteration

Temperature recorded: °C, Humidity recorded: %RH

Results:

Not applicable

Images and plate ID:

Track assignment

System suitability test passed:

Accepted:

Date:

Signature:

Copy - for information only

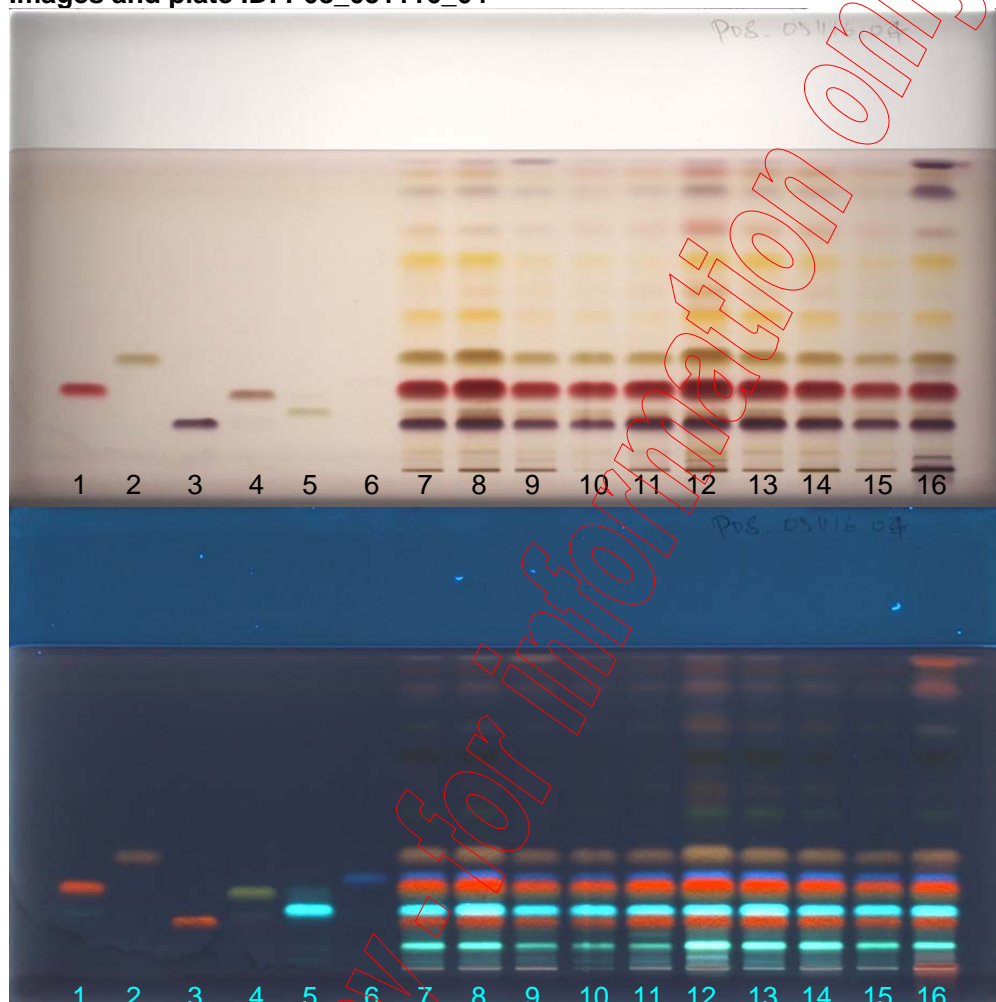
5.3.3 Identification of processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded: 23°C, Humidity recorded: 37%RH

Results:

All processed materials show a similar fingerprint to that of the BRM. The six kavalactone standards are seen in all samples. The overall variable intensity of zones between products is due to the difference in concentration (many products don't specify their content of Kava).

Images and plate ID: P08_051116_04



Track assignment

- | | |
|--------------------------------------|---|
| 1. Kawain | 10. Piper methysticum root paste, solvent free extraction |
| 2. Dihydrokawain | 11. Piper methysticum acetone extract (dry) |
| 3. Methysticin | 12. Piper methysticum tincture |
| 4. Dihydromethysticin | 13. Kava rhizome and root Liquid extract |
| 5. Yangonin | 14. Kava root Liquid extract |
| 6. Desmethoxyyangonin | 15. Kava rhizome Liquid extract |
| 7. Kava BRM | 16. Kava Liquid Caps |
| 8. Kava capules (freeze dried Juice) | |
| 9. Piper methysticum dry extract | |

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

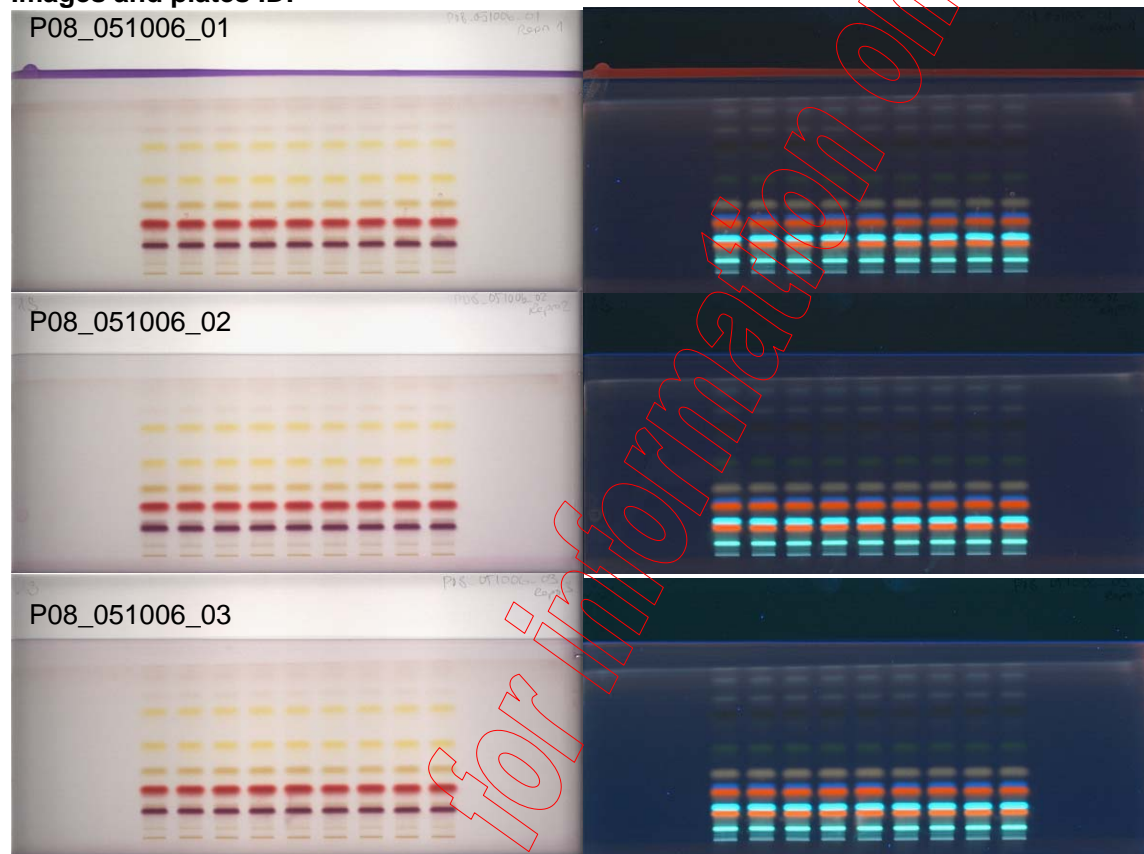
5.4 Repeatability

Temperature recorded: 25.5°C, **Humidity recorded:** 53%RH

Results:

All chromatograms look very similar with respect to number, position, color, and intensity of zones. [Self-impregnated plates]

Images and plates ID:



R_F	P08_051006_01	P08_051006_02	P08_051006_03	ΔR_F
Methysticin	0.17	0.16	0.16	0.01
Kawain	0.29	0.28	0.28	0.01
Dihydrokawain	0.38	0.38	0.39	0.01

System suitability test passed: Yes **Accepted:** Yes **Date:** 06.Oct.2005 **Signature:**

5.5 Intermediate precision

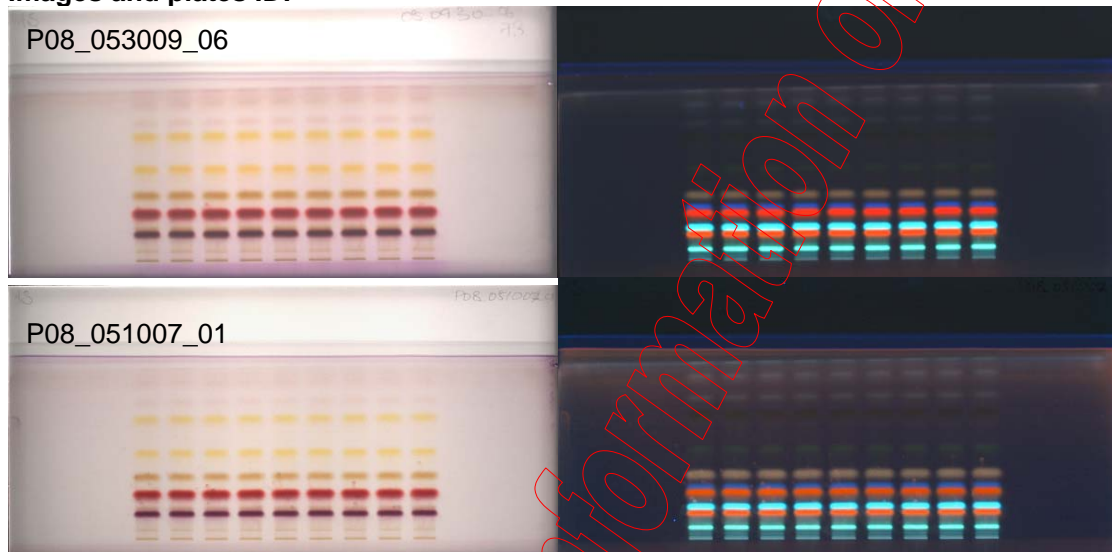
Day 2 Temperature recorded: 25°C, Humidity recorded: 50%RH

Day 3 Temperature recorded: 25°C, Humidity recorded: 53%RH

Results:

All chromatograms look very similar with respect to number, position, color, and intensity.
 [Self-impregnated plates]

Images and plates ID:



R_F	P08_051006_01	P08_050930_06	P08_051007_01	ΔR_F
Methysticin	0.17	0.16	0.15	0.02
Kawain	0.29	0.28	0.26	0.03
Dihydrokawain	0.38	0.38	0.35	0.03

System suitability test passed: Yes Accepted: Yes Date: 07.Oct.2005 Signature:

5.6 Reproducibility

Results:

See FO 70.002.05b "Checklist for secondary lab".

5.7. Robustness

5.7.1 TLC Plate

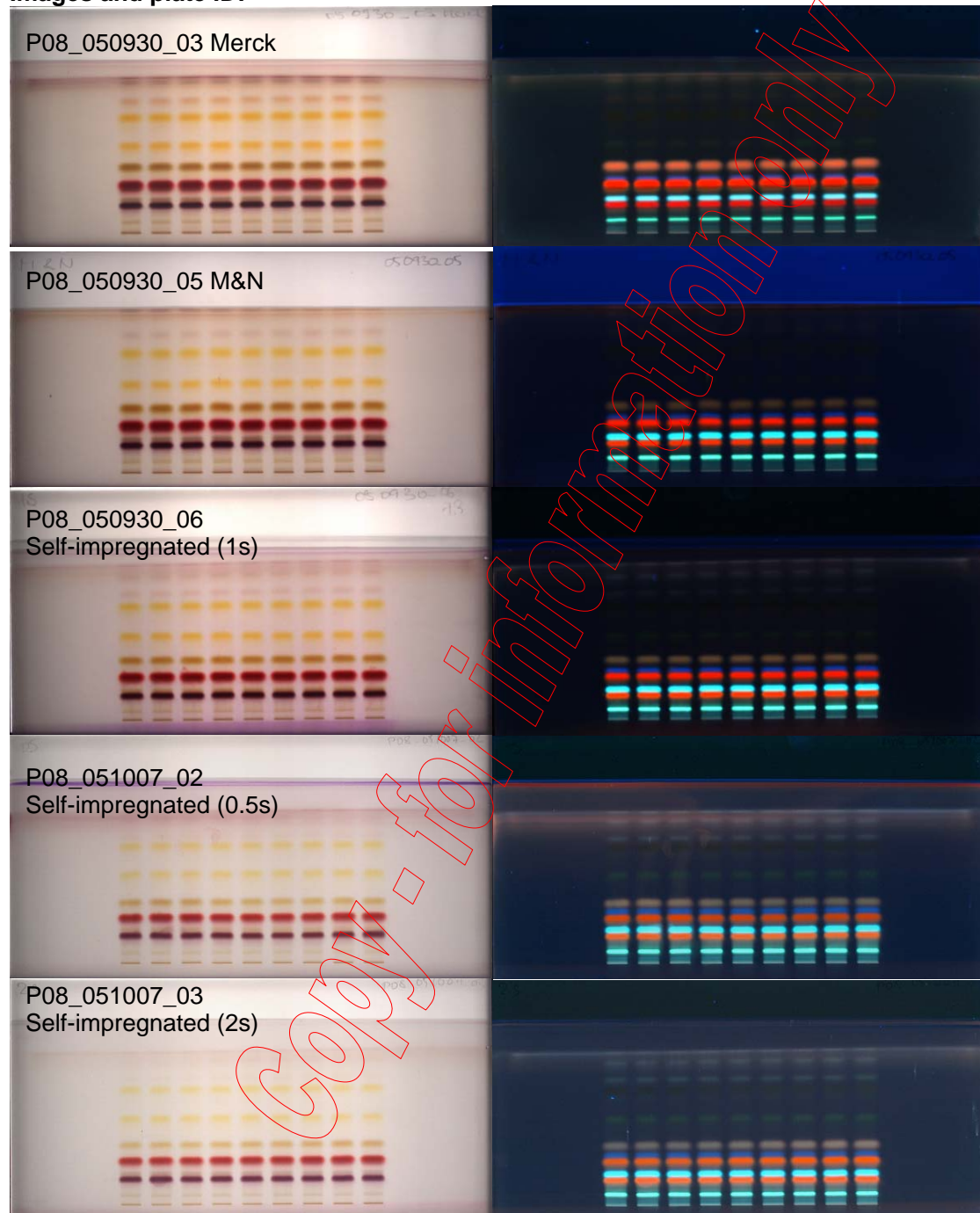
Temperature recorded: 25°C,

Humidity recorded: 50%RH

Results:

No significant difference is seen between the plates. The R_f values are lower on the self-impregnated plate.

Images and plate ID:



Different manufacturers

R_F	P08_050930_03 Merck	P08_050930_04 Macherey&Nagel	P08_050930_06 Self-impregnated (1s)	ΔR_F
Methysticin	0.18	0.18	0.16	0.02
Kawain	0.31	0.30	0.28	0.03
Dihydrokawain	0.43	0.43	0.38	0.05

Different impregnation times

R_F	P08_050930_06 (1s)	P08_051007_02 (0.5s)	P08_051007_03 (2s)	ΔR_F
Methysticin	0.16	0.18	0.16	0.02
Kawain	0.28	0.30	0.28	0.02
Dihydrokawain	0.38	0.40	0.39	0.02

System suitability test passed: Yes **Accepted:** Yes **Date:** 07.Oct.2005 **Signature:**

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5.7.2 Developing distance

Temperature recorded: 25°C,

Humidity recorded: 56%RH

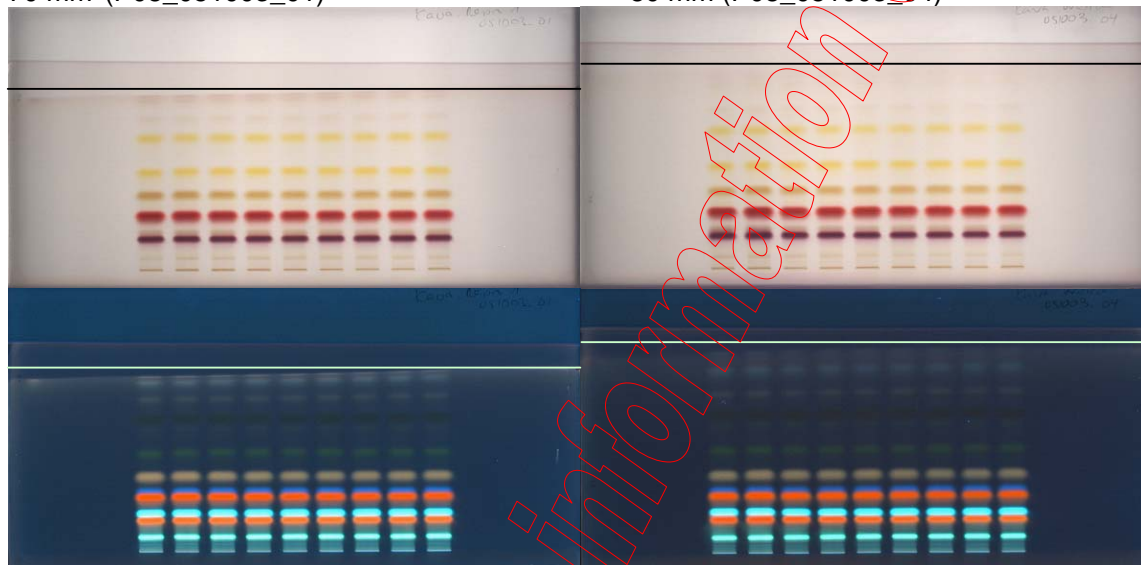
Results:

The separation is not affected by the increased developing distance, however the R_F values are generally lower when the developing distance is increased. [Merck plates]

Images and plate ID:

70 mm (P08_051003_01)

80 mm (P08_051003_04)



R_F	P08_051003_01	P08_051003_04	ΔR_F
Methysticin	0.18	0.16	0.02
Kawain	0.31	0.28	0.03
Dihydrokawain	0.43	0.38	0.05

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

5.7.3 Relative humidity

Temperature recorded: 25°C

Results:

3.5%RH: molecular sieve

38%RH: magnesium chloride

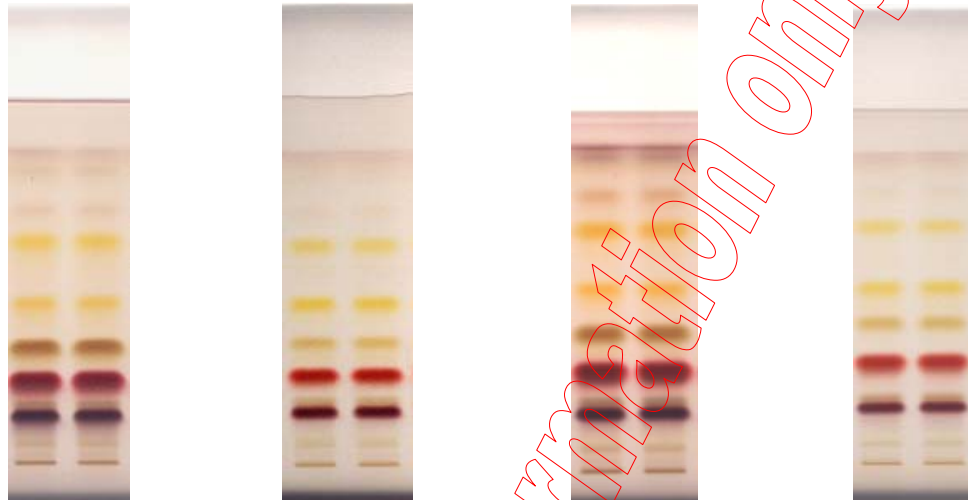
73%RH: sodium chloride

50%RH: ambient humidity in laboratory

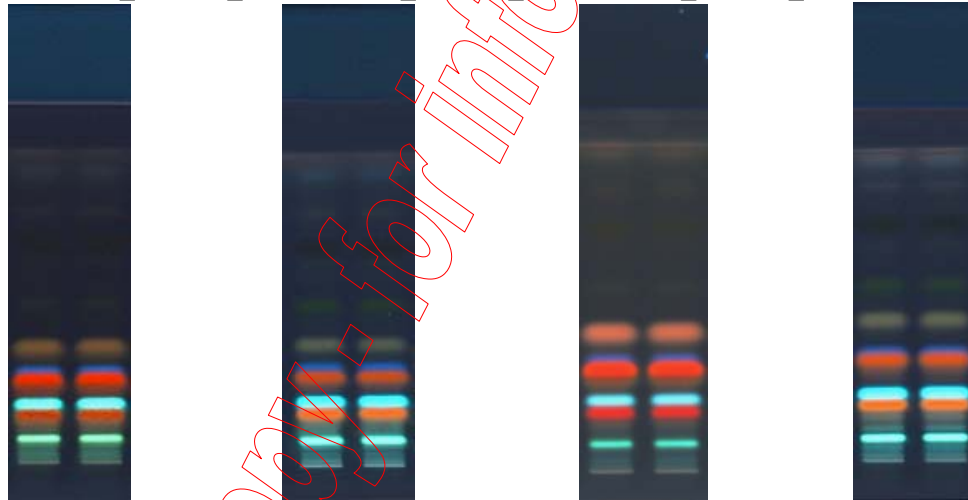
The R_F values increase with increasing humidity. However, the separation of the substances is not influenced by the varying humidity. [Merck plates]

Images and plates ID:

3.5%P08_051005_02 38% P08_051003_05 50%P08_050930_03 73% RH P08_051005_03

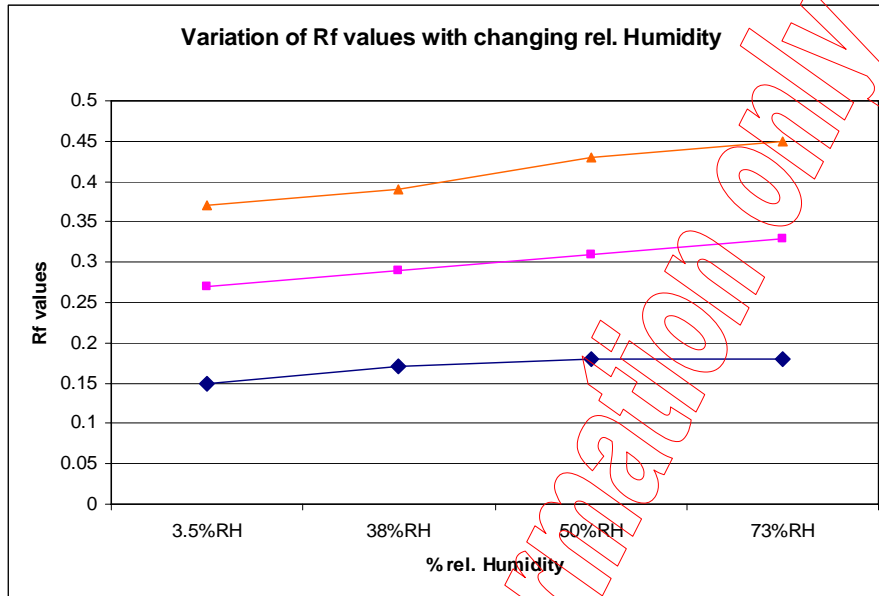


3.5%P08_051005_02 38% P08_051003_05 50%P08_050930_03 73% RH P08_051005_03



R_F	3.5%RH	38%RH	50%RH	73%RH
Methysticin	0.15	0.17	0.18	0.18
Kawain	0.27	0.29	0.31	0.33
Dihydrokawain	0.37	0.39	0.43	0.45

Diagram showing R_f value as function of %RH:



System suitability test passed: Yes Accepted: Yes Date: 10.Nov.2005 Signature:

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Comments

None. The method is valid.

Suggested changes

None

Completed / Printed

Date:

Signed:

Date of review:

Name:

Title:

Signature:

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