

Evaluation and optimization of Identification Method for Eleuthero by HPTLC fingerprint

1. Evaluation of existing methods and goal of the analysis:

Goals: Develop a representative fingerprint for the identification of Eleuthero root (definition USP: *Eleutherococcus senticosus* syn. *Acanthopanax senticosus*). Use Eleutherosides E, B, and E1 as standards.

2. Methods from literature, paper review:

Literature (see Appendix)	Scope	Mobile phase / Stationary phase	Detection using phase
USP	Detection of eleutheroside B	Chloroform, methanol, water (70:30:4 v/v) TLC plate, Silica gel	Antimony chloride
Ph.Eur.5	Detection of eleutheroside B and E (with esculin and catalpol as standards)	Dichloromethane, methanol, water (70:30:4 v/v) TLC plate, Silica gel	Anisaldehyde solution
Wagner TLC Atlas	Detection of eleutheroside B and E and E1, Differentiation of Eleuthero Types A, B, and C	Chloroform, methanol, water (70:30:4 v/v) TLC plate, Silica gel	Vanillin reagent or Antimony chloride
Chin.Ph.	Comparison with botanical reference material and Isofraxidin	Chloroform, Methanol (95:5 v/v); TLC plate, Silica gel (with Gypsum and Carboxymethylcellulose as binders)	UV 254 nm

3. Material

Sample available:

Sample Name	Source / Lot	Authentication
Eleuthero Root, shredded	Removed - proprietary information	yes

Standards (marker compounds) available:

Name	Source
------	--------

Eleutheroside B (syn. Syringin)	ChromaDex 05060-101
Eleutheroside E	ChromaDex 05065-101
Eleutheroside E1	ChromaDex 05064-101

Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB291342
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB302430

Instruments and software

Instrument	Manufacturer
Automatic TLC Sampler 4	CAMAG
DigiStore	CAMAG
TTC 20x20 cm	CAMAG
TTC 20x10 cm	CAMAG
TTC 10x10 cm	CAMAG
TLC Plate Heater III	CAMAG
Immersion Device III	CAMAG
Mill KB5/10	IKA
Centrifuge EBA21	Hettich
Ultrasonic Bath	Telsonic TPC25
Balance	Mettler-Toledo

Software	Manufacturer
WinCATS	CAMAG

4. Optimization of the method

4.1 Mobile phase

The mobile phases for the separation of Eleutherosides E, B, and E1 were found in the literature. The mobile phase of the Ch.Ph. was not evaluated, because the corresponding stationary phase is not commercially available and the focus is not on the separation of Eleutherosides.

The mobile phase of the USP/Wagner yields a chromatogram with clearly distinct zones. The mobile phase of the Ph.Eur.5, although avoiding the use of chloroform, doesn't provide a chromatogram with sufficient separation of the zones close to Eleutheroside B.

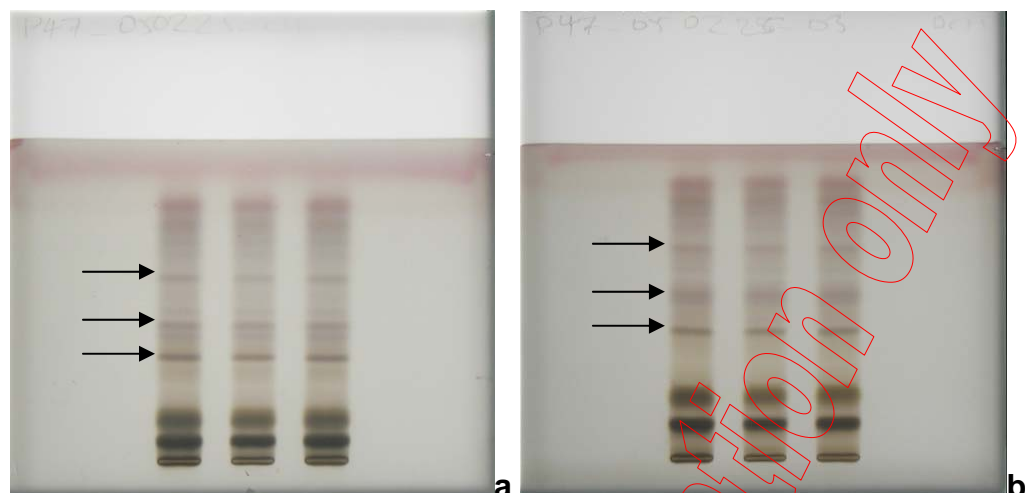


Figure 1

a) mobile phase USP/Wagner, b) mobile phase Ph.Eur.5
Derivatization with sulfuric acid reagent. Eleutheroside E, B, and E1 are marked with an arrow (ascending order).

The following mobile phase will be used: Chloroform, methanol, water (70:30:4 v/v)

4.2 Extraction and application volume

Different sample preparations were tested and compared side by side after chromatography on HPTLC plate. 4 different volumes of each sample solution were applied.

a) Sample preparation according to USP

5 g milled root are extracted under reflux with 25 mL ethanol 30% for 30 min. The filtrate is evaporated to dryness (rotatory evaporator) and redissolved in 10 mL methanol (USP: 5 mL, residue was not completely dissolved).

b) Sample preparation according to Ph.Eur.5

1 g milled root is extracted under reflux with 10 mL ethanol 50% for 1h. The filtrate is evaporated to dryness (rotatory evaporator) and redissolved in 2.5 mL ethanol 40%.

c) Sample preparation according to Wagner&Bladt

1 g milled root is extracted under reflux with 10 mL methanol 50% for 15 min. The filtrate is mixed with 15 mL water-saturated butanol, the butanol layer is evaporated to dryness (rotatory evaporator) and redissolved in 1 mL water.

d) Sample preparation simplified

1 g milled root is extracted by sonication with 10 mL ethanol 50% for 15 min. The solution is centrifuged and the supernatant used as test solution.

Image of derivatized plate white light

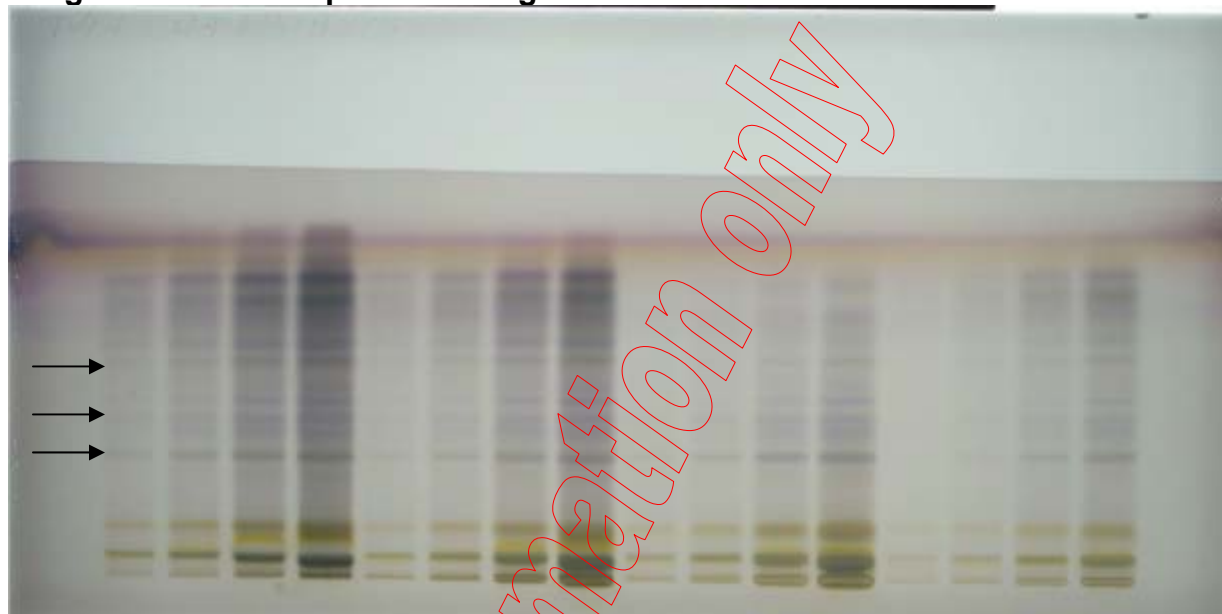


Figure 2: Comparison of extraction methods

1, 2, 5, and 10 μL of solutions a)-d) were applied. Derivatization with anisaldehyde solution. Eleutheroside E, B, and E1 are marked with an arrow (ascending order).

All samples show a similar profile, only the intensity varies. The simplest extraction method will be chosen (last 4 tracks). As the bands are not very intense, the volume of the extraction solvent will be reduced to the half.

Extraction will be performed as follows: 1 g milled root is extracted by sonication with 5 mL ethanol 50% for 10 min. The solution is centrifuged and the supernatant used as test solution. Final concentration: 0.2 g/mL, application volume: 10 μL .

4.3 Developing distance

Variations in developing distance were not tested. The compounds of interest (Eleutherosides E, B, and E1) are separated using the standard developing distance of 6 cm.

4.4 Derivatization

Various derivatization methods from the literature were tested.

USP: Antimony (III) chloride, detection under UV 366 nm.

Ph.Eur.5: Anisaldehyde solution, detection in white light.

Wagner and Bladt: UV 366 nm without derivatization, then antimony (III) chloride (detection under UV 366 nm), or vanillin reagent (detection in white light).

Alternative derivatization: Sulfuric acid solution, detection in white light.

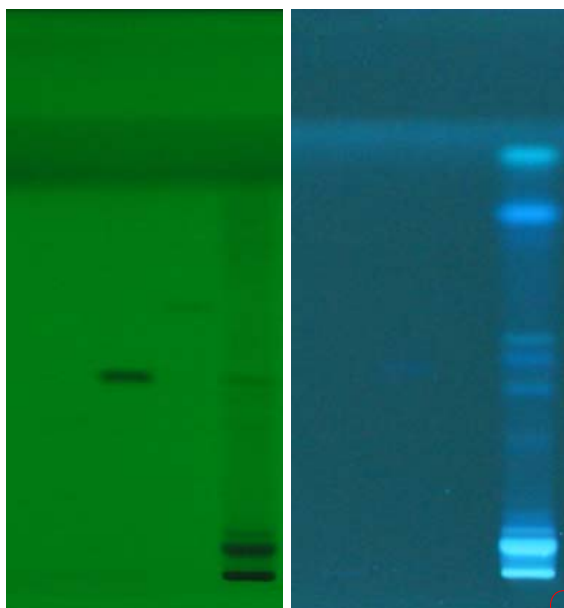


Figure 3a: UV 254 nm and 366 nm (no derivatization)

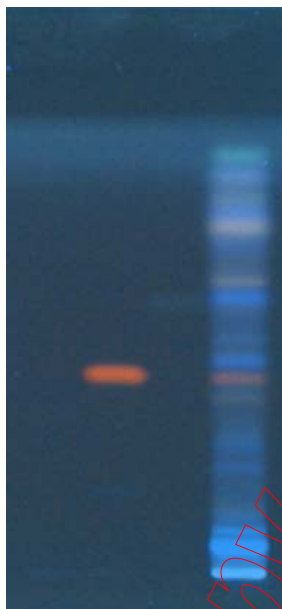


Figure 3b: Derivatization by spraying with antimony (III) chloride (10% in chloroform-methanol 1:1), UV 366 nm

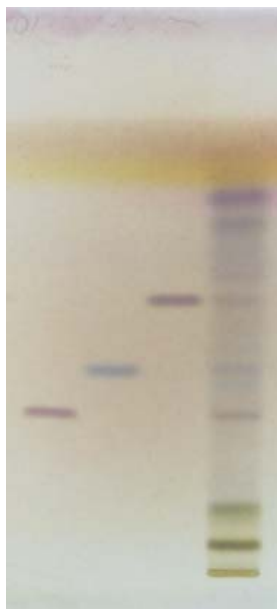


Figure 3c: Derivatization by spraying with anisaldehyde solution and heating at 100°C for 3 min



Figure 3d: Derivatization by dipping in sulfuric acid solution and heating at 100°C for 10 min, white light

All plates:

1. Eleutheroside E
2. Eleutheroside B
3. Eleutheroside E1
4. Eleuthero root

Without any derivatization, the compound Eleutheroside B shows a strong dark band under UV 254 nm. A corresponding zone is seen in the sample. The fluorescing zones seen under UV 366 nm don't correspond to any of the standard substances.

Derivatization with antimony chloride is very specific for Eleutheroside B, but doesn't allow detection of the other substances of interest.

The derivatization with anisaldehyde solution yield a colorful chromatogram, but the background is also colored. Vanillin reagent was not used in this trial. The reproducibility of the color is difficult to achieve with these reagents. Although the colors of the chromatogram obtained after derivatization with sulfuric acid are less diversified, the result is usually more reproducible and stable.

The plate will be examined under 254 nm prior and under white light after derivatization with sulfuric acid.

Copy - for information only

5. Written procedure / Method to be validated

5.1 Preparation of test solutions

1 g of milled root is sonicated for 10 min with 5 mL ethanol-water 1:1. The solution is centrifuged and the supernatant is used as test solution.

5.2 Preparation of reference solutions

1 mg of each eleutheroside (B, E, E1) is dissolved in 1.5 mL ethanol-water 1:1.

5.3 Preparation of derivatizing reagent

Sulfuric acid reagent: 20 mL sulfuric acid are carefully added to 180 mL ice-cold methanol.

5.4 Stationary phase

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

5.5 Sample application

10 µL of test solution and 2 µL of standard are applied each as 8 mm bands, at least 2 mm apart, 8 mm from the lower edge and at least 15 mm from left and right edges of the plate.

5.6 Temperature and humidity

Record temperature and humidity in the laboratory.

5.7 Chromatography

Chamber type: 10x10 cm (or 20x10 cm) Twin Trough Chamber
Configuration: Saturated for 20 min (wetted filter paper in trough opposite to the plate)
Developing solvent: Chloroform, methanol, water (70:30:4), 5 mL (respectively 10 mL) developing solvent per trough.
Developing distance: 70 mm from lower edge of plate (62 mm from application position)
Drying: 5 min with cold air (hair dryer)

5.8 Derivatization

The plate is immersed into reagent for 1 s, then heated at 100°C for 5 min.

5.9 Documentation

- a) Prior to derivatization under UV 254 nm
- b) After derivatization under white light (reflection and transmission)

Appendix

USP

Eleuthero

» Eleuthero is the dried rhizome with roots of *Eleutherococcus senticosus* (Rupr. et Maxim.) (Fam. Araliaceae) [*Acanthopanax senticosus* Harms]. It contains not less than 0.08 percent of the sum of eleutheroside B and eleutheroside E, calculated on the dried basis.

Identification, *Thin-Layer Chromatographic Identification Test* □ 201 □—

Test solution— Comminute about 10 g of Eleuthero, add about 50 mL of alcohol 30% (v/v), and heat under reflux in a water bath for 30 minutes. Cool to room temperature, filter, gently evaporate the solvent, and suspend the residue in 5 mL of methanol.

Standard solution— Prepare a solution of eleutheroside B in methanol containing about 1 mg per mL.

Developing solvent system: a mixture of chloroform, methanol, and water (70:30:4). [NOTE — Saturate the chamber with *Developing solvent system* before the development of the chromatogram.]

Spray reagent— Prepare a solution of antimony trichloride in chloroform having a concentration of about 200 mg per mL.

Procedure— Develop the chromatogram until the solvent front has moved to 15 cm, dry, and spray the plate with *Spray reagent*. Heat the plate at 120° for 10 minutes, and examine it under UV light at 365 nm and in daylight. The chromatogram of the *Test solution* shows a brownish to red zone due to eleutheroside B, corresponding in color and R_F value to the zone exhibited by the chromatogram of the *Standard solution*. A blue zone appears directly above, and a yellow zone appears directly below the red zone. In daylight, a violet band is visible in the lower half sector. Some brownish to yellowish bands occur in the upper sector.

268

10.6 Chromatograms

Eleutherococci radix (rhizoma)

Drug sample	1 Eleutherococci radix (type A) 2 Eleutherococci radix (type B) 3 Eleutherococci radix (type C) 4-12 Eleutherococci radix (commercial drug samples) (n-butanol extracts, 20 µl)
Reference compound	T1 eleutheroside B (syringin) T2 eleutheroside E T3 syringaresinol monoglucoside E ₁ T4 syringaresinol
Solvent system	Fig. 1, 2 chloroform-methanol-water (70:30:4)
Detection	A Without chemical treatment → UV-365 nm B Vanillin-phosphoric acid reagent (VPA, No. 41) → vis C Antimony-(III)-chloride reagent (SbCl ₃ , No. 4) → UV-365 nm

- Fig. 1A** Eleutherococci radix samples 1-3 show phenol carboxylic acids and coumarins as blue fluorescent zones; chlorogenic acid at $R_f \sim 0.05$, lipophilic plant acids and coumarins in the R_f range 0.85-0.95. Their presence and amount varies according to plant origin.
- B** Eleutherococci radix sample 1 and 2 are characterized by the blue to violet-red zones of eleutheroside B (syringin) (T1) at $R_f \sim 0.5$, eleutheroside E (T2) at $R_f \sim 0.35$ and eleutheroside E₁ (T3) at $R_f \sim 0.65$. Syringin (T1) can be absent (e.g. sample 2) or is found in extremely low concentration only.
The amount of blue aglycone zones in the R_f range 0.8-0.95 varies as do the grey zones in the R_f 0.05-0.15 which are partly due to free sugars.
- C** The zone of syringin (T1) fluoresces specifically orange-red with SbCl₃ reagent. Syringin is accompanied by a blue and yellow fluorescent zone directly above and below, respectively.
- Fig. 2** **TLC Synopsis** (VPA reagent, vis)
As demonstrated with the Eleutherococcus samples 4-12, the amount and presence of eleutheroside B at $R_f \sim 0.5$, as well as eleutheroside E at $R_f \sim 0.35$ and its monoglucoside E₁ at $R_f \sim 0.65$, varies depending on origin of the plant and the part of the roots used for investigation. The grey zones in the R_f range 0.05 and 0.2 (e.g. chlorogenic acid, free sugars) and the blue-grey and violet-zones in the upper R_f range are present in varying amounts.

