

Date	Author	Project No.	Project Name
26.Sept.2005	Alison DeBatt	A168	Milk Thistle

Validation Protocol

Validation of Method for the Identification of Milk Thistle 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 Milk Thistle by HPTLC fingerprint is suitable to identify a given sample of plant material as Milk Thistle (*Silybum marianum*) based on its flavonolignan fingerprint.

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

2. General acceptance criteria:

The method is valid if:

- A botanically authenticated sample of Milk Thistle (*Silybum marianum*) 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, then heated under reflux (or in a closed vial) for 5 min at 70°C, 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 and heated under reflux (or in a closed vial) for 5 min at 70°C, 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: dissolve 1 mg each of silybin (= silybinin), silydianin, silychristin, and taxifolin in 10 mL of methanol each. (All reference materials available from ChromaDex.)

4.3 Preparation of derivatizing reagent

Natural Products reagent (NP): 1 g of diphenylborinic acid aminoethylester is dissolved in 200 mL of ethyl acetate.

Macrogol reagent (PEG): 10 g of polyethylene glycol (macrogol) 400 are dissolved in 200 mL of dichloromethane.

4.4 Stationary phase

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

4.5 Sample application

10 µL of test solution, 10 µL of botanical reference solution, and 10 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:	Saturated for 20 min (filter paper wetted with developing solvent in trough opposite to the plate)
Developing solvent:	Chloroform, acetone, formic acid (75:16.5:8.5), 5 mL (respectively 10 mL) of 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 heated on a plate heater (or in an oven) at 100°C for 5 minutes and then dipped, while still hot, into the NP reagent. The plate is dried in a stream of cold air for 5 minutes, then dipped into PEG reagent and heated at 100°C for 5 minutes.

4.9 Documentation

- After derivatization under UV 366 nm
- After derivatization under white light (reflection and transmission)

4.10 Image of chromatograms



Track assignment:

- 1: Silychristin
- 2: Taxifolin
- 3: Silydianin
- 4: Silybin
- 5: *Silybum marianum* (BRM)

4.11 Evaluation of results:

UV 366 nm (after derivatization)

The standard silychristin (track 1, $R_F=0.18$) appears as a green zone, while taxifolin (track 2, $R_F=0.22$) appears as an orange zone. There is a dark green zone seen for silydianin (track 3, $R_F=0.27$), and a bright green zone for silybin (track 4, $R_F=0.35$).

The BRM shows zones corresponding in color and position to those of the four standards. Two faint zones just above the zone corresponding to silybin and several faint zones below the one corresponding to silychristin are also seen. An intense blue fluorescing zone is seen just above the application position.

White light (after derivatization)

The standards silychristin (track 1, $R_F=0.18$), silydianin (track 3, $R_F=0.27$), and silybin (track 4, $R_F=0.35$) appear as faint yellow zones. Taxifolin appears as an orange zone (track 2, $R_F=0.22$).

The BRM shows zones corresponding in color and position to those of the four standards. No other prominent zones are seen except one at the application position.

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 taxifolin under UV 366nm, which is, even when co-eluting with a green zone, clearly separated from the zone corresponding to silychristin

5. Validation

5.1 Materials

5.1.1 Chemicals and solvents

Name	Manufacturer	Quality / Purity
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Methanol	EMD	HPLC
Acetone	Spectrum	HPLC
Formic acid 96%	Spectrum	ACS
Dichloromethane	EMD	HPLC
Chloroform	Acros	ACS
Diphenylborinic acid aminoethylester	Sigma	not specified
Polyethylene glycol / Macrogol 400	Roth	purum
Ethyl acetate	Spectrum	HPLC

5.1.2 Samples and Reference materials

Botanical Reference Material

Name	Source / Batch	Authentication
Milk thistle BRM – <i>Silybum marianum</i> (fruit)	Removed - proprietary information	Yes

Additional samples

Name	Source / Batch	Authentication
<i>Silybum marianum</i> (whole seed - unripe)	Removed - proprietary information	No
<i>Silybum marianum</i> (whole seed - unripe)		No
<i>Silybum marianum</i> (whole seed - ripe)		No
<i>Silybum marianum</i> (whole seed)		No
<i>Silybum marianum</i> (whole seed)		No
<i>Silybum marianum</i> (whole seed)		No
<i>Silybum marianum</i> (whole seed)		No
<i>Silybum marianum</i> (whole seed - ripe)		No

Adulterants

Name	Source / Batch	Authentication
No adulterants available	n/a	n/a

Processed materials

Name	Source / Batch
<i>Silybum marianum</i> (tablets)	Removed - proprietary information
<i>Silybum marianum</i> (tablets)	
<i>Silybum marianum</i> (capsules)	
<i>Silybum marianum</i> (capsules)	
<i>Silybum marianum</i> (liquid extract)	
<i>Silybum marianum</i> (liquid extract)	

Standards (marker compounds, chemical references)

Name	Source / Batch
Silybin (A & B isomers)	Chromadex 19225-755
Silvdianin	Chromadex 19245-031
Silychristin	Chromadex 19240-582
Taxifolin	Chromadex 20065-101

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	QB412660

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 KB5/10	IKA Works	03193040
Ultrasonic Bath	Fischer Scientific	9493699
Balance	Sartorius	70805243

5.1.5 Software

Software	Manufacturer	Version
WinCATS	CAMAG	1.3.4 and 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. 10 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. 10 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 10 µL of this solution are applied according to section 4.5 next to the first sample on the set-aside plate, followed by 10 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 1, 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 Milk Thistle 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 Milk Thistle 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:

There are no known adulterants of milk thistle

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 Milk Thistle 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 10 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 the zones corresponding to the four flavono-lignans standards 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 four 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 four 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
30.Sept.2005	Alison DeBatt	A168	Milk Thistle

Validation Results

Validation of Method for the Identification of Milk Thistle by HPTLC Fingerprint

5.2 Stability

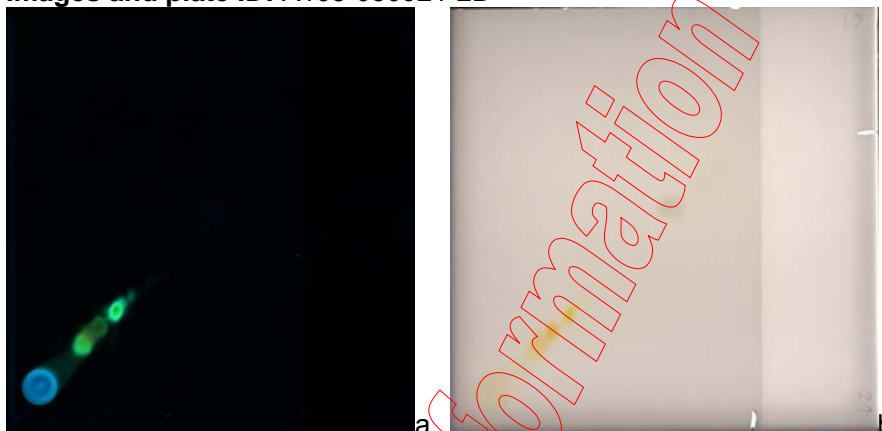
5.2.1 Stability of analyte during chromatography

Temperature recorded: 24°C, Humidity recorded: 54%RH

Results:

No spot is located off the diagonal; therefore the sample is considered stable during chromatography.

Images and plate ID: A168-050921-2D



Accepted: Yes

Date: 21.Sept.2005

Signature:

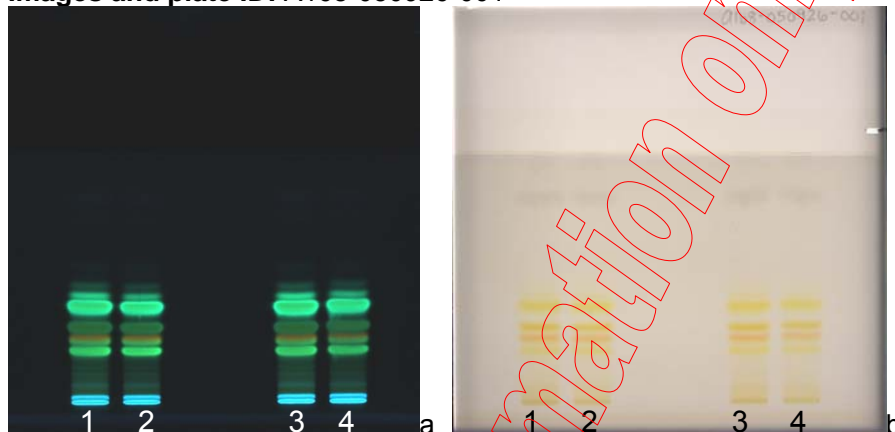
5.2.2 Stability of analyte in solution and on the plate

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

Results:

No difference is 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: A168-050926-001



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: 26.Sept.2005

Signature:

5.2.3 Stability of derivatization/result

Temperature recorded: 24°C, Humidity recorded: 54%RH

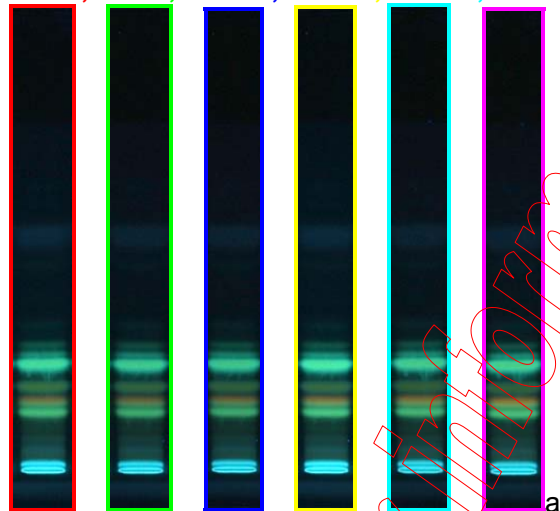
Results:

UV 366 nm: the intensity of the zones varies slightly over time, however no zones appear or disappear.

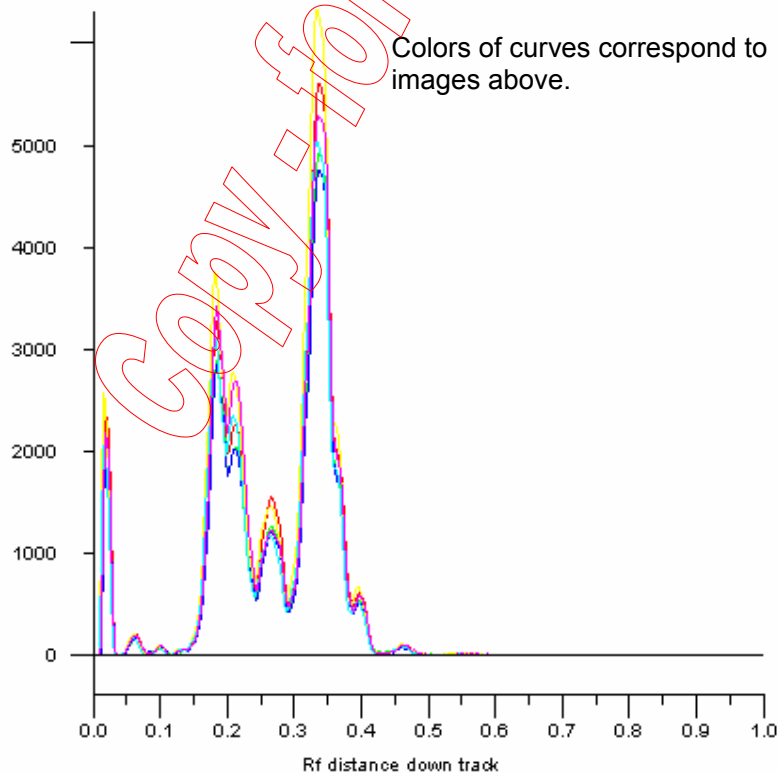
White light: The overall intensity of the fractions increases for about 5 minutes after derivatization and then remains stable for up to one hour.

Images and plate ID A168-050921-001/ Densitograms:

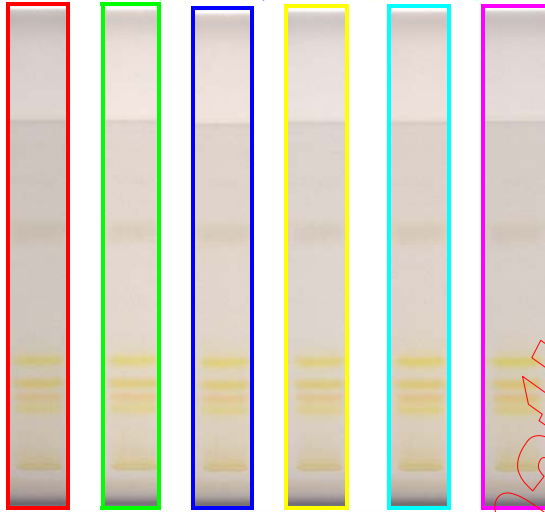
2 min, 5 min, 10 min, 20 min, 30 min, 1h



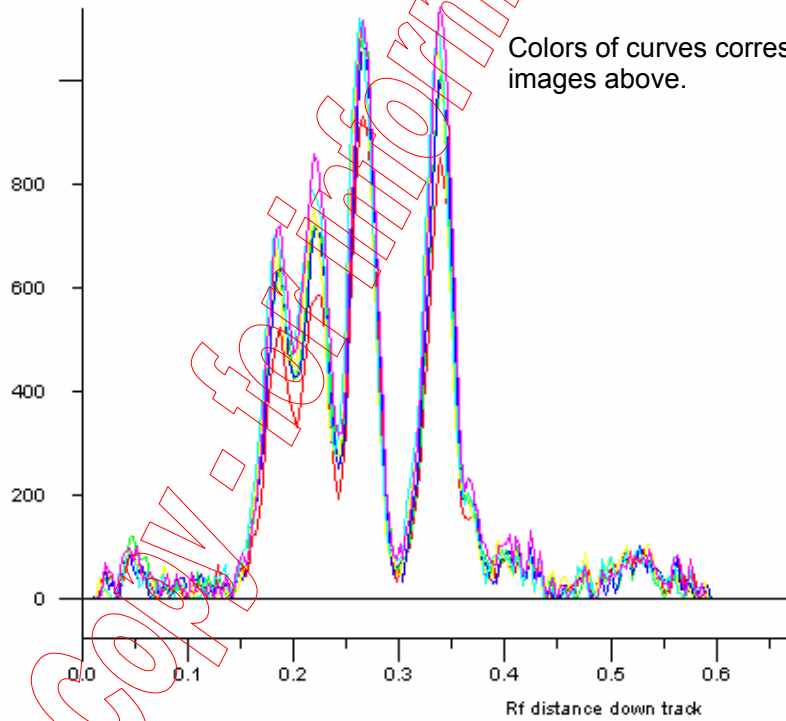
Profile height



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



Profile height



Accepted: Yes

Date: 21.Sept.2005

Signature:

5.3 Specificity

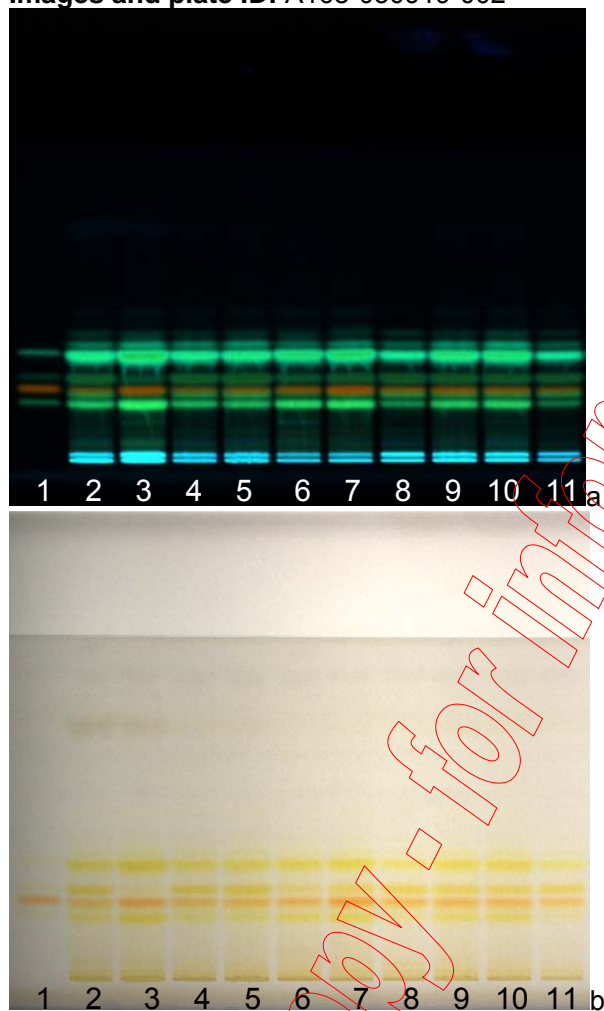
5.3.1 Identification of Milk Thistle samples and processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded: 24°C, Humidity recorded: 56%RH

Results:

The method is specific for milk thistle and all of the raw materials meet acceptance criteria. The ripe seeds (on tracks 8 and 11) show a lower content of flavono-lignans.

Images and plate ID: A168-050919-002



Track assignment

- 1: Flavono-lignans: silychristin, taxifolin, silydianin, and silybin (increasing R_F)
- 2: *Silybum marianum* (BRM)
- 3: *Silybum marianum* (whole seeds)
- 4: *Silybum marianum* (whole seeds)
- 5: *Silybum marianum* (whole seeds)
- 6: *Silybum marianum* (whole seeds)
- 7: *Silybum marianum* (whole seeds)
- 8: *Silybum marianum* (ripe seeds)
- 9: *Silybum marianum* (unripe seeds)
- 10: *Silybum marianum* (unripe seeds)
- 11: *Silybum marianum* (ripe seeds)

System suitability test passed: Yes Accepted: Yes Date: 19.Sept.2005 Signature:

5.3.2 Detection of adulteration

Not applicable

System suitability test passed: **Accepted:** **Date:** **Signature:**

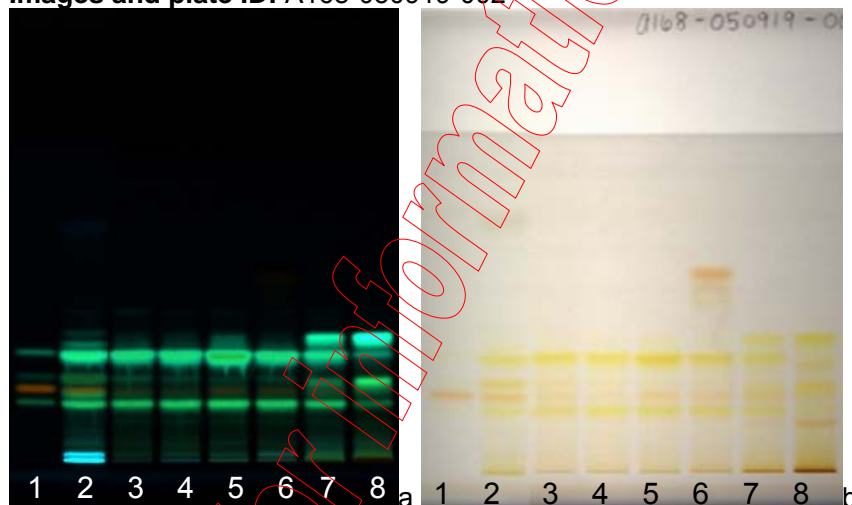
5.3.3 Identification of processed materials by comparison to the Botanical Reference Material (BRM) and chemical references.

Temperature recorded: 24°C, Humidity recorded: 56%RH

Results:

The method is specific for milk thistle and the identification of processed materials; however, the relative intensity of zones corresponding to taxifolin and silydianin is lower in all products. The product on track 8 don't contain taxifolin. The liquid samples on tracks 7 and 8 show additional zones above silybin and between the positions of silydianin and taxifolin. The blue fluorescing zone near the application position is missing in all products (UV 366 nm). The capsule on track 6 shows a reddish band at high R_f (white light).

Images and plate ID: A168-050919-002



Track assignment

- 1: Flavono-lignans: silychristin, taxifolin, silydianin, and silybin (increasing R_f)
- 2: *Silybum marianum* (BRM)
- 3: *Silybum marianum* (tablets) (2 μ L only)
- 4: *Silybum marianum* (tablets) (2 μ L only)
- 5: *Silybum marianum* (capsules) (2 μ L only)
- 6: *Silybum marianum* (capsules) (2 μ L only)
- 7: *Silybum marianum* (tincture) (0.5 μ L only)
- 8: *Silybum marianum* (tincture) (0.5 μ L only)

System suitability test passed: Yes **Accepted:** Yes **Date:** 19.Sept.2005 **Signature:**

5.4 Repeatability

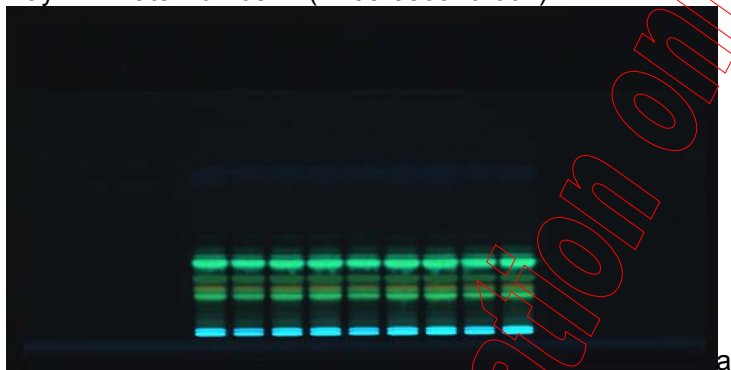
Temperature recorded: 24°C, **Humidity recorded:** 46%RH

Results:

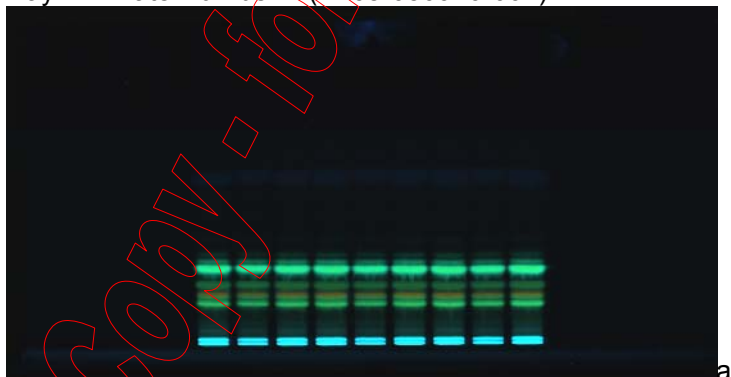
All chromatograms are similar with respect to number, position, color, and intensity of zones.
No disturbances are seen.

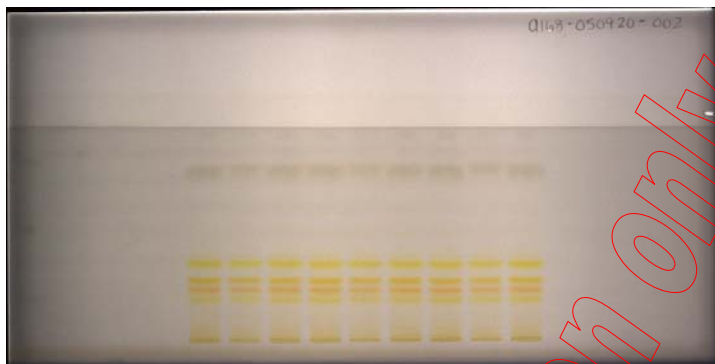
Images and plates ID:

Day 1 – Plate Number 1 (A168-050920-001)



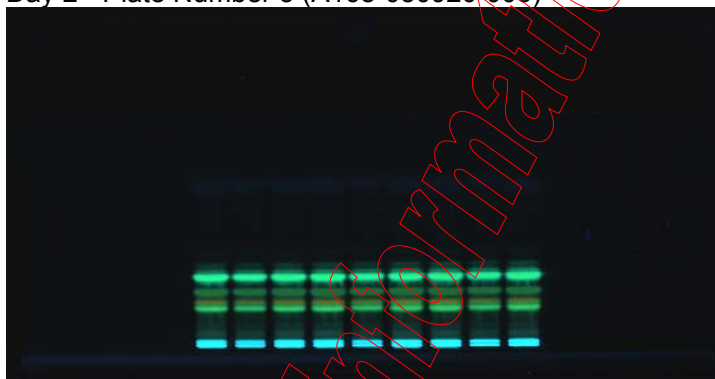
Day 1 – Plate Number 2 (A168-050920-002)



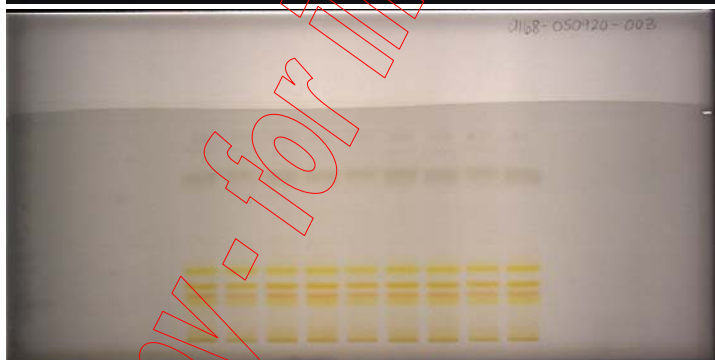


b

Day 2 - Plate Number 3 (A168-050920-003)



a



b

R_F	Plate 1 (A168-050920-001)	Plate 2 (A168-050920-002)	Plate 3 (A168-050920-003)	ΔR_F
Silychristin	0.17	0.17	0.17	0.0
Taxifolin	0.21	0.22	0.21	0.01
Silydianin	0.25	0.26	0.24	0.02
Silybin	0.32	0.33	0.31	0.02

System suitability test passed: Yes Accepted: Yes Date: 20.Sept.2005 Signature:

5.5 Intermediate precision

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

Humidity recorded: 54%RH
Humidity recorded: 51%RH

Results:

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

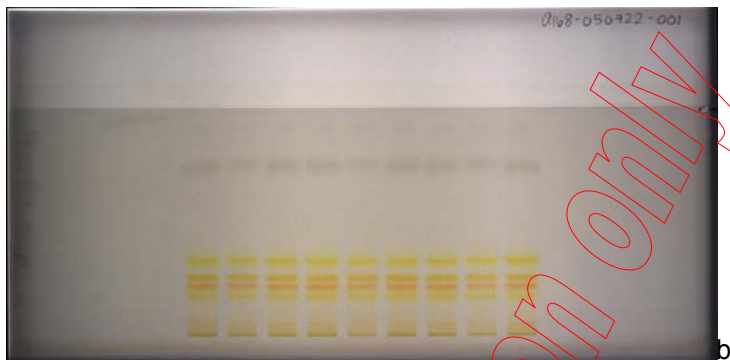
Images and plates ID:

Day 2 (A168-050921-001)



Day 3 (A168-050922-001)





R_F	Day 1 (A168-050920-001)	Day 2 (A168-050921-001)	Day 3 (A168-050922-001)	ΔR_F
Silychristin	0.17	0.18	0.17	0.01
Taxifolin	0.21	0.23	0.22	0.02
Silydianin	0.25	0.27	0.25	0.02
Silybin	0.32	0.33	0.33	0.01

System suitability test passed: Yes Accepted: Yes Date: 22.Sept.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: 50%RH

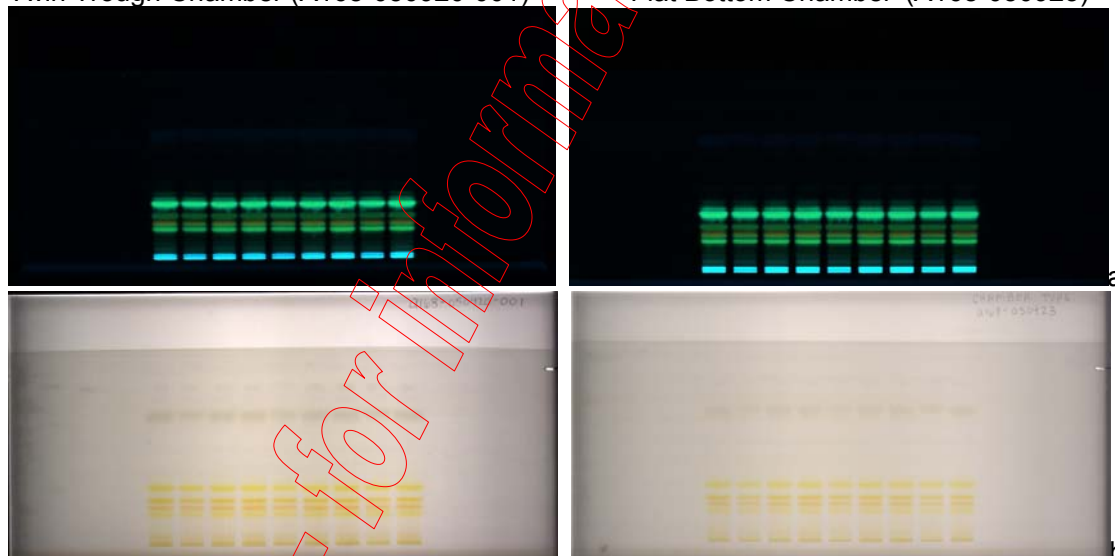
Results:

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 (A168-050920-001)

Flat Bottom Chamber (A168-050923)



R_F	Twin Trough Chamber (A168-050920-001)	Flat Bottom Chamber	ΔR_F
Silychristin	0.17	0.18	0.01
Taxifolin	0.21	0.22	0.01
Silydianin	0.25	0.25	0.0
Silybin	0.32	0.33	0.01

System suitability test passed: Yes Accepted: Yes Date: 23.Sept.2005 Signature:

5.7.2 Developing distance

Temperature recorded: 24°C,

Humidity recorded: 50%RH

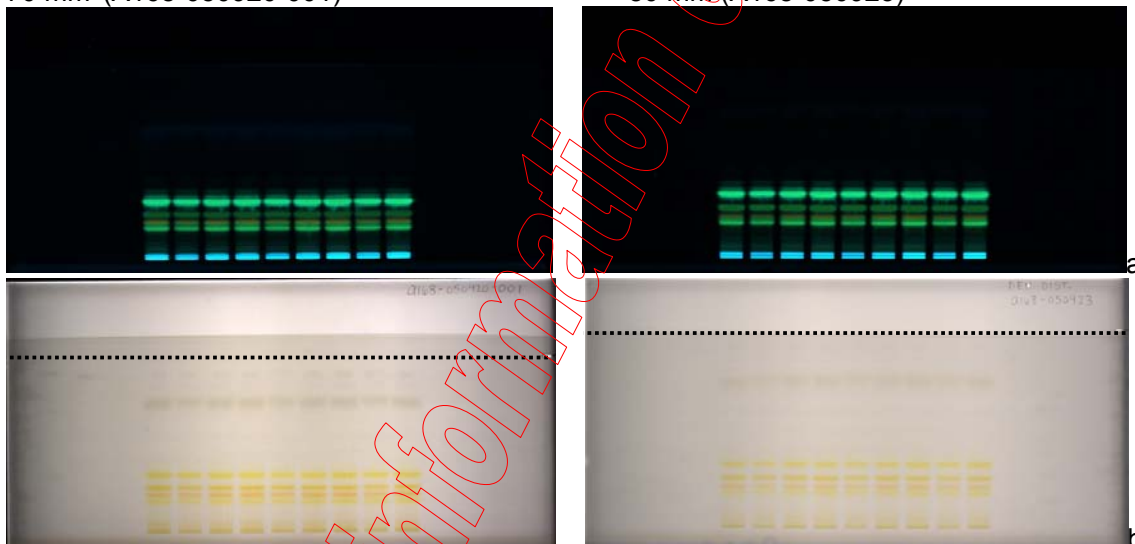
Results:

The increased developing distance does not affect results.

Images and plate ID:

70 mm (A168-050920-001)

80 mm (A168-050923)



R_F	70 mm (A168-050920-001)	80 mm	ΔR_F
Silychristin	0.17	0.17	0.0
Taxifolin	0.21	0.22	0.01
Silydianin	0.25	0.25	0.0
Silybin	0.32	0.32	0.0

System suitability test passed: Yes Accepted: Yes Date: 23.Sept.2005 Signature:

5.7.3 Relative humidity

Temperature recorded: 24°C

Results:

2.5%RH: molecular sieve

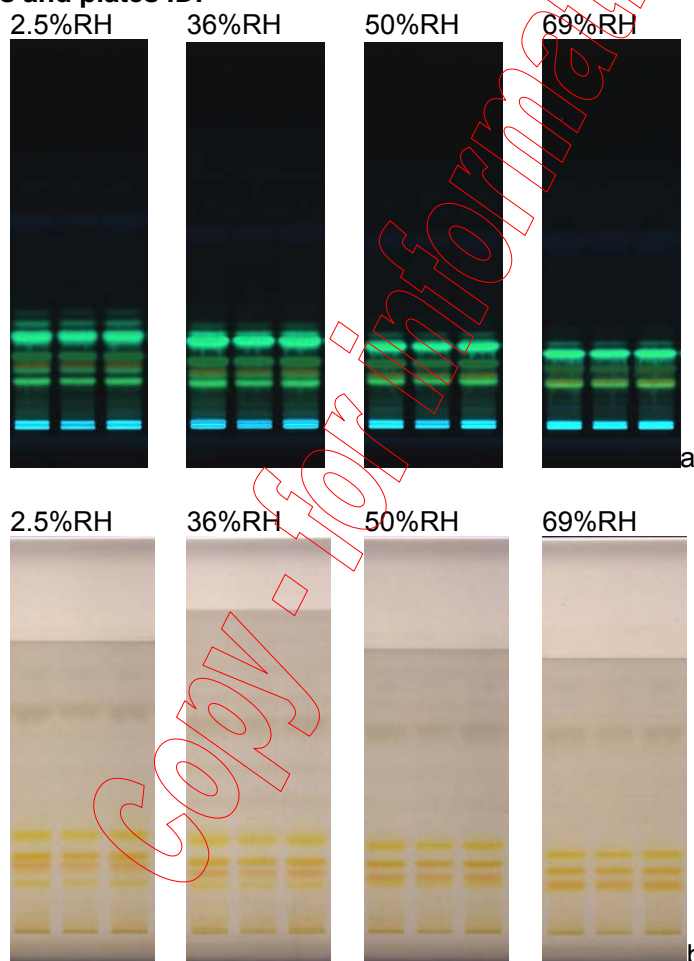
36%RH: magnesium chloride

69%RH: sodium chloride

50%RH: ambient humidity in laboratory

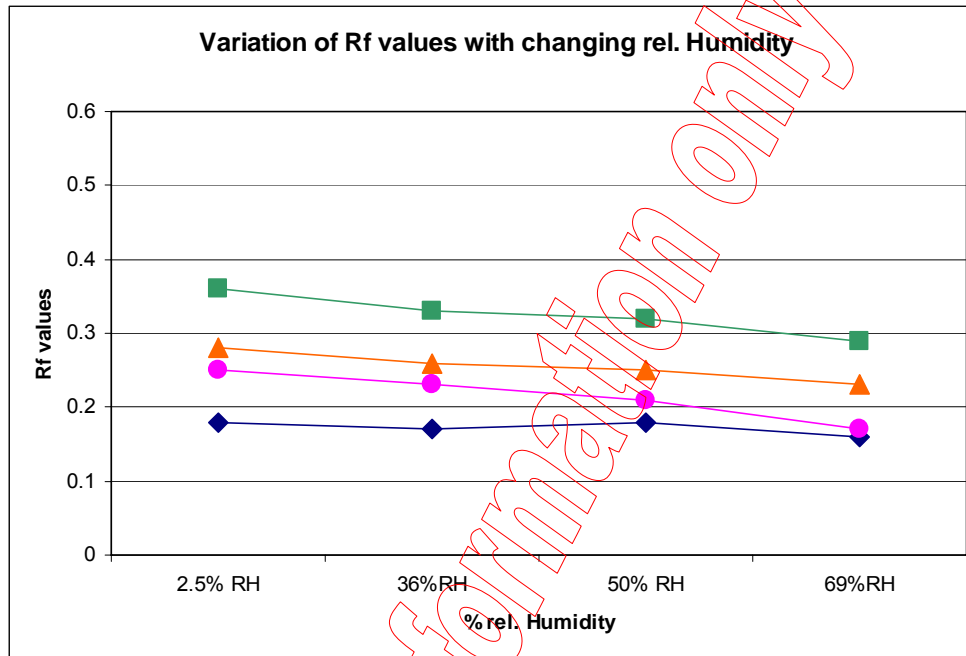
There is a decrease in separation at humidity over 50%RH. Taxifolin particularly is not well separated from the zone below. The plate should not be developed without humidity control when the ambient humidity is above 50%RH. The system suitability test is only met for relative humidity between 2.5% and 50%RH.

Images and plates ID:



R_F	A168-050923 2.5% RH	A168-050923 36%RH	A168-050923 50% RH	A168-050923 69%RH
Silychristin	0.18	0.17	0.18	0.16
Taxifolin	0.25	0.23	0.21	0.17
Silydianin	0.28	0.26	0.25	0.23
Silybin	0.36	0.33	0.32	0.29

Diagram showing R_f value as function of %RH:



System suitability test passed: Yes, between 2.5 and 50 %RH. The plate should not be developed at humidity higher than 50%RH without humidity control.

Accepted: Yes **Date:** 23.Sept.2005 **Signature:**

Comments

The separation is decreased and the system suitability is not met when the plate is developed at humidity higher than 50%RH without humidity control.

Suggested changes

The plate should be conditioned to about 30%RH if the relative humidity in the lab exceeds 50%RH.

Completed / Printed

Date:

Signed:

Date of review:

Name:

Title:

Signature:

Copy - for information only