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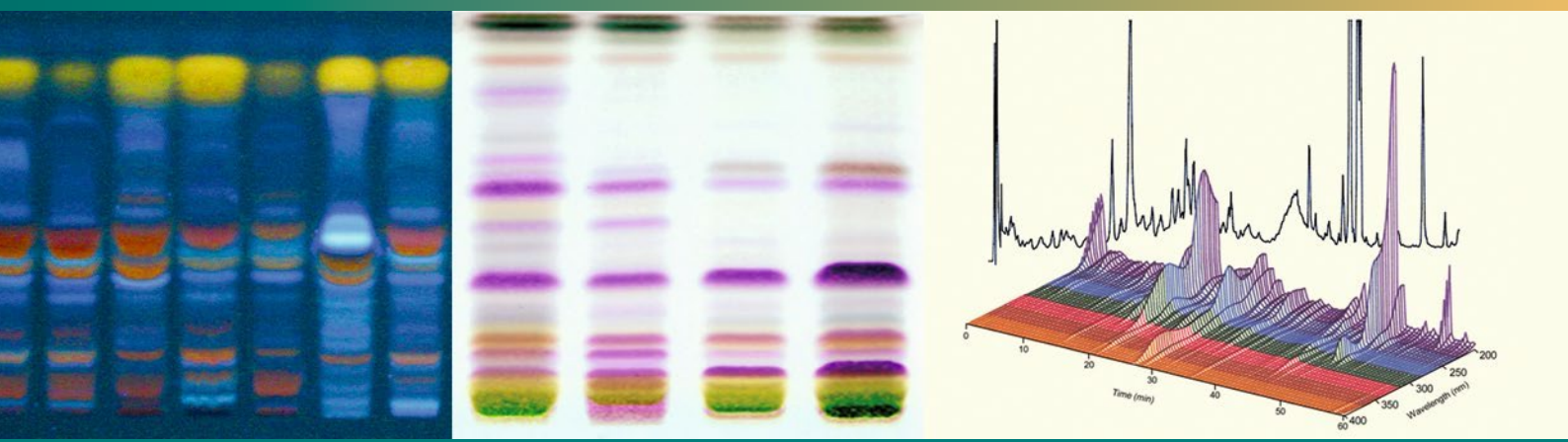
Chromatographic Fingerprint Analysis of Herbal Medicines

Thin-Layer and High Performance Liquid Chromatography of Chinese Drugs



Volume 4

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Erste deutsche Klinik für Traditionelle Chinesische Medizin
Fachklinik für Psychosomatik und Psychotherapie

University Hospital at Beijing University of Chinese Medicine

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- We are deeply indebted to our technical assistants Mrs. Stefanie Püls and Mrs. Talee Barghouti.

Authors' Contributions

- Supervisor and responsible for the correct performance of the Analytical Monographs of Chinese Herbal Drugs Prof. Dr. Dr. h.c. Hildebert Wagner, Special Analytical Laboratory of the Department of Pharmacy of the University of Munich, Butenandtstr. 5, 81377 Munich; e-mail. h.wagner@cup.uni-muenchen.de

Introduction

- **Legislation**
  Among the various prerequisites for a perfect quality proof of these herbal drugs, authentication and safety proof take first precedence. Identification was in former times primarily synonymous with the macroscopic and microscopic botanical authenticity. Since that time, however, chemical composition and particularly the complex entities of the low molecular constituents have become of greater interest for oral medicinal application and thus in evaluating the pharmacological effects and therapeutic efficacy of the plant drug extracts obtained by decoction or other extraction processes.

- Independent of the specific national drug regulations for countries around the world, there is also an international consensus that all TCM drugs must meet certain, stipulated high-quality standards. Additionally, it must be guaranteed that all TCM drugs prescribed by physicians are safe for patients. The safety proof aims mainly to exclude any kind of possible falsifications of the herbal drugs and the limitation of concentrations of heavy metals, aflatoxins and defined microbial adulterations.

- **Applied Methods of the Qualified Proof**
  The main method used is TLC (thin layer chromatography), which allows us to present the visualized main characteristic constituents in the form of coloured TLC photographs. The second method, used globally, is HPLC (high pressure liquid chromatography) in the form of a so-called fingerprint analysis. This technique allows us to detect the complex entities of all low molecular constituents of a plant drug extract, with the advantage that the single constituents can be made visible in the form of peak profiles. Additionally, the single constituents can be quantified by using online recordable UV spectra with the diode array technique. It is also possible to gain preliminary information as to which chemical structure type the single compounds may belong. From this year forward, LC-MS (liquid chromatography-mass spectroscopy) is also available for the analysis of plant extracts whose chemical compositions have previously been only minimally investigated.

- **Publication of the Analytical Monographs of Investigated Herbal Chinese Drugs**
  The following volumes were published by Springer, Vienna and New York, with financial support from the TCM Clinic Bad Kötzting; Wagner, H., Bauer, R., Melchart, D., Xiao, P.-G., Staudinger, A. (Eds.)

  – Vols. I and II (2011) containing 80 analytical monographs
  – Vol. III (2015) containing 23 analytical monographs
  – Vol. IV (2016) containing 22 analytical monographs
  – Vol. V (in preparation; publication scheduled for February/March 2017)

- Note: All single analytical monographs that are already edited can be downloaded at http://www.springer.com/de/book/9783709107621

Prospects for the Improvement of the Quality Proof of Chinese Herbal Drugs

1. **Authenticity of TCM drugs not definitely assessable**
   Some herbal drugs are not yet produced under controlled cultivation but originate from wild collections. Even if they are derived from cultivations, it must be taken into account that they can originate from quite varied climate zones and that they may be harvested under a variety of conditions. Therefore, their chemical authenticity and homogeneity within a defined plant species often cannot be guaranteed. We have thus investigated as many herbal drug samples as we were able to acquire from different districts, climate zones and markets in China, as well as reference drugs from some German herbal drug firms that also import herbal drugs from China.
2. For 5–10% of imported plant drugs from China, we do not receive specific information about the plant part (Flos, Fructus, Semen, Folium, Cortex or Radix and Rhizoma) from which they were collected. Such drugs are specified as "herba" analogues. For these drug samples, it cannot be expected that the TLC and HPLC chemical fingerprints are very homogenous. Not all parts of a herbal drug contain the same chemical constituents. The documentation in the corresponding herbal analytical monographs confirms this judgement (see e.g. Herba Leonuri, Vol. II; Herba Lysimachiae, Vol. III; or Herba Violae, Vol. IV). Therefore, it will be necessary that this discrepancy has to be corrected in the future. Otherwise, it cannot be expected that the results of clinical application can be reproduced.
3. **Uncertain botanical nomenclature**
   The non-uniform nomenclature for the same plant in various regions of China can cause impermissible substitutions or falsifications. This occurred some years ago when the root of *Stephania tetrandra* (Hanfangji) was mistaken for the root of *Aristolochia fangji* (Guanfangji). The latter of both contains the carcinogenic aristolochic acid which can produce severe nephrotoxic side effects. A similar Chinese drug is the tetraploid *Acorus tatarinowii* which differs in a very high content of carcinogenic β-asarone from that of the diploid *Acorus calamus*, known officially in most western countries. Meanwhile special chromatographic methods were developed and described in the analytical monographs to avoid such falsifications.
4. **Great variability of plant species**
   Several herbal drug monographs of the Chinese Pharmacopoeia list more than two species or subspecies and sometimes up to eight species labelled as synonyms, subspecies or subvarieties. It is assumed that all species contain the same constituents in the same amount. In our 20 years running TLC- and HPLC-fingerprint investigations, we have shown that in many cases considerable differences were detectable between the single species and the main official drugs. Correspondingly, it may be suggested that a great number of the "subspecies" do not possess the same pharmacological and therapeutic efficacy. This fact must be recognized and taken into consideration!

Guidelines for the Experimental Work

Source of the Herbal Drugs

As discussed in the preceding paragraph, the herbal drugs must originate from clearly identified botanical species. Additionally, it must be taken into consideration that differences in cultivations, climatic conditions, time of harvest, drying and storing conditions can cause slight chromatographic deviations which cannot be avoided and are normal. Therefore, it is worthwhile to investigate as many herbal drug samples of one species as can be obtained from different geographic and ecological areas.

Extraction Conditions

The chosen extraction procedures should be fast but efficient according to present scientific knowledge and inclusive of the total entity of the low molecular constituents of a herbal drug. This can be achieved in most cases using alcohol (MeOH or EtOH). Additional fingerprints can be obtained by extraction using petroleum ether/hexane or chloroform (for lipophilic compounds) or water/water-acetone mixtures (for tannins, high polymeric procyanidins and amino acids) as solvents. Polysaccharides and proteins can be characterized via their sugar or amino acid fingerprints after enrichment and acidic or enzymatic hydrolysis.

Chromatographic Conditions

Plates/Columns

- For the chromatography TLC- or HPTLC-standardized Silica Gel F254 (Merck) plates, in some specific cases also aluminium oxide- or cellulose-coated plates (Merck) are used. HPTLC plates are precoated with Silica Gel of an average particle size and a narrow size distribution of 5 µm (as opposed to TLC material of 15 µm average particle size and a broader size distribution).
- For all HPLC-analysis reversed phase C-18 or C-8 columns (LiChroCART® 125-4/250-4 LiChrospher® 100 RP-18 (5 µm), Merck or LiChroCART® 125-4/250-4 LiChrospher® 60 RP select B (5 µm), Merck) can be used with a Merck HITACHI L-4500 A Diode Array Detector.

Detection/Solvent System

In the appendix of Volumes 1 and 2 (p. 451/1009), the most used reagents and basic solvent systems in TLC and HPLC are listed for the detection of main structure types of drug constituents in herbal drugs.

Reference Compounds

The availability of reference compounds which are characteristic of any herbal drug and at the same time represent the main pharmacologically active constituents of any plant facilitates the identity (quality) proof of a herbal drug and is a requirement for quantitation determination. If they cannot be isolated in the researcher's own laboratory, some of them can also be purchased from special firms. In Germany the firm PhytoLab in Vestenbergsgreuth (www.phytolab.com) offers many reference compounds which are listed as "marker compounds" in the Chinese Pharmacopoeia.

Reproducibility of the Fingerprint Analysis

If the same technical conditions described are used, it can be expected that even with the use of instruments from other firms, nearly identical TLC and HPLC fingerprints must be obtained. If, however, for any reason, the grade of separation and/or the R*f* and Rt values deviate from those stipulated in the monographs, the sequence and the overall TLC zone and HPLC peak profiles must in any case be identical.

Photography

The TLC chromatograms were developed by a Canon PowerShot G2 digital camera in a CAMAG Reprostar 3 cabinet using winCATS software (www.camag.com).

Cortex Albiziae – *Hehuanpi*

Pharmacopoeia: [1] Pharmacopoeia of the People's Republic of China, English Edition Vol. I, 2010

Official drug: [1] Silktree Albizia Bark is the dried stem of *Albizia julibrissin* Durazz. (Fam. Fabaceae). The drug is collected in summer and autumn, and dried in the sun.

Origin: [2] China, Japan, India, Iran and Ethiopia

Description of the drug: [1] Quilled and semi-quilled, 40–80 cm long, 0.1–0.3 cm thick. Outer surface greyish-brown, somewhat longitudinally wrinkled, some shallowly fissured, with dense and distinct transversal elliptical lenticels, brown or brownish-red, occasionally showing prominent transversal ribs or some large rounded branch scars, and often covered with patches of lichens; inner surface pale yellowish-white, smooth, with fine and dense longitudinal striation. Texture hard und fragile, easily broken, fracture fibrously laminated, pale yellowish-white.

Pretreatment of the raw drug: [1] Foreign matters are eliminated, washed clean, soaked well, cut into slivers or pieces, and dried in the sun.

Medicinal use: [3] It is used as tonic, stimulant and sedative for treatment of melancholy and insomnia, also for treatment of traumatic diseases.

Effects and indications of Cortex Albiziae according to Traditional Chinese Medicine [1–6]

Taste:	Sweet
Temperature:	Neutral
Channels entered:	*Orbis hepaticus*, *o. cardialis*, *o. pulmonalis, o. lienalis*
Effects (functions):	To remove depression and tranquilize the mind. Activate blood and disperse swelling, anti-inflammatory, treating of swelling and pain of the lungs, skin ulcers, and wounds
Symptoms and indications:	Disquietude of heart spirit, depression and insomnia, lung abscess, sore and swelling, pain caused by injuries from falls

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Main constituents: [3, 6–14]

- **Lignan glucosides**

(−)-Syringaresinol-4-O-β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside, syringaresinol tri- and tetraglycoside

- **Lignans and derivatives**

derivatives of 3,4,5- methoxyphenol (lyoniresinol, vomifoliol and icariside E5)

- **Saponins**

Julibroside I-III, A_1–A_4, B_1, C_1, J_1–J_3, J_7 and J_9, J_{16}, J_{17}, J_{21} J_{29}–J_{31}, J_{32}, J_{34}, J_{35}, saponins with prosapogenins machaerinic acid methyl ester, acacic acid lactone, acacigenin B, machaerinic acid lactone, 16-deoxyacacigenin B, julibro triterpenoidal lactone A (16β-isomer of acacic acid lactone) julibrogenin A

- **Flavone glucosides**

7, 3′,4′-trihydroxyflavone, quercetin-3-O-β-D-galactopyranoside, quercetin-3-O-α-L- rhamnopyranoside

- **Other compounds**: syringic acid, β-sitosterol, α-spinasterol-3-*O*-β -D-glucopyranoside

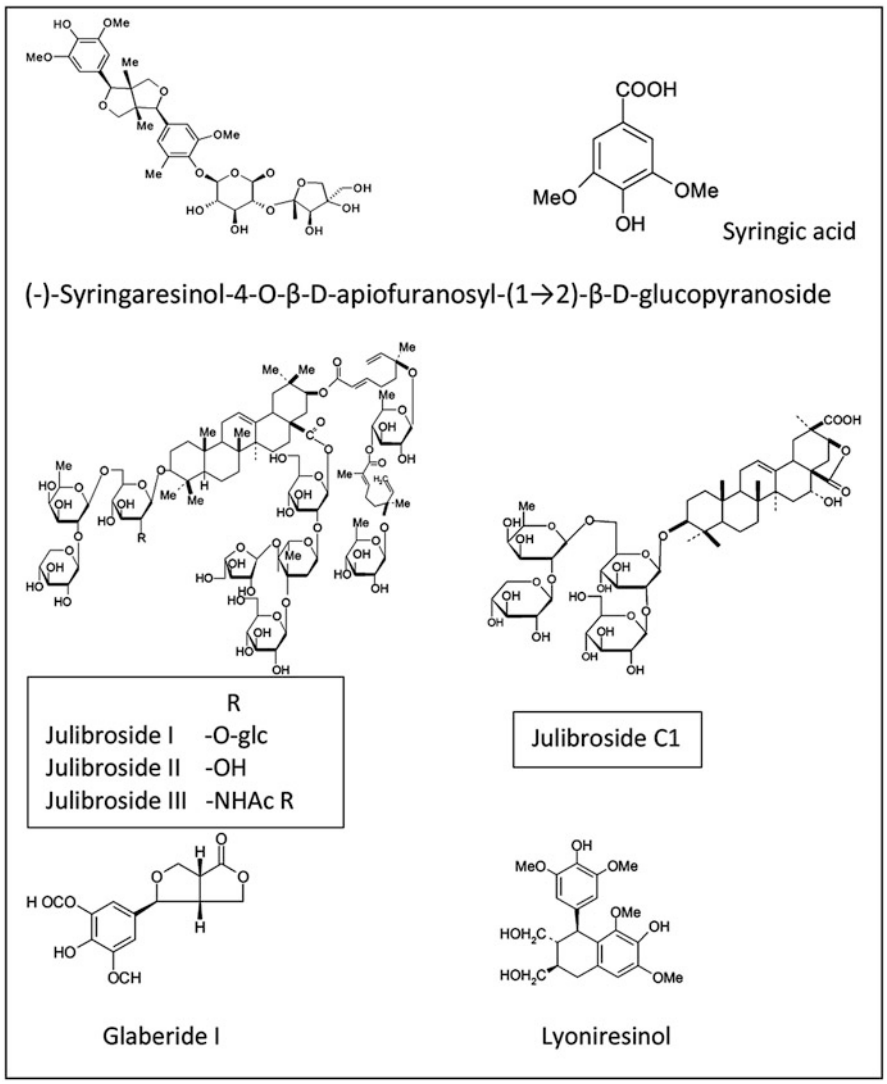

Fig. 1 Formulae of the main constituents of Cortex Albiziae [1, 3, 7, 9, 10]

Reported pharmacological effects: [3–5, 7, 10, 13, 15]

- antioxidant effect
- anti- inflammatory
- sedative effect
- anxiolytic effect
- anti-tumoral effect by induction of apoptosis type (human acute leukemia junket T-cells)

TLC Fingerprint Analysis:

	Drug samples	Origin
1	Cortex Albiziae / *Albizia julibrissin*	Province Henan (China)
2	Cortex Albiziae / *Albizia julibrissin*	Province Shanxi (China)
3	Cortex Albiziae / *Albizia julibrissin*	Province Sichuan (China)
4	Cortex Albiziae / *Albizia julibrissin*	Sample of commercial drug, obtained from HerbaSinica (origin: Hunan China)
5	Cortex Albiziae / *Albizia julibrissin*	Sample of commercial drug, obtained from China Medica (origin: Beichuan, Sichuan)
6	Cortex Albiziae / *Albizia julibrissin*	Sample of commercial drug, obtained from TCM-Clinic Bad Kötzting (Charge: K07.01.2003)

1. **TLC-fingerprint analysis of Saponins and Lignans:**

Reference compounds		R*f* of Fig. 2a	R*f* of Fig. 2b
T1	Syringaresinol diglucoside	0.61	0.71
T2	Oleanolic acid	0.98	–
T3	Saccharose	0.16	–
T4	Syringic acid	–	0.97

1. Extraction: 2 g powdered drug is extracted with 20 ml of 80 % methanol under reflux for 1 h. The extract is cooled, filtrated and evaporated to dryness. The residue is dissolved in 2 ml methanol and filtered over Chromafil® filtration unit, type 0–20 µm/25 mm.
2. Reference compounds: Each 0.5 mg is dissolved in 0.5 ml ethanol
3. Separation parameters:

   Plate: HPTLC Silica gel 60 F_{254}, Merck

   Applied amounts: Cortex Albiziae extracts: 10 µl each

   Reference compounds: 10 µl each

   Solvent system: Chloroform + methanol + glacial acetic acid + water (7.5 + 4 + 1.5 + 1.25)

   Detection: a) Anisaldehyde – sulphuric acid reagent

   0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml sulphuric acid in that order.

   The plate is sprayed with 10 ml and then heated at 110 °C for 7–10 min. Evaluation is carried out under visible light.

   b) Trichloroacetic acid- potassium hexacyanoferrate-iron-III-chloride reagent (TPF)

   1) 25 % trichloroacetic acid in chloroform.

   2) 1 % aqueous potassium hexacyanoferrate mixed with an equal volume of 5 % aqueous iron-III- chloride.

   The plate is sprayed with solution (1) and heated at 110 ° C for 10 min. It is then sprayed with solution (2) and evaluated in VIS.

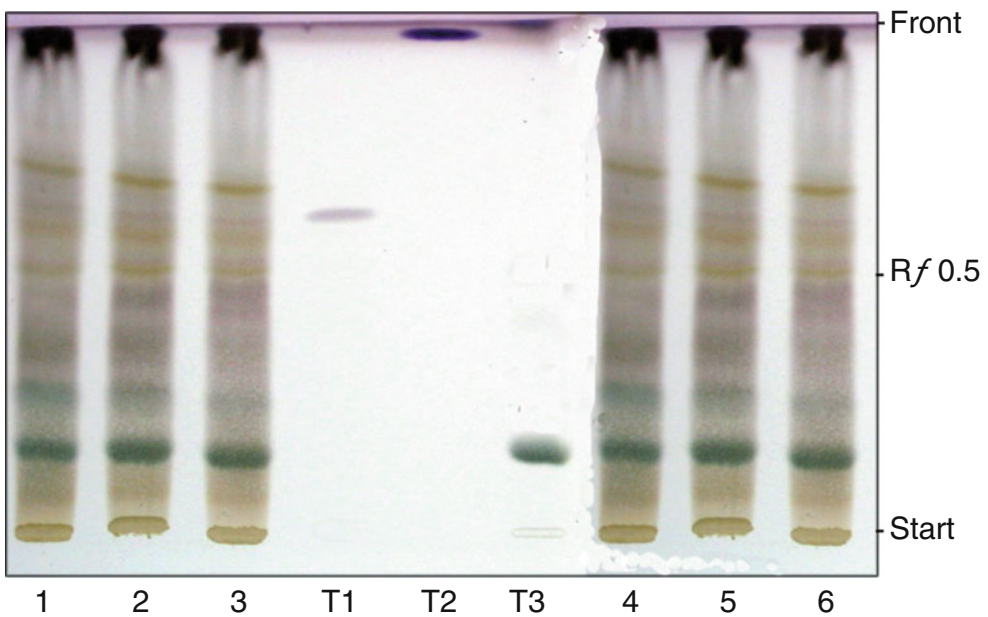

Fig. 2a Thin layer chromatogram of the 80 % methanol extracts of Cortex Albiziae, sprayed with anisaldehyde-sulphuric acid reagent (VIS)

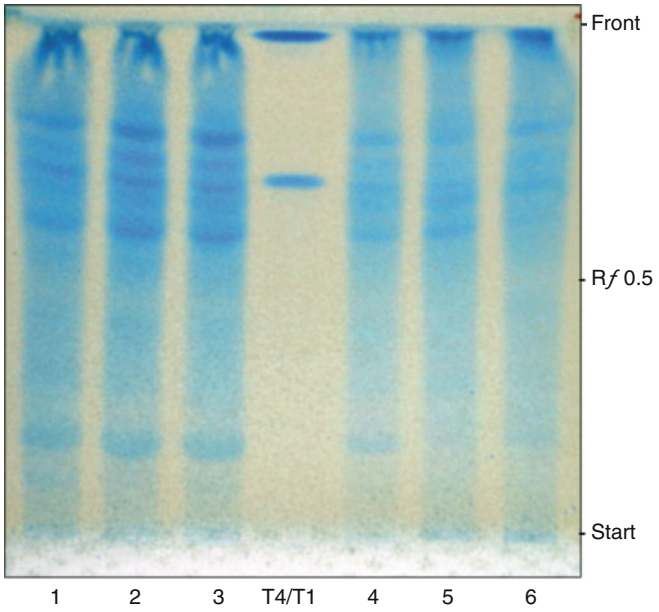

Fig. 2b Thin layer chromatogram of the 80 % methanol extracts of Cortex Albiziae, sprayed with Trichloroacetic acid- potassium hexacyanoferrate-iron-III-chloride reagent (VIS)

4) Description of TLC-fingerprint

Fig. 2a: The TLC is characterised by one dark brown zone at R*f* = 0.98 (Oleanolic acid) and 7–8 weak brown zones from ~ R*f* =0.70 down to R*f* = 0.25. In the first R*f*-range from 0.70 till ~ R*f* = 0.55 violet brown coloured lignan (glycosides) appear with the reference compound syringaresinol diglucoside at R*f* = 0.61. In the second R*f*-range from R*f* = 0.55 till ~ R*f* = 0.15 some of the yellow- brown zones above saccharose could be not assigned to the julibrosides with 3-4 sugar moites. The genuine julibrosides I, II or III (see formula Fig. 1) could not be identified. According to the literature they were never isolated in genuine form and only registered as “prosapogenins” after partial hydrolysis by NMR and MS- spectroscopy!

Fig. 2b: The Cortex Albiziae extracts were developed in the same solvent system but sprayed with the TPF-reagent. The lignan (glucosides) appear in the upper R*f*- range in pigeon blue coloured zone. The saponins cannot be detected with the TPF- reagent.

HPLC-Fingerprint Analysis:

1. Sample preparation: The same extracts used for TLC-fingerprint analysis
2. Injection volume: Cortex Albiziae extracts: 7 µl each
3. HPLC parameter:

Apparatus:	MERCK HITACHI D-6000 A Interface
	MERCK HITACHI L-4500 A Diode Array Detector
	MERCK HITACHI AS-2000 Auto sampler
	MERCK HITACHI L-6200 A Intelligent Pump
Separation column:	LiChroCART® 250-4 LiChrospher® 100 RP-18 (5 µm), Merck
Precolumn:	LiChroCART® 4-4 LiChrospher® 100 RP-18 (5 µm), Merck
Solvent System:	A: 0.1 % H_3PO_4 (Millipore Ultra Clear UV plus® filterd)
	B: Acetonitrile (VWR)
Gradient:	5–18 % B in 15 min., (flow: 0.8 ml/min) 18–40 % B in 35 min., (flow: 0.8 ml/ min) 40–95 % B in 10 min., (flow: 1 ml/min) 95–100 % B in 5 min., (flow: 1 ml/min)
	Total run time: 65 min
Detection:	215 nm

Retention times of the main peaks

Peak	**Rt (min)**	**Compound**
1	6.0	Syringic acid
2	11.0–18.0	Triterpene glucosides
3	21.2	(−)-Syringaresinol-4-O-β-apiofuranosyl- (1 → 2)-β-D-glucopyranosid
4	26.8	Lignan diglucoside
5,6	37.4, 47.21	Triterpene oligoside
7	62.6	α-Spinasterol
8	67.5	Oleanolic acid

4) Description of the HPLC-Figures 3a, 3b and 3c

The extract samples 2,3 and 6 show a very similar peak profile with main peaks in the Rt -range **A** between Rt = 20 and 28 with Peak No. **3** (Rt = 21.2) which could be assigned to (−)-Syringaresinol-4- O-β-apiofuranosyl-(1 → 2)-β-D- glucopyranoside. The prominent peak **4** in the same range (Rt =26.8) might be related to a second lignan glycoside. The peaks in the Rt- range of 11.0–20.0 can be assigned to triterpene oligo-glycosides and the peaks between Rt = 35.0 and 70.0 to sterols inclusive oleanolic acid

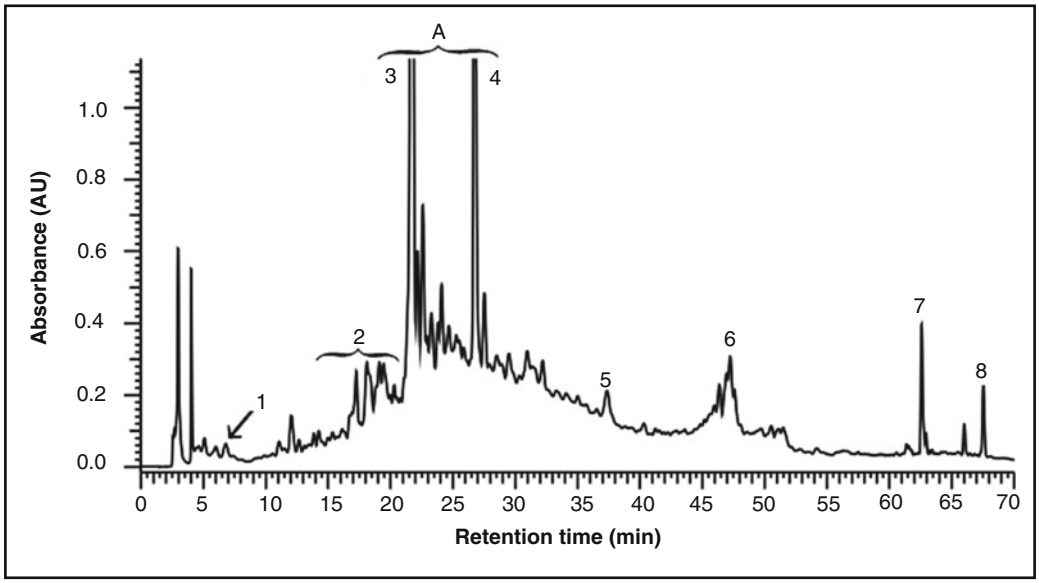

Fig. 3a HPLC-fingerprint analysis of the 80 % methanol extract of Cortex Albiziae, sample 2

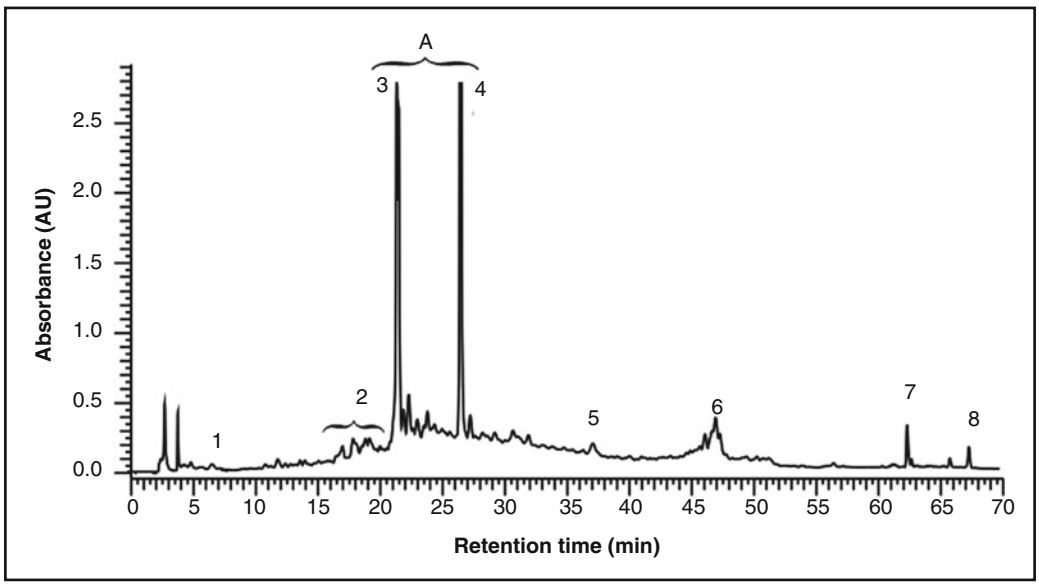

Fig. 3b HPLC-fingerprint analysis of the 80 % methanol extract of Cortex Albiziae, sample 3

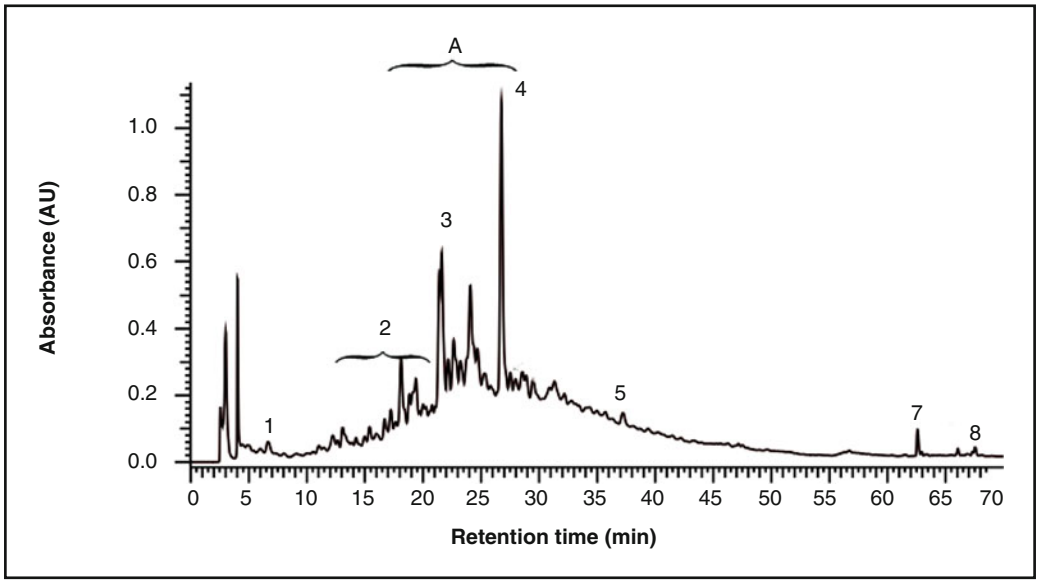

Fig. 3c HPLC-fingerprint analysis of the 80 % methanol extract of Cortex Albiziae, sample 6

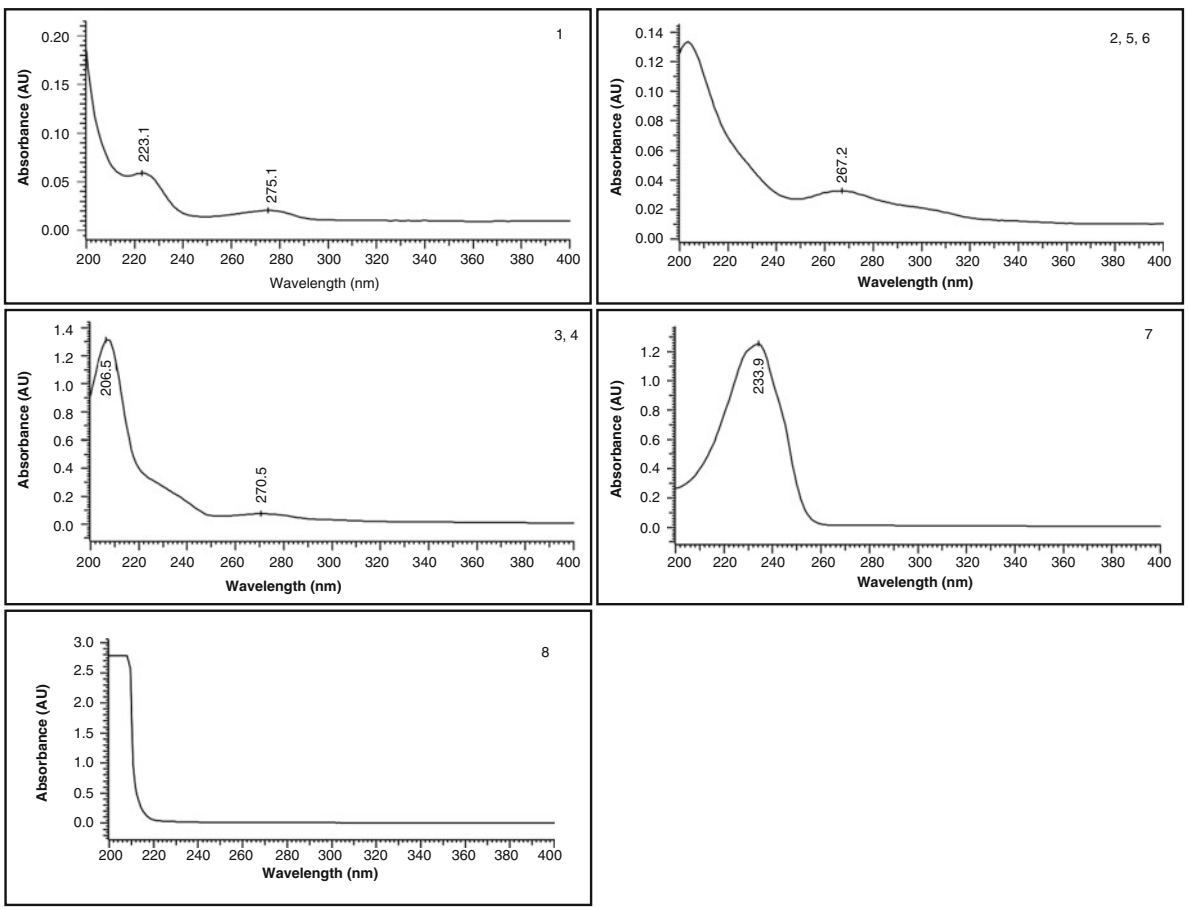

Fig. 4 On line UV-spectra of the main characteristic peaks of Cortex Albiziae

Conclusion

With TLC and HPLC the authentication of Cortex Albiziae can be easily achieved based on the lignan glycosides. The triterpene oligosids are only present in very low concentration, therefore not suitable as marker compounds.

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