

Date	Analyst	Project No.	Project Name
31.01.05	Anne Schibli	P98	Milk Thistle

Evaluation and optimization of methods for identification of Milk Thistle

1. Analytical goal:

The fingerprint should allow the identification of Milk Thistle fruits (*Silybum marianum*) using silybin, silydianin, silychristin, and taxifolin as chemical reference materials. No adulterants have to be considered.

2. Paper review of methods from literature:

Literature (see appendix)	Scope	Mobile phase / Stationary phase	Refer to Figure # below
USP28/NP23	Silydianin	Chloroform, acetone, anhydrous formic acid (75:16.5:8.5)	1a
Ph.Eur.5	Silibinin and taxifolin	Dichloromethane, acetone, anhydrous formic acid (75:16.5:8.5)	1b
Wagner TLC Atlas	Silychristin, silybin, and taxifolin	Chloroform, acetone, anhydrous formic acid (75:16.5:8.5)	1a
USP28/NP23	Silydianin	Chloroform, acetone, anhydrous formic acid (75:16.5:8.5)	1a

3. Experimental evaluation of selected methods

3.1 Materials

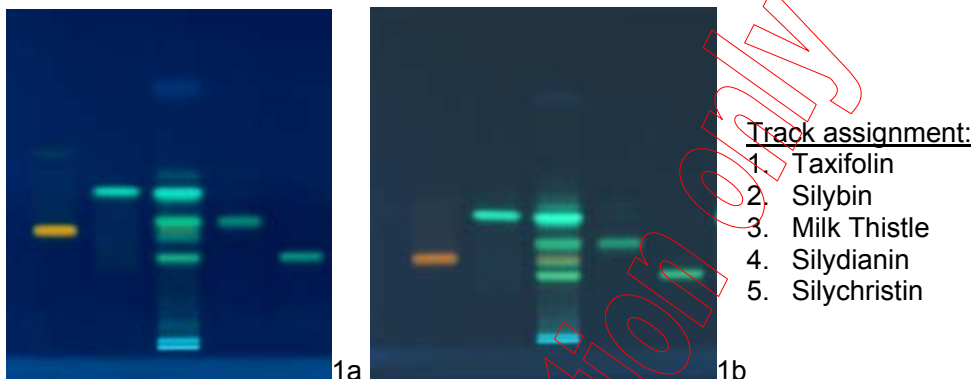
3.1.1 Samples

Sample name	Source / Batch	Authentication	Notes
Milk thistle BRM – <i>Silybum marianum</i>	Removed - proprietary information	Yes	

3.1.2 Standards (marker compounds)

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

3.2 Results and discussion



[changes from original method: use of HPTLC plate]

1a) Ph.Eur.5: Dichloromethane, acetone, anhydrous formic acid (75:16.5:8.5) → the zone corresponding to taxifolin is not well-detected.

1b) USP/NF: Chloroform, acetone, anhydrous formic acid (75:16.5:8.5) → the zone of taxifolin is better separated.

The mobile phase: Chloroform, acetone, anhydrous formic acid (75:16.5:8.5) will be used.

3.3 Conclusions:

Check one

... Method from literature is suitable → continue with section 5

X Method (USP/NF) needs optimization → continue with section 4

... No suitable method is found → refer to SOP 70.002.01 "Evaluation, development, optimization, and validation of methods for identification of medicinal plants and products thereof".

4. Method optimization

4.1 Sample preparation

Different sample preparations were tested and results compared side by side after chromatography on HPTLC plate.

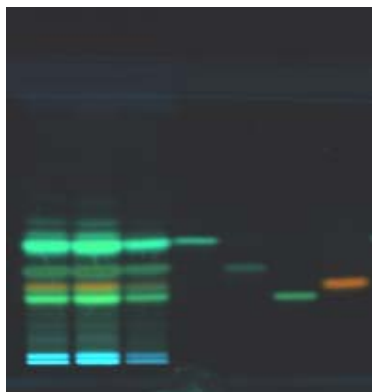
a) **Ph.Eur.5** To 1.0 g of powdered drug add 10 ml of methanol. Heat under reflux in a water-bath at 70 °C for 5 min. Cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 1.0 ml of methanol. **Time required: 30 min**

b) **USP/NF** Transfer about 10 g of finely powdered Milk Thistle, accurately weighed, to an extraction thimble, and cover with a small cotton ball. Transfer the thimble to a continuous-extraction apparatus fitted with a 250-mL round-bottom flask containing 150 mL of solvent hexane, and heat the flask on a heating mantle for 4 hours. Following the extraction, separate the round-bottom flask containing solvent hexane extract from the extraction apparatus, and discard the solvent hexane solution. Remove the adherent solvent hexane from the extraction thimble by drying, and transfer the thimble to an extraction apparatus suitable for hot extraction and fitted with a 250-mL round-bottom flask containing 100 mL of ethyl acetate. [NOTE—Adjust the volume of ethyl acetate, if necessary, to sustain a continuous extraction.] Heat the flask on a heating mantle to allow the solvent to reflux gently. After 8 hours, transfer the extract quantitatively into a 100-mL volumetric flask, dilute with methanol to volume, and mix. Transfer 1.0 mL of this solution to a 25-mL volumetric flask, and dilute with methanol to volume. **Not evaluated because to complicated and time consuming**

c) To 1.0 g of powdered drug add 10 ml of methanol. Heat under reflux in a water-bath at 70 °C for 5 min. Cool and filter. **Time required: 15 min**

d) To 1.0 g of powdered drug add 10 ml of methanol and sonicate for 10 min. Centrifuge. **Time required: 15 min**

Results:



Track assignment:

1. Milk Thistle extraction a), 1 μ L
2. Milk Thistle extraction c), 10 μ L
3. Milk Thistle extraction d), 10 μ L
4. Silybin
5. Silydianin
6. Silychristin
7. Taxifolin

Conclusion:

Extraction will be performed as follows: 1 g milled sample is extracted by heating at 70°C under reflux or in a closed bottle with 10 mL methanol for 5 min. The solution is centrifuged or filtered and the supernatant used as test solution. Application volume: 10 μ L.

4.2 HPTLC methodology

No comparison between TLC and HPTLC was performed. All methods were tested using HPTLC methodology directly.

4.3 Derivatization

The derivatization is done in all methods by spraying the plate with diphenylboric acid aminoethyl ester and subsequently macrogol. However, the description of the derivatization varies.

The Ph.Eur.5 requires heating the plate before spraying: the plate should be warm for derivatization. Diphenylboric acid aminoethyl ester is dissolved in methanol (1%) and macrogol 400 in methanol is used (5%). The evaluation should be done 30 min after the completion of the derivatization.

The USP requires drying the plate in a stream of cold air prior to derivatization. Diphenylboric acid aminoethyl ester is dissolved in methanol (1%) and macrogol 4000 in ethanol is used (5%). The evaluation is done 1h after the completion of the derivatization.

Based on experience, best results are obtained if the plate is be heated prior to the derivatization with diphenylboric acid aminoethyl ester, and treated while still hot.

For reproducibility/standardization reasons, the plates were dipped into the reagents instead of spraying. The composition of the reagents was modified for dipping: Diphenylboric acid aminoethyl ester is dissolved in ethyl acetate (0.5%) and macrogol 400 in dichloromethane is used (5%).

Heating the plate at 100°C for 5 min after the derivatization allowed sparing the waiting time.

Results:



- a) UV 366 nm, no heating after derivatization
- b) UV 366 nm, heating at 100°C for 5 min
- c) white light after heating at 100°C for 5 min

Conclusion

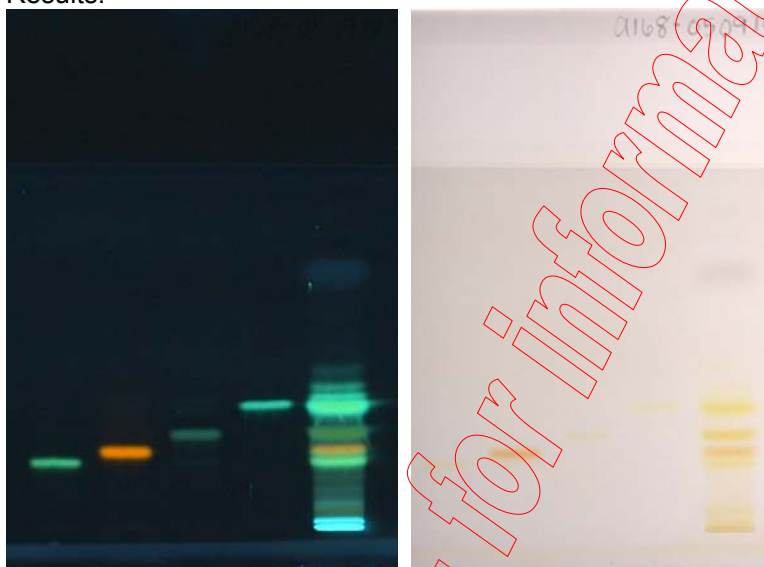
Derivatization will be performed by dipping the plate, heated at 100°C for 5 min, in NP and PEG reagents followed by heating at 100°C for additional 5 min. Detection will be performed under UV 366 nm and under white light.

4.4 Mobile phase

No further optimization.

4.5 Method including all optimized parameters

Results:



Track assignment:

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

Conclusion

Result ok.

4.6 Conclusions

Check one

X Analytical goals achieved → continue with section 5

... Analytical goals not achieved → refer to SOP 70.002.01 "Evaluation, development, optimization, and validation of methods for identification of medicinal plants and products thereof".

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

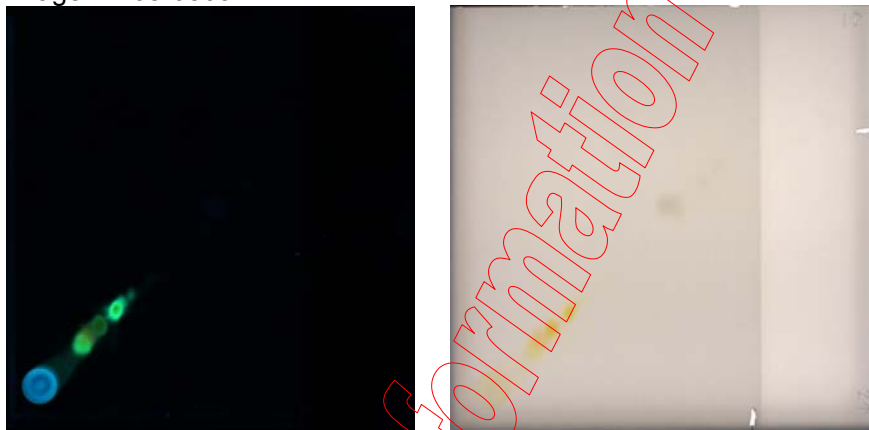
5. Evaluation of stability (pre-validation)

5.1 Stability of analyte during chromatography

Result:

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

Image: A168-050921-2D



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.

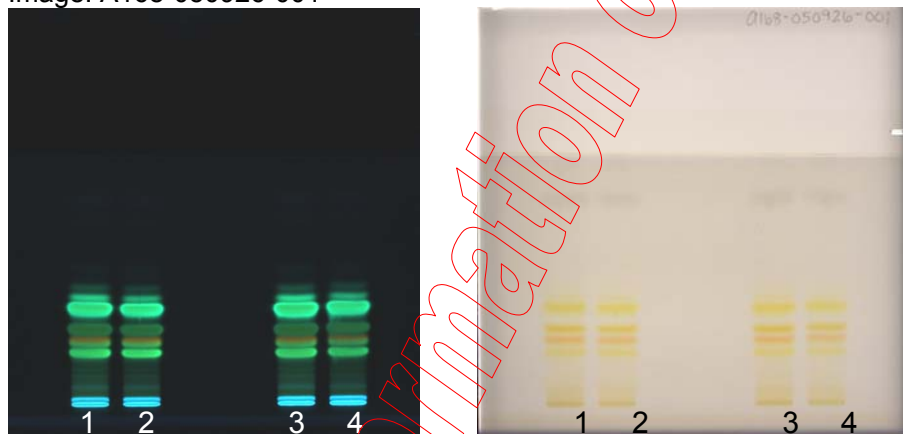
Pass: Yes

5.2 Stability of analyte in solution and on the plate

Result:

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

Image: A168-050926-001



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)

Acceptance criteria:

The sample is stable for at least 3 hours in solution and 3 hours on the plate prior to chromatography if no differences are seen between the four tracks.

Pass: Yes

5.3 Stability of result (for documentation)

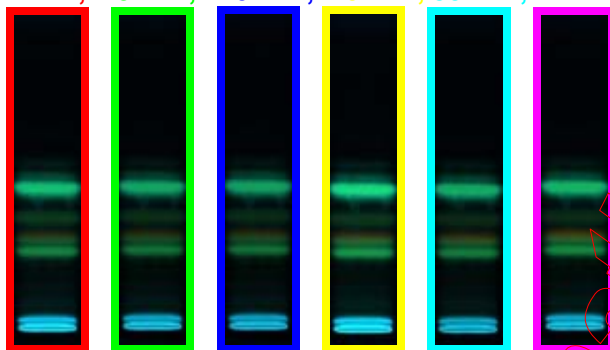
Result:

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 under UV 366 nm: A168-050921-001

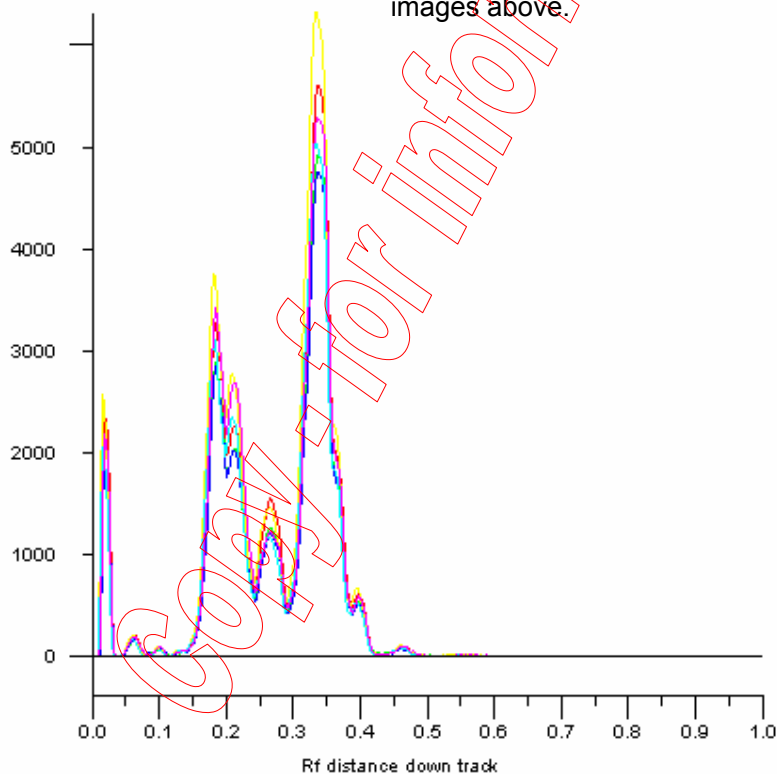
2 min, 5 min, 10 min, 20 min, 30 min, 1h



Densitograms:

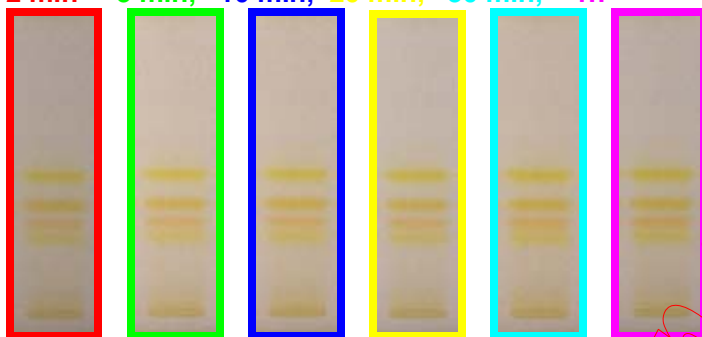
Profile height

Colors of curves correspond to images above.



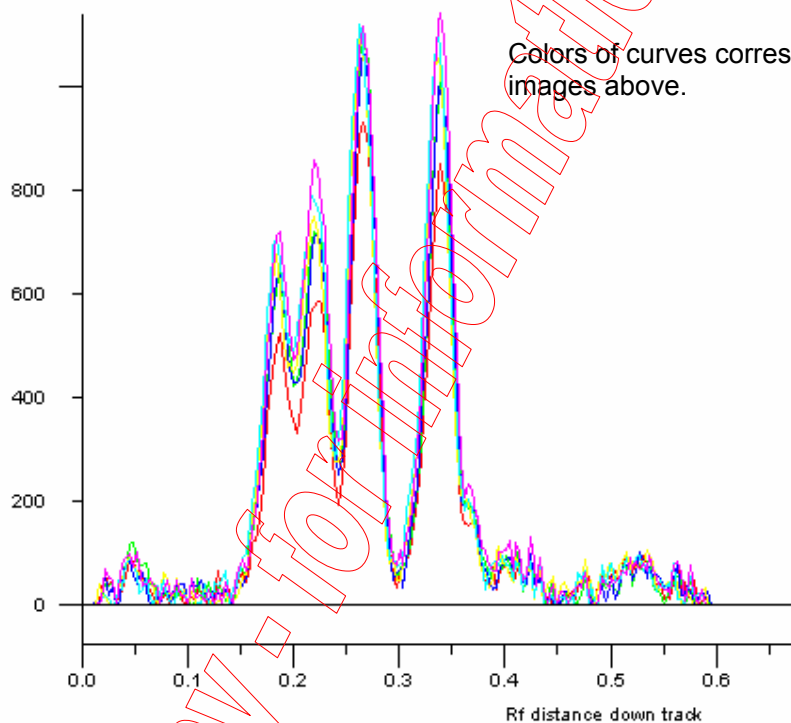
Images under white light: A168-050921-001

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



Densitograms:

Profile height



Acceptance criteria:

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

Pass: Yes

5.4 Conclusion

X Stability tests passed → Use FO 70.002.02 “Method to be validated” for method write up, then validate method according to SOP 70.002.01 “Evaluation, development, optimization, and validation of methods for identification of medicinal plants and products thereof”. If the method is not intended to be validated, use FO 70.002.06 “Application Note” for method write up.

... Stability tests failed → restart with section 4 or refer to SOP 70.002.01 “Evaluation, development, optimization, and validation of methods for identification of medicinal plants and products thereof”.

Printed

Date:

Signed:

Date of review:

Name:

Title:

Signature:

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