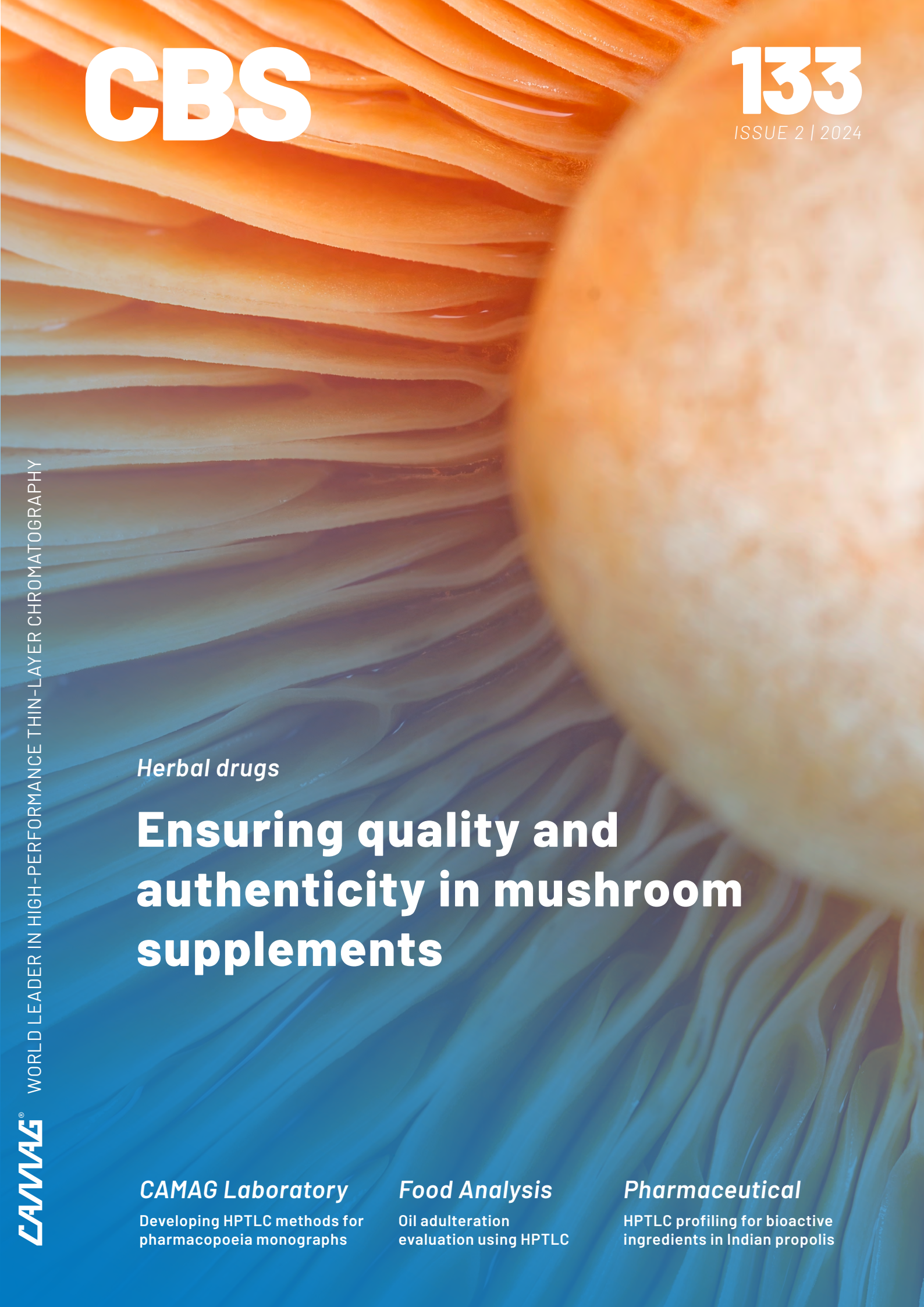
CBS
133
ISSUE 2 | 2024



WORLD LEADER IN HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

CAMAG®

Herbal drugs

Ensuring quality and authenticity in mushroom supplements

CAMAG Laboratory
Developing HPTLC methods for pharmacopoeia monographs

Food Analysis
Oil adulteration evaluation using HPTLC

Pharmaceutical
HPTLC profiling for bioactive ingredients in Indian propolis

CBS
CAMAG BIBLIOGRAPHY SERVICE

No. 133, Issue 2 | 2024

CAMAG Bibliography Service
Edited by Prof. Dr. Melanie Broszat
cbs@camag.com
published by CAMAG Switzerland

Cumulative CAMAG Bibliography Service (CCBS)

Please visit our literature database CCBS at: www.camag.com/ccbs

IN THIS ISSUE

Procedures, applications

CAMAG (Switzerland)
Sonnenmattstrasse 11 | 4132 Muttenz
info@camag.com | www.camag.com

Note from the editor

Dear friends of HPTLC,

welcome to another exciting issue of the CBS Journal as we continue our mission to present important research and advances in the world of HPTLC. This issue brings together several compelling applications from experts around the world that demonstrate the versatility and power of HPTLC in addressing modern analytical challenges.

Our first article delves into the burgeoning world of functional fungi. The team at Nammex is using an innovative HPTLC method to distinguish high-quality mushroom extracts from inferior products on the market. Their research provides a critical tool for improving product authentication in this rapidly growing sector.

Next, we present an insightful article on the development of HPTLC methods for pharmacopoeia monographs. The CAMAG laboratory has long helped to set global standards for the identification of botanical and herbal drugs, and this work continues to shape the quality control of pharmacopoeias worldwide.

In this CBS issue, we also address a key concern in food safety: oil adulteration. The Nestlé research team in Lausanne demonstrates the ability of HPTLC to detect adulteration in edible oils. This method provides a rapid and reliable solution for ensuring authenticity in global food supply chains, underscoring the importance of the technique in both industrial and regulatory contexts.

In the pharmaceutical field, our fourth article focuses on Indian propolis and its bioactive constituents. Researchers from the Poona College of Pharmacy have used HPTLC to determine key neuroprotective compounds in propolis, highlighting the utility of the method in evaluating natural products for therapeutic purposes.

As always, we hope this issue of the CBS Journal inspires and informs your work. We welcome your feedback and look forward to advancing the field of HPTLC together.

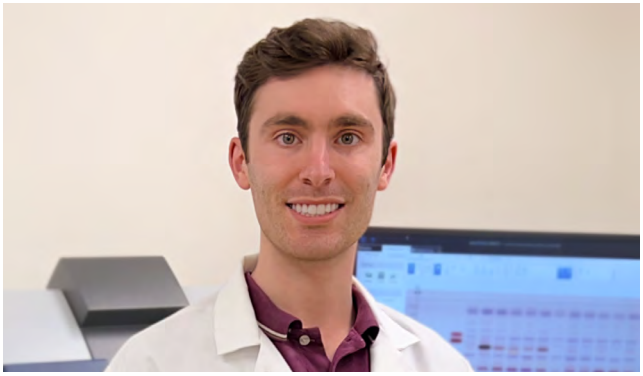
Yours sincerely,

Prof. Dr. Melanie Broszat

Editor CBS

HPTLC for quality differentiation of functional mushrooms

Coleton Windsor (Nammex)



Nammex specializes in the production of high-quality, certified organic mushroom extract powders for the food and dietary supplement (DS) industries. As a result of the rapid growth of the functional mushroom market, we have observed the introduction of many new products of varying quality. Nammex has a long-standing history of leading the industry in product analysis, with a focus on ensuring product authenticity and efficacy [1]. Our laboratory has developed an innovative HPTLC method for the identification and quality control testing of diverse species used in DS products. With this method, we aim to enhance the overall reliability and transparency of quality testing in the industry.

Introduction

The functional mushroom market is experiencing significant growth, driven by factors like increased DS usage and ongoing medical research. Despite the market's size, only one validated HPTLC mushroom identification method has been published (USP *Ganoderma lucidum* monograph) [2], and its indiscriminate use across other species may lead to misidentification, undermining the reliability of the identification process and creating a need for more comprehensive testing solutions.

HPTLC is widely recognized for its effectiveness in botanical identification, making it an ideal method for mushroom analysis. In the absence of validated methods, consumers risk exposure to mislabeled or adulterated products. For instance, products containing tempeh-like mycelium (i.e. vegetative body) fermented grain are often marketed as mushrooms (i.e. fruiting bodies) despite significant compositional differences. Additionally, concentrated mushroom extracts may be deficient in specific marker compounds due to processing conditions.

HPTLC offers a robust, highly selective approach for mushroom differentiation. This new method ensures that characteristic compounds from diverse chemical classes in mushrooms are clearly separated, supporting accurate species identification. The advantages of HPTLC in this context include its specificity, versatility, and ability to detect adulteration in complex products.

Standard solutions

Standard stock solutions are prepared at 0.5 mg/mL in methanol.

Sample preparation

Samples consist of 250 mg of mushroom extract powder or finely milled whole mushrooms. These are extracted in 5.0 mL of methanol, vortexed for 10 s, sonicated for 10 min at room temperature, and centrifuged at 3500 rpm for 10 min. The supernatant is then transferred to vials.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} Premium Purity (Supelco, Merck), 20 × 10 cm are used.

Sample application

10.0 µL of sample solutions and 2.0 µL of standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 15 tracks, band length 8.0 mm, distance from the left edge 20.0 mm, track distance 11.4 mm, distance from the lower edge 8.0 mm.

Chromatography

Plates are developed in the ADC 2, with chamber saturation (with filter paper) for 20 min and after activation at 33 % relative humidity for 10 min using a saturated magnesium chloride solution, development with toluene - methanol - and acetic acid 85:10:5 (*V/V*) to the migration distance of 70 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

The plates are immersed into *p*-anisaldehyde sulfuric acid reagent using the Chromatogram Immersion Device (immersion speed: 5 cm/s, immersion time: 0 s). After derivatization, the plates are heated at 100 °C for 4 min using the TLC Plate Heater.

Documentation

Images of the plates are captured with the TLC Visualizer 3 in UV 254 nm, UV 366 nm, and white light after development, and again after derivatization in UV 366 nm and white light.

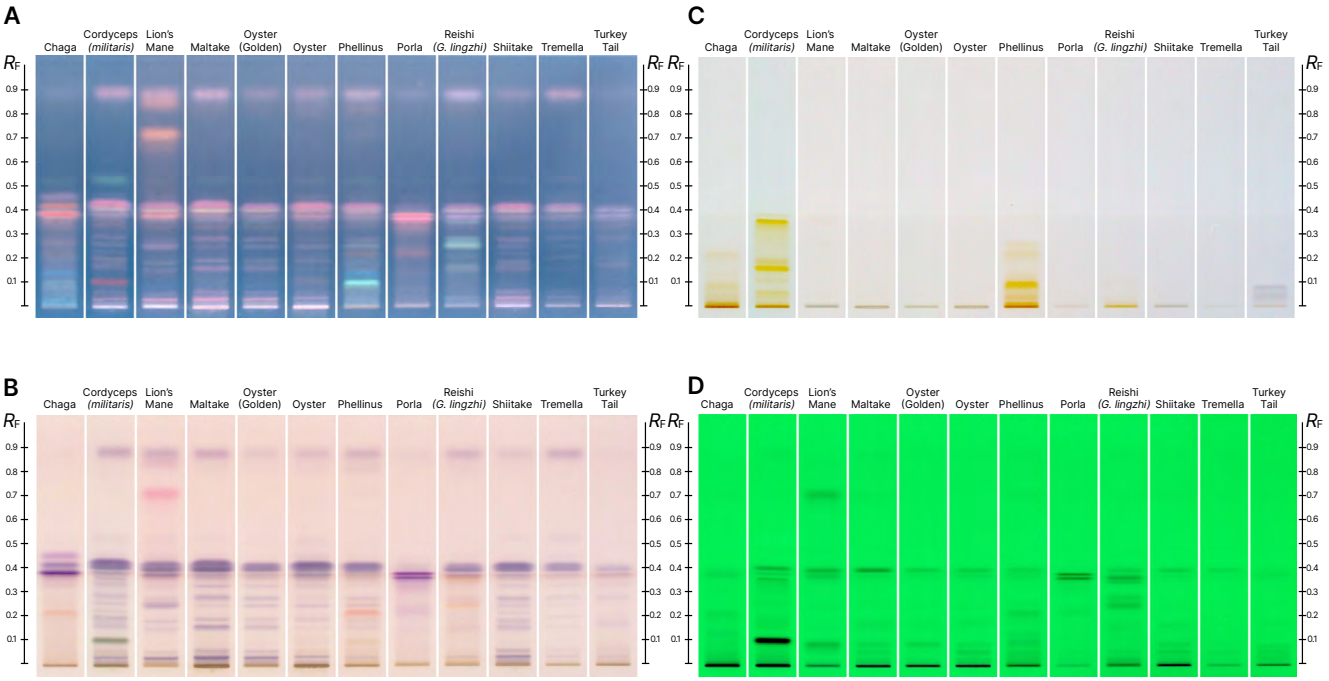
Results and discussion

The high selectivity of the HPTLC method is demonstrated through distinct chromatographic fingerprints obtained for each species. These fingerprints display characteristic bands under multiple detection modes, providing a reliable means of differentiating between species and product types such as mushroom extracts and mycelia fermented grain powders.

Key marker compounds for each species were identified through literature, playing a critical role in distinguishing between the mushroom and the mycelium. Specifically, the mushroom is known to exhibit a different profile of compounds than the mycelium. HPTLC comparisons of mushroom extracts, supported by these chemical markers, effectively demonstrate these differences.

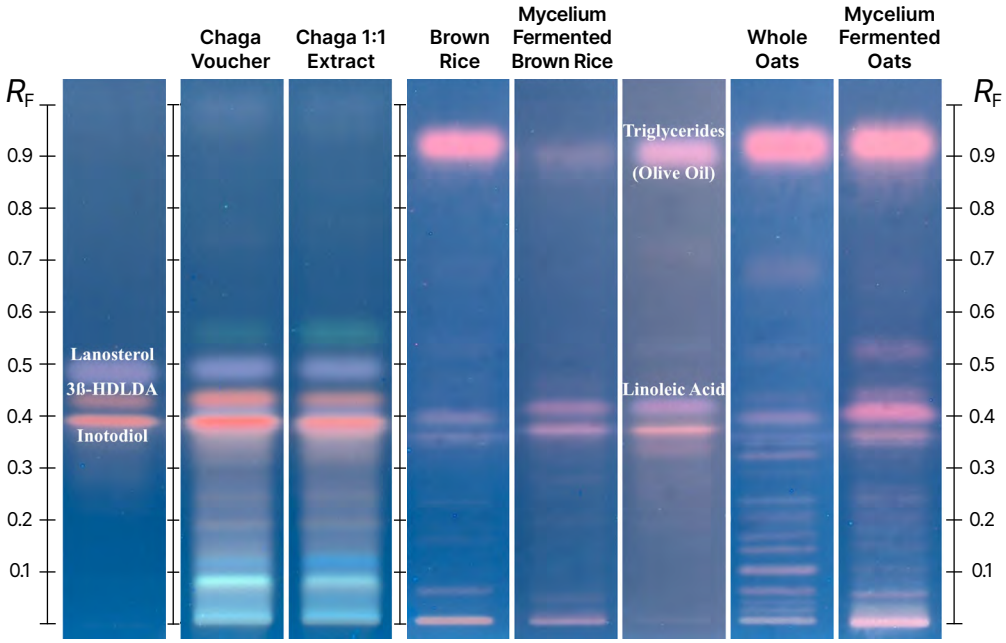
HPTLC comparisons between Chaga conk, pure mycelium, and fermented grain forms reveal significant compositional differences, with fermented grain fingerprints closely matching grain reference materials. Importantly, Chaga triterpenoid markers are absent in fermented grain, which instead shows high concentrations of triglycerides and linoleic acid. These chromatograms highlight the clear differences between Chaga conk, 1:1 extract, brown rice and oats, and fermented grain products, underscoring HPTLC's effectiveness in detecting potential adulteration and verifying product authenticity.

While fermented grain products are expected to contain grain, the lack of sufficient mycelium or relevant compounds, along with unclear labeling practices, raises concerns about product authenticity. Many fermented grain products prominently display "mushroom" on the front label, along with images of mushrooms, but only disclose their myceliated grain content on the back, with some brands failing to identify the grain entirely. This inconsistency in labeling, coupled with the compositional differences identified through HPTLC, underscores the urgent need for more transparent and stringent quality control measures in the mushroom supplement industry.



*HPTLC chromatograms of whole mushroom, conk, or sclerotium vouchers from 12 species, highlighting compositional differences between species under various detection modes. Images after derivatization are shown in UV 366 nm (**A**) and white light (**B**). Chromatograms captured after development are displayed in white light (**C**) and 254 nm UV light (**D**).*

Herbal Drug Analysis



HPTLC comparisons between Chaga conk voucher and fermented grain forms reveal significant compositional differences, with fermented grain fingerprints closely matching grain reference materials. Key Chaga marker compounds – such as inotodiol (R_F 0.39), 3β-HDLDA (3β-hydroxylanosta-8,24-dien-21-al) (R_F 0.43), and lanosterol (R_F 0.49) – are notably absent in fermented grain, which instead shows high concentrations of triglycerides and linoleic acid.

In conclusion, the development of the innovative HPTLC method for the differentiation of functional mushrooms offers a significant advancement in ensuring product authenticity and quality within the growing mushroom supplement market. By providing clear, reliable chromatographic fingerprints for various species, this method enhances the ability to detect adulteration and verify product composition, particularly in distinguishing between mycelia fermented grain-based products marketed as mushrooms. As the market continues to expand, the implementation of robust, transparent quality control measures like HPTLC will be critical in maintaining consumer trust and safeguarding product efficacy.

Literature

[1] Chilton, Jeff. White Paper. Redefining Medicinal Mushrooms: *A New Scientific Screening Program for Active Compounds*. Nammex, 2015. jeff@nammex.com

[2] United States Pharmacopeia (USP). *Ganoderma lucidum* Fruiting Body Monograph. USP 43-NF 38, United States Pharmacopeial Convention, Rockville, MD, 2020.

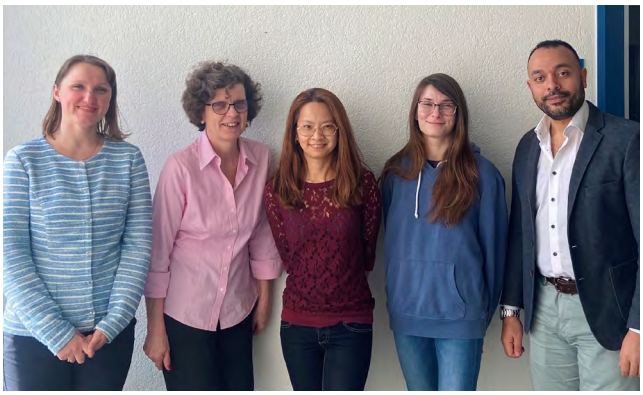
Further information is available on request from the author.

Contact

Coleton Windsor, Nammex, Box 1780, Gibsons, British Columbia, Canada, coleton@nammex.com

Developing HPTLC identification methods for pharmacopoeia monographs

Dr. Kateryna Khokhlova, Ilona Trettin, Dr. Tiên Do, Sonja Drobnjak, Dr. Ehab Mahran



For the past 20 years, CAMAG Laboratory has been a key contributor to pharmacopoeias worldwide, developing identification methods for botanicals, herbal drugs, and extracts. As a pioneer in standard-setting efforts, Dr. Eike Reich played a crucial role as an HPTLC expert in numerous pharmacopoeia committees. Now, as he transitions into retirement, Dr. Reich passes the torch to Dr. Tiên Do and her team, who continue to advance this important work.

Introduction

To effectively support the pharmacopoeia committees, all members of the laboratory undergo extensive training in working with standardized methodologies. Delivering HPTLC methods tailored to the specific requirements of pharmacopoeias involves more than just standardized HPTLC; each scientist must also understand and follow a general method development process. This process encompasses several key stages, illustrated in this paper using the European Pharmacopoeia (Ph. Eur.) monograph on *Epimedium* leaf as an example.

Existing monographs for Epimedium and their accepted species:

Pharmacopoeia	Monograph name	Species included
Korean Ph.	*Epimedium* Herb or leaf	*E. koreanum* Nakai, *E. brevicornum* Maxim., *E. pubescens* Maxim., *E. sagittatum* Maxim., and *E. wushanense* T. S. Ying
Japan Ph.	*Epimedium* Herb or leaf	*E. koreanum* Nakai, *E. brevicornum* Maxim., *E. pubescens* Maxim., *E. sagittatum* Maxim., and *E. wushanense* T. S. Ying
Chinese Ph.	*Epimedium* leaf	*E. koreanum* Nakai, *E. brevicornum* Maxim., *E. pubescens* Maxim., *E. sagittatum* Maxim
Hong-Kong Standards of Chinese Materia Medica	*Epimedium* Herb or leaf	*E. koreanum* Nakai, *E. brevicornum* Maxim., *E. pubescens* Maxim., *E. sagittatum* Maxim

As the preferred chromatographic technique for the identification of herbal drugs, HPTLC aims to determine a characteristic chromatogram (fingerprint) based on the relative position, color, and intensity of specific zones. According to Ph. Eur., HPTLC must adhere to the Ph. Eur.'s general chapter 2.8.25., which specifies all steps and parameters of the HPTLC process. This document describes in detail the specific points relevant to the development of an HPTLC identification method.

Discussion

Developing a suitable identification method involves several steps:

Step 1: definition

The scope of the method must clearly specify the article (e.g. the medicinal plant) to be identified. In addition to the Latin plant name, the definition should include the accepted plant part(s) and the process by which the article is obtained (drying, cutting, extracting, etc.). Ideally, an identification method is specific for the article of the monograph and distinguishes related articles that may be considered adulterants.

Various monographs on "*Epimedium*" target the whole or fragmented dried leaf or herb of several species (see table) according to availability in different markets. The Ph. Eur. monograph on *Epimedium* leaf includes whole or fragmented dried leaf of the major species *E. koreanum* Nakai, *E. brevicornum* Maxim., and *E. pubescens* Maxim., including mixtures thereof.

Acceptance criteria for the herbal drug "*Epimedium* leaf" must include the selected drugs and exclude all others (e.g. *E. sagittatum*).

Step 2: collection of samples

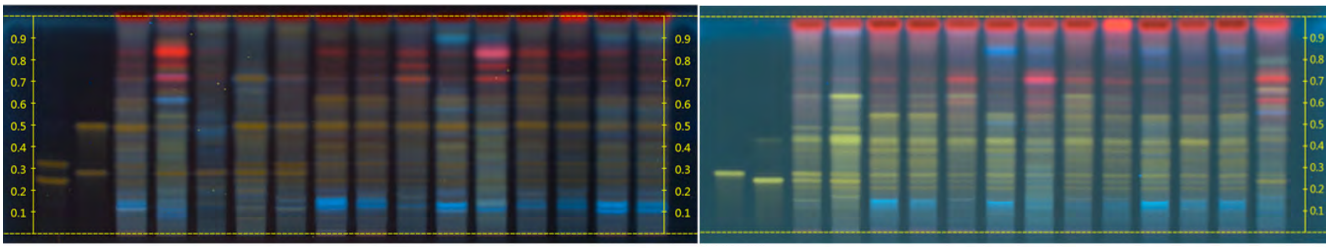
Samples of different origins and related species are collected by the pharmacopeia group and distributed to various collaborating laboratories. Each laboratory also collects its own samples. A wide range of samples is crucial to ensure that the method is applicable to routine analysis of market samples.

Step 3: development / evaluation of HPTLC method(s)

Using standard HPTLC conditions, methods from pharmacopoeias are evaluated for reproducibility, practicality, and fitness for purpose. Other methods can also be considered. For *Epimedium* leaf, several methods have been proposed, each with specific advantages and limitations.

A first proposal was made to the Traditional Chinese Medicine (TCM) Working Party by the Shanghai Institute for Materia Medica (SIMM), using water, formic acid, n-butanol, ethyl acetate 1:1:3:6 (*V/V*) as developing solvent. During the peer review in our laboratory, the R_F values were lower and the colors of zones slightly different.

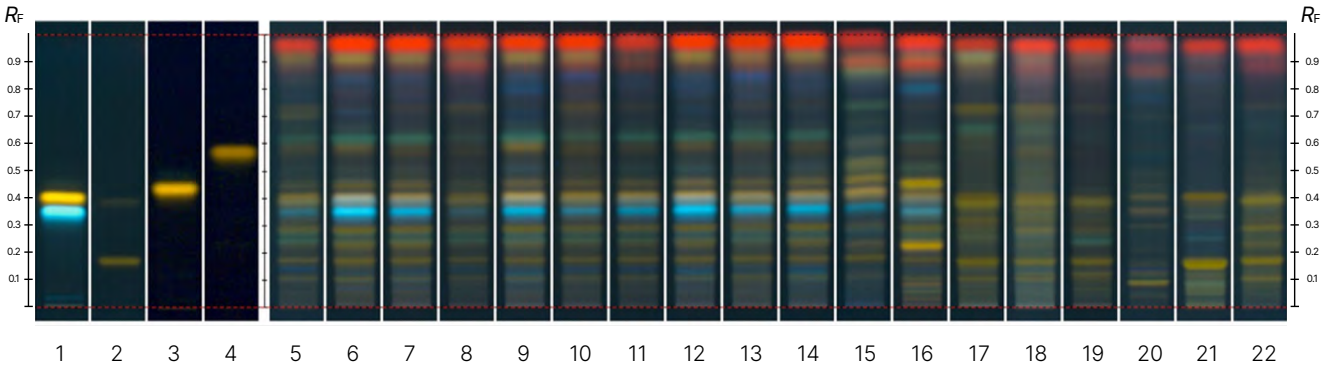
This prompted us to optimize sample preparation, developing solvents, and detection, based on a previously established method for separation of flavonoids, using ethyl acetate - formic acid - water 8:1:1 (*V/V*) and derivatization with NP/PEG reagents.



visionCATS data from SIMM

visionCATS data from CAMAG

Evaluation of the first proposal

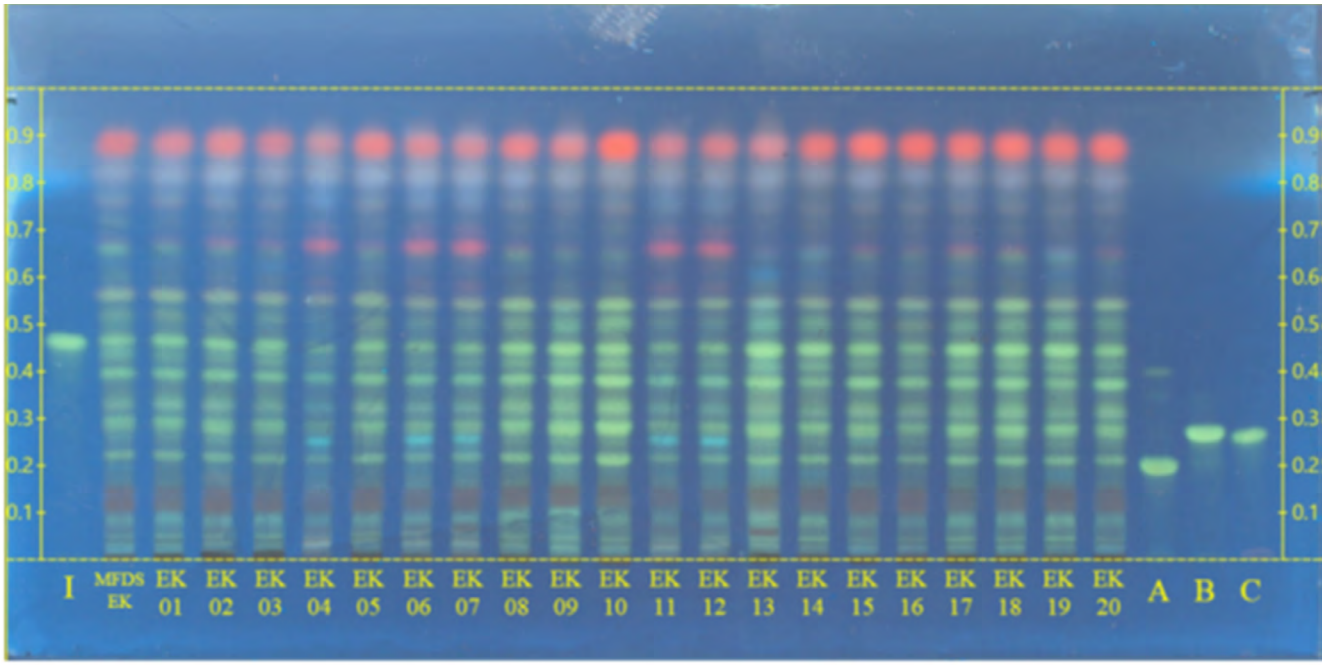


Track	Sample
1	Hyperoside, Chlorogenic acid*
2	Epimedin C
3	Luteolin-7-glycoside
4	Quercitrin
5	S8041 *Epimedium brevicomum*
6	S13780 *Epimedium* – E2
7	S13781 *Epimedium* – E3
8	S13782 *Epimedium* – E4
9	S13783 *Epimedium* – E5
10	S13785 *Epimedium* – E7
11	S13786 *Epimedium* – E8
12	S13787 *Epimedium* – E9
13	S13788 *Epimedium* – E10
14	S13789 *Epimedium* – E12
16	S13790 *Epimedium* – E14
16	S13784 *Epimedium* – E6
17	S13779 *Epimedium*
18	S8038 *Epimedium* Herb-5 (*E. pubescens*)
19	S13778 *Epimedium*
20	S8039 *Epimedium* Herb-6 (*E. koreanum*)
21	S8036 *Epimedium* Herb-3 (*E. wushanese*)
22	S8042 *Epimedium* Herb-9 (*E. sagittatum*)

* with increasing R_F

Second proposal

In parallel, a third method with good reproducibility, using ethanol - ethyl acetate - water 2:1:8 (*V/V*) was developed for consideration by the United States Pharmacopoeia by the Korean group led by Prof. Jang (Kyung Hee University). For compliance with Ph. Eur. Chapter 2.8.25, we included a System Suitability Test (SST) and intensity markers.

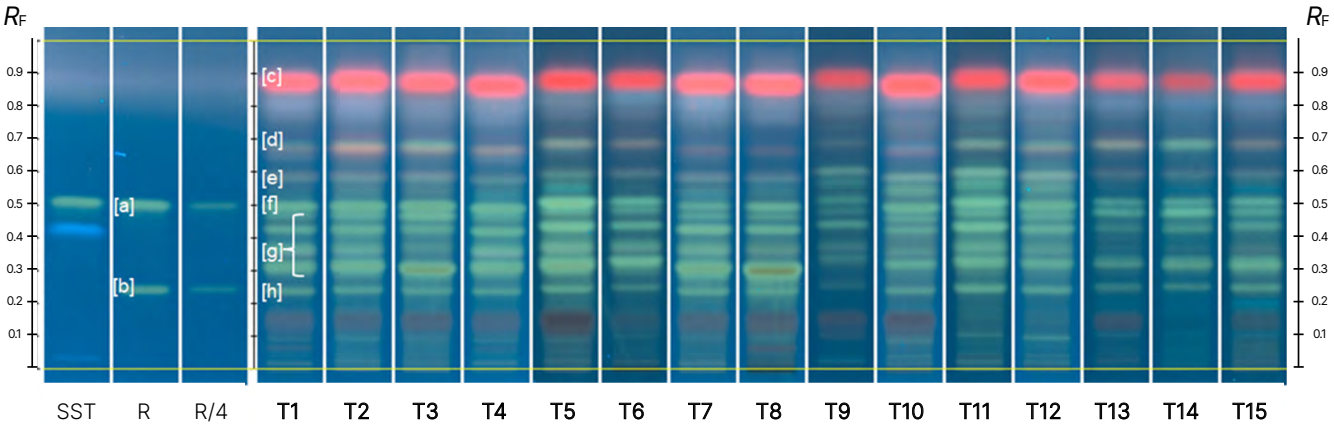


I) Icariin; A) Epimedin A; B) Epimedin B; C) Epimedin C, EK01-EK20) *Epimedium koreanum* leaf; MFDS EK) *Epimedium koreanum* leaf

Third proposal

Step 4: method selection and acceptance criteria definiton

In several iterations, the experts compare the submitted proposals and reach agreement on the most suitable one. With this method, multiple samples are analysed, and the results are described in table format. The data is included in the monograph and published for public comment. In the case of *Epimedium* leaf, species can be clearly discriminated. The result table describes only the features common to the species covered by the monograph.



T1-3 *Epimedium* leaf, T4-6 *Epimedium brevicornum*, T7-8 *Epimedium* leaf, T9-11 *Epimedium koreanum*, T12 *Epimedium* leaf, T13-15 *Epimedium* leaf

Top of the plate	
	[c] A red zone, intense [d] A greenish zone, very faint to equivalent
———	———
[a] Icariin: a green zone	[e] A green zone, faint to equivalent [f] A green zone, faint to intense (icariin) [g] A set of 3-4 green zones, faint to intense
———	———
[b] Epimedin A: a green zone	[h] A green zone, faint to equivalent (epimedin A)
Reference solution (a)	**Test solution**

Data included in the monograph and published for public comment

Step 5: public comments and finalization of method

Comments received from various stakeholders are reviewed by the expert committee before the monograph is presented to the pharmacopoeia commission for adoption. After publication in the Ph. Eur., the HPTLC fingerprints are shown in the EDQM knowledge database.

For CAMAG Laboratory, the involvement in the development and refinement of HPTLC methods not only contributes to global pharmacopoeia standards but also strengthens the scientific rigor and consistency in the identification of herbal drugs. The ongoing collaboration with international groups ensures that these methods are both practical and scientifically sound.

Contact

Dr. Tiên Do, CAMAG, Sonnenmattstrasse 11,
4132 Muttenz, Switzerland, tien.do@camag.com

Oil adulteration evaluation using HPTLC

Paul Rogeboz, Hélia Latado, Ajay Sharma, Neha Chaubey, Shalu Kadian, Enrico Chavez, Tiên Do (CAMAG), Mathieu Dubois, Francesca Giuffrida, Amaury Patin, Maricel Marin-Kuan

The research team at Nestlé Research in Lausanne, Switzerland, develops innovative solutions for food quality and authenticity. Their work, particularly in detecting adulteration in edible oils, plays a key role in ensuring the authenticity of the global food supply chain. By employing advanced chromatographic techniques, the team enhances analytical methods, making a significant contribution to food quality and authenticity. Tiên Do from CAMAG collaborated on this project, contributing to the development of the methods.

Introduction

The evaluation of edible oil authenticity has become increasingly important due to rising incidents of oil adulteration, where low-quality or non-edible oils are mixed with premium oils for economic gain. Such fraudulent practices not only erode consumer trust but also pose health risks. As adulteration methods become more sophisticated, reliable and efficient detection methods are needed.

This study evaluates the use of HPTLC as a cost-effective and efficient tool for monitoring oil authenticity. Both untargeted (fingerprint profiling) and targeted (mineral oil detection) methods were applied to palm, sunflower, and rapeseed oils, demonstrating the capability to detect adulteration at levels between 5% and 25%.

HPTLC offers numerous advantages, including the ability to analyze multiple samples simultaneously with lower solvent consumption. It is also adaptable to different detection protocols and highly reproducible across laboratories. As a result, HPTLC is positioned as an ideal method for industrial applications requiring rapid and user-friendly solutions for oil quality monitoring.

Sample preparation

Edible oils, including sunflower, rapeseed, and palm oil, were collected from various suppliers and prepared for analysis. Authentic oil batches were diluted using cyclopentyl methyl ether (CPME) as the solvent (25.0 µL of oil in 3.0 mL of CPME). The samples were vortexed for 5 seconds, and 1.0 mL of the resulting solution was transferred to a vial for single-use analysis.

Chromatogram layer

HPTLC silica gel 60 F_{254} plates (Merck) were used for vegetable oil analysis, while RP18 F_{254} plates (Merck) were employed for mineral oil adulteration detection. For mineral oil method, the plates were prewashed with methanol and heated at 110 °C for 15 minutes before application.

Sample application

Oil samples were applied as 6.0 mm bands onto the plates using an Automatic TLC Sampler 4.

Chromatography

Plates were developed in the ADC 2 to a migration distance of 70 mm for edible oils and 30 mm for mineral oil detection. A mixture of acetonitrile and CPME (7:3 *V/V*) was used as the developing solvent for vegetable oils, and cyclohexane was used for mineral oil detection. Relative humidity was adjusted to 33% for 10 minutes only for the edible oil method, and chamber saturation was maintained for 20 minutes for both methods.

Post-chromatographic derivatization

After development, chemical derivatization was performed using anisaldehyde reagent for edible oils and primuline reagent for mineral oils. The plates were sprayed with the respective derivatization reagent using the Derivatizer. In the case of anisaldehyde reagent the plates were heated at 100 °C for 3 minutes, and after primuline at 40 °C for 3 min.

Documentation

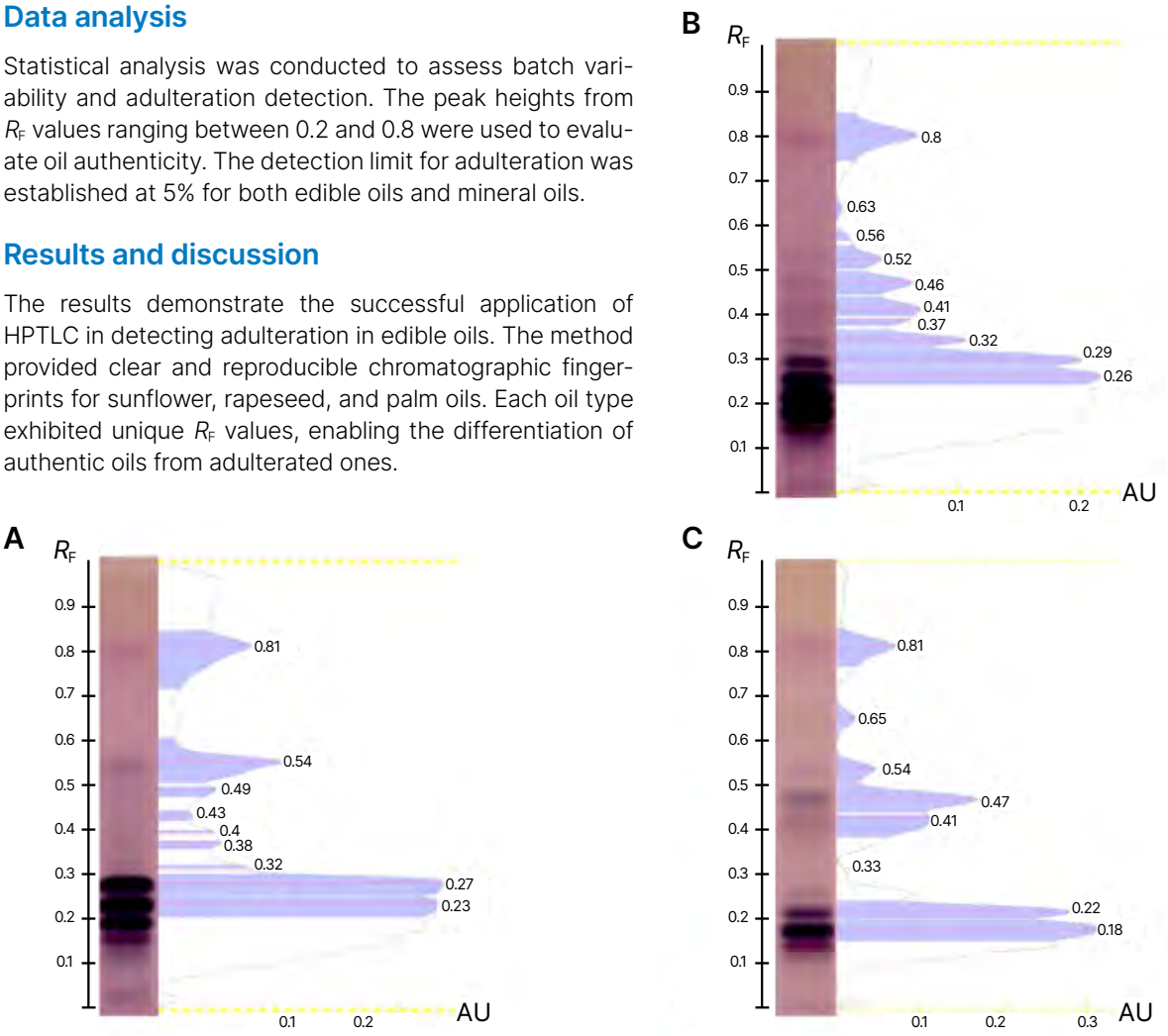
The plates were documented using the TLC Visualizer 2 at UV 366 nm for mineral oils after derivatization with primuline, and in white light (transmission) for edible oils after derivatization with anisaldehyde reagent. Peak profiles from images (PPIs) were analyzed with the *visionCATS* software, and peak heights were recorded to assess the presence of adulterants.

Data analysis

Statistical analysis was conducted to assess batch variability and adulteration detection. The peak heights from R_F values ranging between 0.2 and 0.8 were used to evaluate oil authenticity. The detection limit for adulteration was established at 5% for both edible oils and mineral oils.

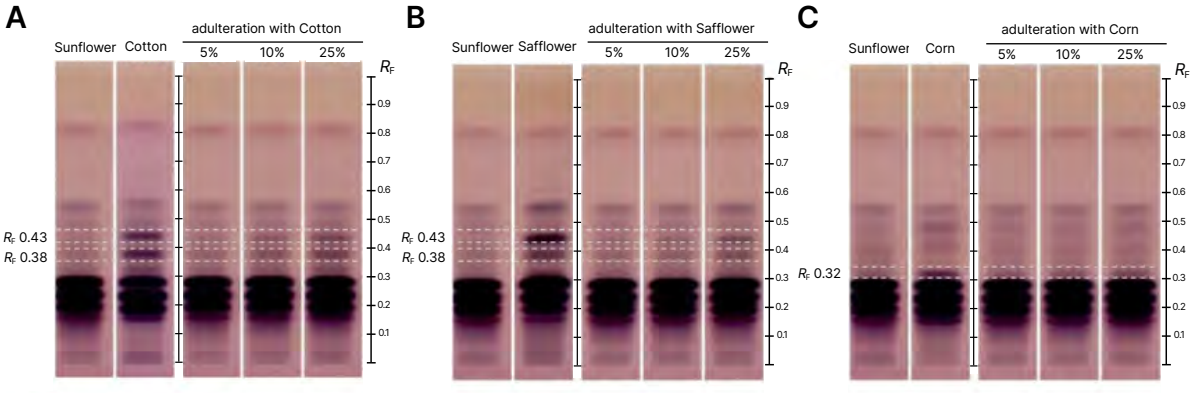
Results and discussion

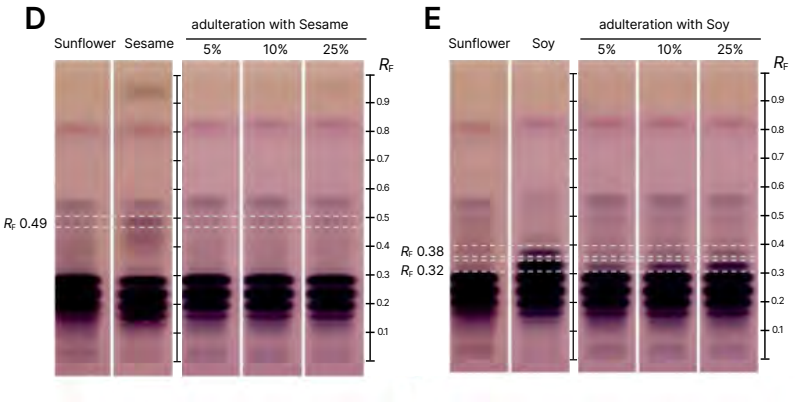
The results demonstrate the successful application of HPTLC in detecting adulteration in edible oils. The method provided clear and reproducible chromatographic fingerprints for sunflower, rapeseed, and palm oils. Each oil type exhibited unique R_F values, enabling the differentiation of authentic oils from adulterated ones.



*Fingerprints of tested oils with corresponding R_F (represented with a red line), HPTLC plate in white light (transmission) after derivatization with anisaldehyde reagent; sunflower oil (**A**), rapeseed oil (**B**), and palm oil (**C**); (https://creativecommons.org/licenses/by/4.0/legalcode)*

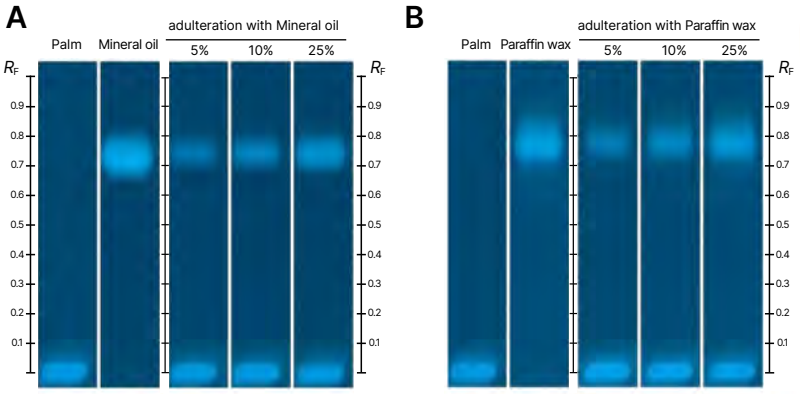
The following HPTLC chromatograms reveal the detection of adulteration in sunflower oil. Samples adulterated with cotton, safflower, corn, sesame, and soy oils were analyzed, and the corresponding R_F values for each adulterant are marked with dashed lines. Adulteration was detected at R_F values specific to each adulterant, such as R_F 0.38 for cotton oil and R_F 0.49 for sesame oil. The clear distinction between authentic and adulterated sunflower oil samples demonstrates the sensitivity of the HPTLC method, which successfully detected adulteration at levels as low as 5%.





*HPTLC chromatograms in white light (transmission) after derivatization with anisaldehyde reagent: Sunflower oil adulterated with cotton oil (**A**), safflower oil (**B**), corn oil (**C**), sesame oil (**D**), and soy oil (**E**) with the corresponding adulteration R_F's (represented with a dash lines); (https://creativecommons.org/licenses/by/4.0/legalcode)*

Adulteration was detected at R_F values around 0.8 for mineral oil and paraffin wax, clearly distinguishing them from the authentic palm oil sample. The high sensitivity of the HPTLC method allowed for the detection of adulteration at levels below 5%, demonstrating its effectiveness in identifying hazardous non-edible oil contaminants such as mineral oils.



*HPTLC chromatograms in UV 366 nm after derivatization with primuline reagent: Palm oil adulterated with mineral oil (**A**) and paraffin wax (**B**); (https://creativecommons.org/licenses/by/4.0/legalcode)*

Conclusion

HPTLC proved to be a valuable tool for detecting adulteration in edible oils, offering a high-throughput, reliable, and relatively simple method. The method is well-suited for industrial applications, ensuring food quality and authenticity in the global edible oil market.

Literature

[1] Paul Rogeboz *et al.* Food Analytical Methods (2024) 17:1336–1347

Further information is available on request from the authors.

Contact

Paul Rogeboz, Société des Produits Nestlé SA, Nestlé Research, 1000, Lausanne, Vers-Chez-Les-Blanc, Switzerland, paul.rogeboz@rd.nestle.com

HPTLC fingerprint profiling for determination of bioactive ingredients in Indian propolis

Sandeep Sankaran, Rahul Dubey, Anushka Bakore, Sathiyanarayanan Lohidasan

Sandeep Sankaran*, PhD Scholar from the Department of Quality Assurance Techniques at Poona College of Pharmacy, BVDU, carried out his research work focusing on the systematic evaluation of the chemical profile and its correlation to neuroprotective activity for Indian bee propolis. The research team under the supervision of Dr Sathiyanarayanan worked comprehensively on deriving the chemical profile of Indian propolis extracts through the HPTLC fingerprinting methodology developed in-house, extending to marker-based standardization and HPTLC-effect-directed analysis.

Introduction

Bee propolis is a valuable yet often neglected therapeutic resource made up of a combination of plant resins gathered during foraging, mixed with the bees' own salivary secretions deposited in the beehives. The chemical composition is highly heterogeneous and depends on the vegetation in and around the hive, climatic conditions, and the bee species. Various analytical techniques have been used to evaluate the quality of propolis, including the use of high-end instruments in combination with chemometric modeling for deriving the complete chemical profile. However, these methods are costly and hard to replicate in quality control labs. A more feasible approach is to standardize based on markers that correlate with the specific biological activity of that propolis variant. The present study was therefore designed to focus on fingerprint profiling for identifying the propolis type, screening for the antioxidant and anticholinesterase components directly on the plate through a new developed, validated and sustainable HPTLC methodology.

To identify the propolis type, a simplified, rapid, low-cost, low-environmental impact, and easily adoptable analytical methodology was developed, extending to the standardization of selected neuroprotective components in Indian propolis. The versatility of HPTLC, with various derivatizing reagents and orthogonal detection capabilities, allows for increased applications. With the advent of thin-layer chromatography-effect directed analysis, it enables direct screening on the TLC plate, establishing preliminary evidence of the biological activities. Thus, this HPTLC method is valuable for rapid chemical profiling and simultaneous screening of antioxidant and anticholinesterase activities of Indian propolis. Also, educating beekeepers about its medicinal value can help them generate additional revenue.

Standard solutions

Stock solutions (1.0 mg/mL) are prepared in methanol, except dimethyl sulfoxide was used for initial solubilization of chrysin. The subsequent working solutions are prepared in methanol, i.e., chrysin (0.10 mg/mL), *p*-coumaric acid (0.05 mg/mL), pinocembrin (0.10 mg/mL), luteolin (0.10 mg/mL), and galangin (0.20 mg/mL).

Sample preparation

Indian propolis extracts and the marketed samples (2.0 mg/mL or 3.0 mg/mL) are prepared by weighing 20.0 mg or 30.0 mg and dissolving in 10.0 mL of ethanol. The samples are sonicated, centrifuged and filtered before TLC analysis.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm are used.

Sample application

1.0-10.0 µL of standard solutions (7-point calibration) and 2.0 and 5.0 µL of sample solutions are applied as bands with the Linomat 5 (with N_2). Plate layout: 15 tracks, band length 6.0 mm, distance from left plate edge 15.0 mm, track distance 11.4 mm, distance from the lower edge 8.0 mm.

Chromatography

Plates are developed in the twin-trough chamber with chamber saturation for 30 min (with filter paper) and development with toluene – ethyl acetate – formic acid 74:26:5 (*V/V*) to the migration distance of 80 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

The developed plate is first heated at 110 °C for 2 min and then placed in the immersion device containing

Natural product reagent (NP or 2-aminoethyl diphenylborinate – 1% (*W/V*) in ethyl acetate). The developed plate is immersed in anisaldehyde sulfuric acid reagent (ASR – prepared fresh by combining 1.0 mL *p*-anisaldehyde with 20.0 mL glacial acetic acid, followed by 170 mL methanol and 10.0 mL concentrated sulfuric acid) and then heated at 100 °C for 5 min. The developed plate is immersed in Ferric chloride solution ($FeCl_3$ – 2 % (*W/V*) in methanol) and then heated for 2 min at 110 °C.

Note: The derivatization was conducted on three different developed plates.

Post-chromatographic bioautography

The developed plate is immersed into a 2,2-diphenyl-1-picryl hydrazyl solution (DPPH - 0.25 % (*W/V*) in methanol), stored in the dark for 30 min. The yellow zones captured against purple background are an indicator of antioxidant components when visualized in white light. The Ellman assay protocol was used wherein the developed plate is first immersed in a solution of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and acetylthiocholine iodide (ATCI) (1 mM DTNB and 1 mM ATCI in buffer A) until the plate was saturated, dried for 5 min and then around 3-4 mL of acetylcholinesterase enzyme solution (*Electrophorus electricus* – AChE - 3 U/mL) is sprayed onto the plate. The white band on the plate is an indicator of acetylcholinesterase inhibition.

Documentation

Images of the plate are captured with the TLC Visualizer 2 in UV 254 nm, UV 366 nm, and white light.

Densitometry

Absorbance measurement is performed with the TLC Scanner 3 and *visionCATS* at 268 nm (chrysin), 297 nm (*p*-coumaric acid and pinocembrin) and 352 nm (luteolin and galangin), slit dimension 5.00 mm x 0.45 mm, scanning speed 20 mm/s, spectra scanned from 200 to 450 nm.

Mass spectrometry

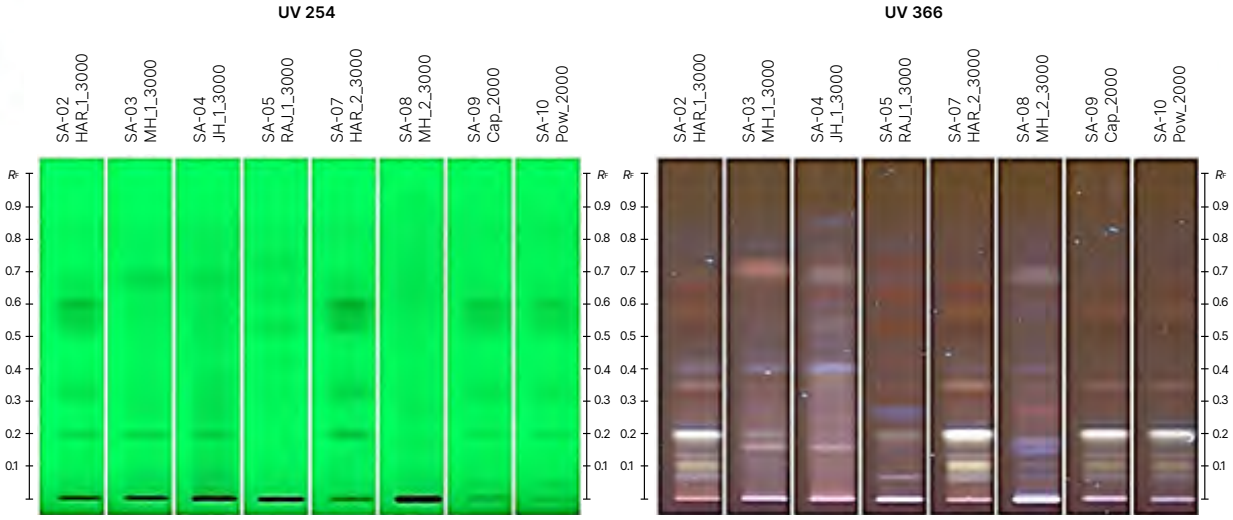
The selected bands are eluted with the TLC-MS Interface 2 at a flow rate of 0.5 mL/min with methanol (with 0.1 % formic acid) into an Electrospray ionization (ESI)-Triple Quadruple Mass Analyzer (Agilent 6460) in the negative ionization mode.

Results and discussion

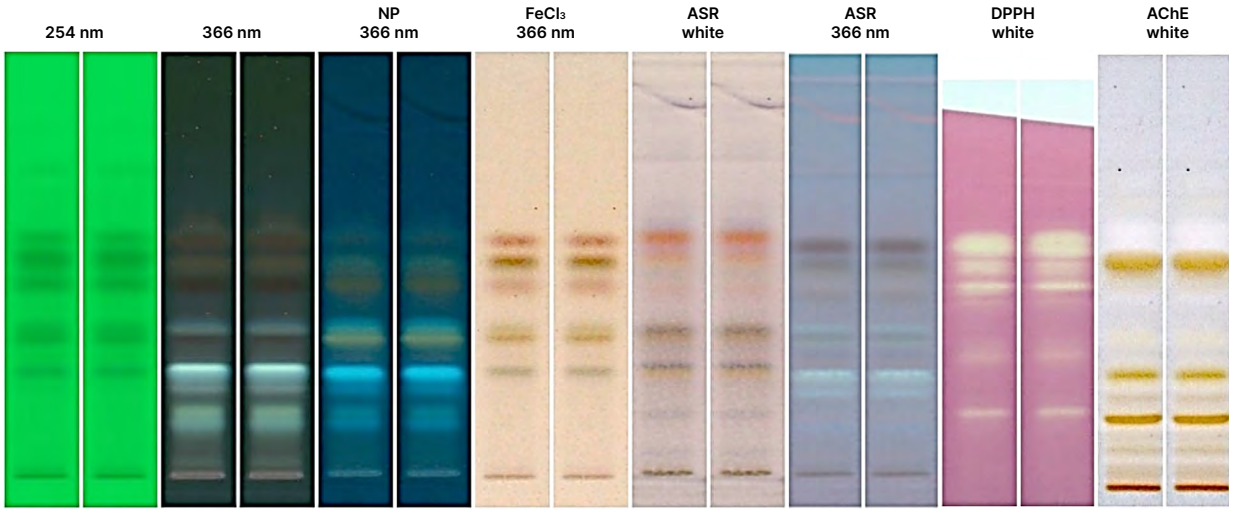
The HPTLC fingerprint image of the various propolis extracts is shown, and the profiles are key indicators of the diversity in vegetation across different regions. The sample coded HAR was mainly of 'O-type' propolis due to the presence of flavonoids like chrysin, galangin, pinocembrin, as well as non-flavonoids like *p*-coumaric acid, matching the characteristic bands of the standard when derivatized with various reagents. Interestingly, the applicability of the method on two marketed products presented a similar fingerprint to that of the HAR extract.

The optimized method is found to be precise (%RSD ≤ 2.0 %), accurate (90–110 %), linear over the concentration ranges (r^2 ≥ 0.995), sensitive and robust resulting in the R_F values of 0.235, 0.353, 0.552, 0.606, and 0.655 for luteolin, *p*-coumaric acid, chrysin, galangin, and pinocembrin, respectively. Pinocembrin (2.30 ± 0.12 % *W/W*) and galangin (5.78 ± 0.30 % *W/W*) are found in the highest concentrations in the HAR sample. The m/z values of the molecular ion and fragment ions from the isolated sample bands matched those of the standards, further confirming the identity of the peaks. The bands with R_F values corresponding to chrysin, galangin, and pinocembrin showed strong antioxidant activity, as indicated by bright yellow zones against a purple background, while the white bands in the extract fingerprint that appeared along the plate following the Ellman's assay are indicative of acetylcholinesterase inhibitors.

Thus, the developed analytical method with orthogonal capabilities can be universally applied to different propolis extracts and formulated propolis products as a quick screening method for fingerprint and neuroprotective profiling.



HPTLC fingerprint image of propolis extracts collected from different regions in India and marketed samples in UV 254 nm and in modified UV 366 nm before derivatization (enhanced contrast)



HPTLC fingerprints of HAR extracts pre- and post-derivatization in different illumination modes

Acknowledgements

The authors would like to express their gratitude to Poona College of Pharmacy (Bharati Vidyapeeth Deemed to be University), Central Bee Research and Training Institute (CBRTI, Pune), All-India Council for Technical Education (AICTE), Anchrom Enterprises Pvt. Ltd. (Mumbai), Bee Basket Enterprises Pvt. Ltd and the Centre of Food Testing Laboratories, (Pune) for all the assistance and support in the work.

Contact

Sandeep Sankaran, Department of Quality Assurance Techniques, Poona College of Pharmacy, Bharati Vidyapeeth (Deemed to be) University, Pune, Maharashtra 411038, India, sandeepsss1992@gmail.com

Literature

[1] Sankaran, S. *et al.* (2024) J Planar Chromat 37 (3), 233–245

[2] Bankova, V. *et al.* (2019) J Apic Res 58, 1–49

[3] Sankaran, S. *et al.* (2023) J Biol Active Prod Nat 13, 76–93.

Further information is available in the article published "Sustainable instrumental thin-layer chromatography-based methodology for standardization of neuroprotective components in propolis collected from India" J Planar Chromat 37, 233–245 (2024). https://doi.org/10.1007/s00764-024-00307-x or on request from the authors.