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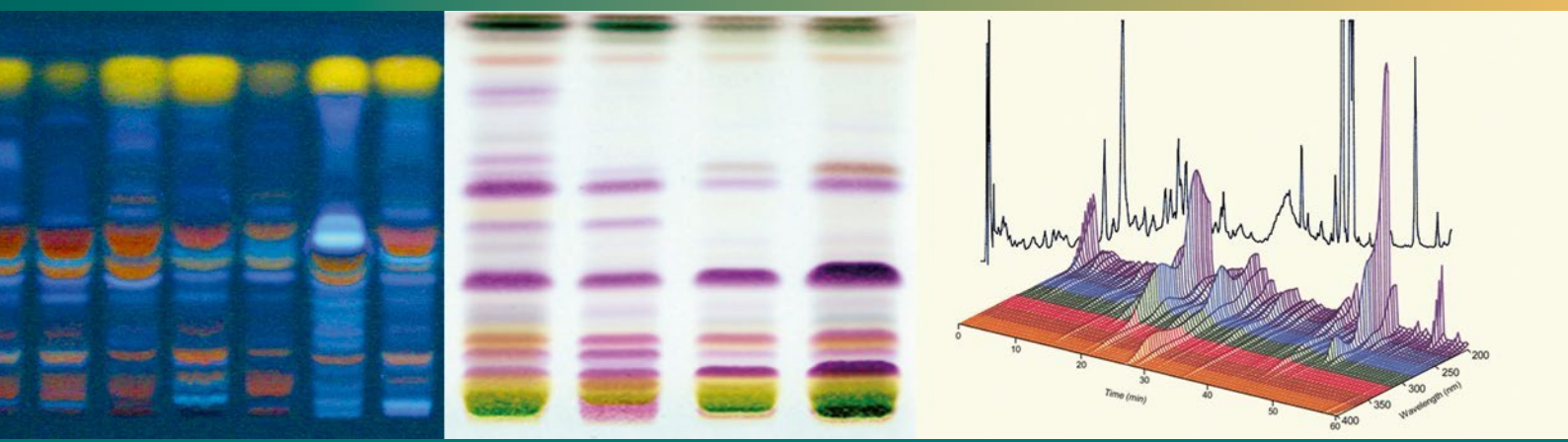
Anton Staudinger · Dieter Melchart *Editors*

# Chromatographic Fingerprint Analysis of Herbal Medicines

## Thin-Layer and High Performance Liquid Chromatography of Chinese Drugs




Volume 5

Springer

TCM-KLINIK BAD KÖTZTING
Erste deutsche Klinik für Traditionelle Chinesische Medizin
Fachklinik für Psychosomatik und Psychotherapie

University Hospital at Beijing University of Chinese Medicine

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# Acknowledgements


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# Authors' Contributions

- Supervisor and responsible for the correct performance of the analytical monographs of Chinese herbal drugs: Prof. Dr. Dr. h.c. Hildebert Wagner, 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 the 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. It is also possible to gain preliminary information as to which chemical structure type the single compounds may belong. Since this year, 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.))

  – Vol. I and II (2011) containing 80 analytical monographs
  – Vol. III (2015) containing 23 analytical monographs
  – Vol. IV (2016) containing 22 analytical monographs
  – Vol. V (2018) containing 20 analytical monographs (Wagner H, Püls S., Barghouti T., Melchart D., Staudinger A. (Eds.))

- 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 near 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 and 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 procyanidines 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 F 254 (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 to Volumes 1 and 2 (pp. 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 Dictamni – *Baixianpi*

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

**Official drug:** [1] Densefruit Pittany Root-bark is the dried root bark of *Dictamnus dasycarpus* Turcz. (Fam. Rutaceae).

The root is collected in spring and autumn, removed from soil and rough bark. The root bark is stripped off and dried.

**Origin:** [2, 3] Mainly in Chinese provinces such as Liaoning, Hebei and Shandong, Anhui, Gansu, Heilongjiang, Henan, Hubei, Jiangsu, Jiangxi, Jilin, Ningxia, Shaanxi, Shanxi, Sichuan, Xinjiang. Also in Korea, Mongolia, Russia (Far East).

**Description of the drug:** [1] Quilled, 5–15 cm long, 1–2 cm in diameter, 2–5 mm thick. Outer surface greyish-white or pale greyish-yellow, with fine longitudinal wrinkles and rootlet scars, frequently with small protruding granular dots; inner surface almost white, with fine longitudinal striations. Texture fragile, dusting on breaking, fracture uneven and somewhat lamellar, when outer layer peeled off, numerous glittering small spots observed on exposing to light. Odour, muttony; taste, slightly bitter.

**Medicinal use:** [4] It is used for the treatment of icterus, carbuncle and abscess and eczema (by external application).

**Effects and indications of Cortex Dictamni according to Traditional Chinese Medicine** [1, 5–13]

| | |
|---|---|
| Taste: | Bitter |
| Temperature: | Cold |
| Channels entered: | *Orbis lienalis*, *O. stomachi*, *O. vesicalis* |
| Effects (functions): | To clear heat and dry dampness, dispel wind and remove toxin. |
| Symptoms and indications: | Dampness-heatsore and toxin, dripping yellow water, eczema, rubella, sore, scabies and tinea, wind-dampness heat impediment, jaundice and red urine. |

**Published constituents:**

- **Furoquinoline alkaloids** [4, 6–13]

  Dictamnine, γ-fagarine, skimmianine, haplopine, isomaculosidine, dasycarine, paltydesmine

- **Limonoids** [4, 6, 7, 9–13]

  Fraxinellone, 6β-hydroxyfraxinellone (= dasycarpol), isofraxinellone, fraxinellonone, obacunone, 7α-acetyl-obacunol, limonin, limonin diosphenol, rutaevin, dictamdiol, calodendrolide, 7α-acetyldihydronomilin, dictamnusine, dictamdiol A+B

- flavonoids (wogonin), coumarins (xanthotoxin), psoralen, sesquiterpenes and their glycosides (e.g. dictamnol, β-elemol, dictamnoside A-I + L-N) [4, 6, 7, 9–14]



H. Wagner et al. (eds.), *Chromatographic Fingerprint Analysis of Herbal Medicines Volume V*, DOI 10.1007/978-3-319-67062-1_1

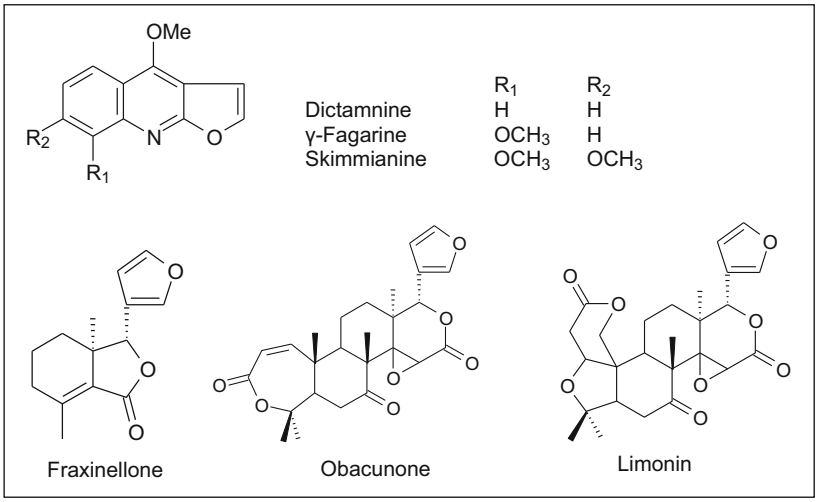


**Fig. 1** Formulae of the main compounds of Cortex Dictamni [4, 7, 8]

**Reported pharmacology:**
- mutagenic [4, 8]
- antifungal [4, 5, 10, 12]
- antimicrobial [7]
- antiplatelet aggregation [4, 7]
- cytotoxicity [4, 7]
- anti-tumor [7]
- anti-inflammatory [7, 9]
- vascular-relaxing effects [7]
- neuroprotective [13]

**TLC fingerprint analysis [15]**

| Drug samples | | Origin |
|---|---|---|
| 1 | Cortex Dictamni/*Dictamnus dasycarpus* | Sample of commercial drug obtained from HerbaSinica (origin: Jilin) |
| 2 | Cortex Dictamni/*Dictamnus dasycarpus* | Sample of commercial drug obtained from China Medica (origin: Guchifeng, Neimenggu) |
| 3 | Cortex Dictamni/*Dictamnus dasycarpus* | Province Liaoning, China |
| 4 | Cortex Dictamni/*Dictamnus dasycarpus* | Province Shandong, China |

| **Reference compounds of Fig. 2a/b** | | R*f* |
|---|---|---|
| n.a. | Fraxinellone | 0.70 |
| n.a. | Dictamnine | 0.41 |
| T1 | Obacunone | 0.20 |
| T2 | Limonin | 0.13 |

n.a. = not applied

1) Extraction: 2.0 g powdered drug are extracted with 10 ml methanol in an ultrasonic bath for 15 min. The extract is filtered, evaporated to dryness and the residue is dissolved in 1 ml methanol.

2) Reference compounds: 1.0 mg is dissolved in 1.0 ml methanol

3) Separation parameters:

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

Applied amounts: Radix Dictamni extracts: each 10 µl

Reference compounds: each 10 µl

Solvent system: Toluene + methanol + glacial acetic acid (10 + 0.5 + 0.1)

Detection: **Anisaldehyde-Sulphuric acid reagent**

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

The plate is sprayed with 10 ml, heated at 105 °C for 10 min, then evaluated in VIS and under UV 366 nm.

**Note:** The reagent has only limited stability and is no longer useable when the colour has turned to red-violet.

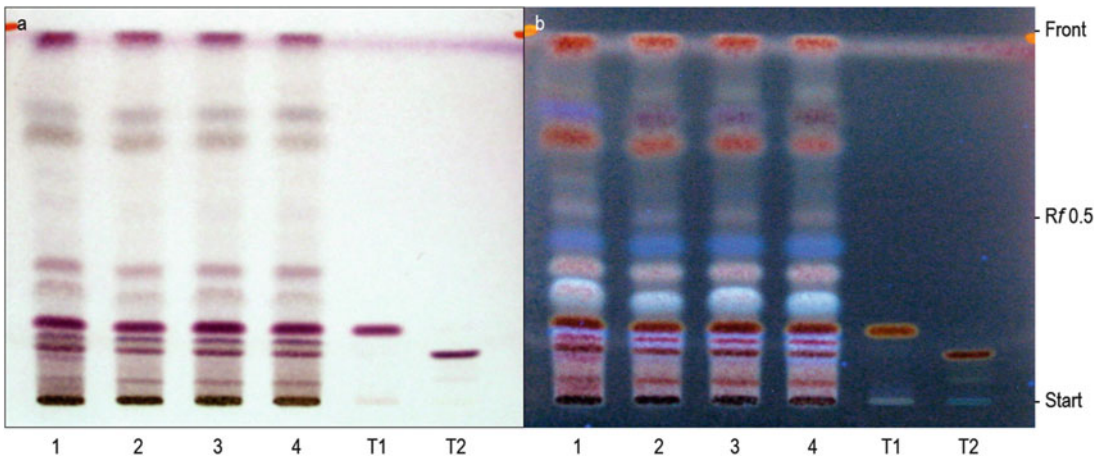


**Fig. 2** Thin layer chromatogram of Cortex Dictamni methanol extracts, sprayed with Anisaldehyde-Sulphuric acid reagent (a = VIS, b = UV 366 nm)

4) Description of **Fig. 2a:**

The TLC of the methanol extracts in VIS is characterized by three violet zones in the deep R*f*-range of which two of them could be identified as obacunone (**T1**) and limonin (**T2**).

Description of **Fig. 2b:**

In this TLC the three violet zones of Fig. 2a are also visible with similar colour. Additionally in the higher R*f*-range appear dictamnine with light blue fluorescent colour at R*f* = 0.41 and fraxinellone at R*f* = 0.70 with red (carmine) colour.

**HPLC-fingerprint analysis:** [15]

| | |
|---|---|
| 1) Extraction: | 2.0 g powdered drug is extracted with 10 ml methanol in an ultrasonic bath for 15 min. The extract is filtered, evaporated to dryness and the residue is dissolved in 1 ml methanol. The extract is filtered over Chromafil®, Type 0.20 µm. |
| 2) Injection volume: | Radix Dictamni extracts: each 10 µl |
| 3) HPLC parameter: | |
| Apparatus: | MERCK HITACHI D-6000 A Interface |
| | MERCK HITACHI L-4500 A Diode Array Detector |
| | MERCK HITACHI AS-2000 Autosampler |
| | 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% phosphoric acid/water (Millipore Ultra Clear UV plus® filtered) |
| | B: acetonitrile (VWR) |
| Gradient: | 15–53% B in 30 min, |
| | 53–63% B in 5 min, |
| | 63–90% B in 25 min |
| | total runtime: 60 min |
| Flow: | 1.0 ml/min |
| Detection: | 210 nm |

Retention times of the main peaks

| Peak | Rt (min) | Compound |
|---|---|---|
| 1 | 26.4 | Limonin |
| 2 | 27.6 | Dictamnine[a] |
| 3 | 31.8 | Obacunone |
| 4 | 33.0 | Fraxinellone[a] |

[a]According to ref. [6, 7, 9]