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CAMAG LABORATORY

Method ID	Identification of Black Cohosh	Author	Date
MOA 005		AS	24.Oct.2005

Only for information

Validated Method

1. Purpose of method

The method for identification of Black Cohosh by HPTLC fingerprint is suitable to identify a given sample of plant material as Black Cohosh (*Actaea racemosa* or *Cimicifuga racemosa*) based on its triterpene fingerprint and its pattern of plant acids.
Adulterants, such as other Cohosh species, *Caulophyllum thalictroides*, and Chinese Sheng Ma, show a different profile.

The method may be used to identify an extract as derived from Black Cohosh (*Actaea racemosa*), provided that the material was made from a single herb and is intended to contain the constituent profile seen in Black Cohosh.

The denominations *Actaea* and *Cimicifuga* are interchangeable.

2. Materials

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

2.1 Chemicals and solvents

Ethanol, methanol, ethyl formate, sulfuric acid 95-97%, formic acid, toluene: all of "for analysis" or HPLC quality, distilled or demineralized water.

2.2 Samples and reference materials (optional)

Botanically authenticated and freshly dried *Cimicifuga racemosa* (*Actaea racemosa*) rhizoma.
Actein, chlorogenic acid, caffeic acid, isoferulic acid [ChromaDex].

2.3 Plates

Glass plates HPTLC Si 60 F_{254}, 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.

3. Description of method

3.1 Preparation of test solutions

3.1.1 Raw materials

Mill each sample to a fine powder. Weigh 500 mg each of powder in individual centrifuge tubes or flasks. Add 10 mL of an ethanol-water mixture (1:1) each and mix well. Sonicate for 10 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 500 mg of raw material in individual centrifuge tubes or flasks. Add 10 mL of an ethanol-water mixture (1:1) each and mix well. Sonicate for 10 min. Centrifuge or filter the solutions and use the supernatants / filtrates are as test solutions.

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 actein in a flask. Add 10 mL of methanol. Individually dissolve chlorogenic acid, caffeic acid, and isoferulic acid in the same way.

3.3 Preparation of derivatizing reagent

Place 180 mL of methanol in a 200 mL glass bottle and cool it down in a water-ice cubes-salt bath or in a freezer. To the ice-cold methanol add slowly and carefully 20 mL of sulfuric acid and mix well. Allow the mixture to cool to room temperature.

3.4 Stationary phase

10x10 cm (or 20x10 cm) glass plates HPTLC silica gel 60 F_{254} (Merck).

3.5 Sample application

Apply 2 µL of test solution, 2 µL of botanical reference solution, and 2 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. Condition the plate to about 5%RH using a suitable device (e.g. ADC 2 with molecular sieve, conditioning tray or desiccator with sulfuric acid).

3.7 Chromatography

3.7.1 Developing solvent

Place 50 mL of toluene, 30 mL of ethyl formiate, and 20 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.

3.7.2 Chamber

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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 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

Document the plate using UV 366 nm illumination.

3.8.2 Derivatization

Turn on plate heater or oven and select temperature (100°C). Charge the tank of the immersion device with 200 mL of reagent. Place plate in holder of immersion device, set parameters (speed: 5, time:0) and press start. Let excess reagent drip off the plate; wipe off the back of the plate with a paper towel. Remove plate from plate holder. Place plate onto plate heater or in oven. Remove hot plate after 5 min and let it cool down to room temperature.

3.8.3 Documentation of derivatized plate

Document the plate using illumination with white light (reflection and transmission).
Document the plate using illumination with UV366 nm.

3.9 Results

Compare the images of the plate obtained under 3.7.1 and 3.7.3 with the images provided under 4.1.1 and 4.1.2. The plate can only be evaluated if it passes the system suitability test (4.3).
Evaluate the results according to the descriptions under 4.2.1 and 4.2.2. The test solution can be identified as Black Cohosh if the fingerprint obtained is similar to that of the BRM. The intensity of the zones may vary, however, the zone corresponding to actein must be seen after derivatization. In comparison to the BRM, the test solution may not show any additional intense zone.

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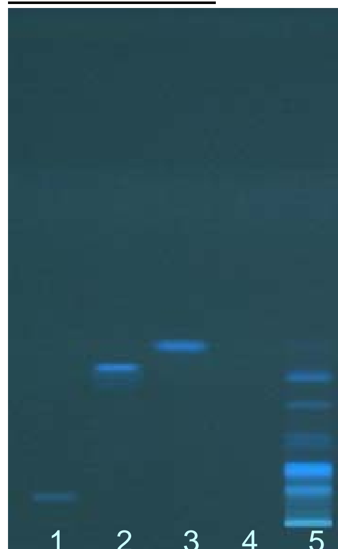
4. Results for comparison

4.1 Image of chromatograms

4.1.1 UV 366 nm



4.1.2 White light and UV 366 nm after derivatization

Track assignment

1. Chlorogenic acid
2. Caffeic acid
3. Isoferulic acid
4. Actein
5. *Actaea racemosa* BRM

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4.2 Description of results:

For comparison with a wide range of adulterants see Appendix.

4.2.1 UV 366 nm

The standards chlorogenic acid (track 1), caffeic acid (track 2), and isoferulic acid (track 3) each show a blue fluorescing band. Actein (track 4) is not seen. The sample shows several blue fluorescing bands of different intensities. The lowest band is slightly above the position of chlorogenic acid. A band is seen just below the position of caffeic acid. A very faint band corresponding to isoferulic acid is seen.

4.2.2 After derivatization

White light

The standards chlorogenic acid (track 1) and caffeic acid (track 2) are not well detected. Isoferulic acid (track 3) and actein (track 4) each show a brownish band. The sample shows one brownish band corresponding to actein and several brownish bands below that zone. There may be brownish zones above the band of actein. One brown zone and two broad blue zones are seen above the application position.

UV 366 nm

The standards chlorogenic acid (track 1), caffeic acid (track 2), and isoferulic acid (track 3) each show a blue to green fluorescing band. Actein (track 4) shows a brownish fluorescing band. The sample shows a cluster of alternating brownish and bluish bands, of which one brown band matches the zone obtained for actein in color and position. One dark zone and two bluish-green zones are seen above the application position.

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4.3 System suitability test:
After derivatization, actein is detected as a sharp and clearly distinguished zone in the sample under white light as well as under UV 366 nm.

5. Approvals

Validation approved:
Date: 07. Sept. 2005, **by:** ER

MOA 005 released:
Date: , **by:** , **Signature:**

Revision history

Creation date
24. October 2005/AS

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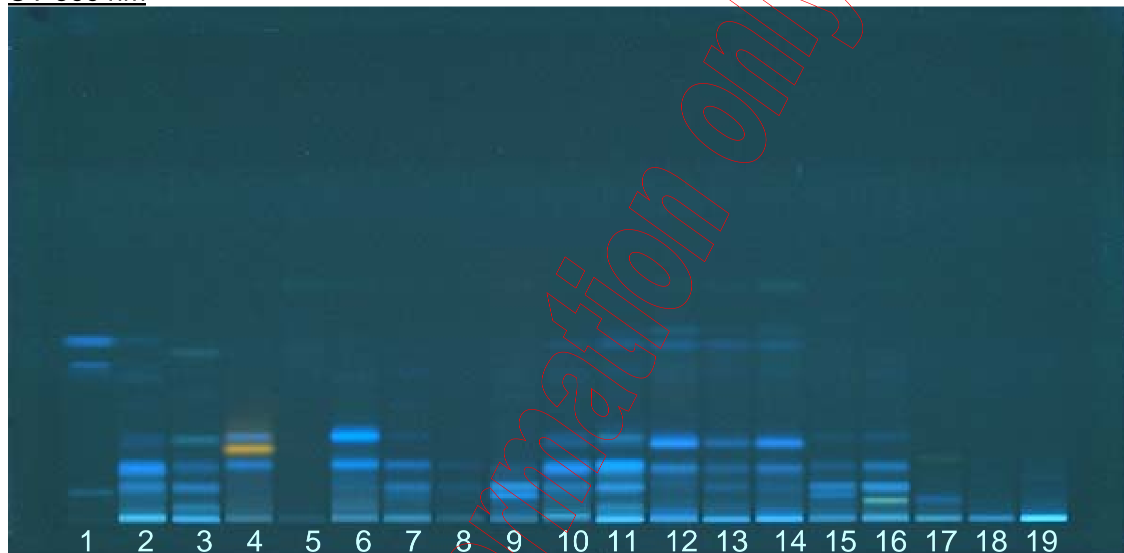
Appendix

Comparison with adulterants of Black Cohosh

UV 366 nm



White light after derivatization

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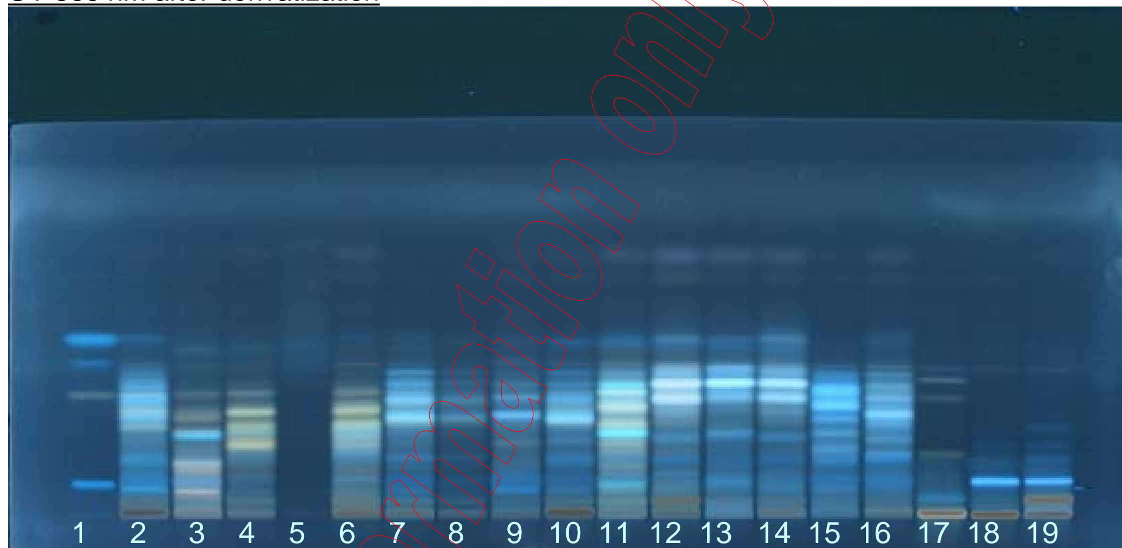
UV 366 nm after derivatization



Track assignment

1. Chlorogenic acid, actein, caffeic acid, and isoferulic acid (increasing R_F values)
2. *Actaea racemosa* BRM
3. *Actaea podocarpa* [probably mislabeled]
4. *Actaea podocarpa* (Yellow cohosh root)
5. Potting soil [mistaken for sample]
6. *Actaea podocarpa*
7. *Actaea pachypoda*
8. *Actaea pachypoda*
9. *Actaea pachypoda* (leaves)
10. *Actaea rubra*
11. *Cimicifuga foetida*
12. *Cimicifuga dahurica*
13. *Cimicifuga dahurica* / Bei Shengma
14. *Cimicifuga heracleifolia* [similar to *Cimicifuga dahurica*, probably mislabeled]
15. *Cimicifuga* sp unknown*
16. *Cimicifuga* sp unknown*
17. *Caulophyllum thalictroides* (Blue Cohosh root)
18. Guang Shengma (no *Cimicifuga*)
19. Guang Dong Sheng Ma

* These samples are about 5 years old.