

Evaluation and Optimization of Method for Identification of Ginseng Species by HPTLC Fingerprint

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

Goals: Develop a fingerprint for the identification of Ginseng root as either *Panax ginseng*, *P. quinquefolium*, or *P. notoginseng* (*syn. P. pseudoginseng*). Ginsenoside Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 are used as standards.

2. Methods from literature, paper review:

Literature (see appendix)	Scope / reference materials	Mobile phase / Stationary phase: TLC plate, Silica gel	Figure	Detection using
USP/NF 20 2 nd suppl.	Escin and arbutin	Ethyl acetate, water, 1-butanol (25:50:100), upper phase	1a, 1b	Anisaldehyde solution
Ph.Forum 30(2)	American ginseng: 2D Chromatography using American and Asian ginseng reference extracts	1—chloroform, methanol, water (13:7:2), lower phase. 2— Water, butyl alcohol, ethyl acetate (5:4:1), upper phase	1c	Anisaldehyde solution
Ph.Forum 30(2)	Asian ginseng: Escin and arbutin	As USP/NF 20	1a, 1b	Anisaldehyde solution
Pharmeuropa (15.3)	Escin and arbutin	As USP/NF 20	1a, 1b	Anisaldehyde solution
Chinese Ph.	Rb1, Re, Rg1	Chloroform, ethyl acetate, methanol, water (15:40:22:10), store at 10°C, use lower layer	1d	Anisaldehyde solution
Plant Drug Analysis, Wagner	Rb1, Rb2, Rc, Rd, Re, Rg1, Rg2	Chloroform, methanol, water (70:30:4)	1e	Vanillin solution
JPC 15(2) 2002	Rb1, Re, Rg1	Chloroform, methanol, water (65:50:10)	1f	Anisaldehyde solution
Modified Chinese Ph.	All available ginsenosides	Chloroform, ethyl acetate, methanol, water (15:40:22:9)	1g	Sulfuric acid reagent
Xie and Yan HRC&CC 10 (1987)	Ginsenosides mixture	Chloroform, ethyl acetate, methanol, water (15:40:22:10), store at 10°C overnight, use lower layer. Plate drying over P ₂ O ₅ .	Not performed (elaborate sample prep and chromatography)	Sulfuric acid reagent

3. Material

Sample available:

Sample Name	Source / Lot	Authentication
Panax ginseng	CAMAG	Yes
American Ginseng powder (Panax quinquefolium)	CAMAG	No
Panax notoginseng	CAMAG	Yes

Standards (marker compounds) available:

Name	Source
Aescin	Merck, K91050101 014
Arbutin	Phyto-Cal Inc., 007A-002
Ginsenoside Re	Pharmaton, K.H.08.95
Ginsenoside Rf	Pharmaton, K.H.08.95
Ginsenoside Rd	Pharmaton, K.H.08.95
Ginsenoside Rg1	Pharmaton, K.H.08.95
Ginsenoside Rg2	Pharmaton, K.H.08.95
Ginsenoside Rb1	Pharmaton, K.H.08.95
Ginsenoside Rc	Pharmaton, K.H.08.95
Ginsenoside Rb2	Pharmaton, K.H.08.95

Plates

TLC plate	Size	Source	Batch
Glass plates HPTLC Si 60 F254	10x10 cm	Merck	OB312657
Glass plates HPTLC Si 60 F254	20x10 cm	Merck	OB302430
Glass plates TLC Si 60 F254	20x20 cm	Merck	OB268992

Instruments and software

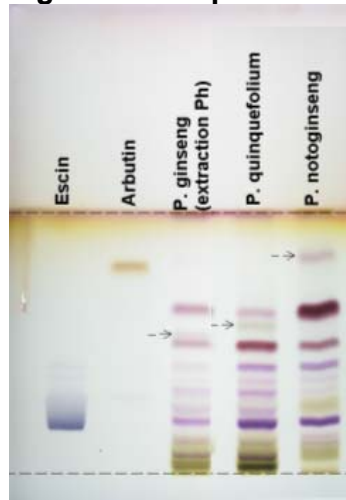
Instrument	Manufacturer
Automatic TLC Sampler 4	CAMAG
DigiStore	CAMAG
TTC 20x20 cm	CAMAG
TTC 20x10 cm	CAMAG
TTC 10x10 cm	CAMAG
TLC Plate Heater III	CAMAG
Immersion Device III	CAMAG
Mill KB5/10	IKA
Centrifuge EBA21	Hettich
Ultrasonic Bath	Telsonic TPC25
Balance	Mettler-Toledo

Software	Manufacturer
WinCATS	CAMAG

4. Optimization of the method

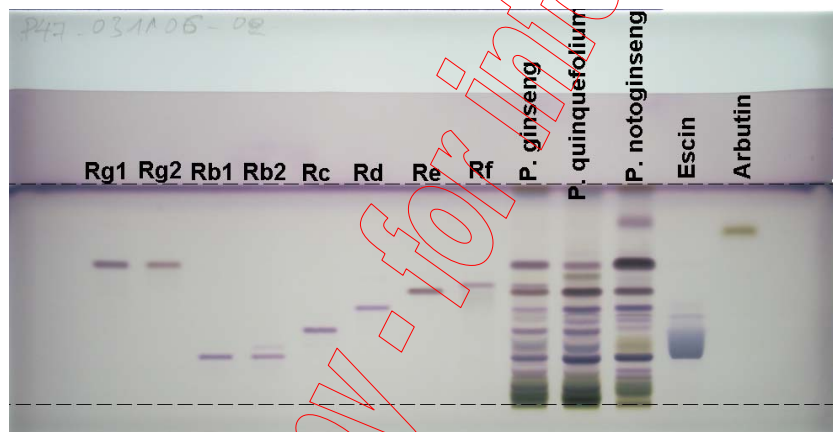
4.1 Mobile phase

Figure 1: Comparison of various mobile phases



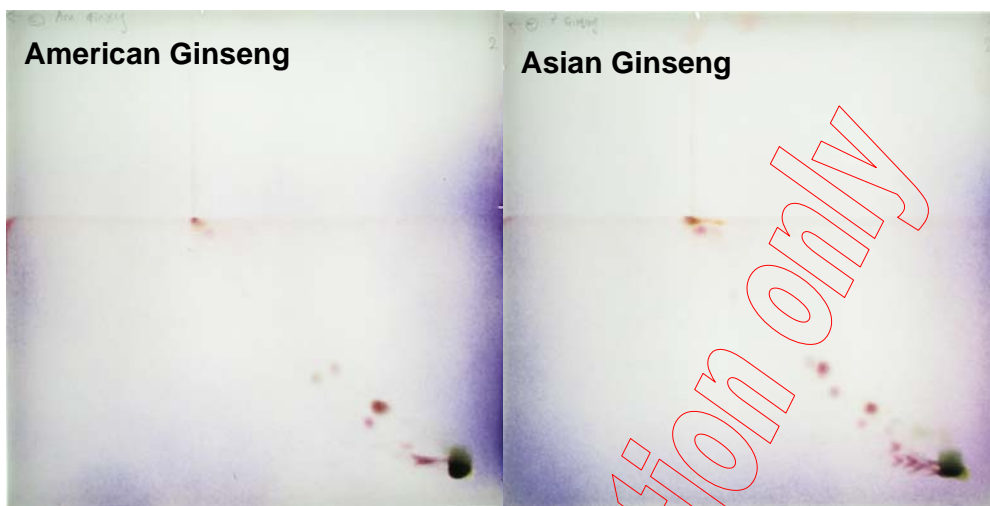
1a, TLC plate

USP/NF 20, 2nd suppl. (Asian ginseng), Ph. Forum 30(2) (Asian ginseng), and Pharmeuropa 15.3: **Developing time 2.5h**



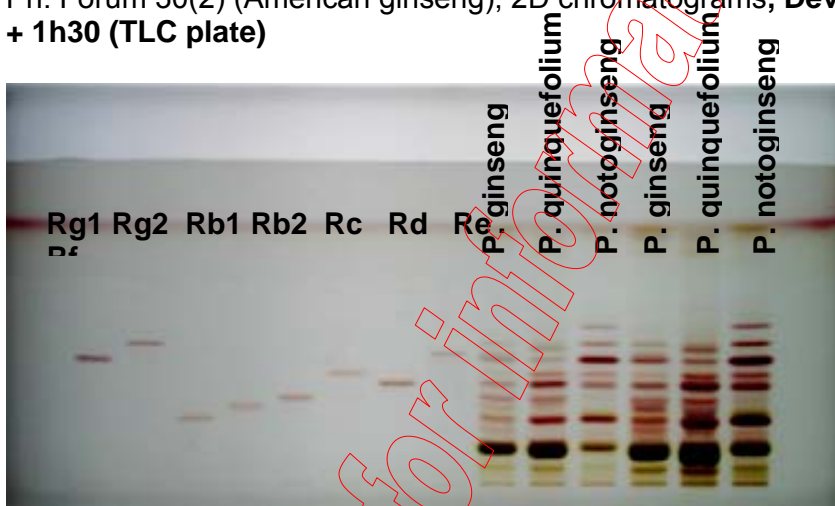
1b, HPTLC plate

Same as 1a, only change to HPTLC plate: **Developing time 1h**



1c

Ph. Forum 30(2) (American ginseng), 2D chromatograms, **Developing time 45 min + 1h30 (TLC plate)**



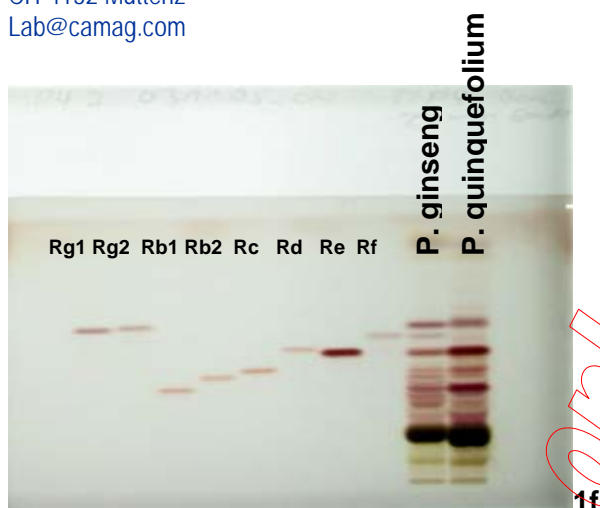
1d

Chinese Pharmacopoeia, **Developing time 20 min (HPTLC plate)**

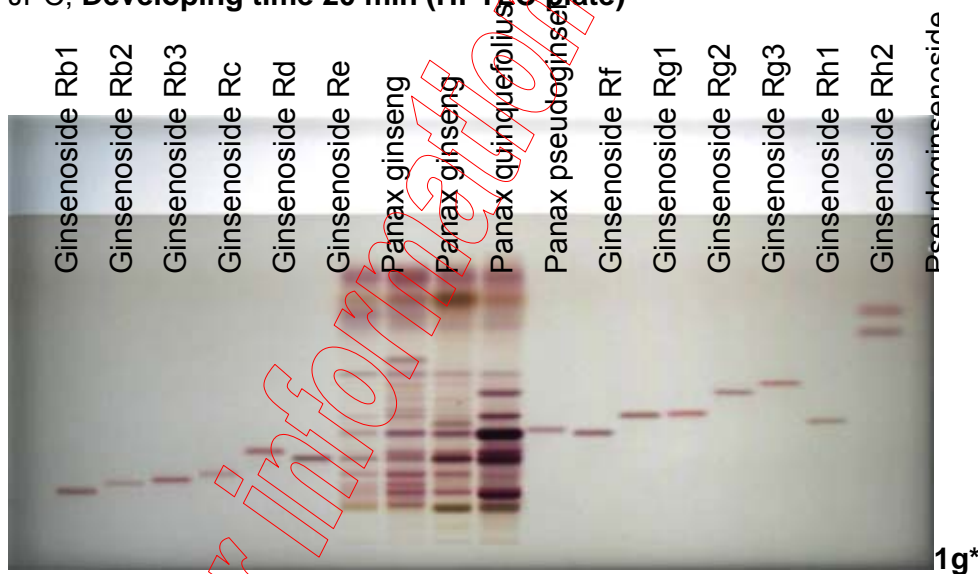


1e

Wagner TLC Atlas, **Developing time 20 min (HPTLC plate)**



JPC, Developing time 20 min (HPTLC plate)



Modified Chinese Ph, 20 min (HPTLC plate)

*The following mobile phase will be used: Chloroform, ethyl acetate, methanol, water (15:40:22:9)

4.2 Extraction and application volume

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

- 1 g of powdered drug is sonicated with 10 mL of methanol for 10 min and centrifuged. **Time required: 20 min**
- 1 g of powdered drug is sonicated with 10 mL of methanol-water (70:30) for 10 min and centrifuged. **Time required: 20 min**
- 1 g of powdered drug is sonicated with 10 mL of absolute ethanol for 10 min and centrifuged. **Time required: 20 min**
- 1 g of powdered drug is sonicated with 10 mL of ethanol-water (90:10) for 10 min and centrifuged. **Time required: 20 min**

- e) 1 g of powdered drug is refluxed in soxhlet with 50 mL of petroleum ether (40-60°C) for 3 h. Petroleum ether is discarded and residue dried. Next day, the residue is refluxed in soxhlet with 60 mL of methanol for 6 h. The methanol phase is dried (rotavap) and the residue dissolved in 10 mL of water. The water is extracted 2x with 20 mL of water-saturated butanol each. The butanol phases are combined and washed 2x with 5 mL of water each. Water phases are discarded (1 mL is kept for test: e1). Butanol is evaporated and the residue redissolved in 10 mL of methanol. **Time required: 12h**
- f) Method ChP: 1 g of powdered drug is refluxed with 40 mL of chloroform to defat. Solution is filtered and chloroform discarded. Residue is dried in air, then moistured with 0.5 mL of water and 10 mL of water-saturated butanol. This mixture is sonicated for 30 min. 3 mL of ammonia 25% are added and the solution mixed, then centrifuged. The supernatant is evaporated to dryness and redissolved in 1 mL of methanol. **Time required: 2h**
- g) Method ChP, without defating: 1 g of powdered drug moisturized with 0.5 mL of water and 10 mL of water-saturated butanol. This mixture is sonicated for 30 min. 3 mL of ammonia 25% are added and the solution mixed, then centrifuged. The supernatant is evaporated to dryness and redissolved in 1 mL of methanol. **Time required: 1h**



Figure 2: Comparison of extraction methods

Application volume: 15 µL of each sample solution

**Extraction will be performed as follows: 1 g of milled root is extracted by sonication with 10 mL of absolute ethanol for 10 min. The solution is centrifuged and the supernatant used as test solution. Application volume: 10µL are sufficient.*

4.3 Derivatization

Various derivatization reagents from the literature were tested.

Most methods require anisaldehyde reagent, which yields violet colored ginsenoside zones (Figure 3a). However, the plate background shows violet and yellow (daylight) or green (UV 366 nm) zones across the plate.

Derivatization with sulfuric acid reagent produce less colored zones in white light, but allows the differentiation of panaxadiol (blue) and panaxatriol (brownish) derivatives under UV 366 nm.

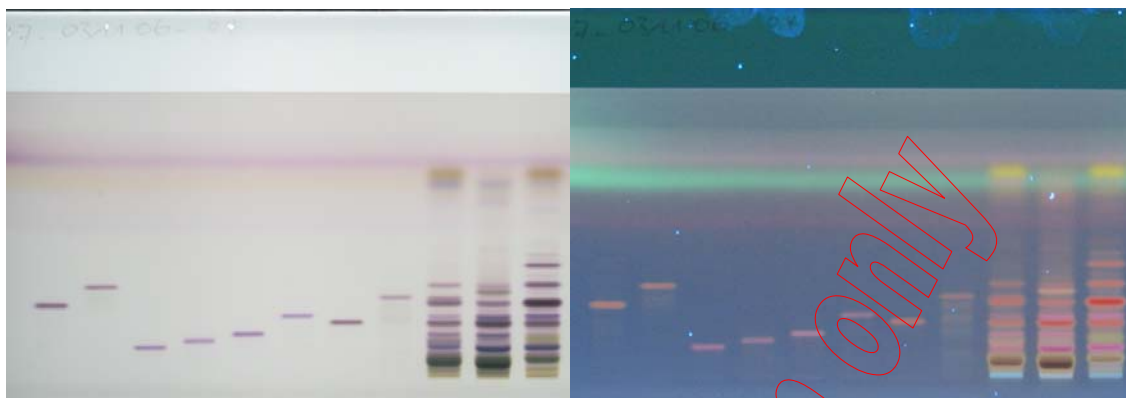
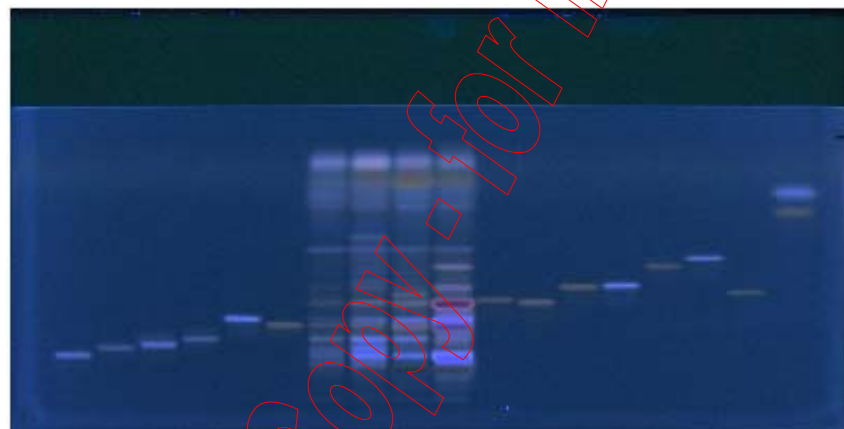


Figure 3a: Derivatization with anisaldehyde reagent (left: white light, right: UV 366 nm)



white light*



UV 366 nm*

Figure 3b: Derivatization with sulfuric acid reagent

**Derivatization will be performed with sulfuric acid reagent.*

4.4 Developing distance

4.5 Stability of sample in the chromatographic system (2D chromatogram).

4.6 Stability of sample in solution and on the plate.

4.7 Influence of humidity/temperature/chamber conditioning.

4.8 Reproducibility of the method.

See Validation protocol.

4. Written Procedure / Method to be validated

4.1 Preparation of test solutions

1 g of powdered raw material is mixed with 10 mL of absolute ethanol, sonicated for 10 min, and centrifuged. The supernatant is used as test solution.

4.2. Preparation of reference solutions

Botanical reference solution: 1 g of powdered raw material is mixed with 10 mL of absolute ethanol, sonicated for 10 min, and centrifuged. The supernatant is used as reference solution.

Chemical reference solutions: 1 mg of each ginsenoside (Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, Rg3, Rh1, Rh2), pseudoginsenoside F11, panaxadiol, and panaxatriol is dissolved in each 5 mL of methanol¹.

4.3. Preparation of derivatizing reagent

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

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 and 5 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.

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, ethyl acetate, methanol, water (15:40:22:9), 10 mL (respectively 20 mL) developing solvent per trough.
Developing distance:	80 mm from lower edge of plate (72 mm from application position)
Drying:	5 min with cold air (hair dryer)

4.8 Derivatization

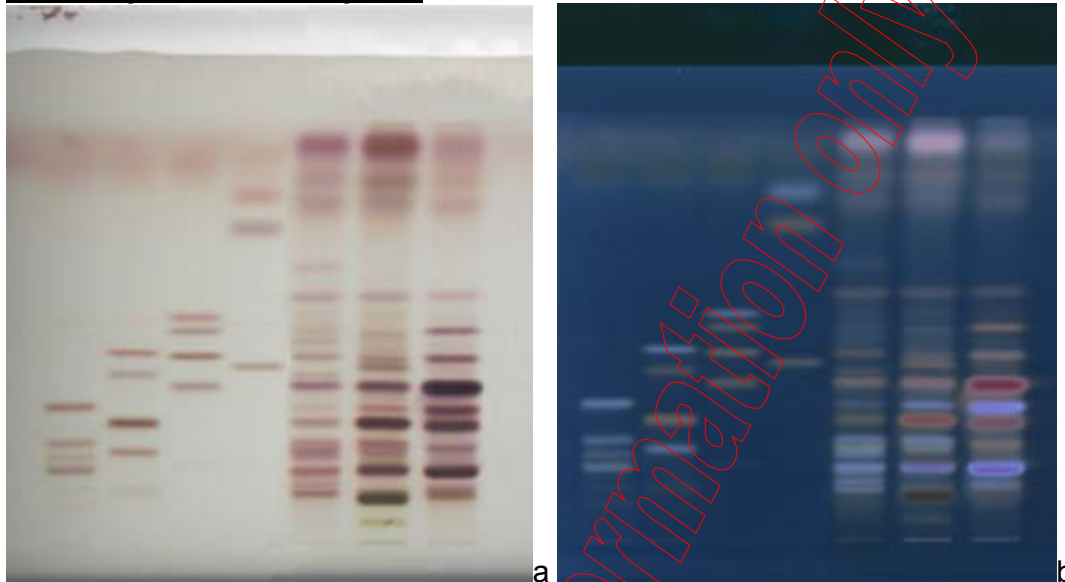
The plate is immersed into reagent for 1 s, then heated at 100°C for 5 min.

¹ Methanol is more common as solvent in other analytical techniques such as HPLC. Using methanol, the standards can also be used otherwise.

4.9 Documentation

- a) After derivatization under white light
- b) After derivatization under UV 366 nm

4.10 Images of chromatograms



- 1: Ginsenosides Rb1, Rb2, Rc, Rd
- 2: Ginsenosides Rb3, Re, Rf, Rg3
- 3: Ginsenosides Rg1, Rg2, Rh1, Rh2
- 4: Pseudoginsenoside F11, panaxatriol, panaxadiol
- 5: *Panax ginseng* (Asian ginseng)
- 6: *Panax quinquefolium* (American ginseng)
- 7: *Panax notoginseng* (syn. *Panax pseudoginseng*)