

Method ID		Author	Date
MOA 008	Identification of Milk Thistle	AS	24.Jan.2006

## Validated Method

### 1. Purpose of method

The method for identification of Milk Thistle seeds (fruits) by HPTLC fingerprint is suitable to identify a given sample of plant material as Milk Thistle (*Silybum marianum*) based on its flavonolignane fingerprint.

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

### 2. Materials

Wear lab coat, protective goggles and gloves at all times when handling chemicals.

#### 2.1 Chemicals and solvents

Methanol, acetone, formic acid, dichloromethane, chloroform, diphenylborinic acid aminoethylester, polyethylene glycol (Macrogol) 400, ethyl acetate.

#### 2.2 Samples and reference materials (optional)

Botanically authenticated and freshly dried Milk Thistle seeds (fruits), and silybin (=silibinin), silydianin, silychristin, and taxifolin (available from ChromaDex).

#### 2.3 Plates

Glass plates HPTLC Si 60 F<sub>254</sub>, 10x10 or 20x10 cm, Merck (Darmstadt, Germany), or others if equivalence was shown.

#### 2.4 Lab ware and instruments

- Analytical mill or mortar,
- ultrasonic bath,
- centrifuge with centrifuge tubes, or suitable set-up for filtration with beakers or small flasks (10 or 20 mL)
- analytical balance,
- graduated pipettes (1, 5, and 10 mL),
- graduated cylinder (50 mL),
- glass bottles (with tightly closing lid, 100 mL and 200 mL),
- TLC Twin Trough Chamber or Flat Bottom Chamber 20x10 cm, alternatively automatic developing chamber,
- sample application device using the spray-on technique (such as Linomat, ATS [CAMAG] or AS 30 [Desaga]),
- chromatogram immersion device [CAMAG],
- plate heater or oven,
- documentation system consisting of an illumination device for UV 254 nm, UV 366 nm, and white light and a video or digital camera,
- suitable TLC software,
- thermometer and hygrometer
- device for humidity control of plates
- lab coat, protective goggles and gloves.

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### **3. Description of method**

#### **3.1 Preparation of test solutions**

##### **3.1.1 Raw materials**

Mill each sample to a fine powder. Weigh 1 g each of powder in individual centrifuge tubes or flasks. Add 10 mL of methanol each and mix well. Close the lid or cap and heat at 70°C for 5 min. Alternatively the samples can also be heated under reflux for 5 min. Centrifuge or filter the solutions and use the supernatants / filtrates as test solutions.

##### **3.1.2 Dry extracts and dry finished products**

Weigh an amount of each extract powder or finished product equivalent to 1 g of raw material in individual centrifuge tubes or flasks. Add 10 mL of methanol each and mix well. Close the lid or cap and heat at 70°C for 5 min. Alternatively the samples can also be heated under reflux for 5 min. Centrifuge or filter the solutions and use the supernatants / filtrates as test solutions.

##### **3.1.3 Liquid extracts and liquid finished products**

Dilute the liquid samples 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 as described under 3.1.1.

#### **3.2 Preparation of reference solutions (optional)**

##### **3.2.1 Botanical reference solution**

As 3.1.1

##### **3.2.2 Chemical reference solutions**

Weigh 1 mg of silybin (= silibinin) in a flask. Add 10 mL of methanol. Individually dissolve silydianin, silychristin, and taxifolin in the same way.

#### **3.3 Preparation of derivatizing reagents**

**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.

#### **3.4 Stationary phase**

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

#### **3.5 Sample application**

Apply 10 µL of test solution, 10 µL of botanical reference solution, and 10 µL of each chemical reference solution 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.

#### **3.6 Temperature and humidity**

Record temperature and humidity in the laboratory. If the relative humidity exceeds 50%RH, condition the plate to about 30%RH using a suitable device.

#### **3.7 Chromatography**

##### **3.7.1 Developing solvent**

Place 75 mL of chloroform, 16.5 mL of acetone, and 8.5 mL of formic acid in a bottle, close lid tightly and mix content by shaking. Larger or smaller amounts of solvent can be prepared once a day.

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### 3.7.2 Chamber

Line one side of a 10x10 cm Twin Trough Chamber with filter paper. Pour 10 mL of developing solvent over the paper, and tilt the chamber to equilibrate solvent level in both troughs, close the lid. Allow the chamber to saturate for 20 min. If using a 20x10 cm chamber, use 20 mL of developing solvent. If using a Flat Bottom Chamber, use enough solvent to cover the bottom with a 5 mm level. If using an automatic chamber, refer to the manufacturer's instructions.

### 3.7.3 Development

Measure and mark on the plate the developing distance of 70 mm from lower edge of plate (62 mm from application position). Open the saturated chamber and introduce the plate with the layer facing the inside, close the chamber and wait for the solvent to reach the mark. Remove the plate from the chamber.

### 3.7.4 Drying

Dry the plate for 5 min with cold air (hair dryer).

## **3.8 Documentation and derivatization**

### 3.8.1 Documentation of non-derivatized plate

Not applicable

### 3.8.2 Derivatization

Turn on plate heater or oven and select temperature (100°C). Heat the plate at 100°C for 5 min. Charge the tank of the immersion device with 200 mL of NP reagent. Immediately dip the plate, while still hot, into the reagent by placing the plate in holder of immersion device. Set parameters (speed: 5, time: 0) and press start. Let excess reagent drip off and wipe off the back of the plate with a paper towel. Remove the plate from plate holder. Allow the plate to dry for 5 minutes inside the hood.

The plate is then derivatized again by dipping, this time in the PEG reagent. Place the twice derivatized plate onto the plate heater (set to 100°C) for 5 minutes. Remove the hot plate after 5 minutes and let it cool down to room temperature.

### 3.8.3 Documentation of derivatized plate

Document the plate using illumination with UV366 nm.

Document the plate using illumination with white light (reflection and transmission).

## **3.9 Results**

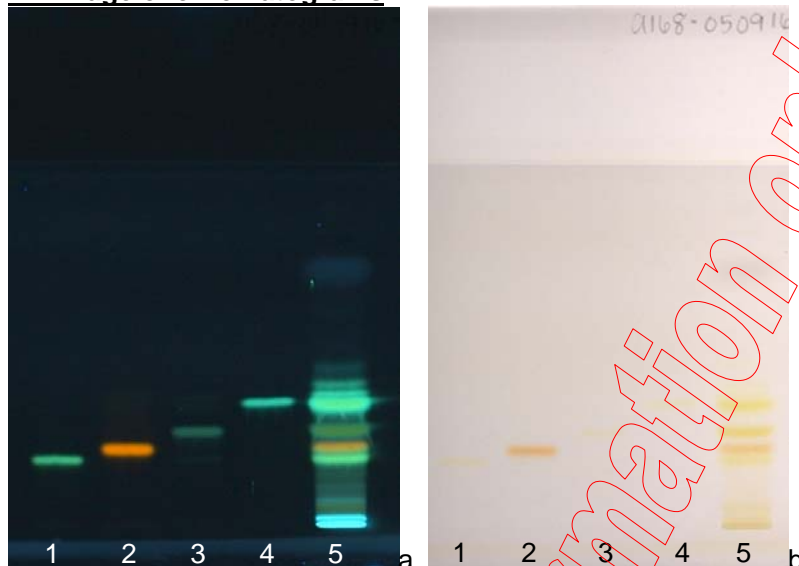
Compare the image(s) of the plate obtained under 3.8 with the image(s) provided under 4.1. The plate can only be evaluated if it passes the system suitability test (4.3).

Evaluate the results obtained with the test solution according to the description under 4.2. The test solution can be identified as Milk Thistle if the fingerprint obtained is similar to that of the BRM. The intensity of the zones may vary, however, the zones corresponding to the four chemical references (silybin, silydianin, silychristin, and taxifolin) must be seen. In comparison to the BRM, the test solution must not show any additional intense zone.

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#### 4. Results for comparison

##### 4.1 Image of chromatograms



##### Track assignment:

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

##### **4.2 Description of results:**

###### a) 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.

###### a) 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.

##### **4.3 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.

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### 5. Approvals

**Validation approved:**

**Date:** 27.January 2006, **by:** ER

**MOA 008 released:**

**Date:** \_\_\_\_\_, **by:** \_\_\_\_\_, **Signature:** \_\_\_\_\_

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### Revision history

Creation date

27.January 2006/AS

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