CBS 123 • SEPTEMBER 2019

# CBS 123

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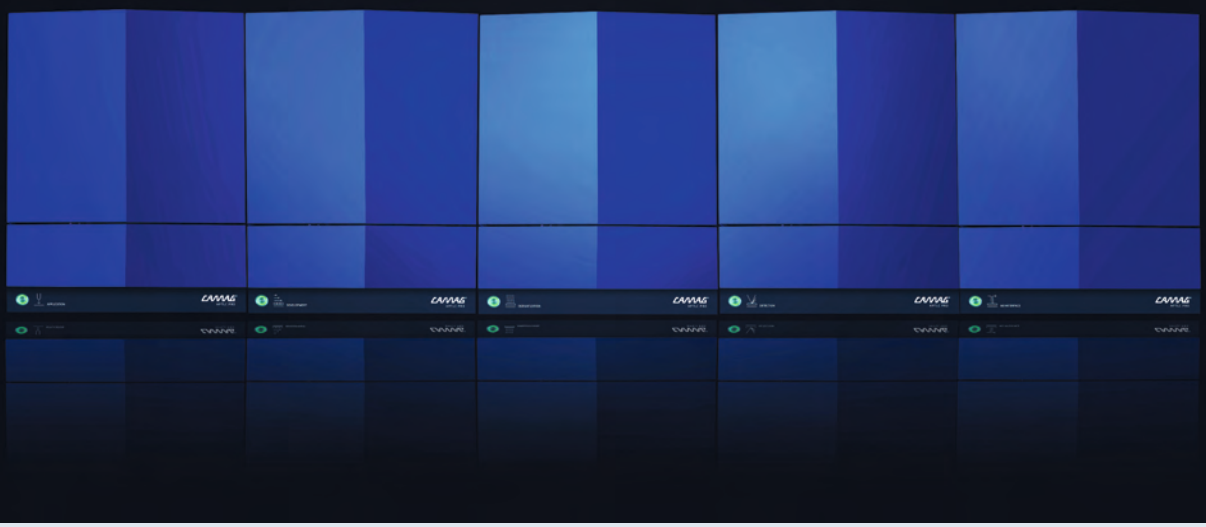
SEPTEMBER 2019

Introducing
CAMAG® HPTLC PRO



Other topics of this issue:
PAHs in road surfaces
Lovastatin and citrinin in red yeast rice products
Analysis of purity of ginkgo products
Pharmacokinetics of berberine
Production and analysis of innovative plant extracts
Chemotaxonomic discrimination of *Clerodendrum* species

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cbs@camag.com
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**IN THIS ISSUE**

**Procedures, applications**



**Products featured in this issue**



**Column: Know CAMAG**



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# Polycyclic aromatic hydrocarbons in road surfaces

*From left: Urs Maier, Alessandra Sodero, Christian Ardizzone, Thomas Glättli*

Bachema AG is a Swiss contract laboratory, which offers chemical and biological tests for water and contaminated sites since its foundation almost 60 years ago. Analytical services for the recycling and landfilling of construction waste have been provided.

## Introduction

Bachema AG quantifies the content of polycyclic aromatic hydrocarbons (PAHs) in the binder of asphalt pavements by GC-MS and HPTLC based on DIN 38407-7:2000-09. Though quantification by HPTLC is fast and reliable, the manual effort required at regular intervals proved to be disadvantageous for operators. Control of plate development and drying time with a stopwatch repeatedly interrupted their workflow. Furthermore, the substitution of dichloromethane by a less toxic alternative was also of interest.

**In the framework of a student internship, Ms. Sodero implemented the Automated Multiple Development (AMD 2) system for separation. After validation and accreditation by Bachema AG, the modernized PAH method runs without dichloromethane, stopwatch and development at –20 °C.**

## Standard solution

PAH mixture (each 2 mg/mL in dichloromethane – benzene 1:1, AccuStandard Z-014G), diluted 1:50, 1:500, and 1:10 000 in toluene. Different amounts of these dilutions were applied to the HPTLC plate to obtain seven standard levels ranged 0.4 to 80 ng/band.

## Sample preparation

Road surface samples were extracted with hot toluene and then diluted according to their binding agent content by an external laboratory (no details available). As positive control, a well known mixture of different real samples was used.

## Chromatogram layer

HPTLC plate silica gel 60 AMD (Merck), 20 x 10 cm, impregnated by immersion for 4 s into a caffeine solution (10 g in 180 mL dichloromethane), followed by drying for 1 min using a hair dryer and at room temperature for 1 h

## Sample application

Automatic TLC Sampler 4, bandwise application, 20 tracks, band length 6.0 mm, distance from left edge 14.0 mm, distance from lower edge 8.0 mm, application volume of 2 µL for sample as well as positive control and 1–5 µL for standard solutions.

## Chromatography

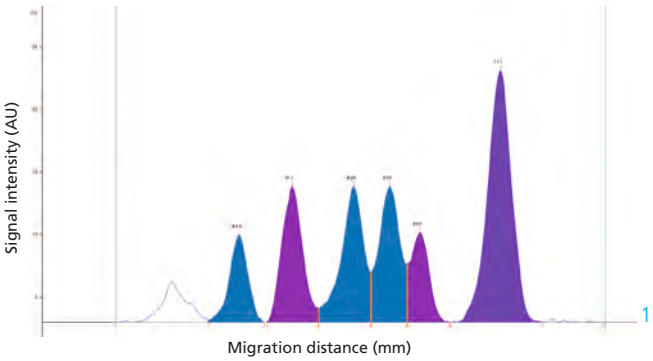
AMD 2 development up to 80 mm with isopropyl acetate – cyclohexane 13:7 (no preconditioning), followed by drying for 2 min

## Densitometry

TLC Scanner 3 and winCATS, fluorescence measurement at 366/>400 nm using the mercury lamp, slit dimension 3.00 mm x 0.20 mm, scanning speed 20 mm/s

## Calibration and processing

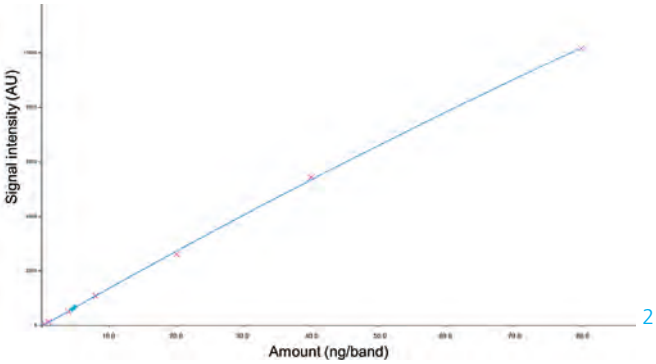
Road surface sample extracts as well as the positive control gave the same $hR_F$ values as the corresponding standards. Calibration was performed in the polynomial working range from 0.4–80.0 ng/band. The correlation of results obtained by the GC-MS, separating 16 PAHs, *versus* HPTLC method, separating six PAHs, was proven with a set of more than 400 samples. A conversion factor was introduced to extrapolate the HPTLC results of six PAHs to the 16 PAHs according to EPA.




1

*Densitogram of a positive control sample (2 µL/band) measured at 366/> 400 nm*

| Substance name | Abbreviation | Migration distance (mm) |
|---|---|---|
| Benzo(g,h,i)perylene | BPE | 33 |
| Indeno(1,2,3-cd)pyrene | IPY | 39 |
| Benzo(a)pyrene | BaP | 46 |
| Benzo(b)fluoranthene | BbF | 50 |
| Benzo(k)fluoranthene | BkF | 53 |
| Fluoranthene | FLT | 62 |




2

*Calibration curve of fluoranthene (0.4–80.0 ng/band, r = 0.9999, sdv = 2.5%)*

## Results and discussion

The method was successfully transferred from manual to automated HPTLC separation, using the AMD 2 system. The optimized method (9 min/sample; 66 samples/day) was faster than the previous HPTLC-FLD and GC-MS methods. It used less toxic solvent and required less manual operation. In contrast, a clean-up by solid phase extraction was required for GC-MS analysis, making it time-consuming (35 min/sample, 30 samples/day). Therefore, step-automated HPTLC-FLD turned out to be the method of choice. Only in few cases, a quantification and confirmation by GC-MS was needed. The recovery rate (sum of PAHs in the positive control) in comparison with the GC-MS method was 103.5%.

Further information is available on request from the authors.

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# Lovastatin and citrinin in red yeast rice products

*Dr. Ines Klingelhöfer and Prof. Dr. Gertrud Morlock*

The Food Science group at Justus Liebig University Giessen, Germany, develops straightforward HPTLC methods as the following one.

## Introduction

Lovastatin (Mevinolin, Monakolin K) is synthesized by *Monascus* fungi strains during fermentation of rice. At that point the carcinogenic, mutagenic and antibiotic mycotoxin citrinin can be formed. Lovastatin acts as lipid-lowering agent and may be present in the water-soluble hydroxy acid (LH) and slightly fat-soluble dehydrolyzed lactone form (LL).

**A newly developed HPTLC method was validated and applied to 19 red yeast rice products, including powders, food supplements, and Chinese proprietary medicines (Xuezhikang and Zhibituo). The simultaneous analysis including sample preparation allows a high throughput of matrix-rich samples (10 min and 0.5 Euro per analysis) [1].**

## Chromatogram layer

HPTLC plate silica gel 60 (Merck), 20 × 10 cm

## Standard solutions

LL (100 µg/mL) and citrinin solutions (10 µg/mL) in acetone; alkaline hydrolysis of LL (200 µL LL of 1 mg/mL, 700 µL acetone and 50 µL 1 N sodium hydroxide) for 30 min at 4 °C; 50 µL 1 N acetic acid added for neutralization (200 µg/mL LH)

## Sample preparation

Samples (30 mg) extracted in 1 mL (if completely water-soluble) or 2 mL water – acetone 2:3 (*V*/*V*), vortexed (30 s) and centrifuged (9600 × *g*, 5 min)

## Sample application

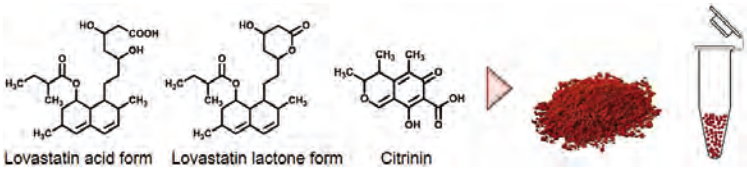
Bandwise with the Automatic TLC Sampler (ATS4), band length 6 mm, track distance 8 mm, application volume 0.5–10 µL/band (standards) and 0.5–20 µL/band (samples)

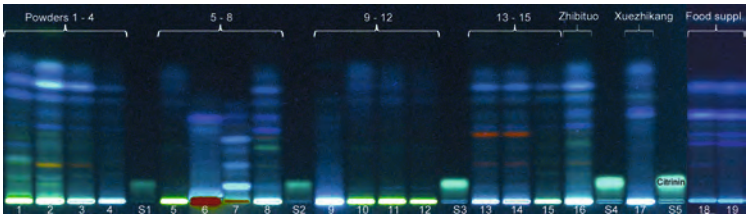
## Chromatography

In Twin Trough Chamber 20 × 10 cm, using *n*-hexane – acetone – 10% acetic acid, 6:4:0.1, up to 65 mm

## Densitometry

TLC Scanner 4 with winCATS, multi-wavelength scan of absorbance of LL/LH at 238 nm and fluorescence of citrinin at 313/>400 nm




*HPTLC chromatogram of red yeast rice products at UV 366 nm (lovastatin not visible)*

## Results and discussion

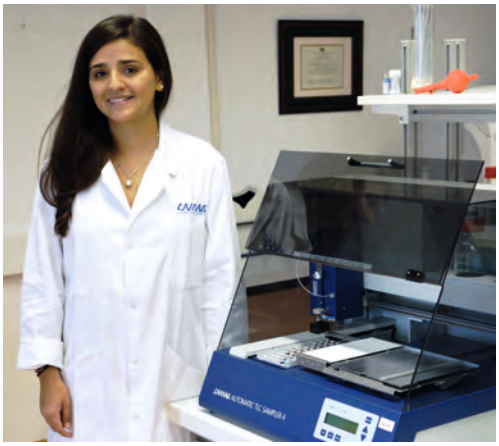
Method validation was successfull. For three analytes at three concentration levels including sample preparation, the overall mean recovery rate was 109.9% ± 5.9% and the mean intermediate precisions were ≤ 2.6% in red yeast rice matrix. For lovastatin analysis, the method was applied to 15 powders (1.5–26.2 g/kg), Zhibituo tablet (2.7 g/kg), Xuezhikang capsule (11.1 g/kg) and two food supplements (40.7 and 41.4 g/kg), which took 10 min and 0.5 Euro per sample [1].

[1] I. Klingelhöfer, G. Morlock, Anal Bioanal Chem 25 (2019) 6655

Further information is available on request from the authors.

Contact: Prof. Dr. Gertrud Morlock and Dr. Ines Klingelhöfer, Justus Liebig University Giessen, Heinrich-Buff-Ring 26–32, 35392 Giessen, Germany, Gertrud.Morlock@uni-giessen.de

# Simplified analysis of purity of ginkgo products



*Débora A. Frommenwiler, CAMAG*

## Introduction

Adulteration of ginkgo products with rutin and quercetin or plants rich in these flavonoids is still a common practice. To produce ginkgo extract compliant to pharmacopoeial standards, about 19 steps are involved with costs estimated at 200–250 $ per kilogram [1]. Adulteration practice ensures that products containing lower than the declared amount of ginkgo extract will still comply with the HPLC assay specifications of the Pharmacopoeias for levels of total flavonoids. This assay includes hydrolysis of the glycosides and quantification of the resulting aglycones (not less than 22–27%). It can be easily falsified by adding bulk rutin/quercetin, sophora fruit/flower bud or buckwheat herb as adulterants. To detect adulterations with rutin and quercetin, the United States Pharmacopoeia (USP) integrated an additional HPLC assay into the USP monograph on ginkgo extract [2,3].

**If identification is performed by comprehensive HPTLC fingerprinting, more information can be obtained from a single analysis. Thus, an additional HPLC assay for rutin and quercetin can be avoided and the overall costs decrease. By utilizing the information contained in the HPTLC fingerprint, identity, absence of adulterants, and compliance with limits for rutin and quercetin can be verified for ginkgo products in a single analysis [4–5].**

## Standard solutions

Methanolic rutin, chlorogenic acid and quercetin solutions (2 mg/10 mL each) for SST; methanolic rutin solution (0.4 mg/mL) and adjusted methanolic extract representing 0.5% quercetin for limit test

## Sample preparation

Products containing ginkgo extract were prepared to contain 10 mg/mL extract in methanol, sonicated for 10 min at room temperature and centrifuged for 5 min

## Chromatogram layer

HPTLC plate silica gel 60 $F_{254}$ (Merck), 20 x 10 cm

## Sample application

Automatic TLC Sampler (ATS 4), 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume 2 µL

## Chromatography

In the Automatic Developing Chamber (ADC 2) with ethyl acetate – acetic acid – formic acid – water 100:11:11:26 after chamber saturation for 20 min (with filter paper) and activation at 33% rel. humidity for 10 min using a saturated solution of magnesium chloride, migration distance of 70 mm (from the lower edge), drying for 5 min

## Post-chromatographic derivatization

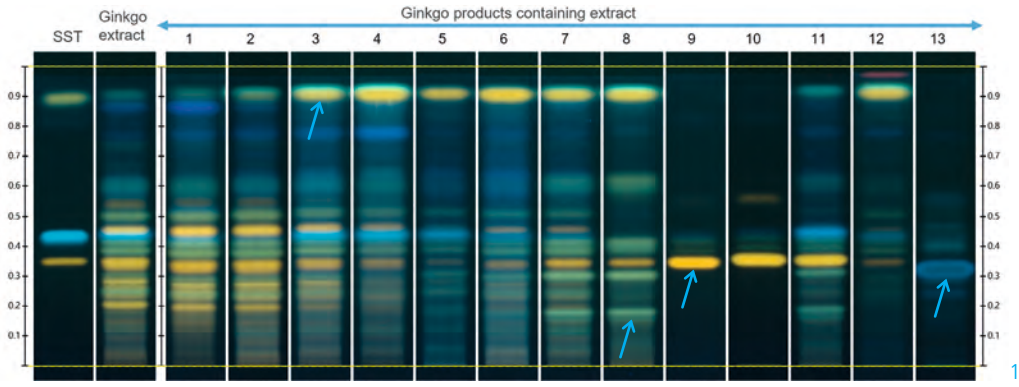
The plate is heated using the TLC Plate Heater (100 °C, 3 min), immersed into NP reagent (1 g 2-aminoethyl diphenylborinate in 200 mL ethyl acetate) using the Chromatogram Immersion Device (immersion speed 3 cm/s, immersion time 0 s), dried under cold air and documented. Then, it is immersed into anisaldehyde reagent (1 mL of *p*-anisaldehyde dissolved in a 200-mL mixture of methanol – acetic acid – sulfuric acid 170:20:10) and heated again.

## Documentation

TLC Visualizer 2 under UV 254 and 366 nm (prior to derivatization), UV 366 nm (after derivatization with NP) and white light (after derivatization with NP and anisaldehyde)

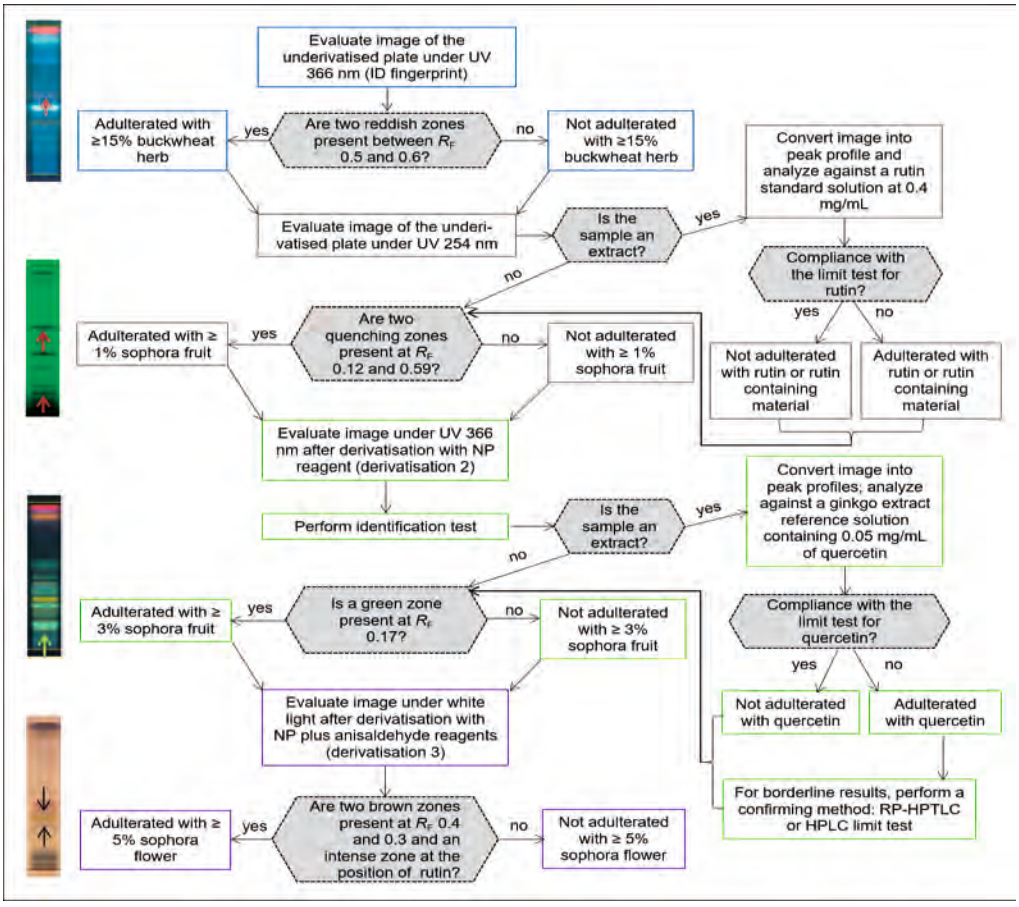
## Results and discussion

The HPTLC method for identification of *Ginkgo biloba* leaf extract from USP was modified. With regard to identification, the majority of investigated ginkgo products (tracks 3–13) yielded either fingerprints fainter than that of the reference extract, intense zones at the position of rutin and/or quercetin, or additional zones not characteristic of ginkgo extract (blue arrows).



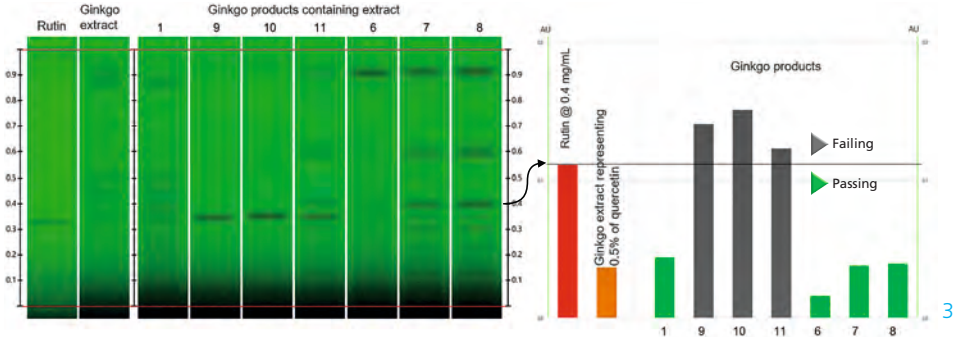

1 *HPTLC fingerprints of ginkgo products at UV 366 nm after derivatization with NP reagent. SST: rutin, chlorogenic acid, quercetin (with increasing $R_F$ values)*

For a purity test, samples of sophora fruit and flower bud, and buckwheat herb were analyzed individually and in mixture with ginkgo extract applying different detection modes and derivatization reagents. The presence of adulterants was detected according to the flow chart.

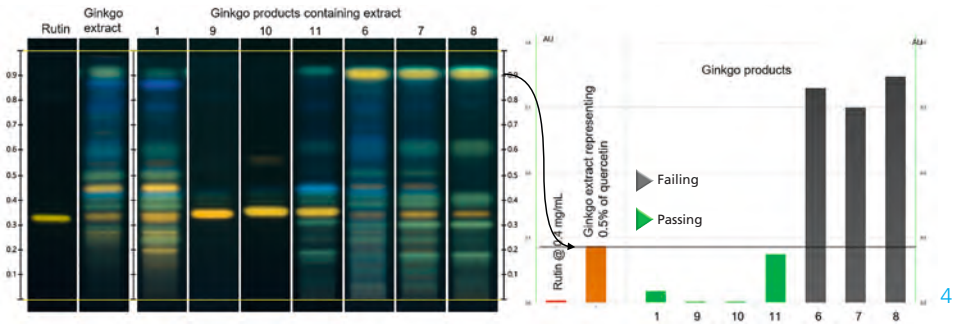



2 *Flow chart for comprehensive HPTLC fingerprinting for identification of ginkgo, ginkgo extract and ginkgo products and detection of adulteration*

For limit tests on rutin and quercetin, images under UV 254 nm prior to derivatization and UV 366 nm after derivatization with NP reagent were converted into peak profiles. Calculations were based on the peak heights of zones at the positions of rutin or quercetin in the standard and sample solutions. Standard solutions were prepared at concentrations corresponding to the maximum limit accepted for rutin and quercetin according to USP.




3 *Limit test for rutin. Chromatograms and respective rutin peak profiles at UV 254 nm prior to derivatization*




4 *Limit test for quercetin. Chromatograms and respective quercetin peak profiles at UV 366 nm after derivatization with NP reagent*

Detected adulterants in ginkgo products:

- GP3 and 4: intense yellow zone at the position of quercetin
- GP5-8: intense yellow zone at the position of quercetin; presence of sophora fruit (green zone); for GP5, faint fingerprint
- GP9 and 10: intense yellow zone at the position of rutin, faint fingerprint
- GP11: intense yellow zone at the position of rutin, presence of sophora fruit (green zone)
- GP12: intense yellow zone at the position of quercetin, faint fingerprint
- GP13: no ginkgo, but 5-hydroxytryptophan

With a few modifications to the detection, the HPTLC method for identification of Ginkgo provided information about the product purity. This simplified the analytical workflow and reduced the number of analyses, if compared to respective USP monographs.


[1] Gafner S. Botanical Adulteration Bulletin January (2018) 1
[2] USP Monograph on powdered ginkgo extract. *In:* Pharmacopoeial Convention 38–NF33 (2015) 6064
[3] Wohlmuth H., *et al.* Phytomedicine 21(6) (2014) 912
[4] Frommenwiler D.A., *et al.* Planta Medica 84 (2018) 465
[5] Frommenwiler D.A., *et al.* Journal of Ethnopharmacology 243 (2019) 112084


Further information is available on request from the author.


Contact: Débora Frommenwiler, CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland, debora.frommenwiler@camag.com

# Know CAMAG

CAMAG has reached its next milestone by launching the "Fully automated sample analysis and evaluation system for routine quality control":

# CAMAG® HPTLC PRO

At the ILMAC in Basel (24–27 September 2019) the first two Modules of this System – HPTLC PRO Module APPLICATION and HPTLC PRO Module DEVELOPMENT – are displayed for the first time to the public. The launch comes together with an all-new website and a new look of our *visionCATS* software (version 3.0)!

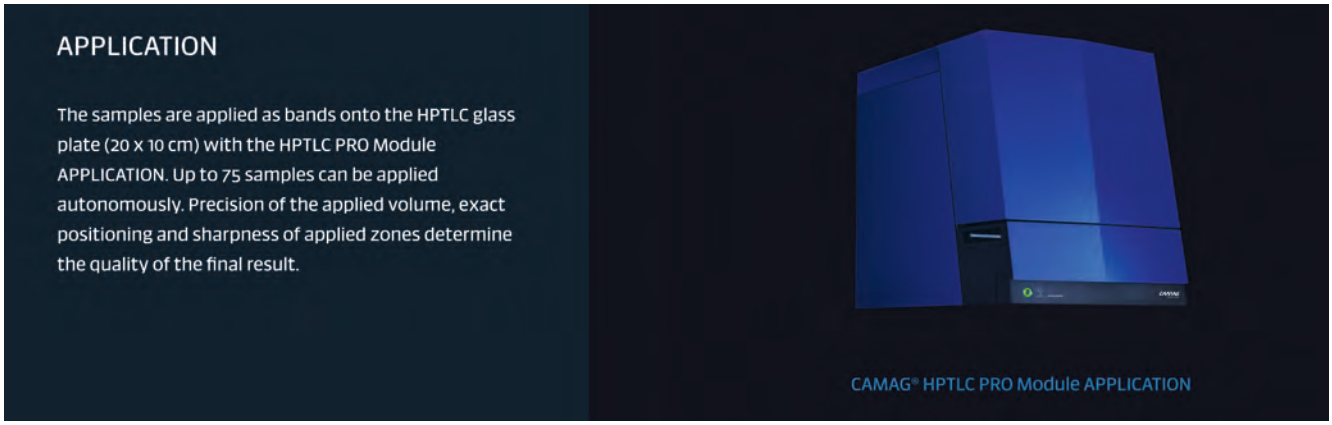
The additional Modules DERIVATIZATION, DETECTION, MS-INTERFACE and PLATE STORAGE will be launched sequentially over the next two years.

The fully automated HPTLC PRO SYSTEM (patented technology) uses HPTLC glass plates (20 x 10 cm) and is best suited for routine quality control of analytes extracted from complex matrices, providing reproducible and reliable results. Without any manual intervention, the HPTLC PRO SYSTEM supports the analysis of up to 75 samples, the use of up to five plates and up to three independent developing solvents.

The new website gives a first impression of the future of HPTLC. You will find the existing instruments and the new ones side by side. HPTLC PRO targets mainly customers with a need for high-throughput, automation and quality control. *visionCATS* – CAMAG's well-known HPTLC software – handles also HPTLC PRO.

This break-through invention intends to make a dream become reality. I hope you will be as excited as we are.

Markus Wyss, Ph.D.
CEO, Head Sales & Marketing




# CBS 123

CAMAG BIBLIOGRAPHY SERVICE

SEPTEMBER 2019


**Remarks about new abstracts added to the CCBS database with this CBS issue**

Over the decades, more than 11000 abstracts have been added to the CCBS database, and with this edition of CBS a further 70 abstracts have been added reporting relevant TLC/HPTLC information from the latest scientific publications in our field.

The characterization of zones of interest by derivatization reactions, mass spectrometry, and nuclear magnetic resonance spectroscopy are strong advantages that can be seen in the added abstracts. For method validation in several studies, the presented linearity still turned out to be problematic (as pointed out previously), if a very limited working range was reported, for example 1:2.5. Or, if the solvent ratios (*e.g.*, 5:6.57:1.69) were different in the meaning (decimal places) of the numbers, which undermines the specified highest accuracy. For the latter, plate activity could have a much greater impact than the digits reported, if it is not kept constant. Also, note the number of decimal places – only significant digits make sense. In the CBS, we have rounded numbers if necessary, because for example an LOD of 1.54 ng/band cannot be reproduced in this accuracy via HPTLC.

Many reviews have been written and recently published on Planar Chromatography. Some covered the individual steps of the TLC/HPTLC technique like plate materials or bioassays in chromatography. Others summarized various hot topics such as counterfeit drugs, cannabis or pesticides. TLC/HPTLC has a lot to offer for quality control along the global product chain.

**Dear friends**

The last issue of CBS was dedicated to the recently deceased Dr. Dieter Jänchen, founder and leader of CAMAG for six decades. With his life's work he created the basis for the present. A fresh breeze has been blowing since this year. After 122 bilingual English/German CBS issues, the CBS will be issued exclusively as an English version. In this issue, the new HPTLC PRO System, which supports high-throughput analysis and plate transfer between modules, is featured on page 8. Together with a new website and version of the *visionCATS* software, CAMAG is thus well prepared for the next decade.

Two interesting applications in this issue come from industry, one about polyaromatic hydrocarbons and the other about bio-enriched plant extracts. In addition, research departments developed analyses for red yeast rice products, the Ayurvedic formulation *Pushyanuga Churna* and discriminated *Clerodendrum* species. The application of the CAMAG laboratory illustratively shows how to test the purity of ginkgo products with the same method as used for identification.

HPTLC sessions took place this year at the HPLC 2019 in Milan and the 12th Balaton Symposium in Siófok, with more to come at the Eastern Analytical Symposium in New Jersey.

The next International Symposium on HPTLC will take place in Ljubljana, Slovenia in the week from June 29th to July 3rd 2020. Further information will be published soon.

Kind regards,


Gertrud Morlock
cbs@camag.com



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# THE CBS CLASSIFICATION SYSTEM

1. **Reviews and books**
   - i) Books on TLC
   - ii) Books containing one or several chapters on TLC
   - c) Books containing frequent TLC information spread over several chapters of other information
2. **Fundamentals, theory and general**
   - i) General
   - ii) Thermodynamics and theoretical relationship
   - c) Relationship between structure and chrom. behaviour
   - d) Measurement of physico-chemical and related values
   - v) Optimization of solvent systems
   - vi) Validation of methods
3. **General techniques** (unless they are restricted to the application within one or two classification sections)
   - i) New apparatus/techniques for sample preparation
   - ii) Separation material
   - c) New apparatus for sample application/dosage
   - d) New apparatus/techniques for chromatogram development
   - v) New apparatus/techniques for pre- or post-chromatographic derivatization
   - vi) New apparatus/techniques for quantitative evaluation
   - vii) New apparatus/techniques for other TLC steps (distinguished from section 4)
4. **Special techniques**
   - i) Automation of sample preparation/application
   - ii) Automation of complex chromatogram developing techniques
   - c) Automation, computer application in quantitative chromatogram evaluation
   - d) Combination of TLC with other chromatographic techniques
   - e) Combination of TLC with other (non-chromatographic) techniques...MS, IR...etc.
5. **Hydrocarbons and halogen derivatives**
   - i) Aliphatic hydrocarbons
   - ii) Cyclic hydrocarbons
   - c) Halogen derivatives
   - d) Complex hydrocarbon mixtures
6. **Alcohols**
7. **Phenols**
8. **Substances containing heterocyclic oxygen**
   - i) Flavonoids
   - ii) Other compounds with heterocyclic oxygen
9. **Oxo compounds, ethers and epoxides**
10. **Carbohydrates**
    - i) Mono- and oligosaccharides, structural studies
    - ii) Polysaccharides, mucopolysaccharides, lipopolysaccharides
11. **Organic acids and lipids**
    - i) Organic acids and simple esters
    - ii) Prostaglandins
    - c) Lipids and their constituents
    - d) Lipoproteins and their constituents
    - e) Glycosphingolipids (gangliosides, sulfatides, neutral glycosphingolipids)
12. **Organic peroxides**
13. **Steroids**
    - i) Pregnane and androstane derivatives
    - ii) Estrogens
    - c) Sterols
    - d) Bile acids and alcohols
    - e) Ecdysones and other insect steroid hormones
14. **Steroid glycosides, saponins and other terpenoid glycosides**
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    - ii) Essential oils
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    - ii) Catecholamines and their metabolites
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    - ii) Peptides and peptidic proteinous hormones
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20. **Enzymes**
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    - ii) Nucleic acids, RNA, DNA
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    - d) Pyridine derivatives
    - e) other N-heterocyclic compounds
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    - c) Coordination compounds
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    - ii) Phosphorus insecticides
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    - d) Herbicides
    - v) Fungicides
    - vi) Other types of pesticides and various agrochemicals
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    - d) Soil pollution
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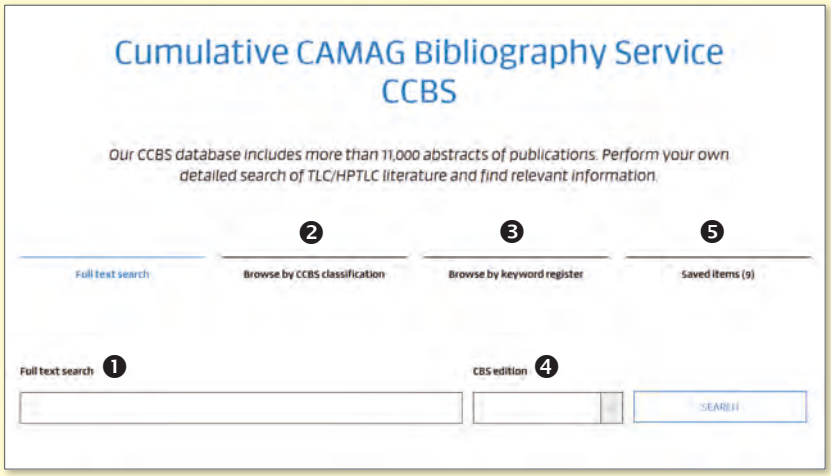


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## Monday, November 18, 2019

### MORNING SESSION (INTERNATIONAL): NOVEL CONCEPTS & UNIQUE APPLICATIONS OF HPTLC

**HPTLC Today and in the Future**
Bernd Spangenberg
Offenburg University of Applied Sciences, Germany

**Recent Contributions of HPTLC to the Analysis of Complex Samples Covering Wide Ranges of Polarity. The Cases of Biofuels, Petroleum and Lipidomic Analysis**
Vicente Cebolla
Spanish National Research Council, Spain

**The Capabilities of Effect-Directed Analysis with HPTLC in Environmental Analysis**
Stefan Weiß
Special Purpose Association for Long Distance Water Supply of the State of Baden-Wuerttemberg, Germany

**Comprehensive HPTLC Fingerprinting – A New Approach to Quality**
Eike Reich, The International HPTLC Association

### AFTERNOON SESSION (NORTH AMERICA): HPTLC IN PRACTICE IN NORTH AMERICA

**HPTLC in the Quality Control of Botanical Supplements: Identification and Beyond**
Melissa Daoust, Traditional Medicinals, Inc.

**Non-Botanical Applications of HPTLC**
Martha Jennens, Eurofins Food Integrity & Innovation

**HPTLC Identification and Quantification of Cannabinoids in Cannabis Samples of Different Chemovarieties**
Mohamed Radwan
National Center for Natural Products Research, School of Pharmacy, The University of Mississippi

**The Validation and Transfer of HPTLC Methods Across Multiple Laboratories**
Chris Lenderink, Amway

**HPTLC in Screening for Undeclared Ingredients**
Anton Bzhelyansky, USP

**The International HPTLC Association**
Maged Sharaf, The International HPTLC Association

# Pharmacokinetics of berberine from *Pushyanuga Churna*

*Prof. Dr. Sunita Shailajan*

As Head of the Botany Department, Professor Dr. Shailajan is in charge of the Herbal Research Lab and Animal Testing Centre at Ramnarain Ruia Autonomous College, Mumbai, India. Her expertise is in the development of phytochemical fingerprints of various medicinal plants and traditional formulations using HPTLC. She has also been active in evaluating plant medicines for the management of female reproductive disorders using validated bioanalytical methods to quantitate phytoestrogens and biomarkers from polyherbal formulations. Recently, her PhD student Yugandhara Patil won a Poster Prize at HPTLC 2018 Bangkok.

## Introduction

The complex Ayurvedic powder formulation *Pushyanuga Churna* consists of twenty-five plant ingredients and one mineral. It is prescribed in the clinical treatment of a wide range of female reproductive disorders (Ayurvedic Pharmacopoeia of India, 2008). Validated HPTLC methods have been reported to quantify the presence of phytoconstituents like ursolic acid, β-sitosterol, lupeol, gallic acid, bergenin, quercetin and berberine in *Pushyanuga Churna* [1]. The synergistic action of these phytoconstituents probably contributes to the therapeutic efficacy of the formulation. The natural alkaloid berberine has many pharmacological effects like lowering the blood glucose and lipid levels, increasing the insulin sensitivity or regulating singularly the menstrual pattern and ovulation rate in woman [2].

***Cissampelos pareira* and *Berberis aristata* are the only two sources of berberine in *Pushyanuga Churna*. There are several reports on pharmacokinetics of berberine from biological matrices using HPLC, but none using HPTLC. Hence, due to the diverse advantages of HPTLC, the pharmacokinetic profile of the bioactive marker berberine was studied using HPTLC after the oral administration of aqueous preparations of *Pushyanuga Churna* to female Wistar rats.**

## Sample preparation

An aqueous slurry of *Pushyanuga Churna* was prepared by vortexing and agitating the formulation overnight and administered orally to fasting rats (2000 mg/kg body weight). Blood was withdrawn at definite time intervals, and berberine was extracted with dichloromethane from the processed plasma. After protein precipitation and centrifugation, the organic layer was evaporated under nitrogen. The residue was reconstituted with 200 µL methanol.

## Chromatogram layer

HPTLC plate silica gel 60 $F_{254}$ (Merck), 20 x 10 cm, pre-washed with methanol, heated for 5 min at 110 °C

## Sample application

Linomat 5, band length 7 mm, distance from lower edge 10 mm, application volume 10 µL for sample solutions

## Chromatography

Development with toluene – ethyl acetate – methanol – formic acid 6:6:2:1 in a Twin Trough Chamber after 20 min chamber saturation, up to the migration distance of 85 mm

## Documentation

Under UV 366 nm using the Reprostar 3 documentation system

## Densitometry

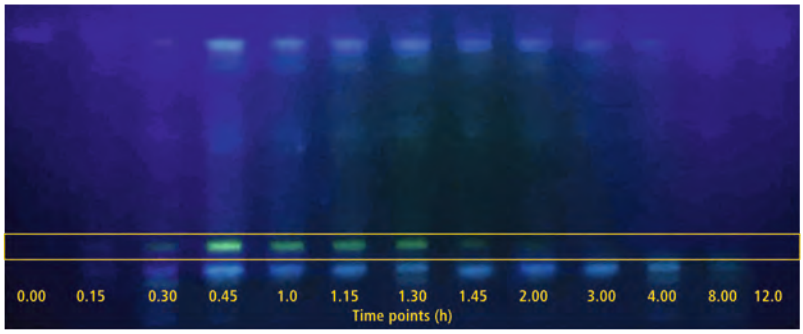
TLC Scanner 4 and winCATS, fluorescence measurement at 366/>400 nm (mercury lamp), slit dimension 6.00 mm x 0.45 mm, scanning speed 20 mm/s

## Results and discussion

The estimation of berberine using the developed HPTLC method was validated according to US-FDA bioanalytical guidelines as follows:

| Parameters | Berberine |
|---|---|
| $hR_F$ | 21 |
| Linearity | 0.1–20 µg/mL |
| LOD/LOQ | 0.05/0.1 µg/mL |
| Regression equation, $R^2$ | y = 1136x + 64.8, 0.9930 |
| System suitability | CV < 2% |
| Intra/Inter-day precision | CV < 2%, nominal within 85–115 % |
| Stability and ruggedness | CV < 2%, mean difference within ± 5% |
| Recovery | within 95–105% |
| Process efficiency | 85% |

The pharmacokinetic parameters were evaluated from blood plasma withdrawn from rats at different time points up to 12 h post administration of *Pushyanuga Churna*.



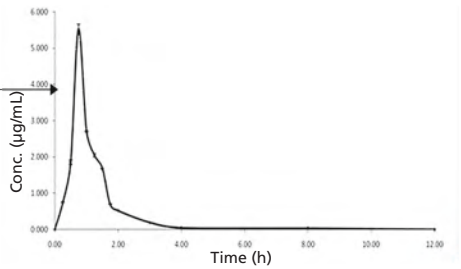

1

*HPTLC chromatogram at UV 366 nm of plasma samples taken over 12 h*

WinNonlin® 6.3 Phoenix™ computer software (version 4.3, Pharsight Corporation, Mountain View, CA, USA) was used for evaluation of the pharmacokinetic parameters and a non-compartmental model was used to analyze the plasma drug concentration time curve data by employing the linear log trapezoidal method. It was observed that the concentration of berberine consistently rised in the blood from 0.15 h up to its maximum concentration at 0.45 h ($T_{max}$). Berberine was rapidly eliminated from the body within 2 h and was complete eliminated from the system after 12 h post administration.

*Pharmacokinetic parameters for berberine from plasma*

| Parameters | Estimate |
|---|---|
| $C_{max}$ (µg/mL) | 5.52 |
| $T_{max}$ (h) | 0.75 |
| Lambda Z ($K_{el}$) | 0.69 |
| HL_Lambda_z ($T_{1/2}$) | 1.01 |
| AUC Last ($AUC_{0-t}$) | 4.53 |
| AUC all | 4.60 |
| AUC Inf_obs ($AUC_{0-\infty}$) | 4.58 |




2

The detection of berberine in plasma of rats administered with *Pushyanuga Churna* highlights the feasibility of HPTLC as the method of choice for the estimation of berberine concentration in rat plasma. Berberine, along with the developed method, can also be proposed as an established marker to monitor the absorption, distribution, metabolism and excretion (ADME) processes of herbal formulations like *Pushyanuga Churna*.

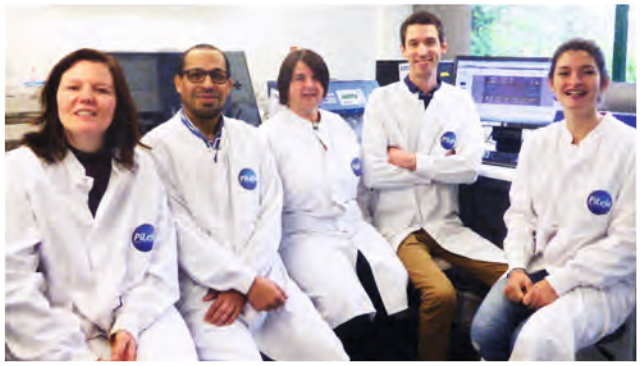
[1] L. Li *et al.* PLoS ONE 10 (2015). DOI: 10.1371/journal.pone.0144072

[2] S. Shailajan *et al.* J AOAC Int 102 (2019) 1003. DOI: 10.5740/jaoacint.18-0380

Further information is available on request from the author.

Contact: Prof. Dr. Sunita Shailajan, Department of Botany, Ramnarain Ruia Autonomous College, Matunga, Mumbai - 400 019, India, sunitashailajan@gmail.com

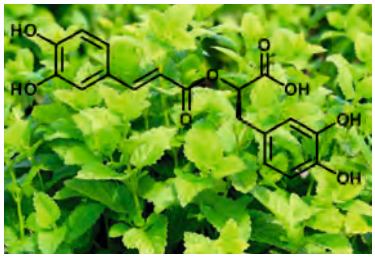
# Analysis of innovative plant extracts



*From left: Anaïs Escalon, Mustapha Aboussif, Dr. Valérie Bardot, César Cotte (PiLeJe Industrie), Lucile Berthomier (Sigma-Clermont)*

The French company PiLeJe Industrie develops liquid and dry ingredients using patented procedures or in-house developed processes. All stages are covered from generating innovative ingredient concepts to producing and validating these in scientific studies:

- Sourcing (search, selection and traceability) of plants in accordance with target market regulations
- Transformation using numerous procedures and specially designed technology, such as the patented ipowder® production process [1]
- Plant analysis for optimum quality using a range of analytical techniques including HPTLC with fingerprints and quantification of active compounds in the plant and its extracts.




1 *Melissa officinalis L. leave rich in RA*

## Introduction

*Melissa officinalis* L. is well-known for its beneficial effects in anxio-depressive states and for its gastrointestinal protective activity. The developed lemon balm ipowder® is made from the infusion of dry *M. officinalis* L. leaves concentrated to the same plant material.

**HPTLC analysis of the fresh leave extract was used to determine the optimal harvesting period. HPTLC analysis of the dry leave extract showed that the ipowder® technology increased the concentration of the major compound rosmarinic acid (RA), while preserving the phytochemical profile of the native plant and its antioxidant properties.**

## Standard solution

RA dissolved in ethanol – water 1:1 (0.05 mg/mL)

## Sample preparation

Ground fresh leaves (1 g) were extracted with 25 mL methanol by sonication and filtered. Ground dry leaves or ipowder® (0.2 g) were extracted with 200 mL ethanol – water 1:1 by sonication and filtered. For fingerprint analysis, a higher concentrated sample solution was used (1 g/100 mL).

## Chromatogram layer

HPTLC plate silica gel 60 $F_{254}$ (Merck), 20 × 10 cm

## Sample application

Automatic TLC Sampler (ATS 4), 20 tracks, band length 8.0 mm, distance from left edge 14.0 mm, distance from lower edge 8.0 mm, application volumes of 5 or 10 µL for sample and 2–8 µL for RA solutions

## Chromatography

In the Automatic Developing Chamber (ADC 2) with chamber saturation (with filter paper) for 20 min and after activation at 33% relative humidity for 10 min using a saturated solution of magnesium chloride, development with and with toluene – ethyl formate – formic acid 6:4:1 for quantification ethyl acetate – water – acetic acid – formic acid 100:27:11:11 for fingerprint analysis to the migration distance of 70 mm (from the lower edge), followed by drying for 10 min

## Post-chromatographic derivatization

1. Immersion in natural product reagent (NP; immersion speed 5 cm/s, immersion time 0 s), drying under a stream of cold air, immersion in polyethylene glycol reagent (PEG; same conditions), followed by drying

2. Immersion in 0.5 mM methanolic 2,2-diphenyl-1-picrylhydrazyl solution (DPPH*; immersion speed 3 cm/s, immersion time 5 s), drying at room temperature in the dark for 90 s and then at 60 °C for 30 s

## Documentation

TLC Visualizer at UV 254 nm, UV 366 nm (NP/PEG) and under white light illumination (DPPH)

## Densitometry

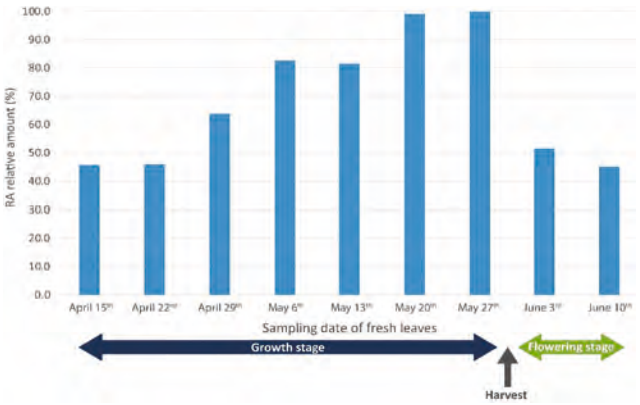
TLC Scanner 4 and *visionCATS*, absorption measurement at 330 nm, UV/VIS spectra recorded from 200 to 700 nm

## HPLC-UV

Using a Waters HPLC-PDA system, separation of 10 µL sample extract ($n$ = 3) on a symmetry C18 cartridge, (100 Å, 5 µm, 4.6 mm x 250 mm) with a 2% aqueous acetic acid solution (A) and acetonitrile (B): 0–20 min from 90–10% (A-B) to 50–50%, 20–21 min to 10–90% and 21–40 min isocratic (90% B), detection at UV 352 nm, RA calibration from 25 to 400 mg/L

## Results and discussion

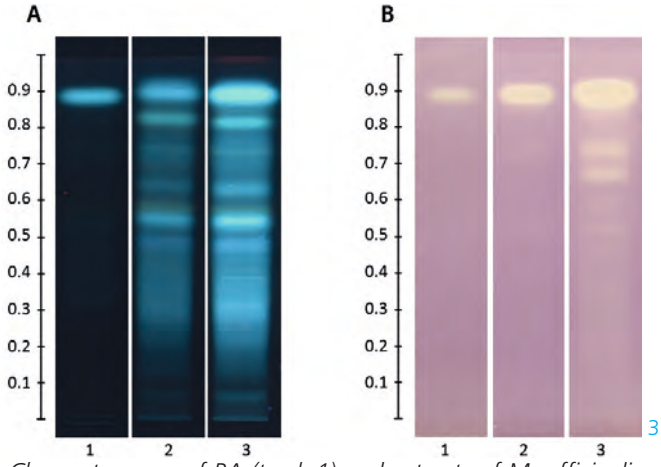
The quality of the extracts depends mostly on the plant quality. A controlled selection of the plant harvest was necessary. Weekly quantifications of RA by HPTLC in ground fresh leave extracts of the same crop figured out the end of May to be the most appropriate period for harvesting *M. officinalis* leaves.



2

*Evolution of the relative RA amount during M. officinalis growth*

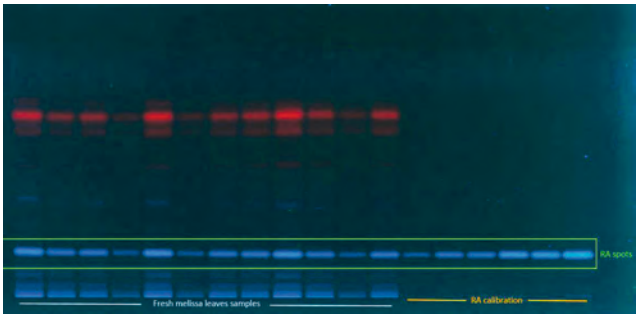
The HPTLC comparison between *M. officinalis* ground dry leave and ipowder® profiles showed that the ipowder® fingerprint was equivalent to that of the ground dry leaves, but with a higher concentration of RA. Both ground dry leaves and ipowder® had antioxidant activities, RA being the major marker of this activity.



3

*Chromatograms of RA (track 1) and extracts of M. officinalis ground dry leave (track 2) and ipowder® (track 3) at UV 366 nm (A) and under white light illumination after DPPH* (B) for fingerprint analysis*

By HPTLC and HPLC similar quantities of RA were measured. The RA concentration was two fold higher in the ipowder® compared to the ground dry leaves. These analyses confirm the enrichment of the extract with the ipowder® process.



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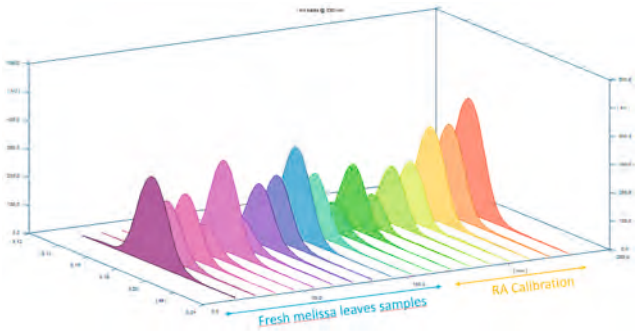
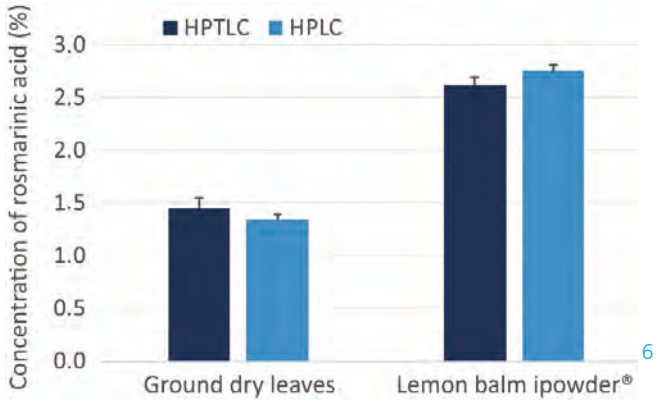


5

*HPTLC chromatogram at UV 366 nm for quantification of RA in melissa leave extracts and respective densitograms*




6

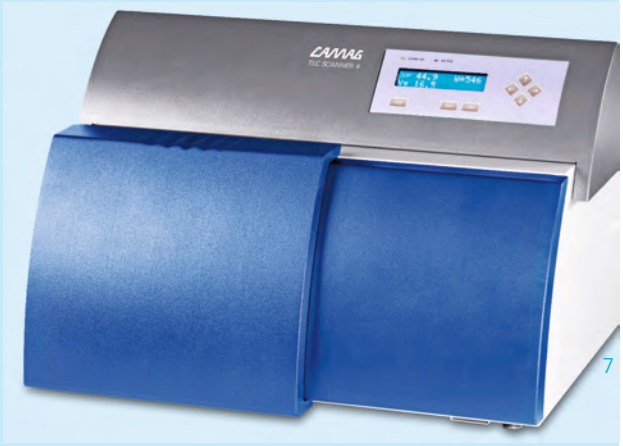
*Percentage of RA in lemon balm ipowder® and M. officinalis ground dry leave extracts by HPTLC versus HPLC*

HPTLC is a powerful analytical tool for quality control and development of ingredients. It is rapid and easy to perform at the different stages: sourcing and selection of raw material, process comparison and characterization of plant extracts. It is a good alternative to HPLC for the quantification of secondary metabolites. The use of HPTLC shows that the ipowder® technology maintains the fingerprint of the native plant and concentrates secondary metabolites.

[1] M. Dubourdeaux. Patent no. EP2080436 A2 (2009)

Further information is available on request from the authors.

Contact: Dr. Valérie Bardot, Naturopôle Nutrition Santé, Les Tiolans, 03800 Saint-Bonnet de Rochefort, France, v.bardot@pileje.com



7

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# Rapid chemotaxonomic discrimination of *Clerodendrum* species

*From left: Madhumita Srivastava, Priyanka Maurya, Sonali Mishra, Narendra Kumar and Dr. Karuna Shanker*

**Developing a fast separation and densitometric evaluation, a large number of samples were analyzed simultaneously. Solvent consumption of up to 15 mL per plate (0.8 mL/sample) was low. The densitometric method was suited for batch-to-batch process control, quality control and detection of adulterations of raw powdered/processed *Clerodendrum* species intended for therapeutic purposes.**

The research group of Dr. Shanker, Principal Scientist in the Analytical Chemistry Department, Central Institute of Medicinal and Aromatic Plants CSIR, Lucknow, India, works on the isolation, characterization and identification of phytochemicals from plants used in the Indian system of medicine *Ayurveda*. Employing different liquid-chromatographic techniques, well-characterized phytochemicals are recognized either as bioactive or chemical markers. These are used to define the therapeutic quality of plant-derived products or as a chemotaxonomic tool for the screening of elite cultivars. Marker-based validated TLC/HPTLC methods are used for high-throughput screening of germplasm and selection of elite chemotypes for medicinal plants in the medical plant variety development program.

## Introduction

TLC/HPTLC has emerged as alternative to other analytical tools and is a globally accepted technique to characterize phytochemicals, crude drug extracts and their formulations. This technique is ideally suited for the screening of plant extracts, if compared to HPLC analysis. Three closely related *Clerodendrum* species *C. inerme* (CI), *C. multiflorum* (CM) and *C. viscosum* (CV) were distinguished *via* the four marker compounds 24β-ethylcholesta-5,22*E*,25-triene-3β-*O*-D-glucoside (1/ECTOG), clerodinin-A (2/CDA), 24β-ethylcholesta-5,22E,25-triene-3β-ol (3/ECTO) and lupeol (4/LU). The chemotaxonomic differentiation was established by similarity analysis of the obtained TLC fingerprints using hierarchical clustering analysis (HCA) and principal component analysis (PCA) [1].

## Chromatogram layer

TLC plate silica gel 60 $F_{254}$ pre-washed using methanol and dried at 80 °C for 5 min

## Standard solutions

Stock solutions (1.0 mg/mL) of the four marker compounds were diluted with methanol and filtered (0.45-µm nylon filter) to obtain standard mixture solutions (20–100 µg/mL).

## Sample preparation

Dried, powdered plant material (100 mg) was extracted with methanol (3 10 mL) by ultrasonication at 40±2 °C for 15, 30 and 45 min. Each pooled extract (30 mL) was concentrated under vacuum and re-dissolved in 1.0 mL methanol.

## Sample application

Linomat 4, band length 5 mm, distance from both the lower and left edge 10 mm, track distance 20 mm, application volume 5 µL each

## Chromatography

Development with toluene – ethyl acetate – methanol – acetic acid 6.5:3:1:0.5 in the twin trough chamber after 10 min chamber saturation, migration distance 90 mm from lower plate edge

## Post-chromatographic derivatization

Immersion into anisaldehyde – sulfuric acid – acetic acid reagent (1:5:95) using the Chromatogram Immersion Device (immersion speed 5 cm/s, immersion time 3 s), followed by drying in cold air for 10 min and heating at 135 °C for 7 min using the TLC Plate Heater.

## Documentation

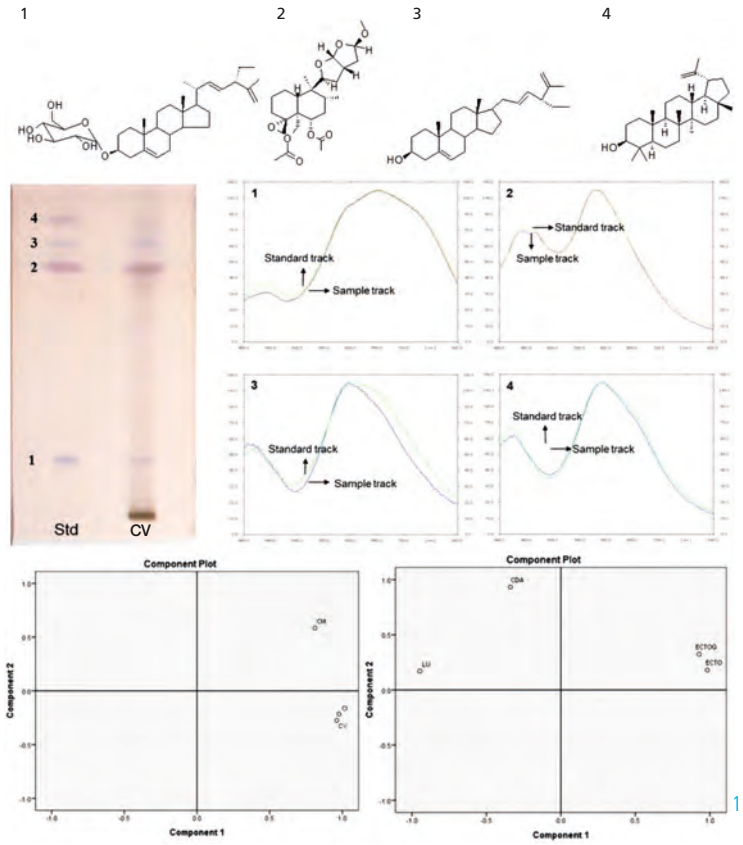
With the DigiStore 2 under white light

## Densitometry

TLC Scanner 4 with winCATS, spectra recording from 400 to 800 nm, absorbance measurement at 560 nm, slit dimension 6.00 mm x 0.45 mm, scanning speed 20 mm/s

## Results and discussion

The PRISMA model was applied for mobile phase development. The final method was validated according to current ICH guidelines [2]. The taxonomic differentiation was established by similarity analysis of the obtained fingerprints. The developed and validated method was applied for the qualitative and quantitative analysis of the chemical markers (1/ECTOG, 2/CDA, 3/ECTO, and 4/LU) in the three *C.* species. The targeted compounds were separated without any interference by the sample matrix, and specificity was also confirmed by FIA-MS.




*Chromatogram of standard mixture (track 1) and methanolic CV extract (track 2), overlaid VIS spectra of standard/sample zones and PCA plots distinguishing the species*

Linear calibration was performed in the range of 100–500 ng/band with correlation coefficients between 0.9937 and 0.9985. The mean precisions (%*RSD*, $n = 3$) were evaluated via three replicates, each at 100, 300 and 500 ng/band on the same day (intra-day precision from 1.3% to 2.5%) and on three consecutive days (inter-day precision from 1.1% to 2.3%). Good recoveries between 99.0% and 101.0% were obtained at a low, medium and high concentration levels for each marker compound in the given sample matrix.

The taxonomic differentiation by similarity analysis of the fingerprints (calculated as correlation and congruence coefficients of 0.26–0.86) demonstrated the poor similarities. PCA resulted into two PC loadings and divided the three species into two groups. PC1 separated CM from CI and CM (explaining 85.48% of variance), mainly due to the distribution of two triterpenoids. The three phytochemicals ECTOG, ECTO and CDA were found as potential taxonomic markers for the differentiation of *Clerodendrum* species.


[1] ICH,Q2 (R1): Validation of Analytical Procedures: Text and Methodology, 2005

[2] S. Madhumita *et al.* K. J Planar Chromatogr, (2019), 32(3), 211–222


Further information is available on request from the authors.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