

Evaluation and optimization of Identification Method for Licorice by HPTLC fingerprint

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

Goals: Develop a fingerprint for the analysis of Licorice root (definition USP: *Glycyrrhiza glabra* or *G. uralensis* root). Establish whether both species can be distinguished from each other. Use glycyrrhizic acid ammonia salt (ammonium glycyrrizate) as a standard.

2. Methods from literature, paper review:

Methods from literature, paper review:

Literature (see appendix)	Scope	Mobile phase / Stationary phase	Refer to Figure #
USP PF	Analysis/detection of glycyrrhizic acid	Butyl alcohol, water, acetic acid 7:2:1 / Silica gel (conventional)	1
Ph.Eur.4	Analysis/detection of glycyrrhetic acid after hydrolysis of glycyrrhizic acid	concentrated ammonia, water, alcohol, ethyl acetate 1:9:25:65 / Silica gel (conventional)	2
Indian Herbal Pharmacopoeia	Analysis/detection of glycyrrhetic acid after hydrolysis of glycyrrhizic acid	Toluene, ethyl acetate, acetic acid 12.5:7.5:0.5 / Silica gel (conventional)	3
Wagner TLC Atlas	Separation of saponins	Chloroform, ethanol, water, ammonia 60:32:12:8 / Silica gel (conventional)	4
	Analysis/detection of glycyrrhetic acid	As Ph.Eur 4	2
	Flavonoids	Ethyl acetate, acetic acid, formic acid, water 100:11:11:26 / Silica gel (conventional)	--
Chinese Ph.	Flavonoids	Ethyl acetate, acetic acid, formic acid, water 15:1:1:2 / Self-coated plates containing NaOH	-- (no possibility of plate coating)
Adaptation of Chinese Ph.	Flavonoids	Ethyl acetate, acetic acid, formic acid, water 15:1:1:2 / HPLTC silica gel	5

3. Material

Samples available:

Sample Name	Source	Authentication	Notes
Licorice Root powder (<i>G.glabra</i>)	Phytolab, 2701303	Yes	Sample distributed to all collaborating labs
<i>Glycyrrhiza uralensis</i> Fisch.	China Nikyang Ltd	Yes	Herbarium
<i>Glycyrrhiza glabra</i>	China Nikyang Ltd	Yes	Herbarium
Commercial Licorice Slices 2 mm x 3-4 cm (<i>G. glabra</i> ?)	China Nikyang Ltd	No	Market China
<i>Glycyrrhiza glabra</i> Root AUIZ	Received from INA 08/03	Yes	
<i>Glycyrrhiza uralensis</i> Root TCM Collection No 435	Received from INA 10/03	Yes	
<i>Glycyrrhiza glabra</i> Root AUIZ	Botanical Liaisons AV12	Yes	
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-1	?	South Hungary
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-2	?	South Hungary
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-3a	?	Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-3b	?	Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-4a	?	South Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-4b	?	South Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-4c	?	South Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-5	?	Hungary
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-6	?	South Italy
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-11	?	
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-12	?	Russia
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-13	?	Ukraine
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-14	?	
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-15	?	
<i>Glycyrrhiza glabra</i> Root	VitaPlant, VIP'GG-16	?	

Standards (marker compounds) available:

Name	Source
Ammonium glycyrrhizate (=glycyrrhizic acid)	Roth, lot 23236047

Adulterants available:

Since no adulterants for Licorice root are known and reported to in the literature, no other plant was included in this trial. Two Licorice species (*G. glabra* and *G. uralensis*) are included.

Processed materials available:

Name	Source	Notes
Licorice root capsules, 450 mg	Received from INA 07/03	Root powder (no extract)
DGL Licorice extract Lozenge 400 mg	Received from INA 07/03	De-glycyrrhinated extract
Licorice + herbal blended extract (liquid)	Received from INA 07/03	Concentration unknown

Plates

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

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

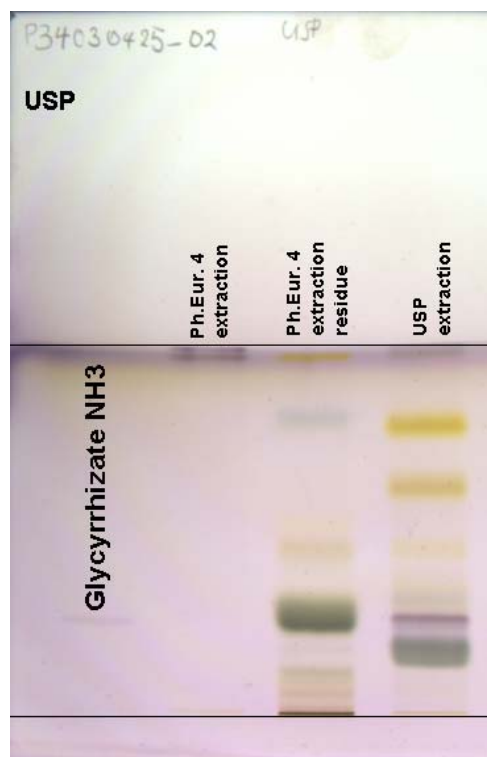


Figure 1: USP
Developing distance/time: 10cm/3h



Figure 2: Ph.Eur.4
Developing distance/time: 15cm/1h h20

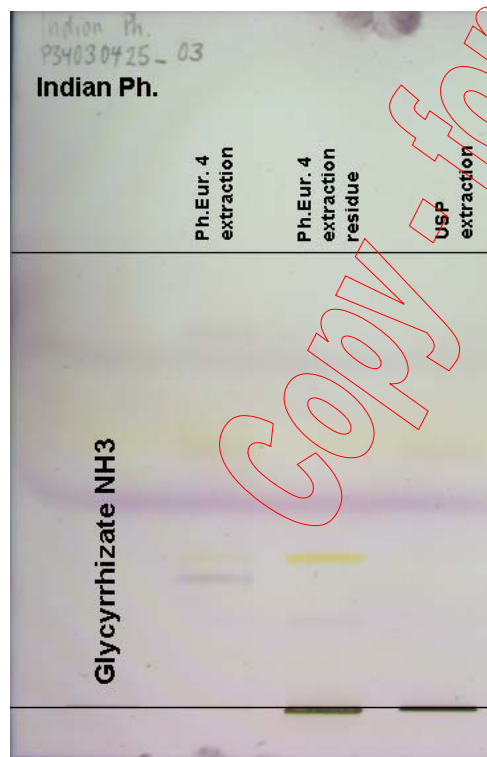


Figure 3: Indian Herbal Ph.

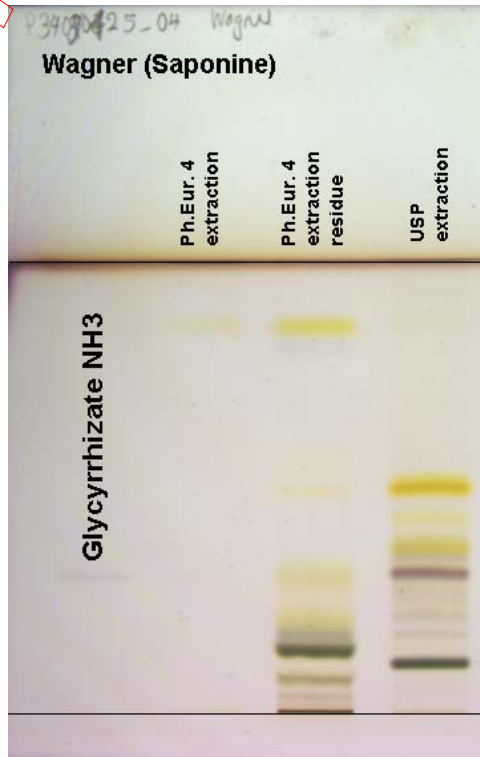


Figure 4: Wagner (saponins)

Developing distance/time: 12 cm/40 min Developing distance/time: 12cm/1h15

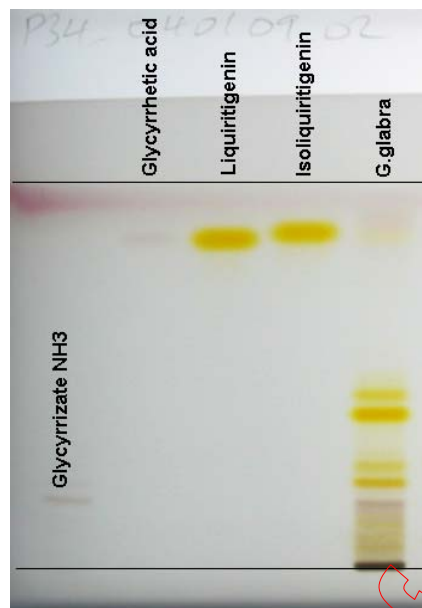


Figure 5: Modification of Chinese Ph.

Developing distance/time: 6 cm/15 min (other standards were included for testing purpose).

The choice of the mobile phase and stationary phase was made according to the following criteria:

- *Generating a fingerprint (sequence of colored zones): USP, Wagner, and Chinese Ph. methods provide clear fingerprints.*
- *Ability to detect glycyrrhizic acid (Rf value, detection in the sample, and separation from other compounds): Ph.Eur.4 and Indian Herbal Ph. methods are not suitable.*
- *Easiness of use (rapidity): All methods using conventional TLC plates, especially the USP method, are very time consuming.*
- *Safety: Wagner method uses chloroform.*

*The best performance was given by the modified Chinese Ph. method.
This method will be used.*

Sample used: Licorice root, milled (*Glycyrrhiza glabra*), Phytolab, 2701303

4.2 Extraction and application volume

Extraction following USP: 2g sample are extracted with 10 mL of ethanol-water 70:30 on a water bath for 5 min.

Alternative 1: 0.5g sample are sonicated with 10 mL of ethanol-water 70:30 for 10 min.

Alternative 2: 0.5g sample are sonicated with 10 mL of ethanol for 10 min.

Alternative 3: 0.5g sample are sonicated with 10 mL of methanol for 10 min.



Figure 7: Comparison of extraction methods

0.5, 1, 2, and 5 μL of each solution were applied. Sample used: Licorice root, milled (*Glycyrrhiza glabra*), Phytolab, 2701303

The USP extract is 4x more concentrated than the alternative extracts. 0.5 μL of USP extract should be equivalent to 2 μL of the alternatives. Only alternative 1 gives similar intensity of zones. Alternative 2 does not extract glycyrrhizic acid (arrow).

The alternative extraction method 1 will be used, because of the easiest preparation, and 2 μL of extract applied.

4.3 Developing distance

In this example, variations in developing distance were not tested. The compounds of interest (glycyrrhizic acid) are separated.

4.4 Derivatization

USP: no derivatization, detection under UV 254 nm. Anisaldehyde or sulfuric acid reagents are referred to in the literature.



Figure 8a: UV 254 nm (no derivatization)
Arrow: Glycyrrhizic acid



Figure 8b:
Derivatization by dipping in sulfuric acid solution and heating at 100°C for 3 min
Glycyrrhizic acid is not detected



Figure 8c:
Derivatization by dipping in sulfuric acid solution and heating at 100°C for 10 min
Arrow: Glycyrrhizic acid

Sample used: Licorice root, milled (*Glycyrrhiza glabra*), Phytolab, 2701303

No images of plate derivatized with anisaldehyde reagent. Results are similar to those with sulfuric acid reagent. However, sulfuric acid yields usually more reproducible and stable results. Therefore, the plate will be looked at under 254 nm prior and under white light after derivatization with sulfuric acid. Heating step must be 10 min in order to complete color development of glycyrrhetic acid.

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



Figure 9: 2D Chromatogram of a Licorice sample. Left: prior to and right: after derivatization by dipping in sulfuric acid solution. Arrow: glycyrrizic acid. Sample used: Licorice root, milled (*Glycyrrhiza glabra*), Phytolab, 2701303

The sample is stable in the chromatographic system.

3.5 Stability of sample in solution and on the plate.

3.6 Influence of humidity/temperature/chamber conditioning.

3.7 Reproducibility of the method.

Not investigated in this example.

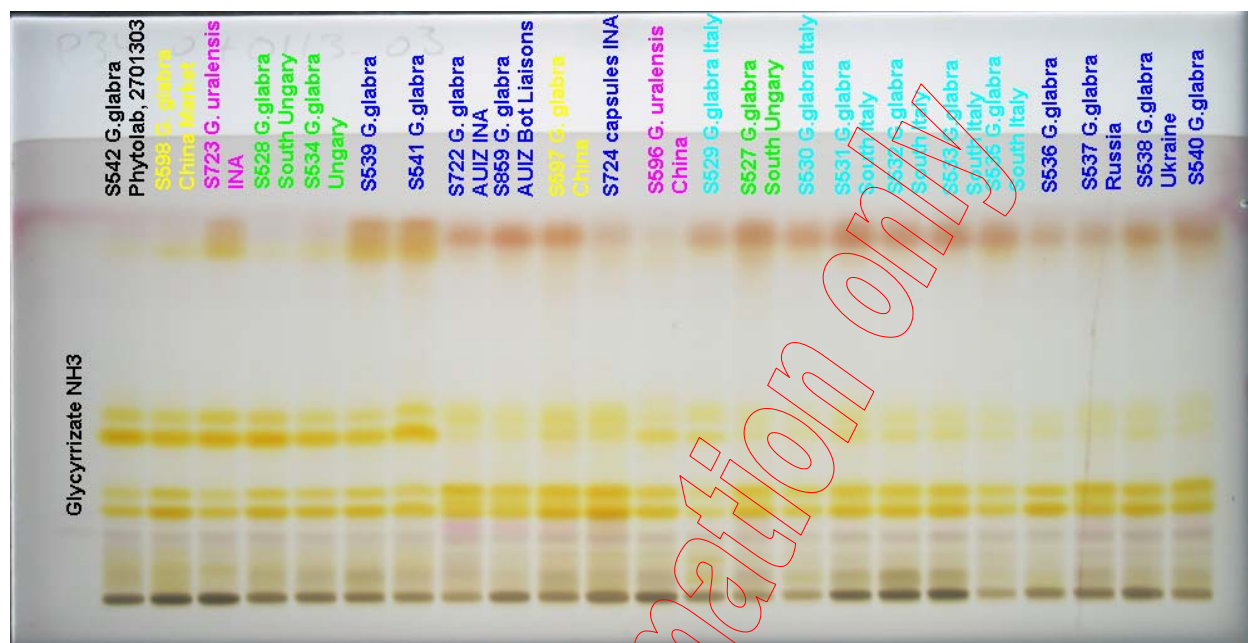


Figure 6: Comparison of all samples

All samples contain the marker ammonium glycyrrhizate.

The presence of 2 clusters of fingerprints is apparent, independent of the denomination of the samples. All samples seem to contain the same compounds, but in different relative amounts.

It is impossible to link one fingerprint with one species.

The Licorice root capsule was the only processed material which gave results using this method. The method didn't work for the DGL-lozenges and for the liquid blended extract provided. Those samples were not included in this trial.

5. Written procedure / Method to be validated

5.1 Preparation of test solutions

0.5 g of milled root (or enough product equivalent to that amount) are sonicated for 10 min with 10 mL ethanol-water 70:30. The solution is centrifuged and the supernatant is used as test solution.

5.2 Preparation of reference solutions

1 mg ammonium glycyrrhizate is dissolved in 10 mL ethanol-water 70:30.

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

2 µL of test solution and 10 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: Ethyl acetate, formic acid, acetic acid, water (15:1:1:2); 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 10 min.

5.9 Documentation

- a) Prior to derivatization under UV 254 nm
- b) After derivatization under white light

Appendix

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PHARMACOPEIAL PREVIEWS

Pharmacopoeial Forum
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pharmacopoeias recognize a third: *G. inflata*. Reviewers are encouraged to submit comments on whether *G. inflata* is used interchangeably with *G. glabra* and *G. uralensis*, and whether it is equivalent in quality. The liquid chromatographic procedure in the test for *Content of glycyrrhizic acid* is based on analyses performed with the Develosil ODS 5- μ m brand of L1 column. The typical retention time for glycyrrhizic acid is 9.5 minutes.

7L00500 (NAT) RTS—23898-01

Add the following:

Licorice

» Licorice consists of the roots, rhizomes, and stolons of *Glycyrrhiza glabra* Linné or *Glycyrrhiza uralensis* Fisher (Fam. Leguminosae). It contains not less than 2.5 percent of glycyrrhizic acid (C₄₂H₆₂O₁₆), calculated on the dried basis.

Packaging and storage—Preserve in well-closed containers. Store in a cool, dry place.

Labeling—The label states the Latin binomial name and, following the official name, the part of the plant contained in the article.

USP Reference standards (11)—*USP Glycyrrhizic Acid RS*.

Botanic characteristics—

Macroscopic—The terrestrial stem is nearly cylindrical, 0.5 to 3.0 cm in diameter and over 1 m in length; externally dark brown to red-brown, longitudinally wrinkled; often has lenticels, small buds, and scaly leaves. The transverse section reveals a rather clear border between phloem and xylem, and a radial structure that often has radiating splits.

Microscopic—The transverse section reveals several yellow-brown cork layers, and a layer of phelloderm that is 1 to 3 cells thick. The cortex exhibits medullary rays, and obliterated sieve portions radiate alternately. The phloem exhibits groups of phloem fibers, which are surrounded by crystal cells, with thick but incompletely lignified walls. The vessels are accompanied by xylem fibers, which are surrounded by crystal cells, and by xylem parenchyma cells. The parenchyma cells contain starch grains, and often contain single crystals of calcium oxalate.

Identification, Thin-Layer Chromatographic Identification Test (201)—

Test solution—Add 10 mL of a mixture of alcohol and water (7:3) to 2.0 g of pulverized Licorice, heat by shaking on a water bath for 5 minutes, cool, and filter.

Standard solution—Dissolve 5 mg of USP Glycyrrhizic Acid RS in 1 mL of a mixture of alcohol and water (7:3).

Application volume: 2 μ L.

Developing solvent system: a mixture of butyl alcohol, water, and glacial acetic acid (7:2:1).

Procedure—Proceed as directed in the chapter, except to develop the chromatogram in an unsaturated chamber to a length of about 10 cm. Examine the plate under UV light at a wavelength of 254 nm. The chromatograms show a dark purple zone, among other spots, due to glycyrrhizic acid at an *R_f* value of about 0.4.

Microbial limits (201)—It meets the requirements of the tests for absence of *Salmonella* species and *Escherichia coli*. The total bacterial count does not exceed 10⁷ per g, the total combined molds and yeasts count does

Ph. Eur. 4

DEFINITION

Liquorice root consists of the dried unpeeled or peeled, whole or cut root and stolons of *Glycyrrhiza glabra* L. It contains not less than 4.0 per cent glycyrrhizinic acid (C₄₂H₆₂O₁₆, Mr 823), calculated with reference to the dried drug.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. To 0.50 g of the powdered drug (180) in a 50 ml round-bottomed flask add 16.0 ml of water R and 4.0 ml of hydrochloric acid R1 and heat on a water-bath under a reflux condenser for 30 min. Cool and filter. Dry the filter and the round-bottomed flask at 105 °C for 60 min. Place the filter in the round-bottomed flask, add 20.0 ml of ether R and heat in a water-bath at 40 °C under a reflux condenser for 5 min. Cool and filter. Evaporate the filtrate to dryness. Dissolve the residue in 5.0 ml of ether R.

Reference solution. Dissolve 5.0 mg of glycyrrhetic acid R and 5.0 mg of thymol R in 5.0 ml of ether R.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of concentrated ammonia R, 9 volumes of water R, 25 volumes of alcohol R and 65 volumes of ethyl acetate R. Allow the plate to dry in air for 5 min and examine in ultraviolet light at 254 nm. The chromatograms obtained with the test solution and with the reference solution show in the lower half a quenching zone due to glycyrrhetic acid. Spray the plate with anisaldehyde solution R, and heat at 100 °C to 105 °C for 5 min to 10 min. Examine in daylight. The chromatogram obtained with the reference solution shows in the lower half the violet zone of glycyrrhetic acid and in the upper third the red zone of thymol. The chromatogram obtained with the test solution shows in the lower half of violet zone corresponding to the zone of glycyrrhetic acid in the chromatogram obtained with the reference solution and a yellow zone (isoliquiritigenine) in the upper third under the zone of thymol in the chromatogram obtained with the reference solution. Further zones may be present.

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TLC IDENTITY TEST

Test solution : Shake 1g of the drug with 20ml chloroform for 15 min . Filter and discard filtrate. Reflux the marc for 1 hr. with 30 ml of 0.5 M H₂SO₄. Cool and shake the unfiltered mixture with chloroform (2 x20ml) and concentrate the combined CHCl₃ extract. Dissolve the residue in 1.0 ml CHCl₃ : MeOH (1:1) mixture.

Reference solution : Reflux 5 mg of glycyrrhizin with 20 ml 0.5 M H₂SO₄. Cool and extract with chloroform (2 x 10 ml). Evaporate the combined CHCl₃ extract and dissolve the residue in 1.0 ml CHCl₃ : MeOH (1:1) mixture.

Solvent system : Toluene : ethyl acetate : gl. acetic acid (12.5: 7.5: 0.5)

Procedure : Apply 5 µl each of Test solution and Reference solution in two different tracks on a precoated silica gel G F₂₅₄ plate (5 x 15cm) of uniform thickness (0.2 mm). Develop the plate in the solvent system to a distance of 12 cm.

Scanning: Scan densitometrically at 254nm both reference and test solution tracks and record the fingerprint profiles.

Visualization of spots :

- (i) Under UV 254 nm.
- (ii) Spray the plate with Anisaldehyde-sulfuric acid reagent and heat at 110°C for 5-10 min.

Evaluation :

- (i) Under UV 254 nm light (before spraying): Two spots (0.41, 0.45) exhibiting quenching are visible in the sample solution track, one of which (Rf 0.41) corresponds to glycyrrhetic acid of reference track.
- (ii) In daylight (after spraying) : Glycyrrhetic acid is visible as a dark violet spot in both reference and test solution tracks. Other spots visible in the test solution includes two dark yellow spots (Rf. 0.45, 0.49), two violet spots (Rf 0.27, 0.70) and a dark blue spot running along with the solvent front.

Wagner and Bladt

Liquiritiae radix

Drug sample	1 Liquiritiae radix	(ethanolic extract, 20 µl)	
Reference compound	T1 glycyrrhizin	T3 glycyrrhetic acid	
	T2 aescin	T4 rutin ($R_f \sim 0.3$)	▶ hyperoside ($R_f \sim 0.55$)
Solvent system	Fig. 10A chloroform-glacial acetic acid-methanol-water (60:32:12:8)		→ saponins
	B ethyl acetate-ethanol-water-ammonia (65:25:9:1)		→ glycyrrhetic acid
	C + D ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26)		→ flavonoids
Detection	A,B Anisaldehyde-sulphuric acid reagent (AS No. 3)		→ vis.
	C Natural products-polyethylene glycol reagent (NP/PEG No. 28)		→UV-365 nm.
	D 50% ethanolic H ₂ SO ₄ (No. 37)		→vis.

- Fig. 10A** **Liquiritiae radix** (1) shows six to seven blue, violet and brown zones in the R_f range 0.1–0.65 in solvent system A. The main saponin glycyrrhizin is detected with AS reagent as a violet zone in the R_f range 0.35–0.4 (T1, R_f similar to aescin/T2), directly below a major brown zone (flavonoids and chalcones).
- B** The aglycone glycyrrhetic acid (T3), which migrates in solvent system A to the solvent front, is found in solvent B at $R_f \sim 0.45$.
- C** The flavanon glycosides and chalcones are separated in solvent C. They fluoresce with NP/PEG reagent yellow-white (R_f 0.15–0.3) and dark green ($R_f \sim 0.4$ and $R_f \sim 0.75$) in UV-365 nm.
- D** With sulphuric acid the flavanon glycosides (e.g. liquiritin, liquiritoside) and the corresponding chalcones appear as characteristically orange-yellowish brown zones (vis).
- Note:* For the detection of glycyrrhizin, see also Chap. 15.

Chinese Pharmacopoeia

Radix Glehniae (北沙参, Beishashen)

Coastal Glehnia Root

Coastal Glehnia Root is the dried root of *Glehnia littoralis* Fr. Schmidt ex Miq. (Fam. Umbelliferae). The drug is collected in summer and autumn, removed from rootlet, washed clean, partially dried in the air, treated with boiling water, peeled and dried, or dried immediately after washing.

Description Slenderly cylindrical, branching occasionally. 15~45 cm long, 0.4~1.2 cm in diameter. Externally yellowish-white, slightly rough, occasionally with patches of cork adhering, or yellowish-brown when unpeeled. Finely wrinkled longitudinally, and with brownish-yellow spotted rootlet scars. Top usually with yellowish-brown remains of rhizome. The upper part somewhat thin, the middle part relatively thick, and the lower part tapering. Texture fragile, easily broken, fracture yellowish-white in bark and yellow in wood. Odour, characteristic; taste, sweetish.

Identification Transverse section: Cortex of several layers of parenchymatous cells, scattered with secretory canals. Cork visible when unpeeled. Phloem broad, rays distinct, sieve tube groups collapsed in the outer part and appearing as a narrow band; secretory canals scattered, 20~65 µm in diameter, containing yellowish-brown secretion, surrounded by 5~8 secretory cells. Cambium in a ring. Xylem rays 2~5 cells wide; most vessels arranged in V-shape; parenchymatous cells containing gelatinized starch granules.

Processing Remove remains of stems and foreign matter, soften slightly, cut into sections, and dry.

Action To replenish *yin* of the lung and stomach, remove heat from the lung, and promote fluid secretion.

Indications Dry cough caused by heat in the lung; bloody sputum in phthisis; thirst in febrile diseases.

Usage and dosage 4.5~9 g.

Precaution Incompatible with Rhizoma et Radix Veratri.

Storage Preserve in a ventilated and dry place, protected from moth.

Radix Glycyrrhizae (甘草, Gancao)

Liquorice Root

Liquorice Root is the dried root and rhizome of *Glycyrrhiza uralensis* Fisch., *Glycyrrhiza inflata* Bat. or *Glycyrrhiza glabra* L. (Fam. Leguminosae). The drug is collected in spring and autumn, removed from rootlet, and dried in the sun.

Description Root of *Glycyrrhiza uralensis* Roots cylindrical, 25~100 cm long, 0.6~3.5 cm in diameter. The outer bark loose or tight. Externally reddish-brown or greyish-brown, obviously longitudinally wrinkled, furrowed, lenticellate, and with sparse rootlet scars. Texture compact, fracture slightly fibrous, yellowish-

white, starchy, cambium ring distinct, rays radiate, some with clefts. Rhizomes cylindrical, externally with bud scars, pith present in the centre of fracture. Odour, slight; taste, sweet and characteristic.

Root of *Glycyrrhiza inflata* Roots and rhizomes woody and stout, some branched, the outer bark rough, mostly greyish-brown. Texture compact, more lignified, fibres and less starchy. Rhizomes with more and large adventitious buds.

Root of *Glycyrrhiza glabra* Texture of root and rhizomes relatively compact, some branched, the outer bark not rough, mostly greyish-brown, lenticels small and indistinct.

Identification (1) Transverse section: Cork consisting of several layers of brown cells. Cortex relatively narrow. Phloem rays broad, mostly curved, frequently with clefts; most phloem fibres in bundles, unligified or slightly lignified, surrounded by parenchymatous cells containing prisms of calcium oxalate; sieve tube tissue often pressed to be collapsed. Fascicular cambium distinct. Xylem rays 3~5 cells wide; vessels frequent, up to 160 µm in diameter; xylem fibres in bundles, surrounded by parenchymatous cells containing prisms of calcium oxalate. Roots without pith at the centre; rhizomes possessing pith at the centre. Powder: Brownish-yellow. Fibres in bundles, 8~14 µm in diameter, thick-walled, slightly lignified, surrounded by parenchymatous cells containing prisms of calcium oxalate, forming crystal fibres. Prisms of calcium oxalate frequent. Bordered pitted vessels large, reticulated vessels rare. Cork cells reddish-brown, polygonal, slightly lignified.

(2) To 1 g of the powder add 40 ml of ether, heat under reflux on a water bath for 1 hour, filter. Heat the residue under reflux in 30 ml of methanol on a water bath for 1 hour and filter. Evaporate the filtrate to dryness and dissolve the residue in 40 ml of water. Extract the aqueous solution with three 20-ml quantities of *n*-butanol. Combine the *n*-butanol solutions, wash with water for 3 times and evaporate on a water bath to dryness, dissolve the residue in 5 ml of methanol as the test solution. Prepare a solution of 1g of Radix Glycyrrhizae reference drug in the same manner as the reference drug solution. Dissolve ammonium glycyrrhizate CRS in methanol to produce a solution containing 2 mg per ml as the reference solution. Carry out the method for thin layer chromatography (Appendix V B), using silica gel G prepared with 1% solution of sodium hydroxide as the coating substance and ethyl acetate formic acid-glacial acetic acid-water (15:1:1:2) as the mobile phase. Apply separately to the plate 1~2 µl of each of the three solutions. After developing and removal of the plate, dry it in air. Spray with 10% solution of sulfuric acid in ethanol. Heat at 105°C to visualize clearly, and examine under ultraviolet light (365 nm). The fluorescent spots in the chromatogram obtained with test solution correspond in position and colour to the spots obtained with the reference drug solution; an orange fluorescent spot in the chromatogram obtained with the test solution corresponds in position and colour to the spot in the chromatogram obtained with the reference solution.

Water Carry out the method for Determination of water (Appendix IX H, method 1), not more than 12.0%.

Total ash Not more than 7.0% (Appendix IX K).

Acid-insoluble ash Not more than 2.0% (Appendix IX K).

Residual organochlorine pesticide Hexachlorocyclohexa (total BHC) is not more than 0.00002%. Chlorophenoths (total DDT) is not more than 0.00002%. Pentachloro nitrobenzene(PCNB) is not more than 0.00001% (Appendix IX Q).

Assay Carry out the method for high performance liquid chromatography (Appendix VI D).